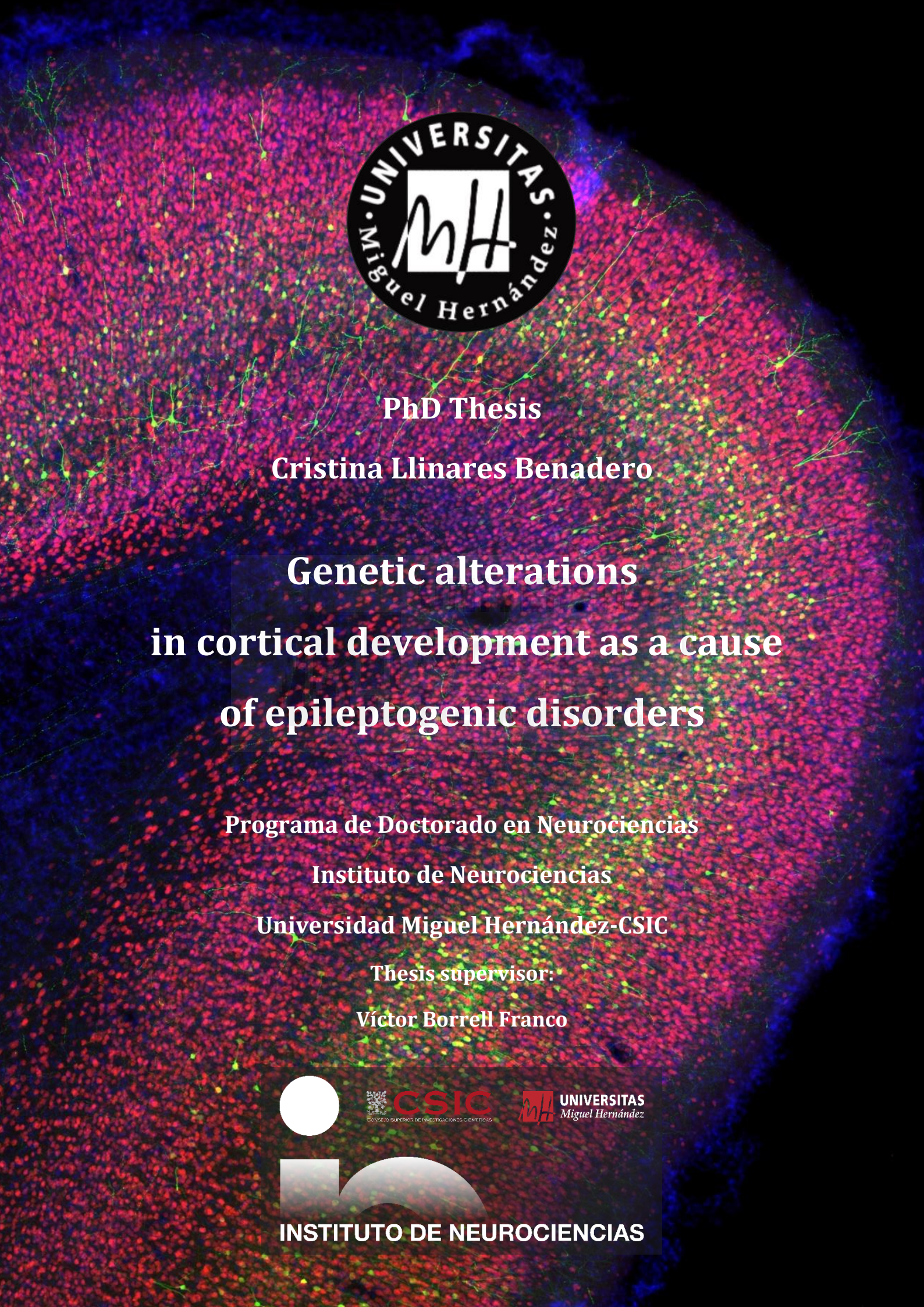

PhD Thesis

Cristina Llinares Benadero

Genetic alterations in cortical development as a cause of epileptogenic disorders

Programa de Doctorado en Neurociencias

Instituto de Neurociencias

Universidad Miguel Hernández-CSIC

Thesis supervisor:

Víctor Borrell Franco

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Genetic alterations in cortical development as a cause of epileptogenic disorders

Memoria de Tesis Doctoral

Cristina Llinares Benadero

Director de Tesis

Víctor Borrell

San Juan de Alicante, 2019

Programa de Doctorado en Neurociencias

UNIVERSITAS
Miguel
Hernández

San Juan de Alicante, 2019

A QUIEN CORRESPONDA:

Esta tesis se presenta por compendio de publicaciones, incluyendo las siguientes publicaciones científicas publicadas durante la realización de la misma:

- Fernández V*, Llinares-Benadero C*, Borrell V. "*Cerebral cortex expansion and folding: what have we learned?"*. EMBO Journal. 2016 May 17;35(10):1021-44. doi: 10.15252/embj.201593701.
- C. Llinares-Benadero and V. Borrell (2019). "*Deconstructing cortical folding: genetic, cellular and mechanical determinants*" Nature Reviews Neuroscience 2019 Jan Epub doi: 10.1038/s41583-018-0112-2.

Atentamente,

Cristina Llinares Benadero

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A QUIEN CORRESPONDA:

Dr. Víctor Borrell Franco, Investigador Científico de la Agencia Estatal Consejo Superior de Investigaciones Científicas,

Autoriza la presentación de la Tesis Doctoral por compendio de publicaciones titulada *"Genetic alterations in cortical development as a cause of epileptogenic disorders"*, realizada por Dª Cristina Llinares Benadero (DNI 48621953E) bajo su inmediata dirección y supervisión en el Instituto de Neurociencias de Alicante, centro mixto CSIC-UMH, y que presenta para la obtención del grado de Doctor por la Universidad Miguel Hernández.

Y para que así conste, y a los efectos oportunos, firma el presente Certificado en San Juan de Alicante, en enero de 2019.

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Prof. Miguel Valdeolmillos López, Coordinador del Programa de Doctorado en Neurociencias del Instituto de Neurociencias, Centro Mixto de la Universidad Miguel Hernández-UMH y la Agencia Estatal Consejo Superior de Investigaciones Científicas-CSIC,

CERTIFICA:

Que la Tesis Doctoral *"Genetic alterations in cortical development as a cause of epileptogenic disorders"* ha sido realizada por Dª Cristina Llinares Benadero (DNI 48621953E) bajo la dirección del Dr. Víctor Borrell Franco y da su conformidad para que sea presentada a la Comisión de Doctorado de la Universidad Miguel Hernández.

Para que así conste a los efectos oportunos, firma el presente certificado en San Juan de Alicante en enero de 2019.

Fdo: Miguel Valdeolmillos

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Quan surts per fer el viatge cap a Ítaca,

has de pregar que el camí sigui llarg,

ple d'aventures, ple de coneixences.

Has de pregar que el camí sigui llarg,

que siguin moltes les matinades

que entraràs en un port que els teus ulls ignoraven,

i vagis a ciutats per aprendre dels que saben.

Tingues sempre al cor la idea d'Ítaca.

Has d'arribar-hi, és el teu destí,

però no forcis gens la travessia.

És preferible que duri molts anys,

que siguis vell quan fondegis l'illa,

ric de tot el que hauràs guanyat fent el camí,

sense esperar que et doni més riqueses.

Ítaca t'ha donat el bell viatge,

sense ella no hauries sortit.

I si la trobes pobra, no és que Ítaca

t'hagi enganyat. Savi, com bé t'has fet,

sabràs el que volen dir les Ítaques.

K. Kavafis (musicat per Lluis LLach)

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Después de tanto tiempo, cuesta creer que ha llegado el día de poner por escrito todo el inmenso agradecimiento que siento por las cosas vividas durante estos últimos cinco años. Me encantaría poder hacer un recorrido detallado por cada una de las personas que me han ayudado, en qué medida y en qué forma lo han hecho. Sin embargo, de hacer algo tan exhaustivo (que sería lo más justo), los que me conocéis sabéis que es muy probable que de esta forma éste fuera el apartado más largo de la tesis, si me pongo a añadir ejemplos y anécdotas (a ver algunos habrá, debo ser fiel a mi estilo). Empezaré por el principio y, como Jack el Destripador, iré por partes.

En primer lugar, quiero dar las gracias a Víctor. Recuerdo perfectamente cuando me llegó el mail ofreciéndome la positibilidad de hacer una entrevista para hacer el doctorado en su grupo. Lo que no sabía es que era un correo que iba envuelto en oro. Después vine aquí y hablé con todo el mundo. En algún momento esto debió convencerles, porque al final tuve la gran SUERTE de hacer aquí la tesis. Víctor, muchísimas gracias por esta increíble oportunidad. Desde que estoy aquí, he tenido la buena suerte de formar parte de un equipo maravilloso, no sólo a nivel profesional sino también en el aspecto humano porque habéis sido compañeros, pero sobretodo sois amigos. Víctor, quiero darte las gracias por todo lo que has hecho por mí desde que llegué aquí. Fácilmente haces sentir a la gente integrada y bienvenida en el laboratorio. Gracias por tener siempre la puerta abierta. Desde el principio he sentido la libertad de decirte lo que pensaba y plantearte cualquier cuestión. Gracias por darme libertad y confianza, por escuchar mis ideas y tenerlas en cuenta. Gracias por todas las oportunidades que me ha dado estar aquí, por exigirme y también por valorarme.

Desde que vine, la gente del labo ha ido yendo y viniendo. Quiero daros las gracias a Isabel y a Marian, por enseñarme, por ayudarme y por vuestros consejos. Aunque ahora ya no estéis en el labo, vuestra experiencia y vuestros precedentes marcaron una tendencia en este labo del que todos nos hemos ido nutriendo, tanto en esfuerzo como en compañerismo.

Hugo, uno de los miembros infalibles del club del fin de semana. Como me acuerdo de la calma que tenías, pero de lo mucho que trabajabas también. Recuerdo quedarme hasta tarde contigo y luego que me dijeras con ese acento italiano: “Ahora, lo que hay que hacer es cenar algo muy bueno”, dicho con el tono apropiado. Gracias por tu ayuda y por transmitirme tranquilidad. Recuerdo cuando empecé a electroporar. Cualquiera que me viera podía ver cuánto me temblaban las manos (aunque Sabrina podría decir que era a los demás a los que les temblaban los ojos; pero yo sé que no). Me dijiste: “imagínate que estás haciendo un bocadillo”, refiriéndote a que fuera pensando por pasos como hacerlo. Y funcionó. Desde el principio fuiste amable conmigo y curiosamente la comida fue un factor de conexión, como aquella pizza y aquel ragú: imposibles de olvidar.

Alguien que también entra en esa categoría es Esther Picó. Esther, gracias por ser desde el primer día un apoyo. Gracias por contagiarme con tu buen humor y tu sentido común, por compartir tu sabiduría, científica y popular. Gracias por hacerme ver que “el Nobel puede esperar” y que por muy mal que pareciera que fueran las cosas “peor es ser puta”. Para mí ha sido superimportante tenerte en este laboratorio y durante buena parte de mi tesis, y sigo notando cada día el hecho de que no estés. Muchas veces me acuerdo de ti y de tus

“escúchameee”, con ese gesto característico tuyo y sonrío pensando en la suerte que he tenido de haber coincidido contigo en tiempo y en espacio.

Si hablamos de sabiduría, no podemos dejar de pensar también en Trini. Trini ha sido un placer trabajar contigo, ver de cerca tu profesionalidad y sobretodo tu calidad humana. Gracias por aportar salero a este laboratorio, por tu buen rollo y por ayudar a todo el mundo con tu amplia experiencia. Esta energía tan positiva también ha venido de Pep. Ay Pep, Pep… Tenerte en el labo ha sido genial. Emanas tranquilidad y buen humor. Gracias por tu sonrisa constante y contagiosa, Gracias por tu complicidad y por tu sentido del humor. Es una pena que la UMH no nos deje tenerte siempre y de continuo.

Hablando de personas que han pasado por este labo y que han compartido parte de este viaje, no puedo dejar de nombrar a dos visitantes muy importantes: Valentina y Ana. Valentina, tu llegada no podría haber sido en mejor momento. Fuiste un soplo de aire fresco y agradezco todos los momentos que hemos vivido desde entonces, hasta estar en tu casa nueva y comer risotto casero. Uz, no sé cómo es posible, pero desde el primer día que estuviste aquí sentí una confianza inmediata. Gracias por tener tantas ganas de venir a Alicante porque el tenerte aquí me dio muchísima energía y perspectiva. Gracias por apoyarme, inculso desde París, por todos tus audios, por tranquilizarme muchas veces y por ofrecerme naturalidad y confianza.

Otra hornada de personas con sus ilusiones renovadas fue llegando, aportando su granito de arena a esta locura a la que cariñosamente llamamos tesis. Kaviya, thank you for being so understanding and for your quietness. I know the path was hard for you too, but you are almost finishing it! Lucía, gracias por aguantar mis suspiros y bufidos de desesperación, que sé que han sido muchos en los últimos meses. Salma, muchas gracias por tu alegría y tu sonrisa, por haberte preocupado por mí, especialmente durante este último tramo. Gracias por tus detalles y por haber cuidado de mí, por transmitirme tu cariño con cualquier gesto. Alex, gracias por tu interés y por tus ganas, por tu espíritu y tu compañerismo. Gracias por ayudarme cuando lo he necesitado.

Jorge, gracias por abrirnos un poco la mente a todos con experimentos y maquinaria muy ajena al día a día del resto de nosotros. Gracias por tu carisma, por preocuparte por los demás y por tu buen humor. Por tus saludos por la mañana, aunque el 90% me asustaran. Te juro que no es nada personal, pero se ve que siempre ha coincidido que me pillabas en periodo de máxima concentración o de máximo empanamiento, jajajja.

Bea, muchas gracias por estar pendiente de todo siempre, de todos los papeles, de todo lo que hay que justificar, siempre cuidando de nosotros como sea. Gracias por haberme aguantado en más de un momento de desesperación, por aguantar mi verborrea, que es mucha. Gracias por tu paciencia, por tu ayuda constante y por tu asesoramiento. Tenemos mucha suerte de contar contigo en el labo.

Ana, gracias por estar siempre dispuesta a todo, por tu inacabable energía y cariño. Gracias por tener siempre un sí en la boca, antes que un no; por dar siempre la oportunidad de hablar o escuchar, por no tener nada en cuenta y por ofrecer siempre tu ayuda. Gracias por los buenos momentos.

Adriii, ¡bonico! Resulta curioso pensar que, muy al principio, ¡¡me intimidabas mucho!! Me daba mucho reparo decirte o preguntarte cualquier cosa porque te veía superserio. Pero luego fui descubriendo al Adri de verdad, un compañero con todas las letras, una persona con muy buenas ideas, pero sobretodo alguien con muy buen corazón. Adri, gracias por tu sinceridad, por tu ayuda y por ir siempre más allá. Gracias por tener una libreta sólo para apuntar los pinchos y las cervezas para hacer que cada una de ellas cuente.

Esther, recuerdo cuando empezaste en este labo. El primer comentario que me hiciste era sobre el T-Rex que tengo en mi bancada, por lo visto a ti también te encantaban los dinosaurios. ¡Eso ya era un punto a tu favor! Cuando llevabas aquí unos días, recuerdo ir a Esther Picó y decirle: "Me gusta un montón como trabaja y además me cae muy bien. ¿Tú la conocías?" La Picó contestó con contundencia: "Of course, la recomendé yo". Me reí y entonces lo entendí todo. Tuvimos la suerte de tener un periodo de Esther$^{2:}$ exponencialmente geniales. Esther, no puedo empezar a darte las gracias por todo lo que has hecho por mí, por todo lo que me has aguantado. Sé que suena a tópico, pero de verdad, tu apoyo este año ha sido vital para mí. Gracias por todas las charlas, por poder contar contigo en cualquier momento, por escuchar todas mis rayadas. Gracias por todos los Bonobos, por tu confianza y tus abrazos. Gracias por darme la mano cuando estaba en la mierda. Gracias por nuestra sincronización, por comprenderme. Por cierto, eres **impresionante** (leído con tu tono).

Camino, Camino. Probablemente la palabra que más he dicho durante la tesis, el nombre más suspirado. Incluso llegaste a decirme "¿Te tranquiliza decir mi nombre? Porque a algunas personas les causa taquicardía." Ay Camino, sí, me ha dado mucha paz decir tu nombre. Aunque desde que te conozco has tenido muchos otros: Caminito, de Juan, Shiba, Anksunamun... ¡Y es que mira que te gustaba poco que te tocara, eh! Pero a mí me pareces tan entrañable que no podía dejar de mostrarte mi cariño con cualquier gesto. Nunca había conocido a nadie como tú. Como te he dico muchas veces "Ay, como eres, eh" a lo que tú respondías "¿Cómo soy?" y yo te contestaba "Pues única en tu especie, Camino". Recuerdo el día que empecé, cuando Víctor me dijo que iba a estar a tu lado. La verdad es que me intimidó en plan… ¡oh Dios mío, al lado de la PostDoc! Sin saberlo, éste era otro de los regalos de Víctor, aunque me di cuenta muy rápido. Camino, no puedes empezar a imaginar lo que ha significado para mí tenerte a mi lado cada día, metafórica y literalmente. Desde el principio me has enseñado, estar contigo me ha dado fuerza. Gracias por dejarme conocerte, por abrirte poco a poco, de té en té. Gracias por todo tu apoyo y por tu cariño. Siempre te he sentido ahí, hasta los días en los que ninguna tenía ganas de hablar, sabía que estabas, que sabías lo que me pasaba de alguna forma. Gracias por escucharme, por tus consejos. Gracias por un abrazo inolvidable. Gracias por preocuparte por mí desde el primer día. No te imaginas cuánto te echo de menos, cuánto pienso en la suerte que he tenido de caminar contigo esta etapa de mi vida y cuánto me gustaba estar junto a ti. Hasta darte los buenos días y contarte cualquier cosa me ha hecho feliz. Echo de menos hasta tus "uhum". Gracias por la complicidad, por comunicarte con la mirada. Gracias por conectar conmigo, por todas las bromas. Gracias por tus días de "voy a tomarme otro café porque éste ha pasado por mi vida". Gracias por lo buena que eres, por tu condición de coulan misterioso. Gracias por estar conmigo hasta el final.

Mon, eres una de las personas por las que la palabra gracias debería reinventarse para que pudiera abarcar todo lo que significa tratándose de ti. No hay ningún momento durante los años de la tesis del que no hayas formado parte. Eres la persona con la que he vivido todo, todas las emociones y experiencias he tenido el privilegio de vivirlas y compartirlas contigo. Hemos bailado con el coche. Hemos operado huronas juntas. Hemos llorado de frustración y de nostalgia y también de la risa. Hemos cantado juntas, hemos compartido ilusiones y viajes. Con todo, siempre hemos podido contar la una con la otra en cualquier situación. Gracias por todos los pitis, todas las charlas en la pasarela, por todas las veces que el reloj ha marcado las beer o'clock. Gracias porque tu sitio fuera un punto de referencia, un lugar al que acudir para encontrar apoyo en los momentos más duros, pero también para celebrar los logros y alegrías del trabajo y de la vida. Tenerte como compañera y verte desde "the other side" al ritmo de Adele ha sido un honor y, sin duda, un pilar que me ha permitido seguir día a día. Gracias por compartir conmigo tu sonrisa, por tu facilidad para motivar la mía. Gracias por creer en mí, por confiar en mí y apoyarme. Gracias por estar conmigo también desde la distancia, sumando pitis virtuales. Sigo escuchando en mi cabeza tu voz desde la distancia diciéndome "lo vas a conseguir". Gracias por tantas experiencias inolvidables, por ser mi compañera en el sentido más amplio de la palabra, pero sobretodo, gracias por ser mi amiga. Gracias por todas las locuras, por vivir conmigo al límite de la ley que ha caracterizado la tesis. Gracias por hacerme ser mejor persona, por hacerme dar lo mejor de mí, por inspirarme cada día con tu cariño y con tus ganas. Lo que intento decir con todo esto es un inmenso gracias, este viaje no habría sido lo mismo sin ti. Has marcado la diferencia en este camino y en mí. Te quiero.

Durante todo este tiempo, he contado con mucho apoyo y también buena parte de él ha venido de otros rincones del INA. La verdad es que casi todo el mundo ha contribuido de alguna forma, aunque sea con un "buenos días" o una sonrisa por el pasillo. A veces no nos damos cuenta de lo importante que es eso, pero dan un plus al día a día. Tenemos una suerte inmensa al trabajar en un sitio en el que al mandar un mail son muchas las manos que ofrecen su ayuda. Por eso quiero darte las gracias Joan Galcerán, por estar siempre dispuesto a compartir tu inmenso conocimiento y tu pasión por la biología molecular. Gracias por tu paciencia y por tu curiosidad. Gracias también Giovanna por tu ayuda con el multifotón. Y gracias Verona por tu entusiasmo, tu sonrisa y esa energía tan positiva y motivadora que transmites.

Requieren una mención especial nuestro laboratorio hermano, los Guillerminos, con los que hemos compartido todo tipo de vivencias. Cuando hemos tenido que pedir algo, siempre he pensado "pregúntale a los de Guille". Quiero daros las gracias a todos por ser tan geniales y por adoptarme en muchas ocasiones, por todas las risas y todo del cariño que transmitís. Sois un grupo asombroso, a nivel tanto profesional como personal, y he tenido una suerte increíble por poder contar con vosotros, por teneros a tan solo una planta de distancia: Gracias a Alejandro, por tu sinceridad y tu buen fondo, gracias a Ana, a Belén, a Rafa, a Noe, a Irene y a Leti. Gracias Luis, por tu gran corazón, por preocuparte por mí, por hacerme reír y sonreírme detrás de tu poblada barba. Gracias Mar, por ser como eres, una combinación asombrosa de coraje y ternura, que asoma como tu lunar en la nariz. Resultas divertida y entrañable, del tipo de persona que cualquiera quisiera tener cerca.

Kika, muchas gracias de corazón, por todo. Gracias por acordarte de mí para cualquier cosa. Gracias por ser así, por hacer las cosas fáciles, por dar buen rollo, por ser tan buena persona. Gracias por dejar que te toque, aunque sea mirándome mal con tu expresión particular de "¿qué haces?" en la cara. Gracias por ayudarme y por escucharme, por bajar mi nivel de ralladura de limón. Gracias por hacerme sentir siempre cómoda, por compartir la peor musaka de la historia que daría lugar a un gran momento para recordar. Gracias por todas las experiencias. Gracias por tu sentido del humor, por tus contagiosos "estásssssssssssssssssssssssssssssssssssss", tus "clarooo" y por tus "se le complica", que ya han llegado a formar parte de mi repertorio e incluso de otros Borrelianos. Gracias por tu risa, esa característica que se nota que te sale de la boca del estómago. Cuando te escucho reir así, me inunda una sensación de alegría y bienestar.

¡Alv! Otra de las piezas importantes durante toda esta etapa. Gracias por tu buen fondo, por tus anécdotas, por estar siempre dispuesto a escuchar o a echar una mano con lo que sea. Gracias por ese gesto tuyo (tuyo heredado de tu padre porque lo hace igual que flipas). Eres un tio genial, que da una atmósfera de comodidad y confianza. Gracias ☺.

Para acabar de nombrar a lo que concierne al INA, no puedo dejar pasar esta oportunidad para dar las gracias a todas las ratonas y huronas que he conocido durante estos años. Gracias a todas las que disteis a luz y cuidasteis de vuestras crías, gracias por ser buenas madres. Pero también gracias a todas aquellas que no lo hicisteis, porque fuisteis parte de una lección importante: lidiar con la frustración como parte de la tesis.

Además de la mucha gente de la buena que me ha rodeado con su apoyo y su cariño a lo largo y ancho de este Instituto, también he contado con el apoyo incondicional de personas que, aunque no lo habitaban como nosotros, han sido también sufridores de toda esta aventura. Empezaré por decir que, aunque como versa nuestro grupo de whatsapp, lo mejor de la vida es comer y dormir (sinceramente, yo siempre me quedaré con comer), sin duda las personas que lo forman están a la altura de cualquier plato gourmet. Ellas son como estrellas Michelín que me cayeron del cielo durante mi etapa universitaria y que hoy en día siguen a mi lado pese a que nos separen algunos kilómetros, personas extraordinarias. Lau, gracias por tu positivismo, por tu entusiasmo por la vida. Vivir contigo fue una de las mejores experiencias que he tenido, me devolviste la confianza en mí misma, me llenaste de energía y, la verdad, me cambiaste. Tenerte cerca me hizo ser más cariñosa porque estar contigo hacía todo tan natural, que supongo que dejó de darme miedo o vergüenza demostrarlo con un abrazo, con una palabra o simplemente con algún gesto físico. Itsi, gracias por tu determinación, por tus principios y por tu carácter. Gracias por ser eres, por tus "chaíto" y tus "twinnnns". Melania, estoy muy agradecida de haberte podido conocer mejor y de compartir contigo tantos momentos. Eres de esas personas que hace todo fácil. Gracias por tu punto alocado, por tu humor y por tu vitalidad. Iraia, gracias por tu sencillez, gracias por tu bondad y por asegurarme el verte al menos una vez al año con tus veraneos en la Vila. Ester, gracias por no haber estudiado arquitectura. Gracias por sentarte junto a mí el primer día. Creo que fue una gran metáfora que nos regaló el destino, pues has estado ahí desde el princpio. Gracias por tu nobleza y tu sinceridad. Gracias por el peso de tus palabras. Eres de esas personas que habla poco, pero lo que dice es contundente y sale del corazón. Gracias por llegar al mío. Chicas, gracias infinitas a todas por aparecer, por todo lo que

hemos compartido, por vivir conmigo amagades primaveres. Gracias por vuestro apoyo incondicional, por compartir nuestra gran pasión (¡¡comer!!) y por hacer de la ciudad Condal mi segundo hogar. Gracias por quererme con todas las letras, de principio a fin. Mila Esker!

También tuve mucha suerte durante el Máster, donde tuve grandes hallazgos. Gracias a los viajadores del tranvía y compañía por vuestro apoyo; Jose Miguel, Dani, Paula, Eva, María y Ángeles. Aún recuerdo el momento en el que os planteé mi gran dilema de qué hacer en uno de nuestros encuentros. Gracias por ayudarme a aclararme. Gracias también a Sole y a Sara, las SS, las primísimas, por ser tan buenas personas e impulsarme y darme la certeza de cuál era la atmósfera de trabajo que quería para mí, y que he tenido la suerte de conseguir. Gracias Nerea, por todo tu apoyo, por todas nuestras charlas, por nuestras tardes de sofá, nuestras risas de cansancio, nuestros planes de qué comer el domingo cuando todavía era jueves. Gracias por cuidarme tanto, por encajar conmigo, gracias en definitiva por aparecer. Gracias por apoyarme en aquellas semanas decisivas, por darme una casa llena de confianza y cariño. Debo decirte que esta historia empezó también gracias a ti.

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Si hay alguien que merienda fuerte, esa es Júlia. Júlia, no sé por dónde empezar a darte las gracias. Desde que tengo memoria siempre has estado ahí, literalmente. No sé que situación extraordinaria debe darse para conocer a alguien de bebés y que luego la vida quisiera que coincidiéramos en el mismo colegio e instituto y nos hiciéramos amigas. Júlia, gracias por ser una de las personas que mejor me conoce, gracias por quererme de esa forma tan incondicional, tan pura, tan real. Gracias por arroparme con tus abrazos, por sonreírme desde cualquier lugar del mundo en el que has estado. Gracias por escucharme y comprenderme, por compartir tus vivencias conmigo. Gracias por tus frases llenas de sabiduría. No puedo olvidar particularmente una, que fue el preludio de lo que me trajo aquí. Ante una negativa me dijiste: “A veces un ‘no’ abre más puertas que un ‘sí’”. Y poco después se abrió la puerta del Borrell Lab. Gracias por darme siempre fuerzas y esperanzas, por creer en mí. Te quiero.

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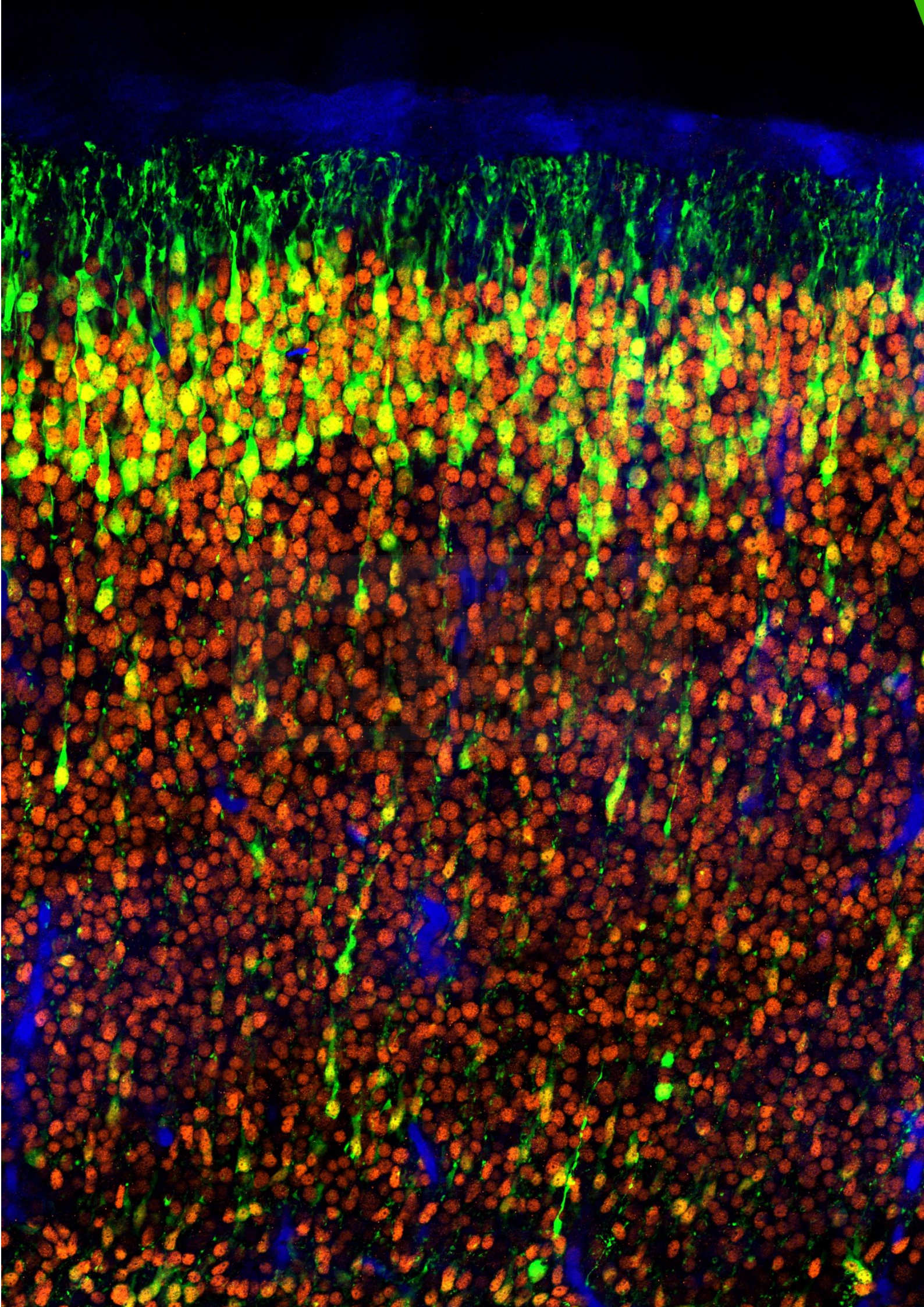

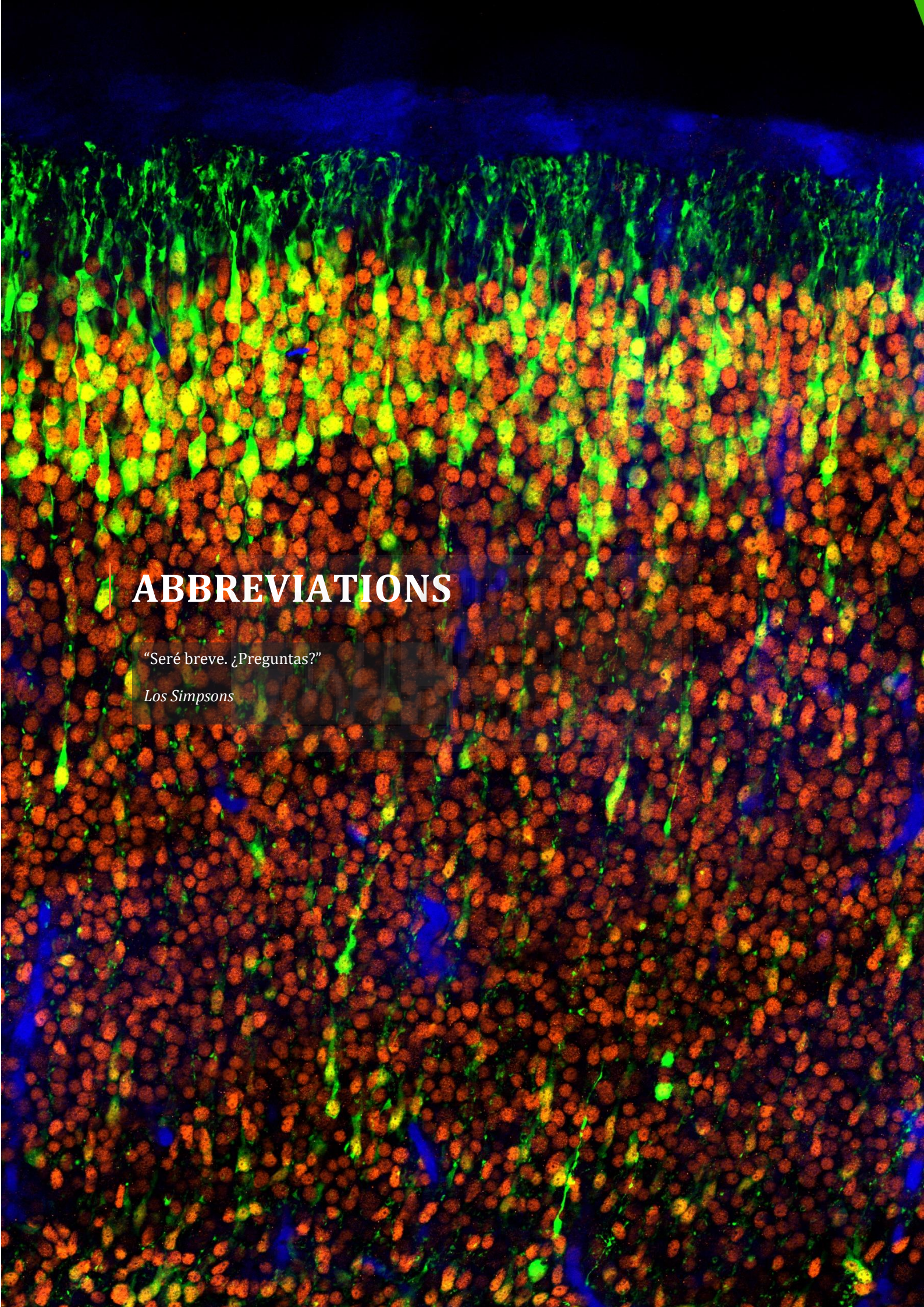

ABBREVIATIONS

> "Seré breve. ¿Preguntas?"
>
> *Los Simpsons*

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aRGCs	apical Radial Glia Cells
β-Cat	beta catenin-1
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BrdU	5-bromo-2'-deoxyuridine
bRGCs	basal Radial Glial Cells
CP	Cortical Plate
CRs	Cajal-Retzius cells
Cux1	Cut Like homeobox 1
DAPI	4′,6-diamidino-2-phenylindole
DEGs	Differentially Expressed Genes
Dl1	Delta 1
Eml1	Echinoderm Microtubule Associated Protein-Like 1
FlnA	Filamin A
Fw	Forward
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GOF	Gain-of-function
IPCs	Intermediate Progenitor Cells
IRES	internal ribosome entry site
ISVZ	Inner Subventricular Zone
IZ	Intermediate Zone
KD	Knock Down
KO	Knock Out
MCDs	Malformations of Cortical Development
mTOR	mammalian target of rapamycin
MZ	Marginal Zone
NBT	Nitroblue Tetrazolium
OE	Overexpresion
OSVZ	Outer Subventricular Zone

Par3	Partitioning defective 3
Pax6	Paired box 6
pCAG	CAG promotor
PH3	phosphohistone 3
PIK3	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIK3R2	Phosphoinositide-3-Kinase Regulatory Subunit 2
PVNH	Periventricular Nodular Heterotopia
RGCs	Radial Glia Cells
SBH	Subcortical Band Heterotopia
shRNA	short-haipin RNA
Tbr2	T-box brain protein 2
VZ	Ventricular Zone
WM	White matter
WT	Wild type

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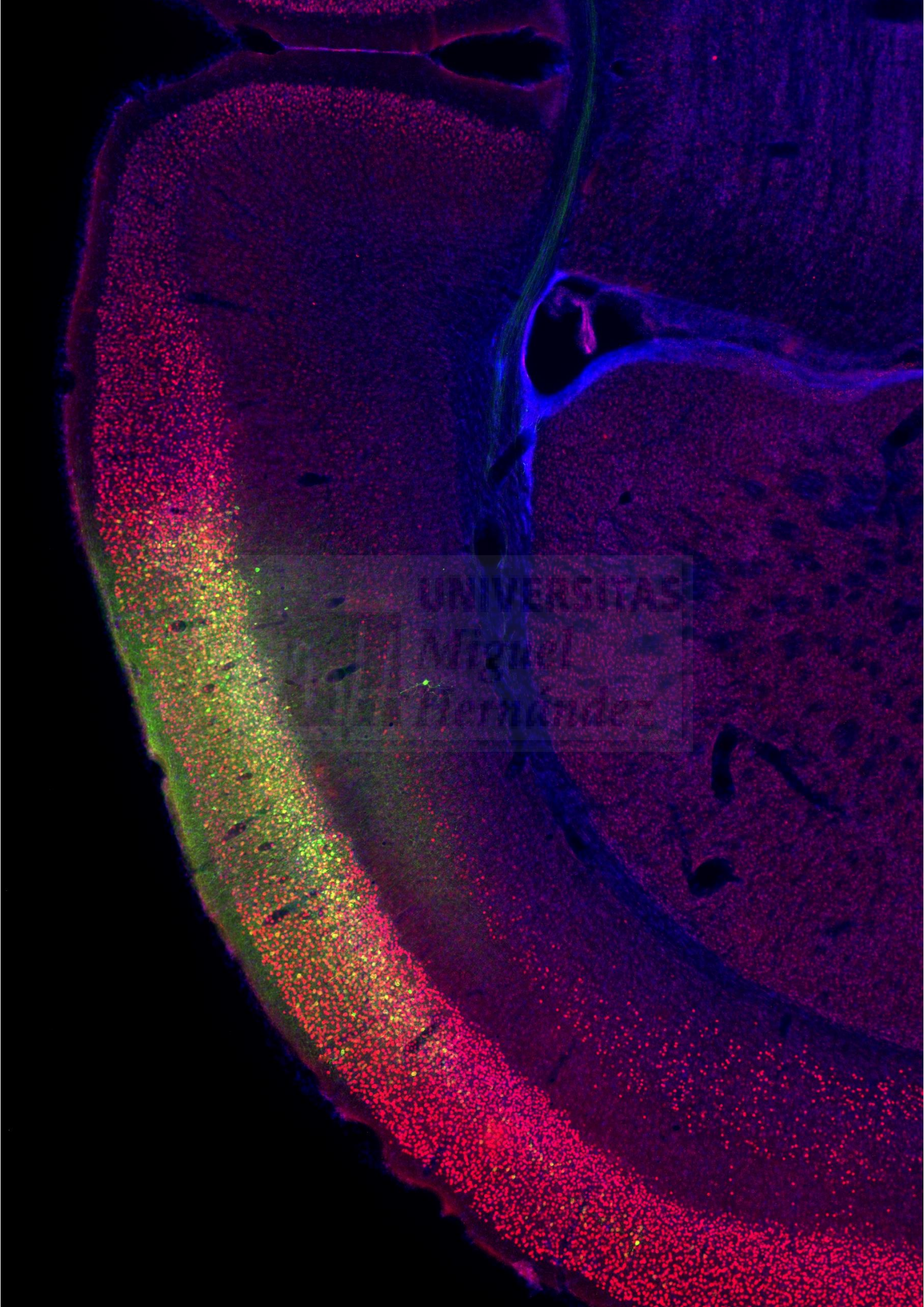

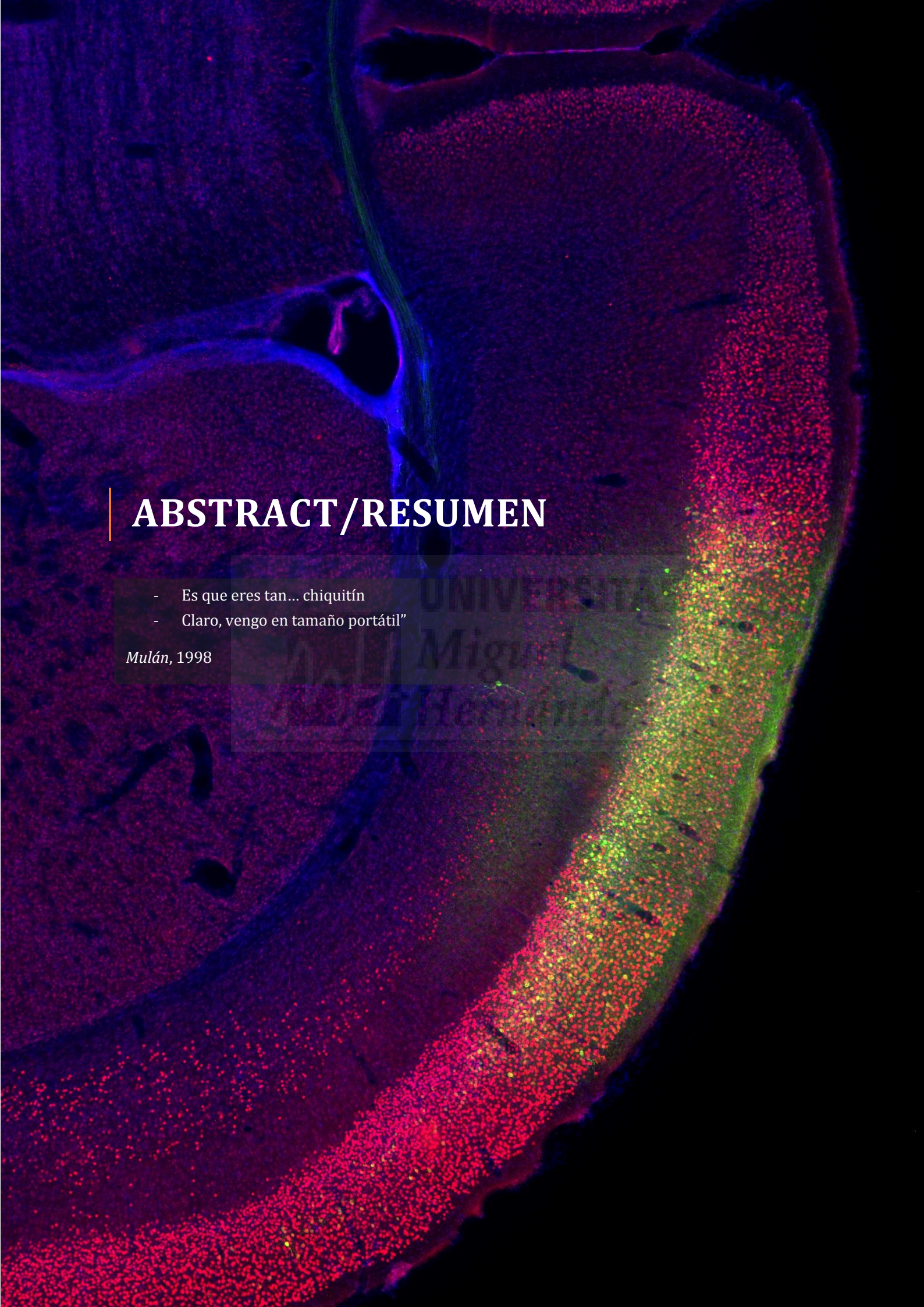

ABSTRACT/RESUMEN

- Es que eres tan… chiquitín
- Claro, vengo en tamaño portátil”

Mulán, 1998

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ABSTRACT

During embryogenesis, the developing cerebral cortex undergoes a dramatic expansion and folding. Alterations in cortical folding cause intellectual impairment and epileptogenic disorders, demonstrating its significant functional relevance. Some of the most common pathologies are polymicrogyria, the formation of multiple small folds; and periventricular nodular heterotopia (PVNH), where neurons accumulate ectopically in the vicinity of the telencephalic ventricles forming nodules, which act as epileptic foci. Mutations in *PIK3R2* and *FLNA* are found causative for these pathologies, respectively.

A novel gain-of-function mutation was identified in *PIK3R2*, a gene coding for a regulatory subunit in the (PI3K)-AKT-mTOR pathway, causing bilateral polymicrogyria in patients. To understand the cellular mechanisms underlying this pathology, we performed functional experiments in developing mice and ferrets by overexpressing *PIK3R2* with a mutation found in patients, or its wild-type counterpart. We found that overexpression of PIK3R2 in mouse embryos by *in utero* electroporation caused an increase in proliferation of Radial Glia (RGCs) and Intermediate Progenitor Cells (IPCs), leading to their greater self-amplification. Migration of cortical neurons was also impaired, resulting in their accumulation of cells in deep cortical layers at embryonic and postnatal stages. Similarly, in ferret visual cortex the overexpression of Pik3r2 causes a delay in neuronal migration, a defect observable in long-term experiments in which juvenile animals showed an increase in cells populating cortical layers V and VI with respect to controls. In contrast, when the manipulation was performed in the parietal cortex, we detect a greater neurogenesis and an ectopic positioning of neurons at embryonic stages, which was consolidated in juvenile ferrets, presenting neuronal periventricular heterotopias and an overmigration defect altering cortical surface. Our findings demonstrate that increased levels of PIK3R2 alters progenitor cell proliferation and neuronal migration during cortical development in an area-specific manner, which suggests a different robustness of cortical areas.

Periventricular nodular heterotopia (PVNH) has been associated to mutations in the *FLNA* gene. Here, we analyzed the expression pattern of *FlnA* in cortical development of mouse and ferret, and we observed the highest levels of expression in germinal layers in both animal models. Importantly, *FlnA* expression in ferret was heterogeneous along the Outer Subventricular Zone (OSVZ), being higher in the prospective gyri compared to sulci. Next, we performed overexpression and knock down experiments by *in utero* electroporation of full length *FlnA* and *FlnA*-shRNA, respectively, in mouse embryos. Overexpression of FlnA causes a delay in neuronal migration at embryonic stages. Additionally, this retention is maintained postnatally (P21), when we can find accumulation of neurons resembling periventricular nodules. Upon *FlnA* knock down, we find that those neurons that finally migrate fail to acquire their proper laminar position, ending misplaced in superficial positions. An alteration in

neuronal migration was observed also in postnatal ferrets electroporated with *FlnA* knock down, but not in overexpression conditions. However, overexpression of *FlnA* causes a disruption of the apical lamina resulting in premature cell delamination or death, although this do not cause the formation of periventricular nodules.

Finally, we analyzed the expression pattern of a gene involved in subcortical band heterotopia, *Eml1*, during ferret cortical development. We observed that it is expressed in germinal layers and in the cortical plate, but in contrast to the other gene that we have analyzed, the highest expression in the germinal layers starts later in development, contrary to what has been previously observed in mouse. Overexpression experiments performed in ferret visual cortex showed a slight neuronal migration defect at early postnatal stages that was self-corrected later during development.

RESUMEN

Durante la embriogénsis, el desarrollo de la corteza cerebral sufre una dramática expansión y plegamiento. Alteraciones en el plegamiento cortical causan discapacidad intelectual y desórdenes epilépticos, demostrando su importancia funcional. Algunas de las patologías más comunes son polimicrogiria, la formación de múltiples pequeños giros; y heterotopia nodular periventricular, en la que las neuronas se acumulan ectópicamente en la vicinidad de los ventrículos telencefálicos formando nódulos que actúan como foco epiléptico. Mutaciones en *PIK3R2* y *FLNA* se han definido causantes de estas patologías, respectivamente.

Una nueva mutación de ganancia de función ha sido identificada en *PIK3R2*, un gen codificante para la subunidad reguladora de la cascada PI3-AKT-mTOR, causando polimicrogiria bilateral en pacientes. Para entender los mecanismos celulares que subyacen en esta patología, realizamos experimentos en ratones y hurones en desarrollo sobreexpresando *PIK3R2* con la mutación hallada en pacientes, o su forma "salvaje". Hemos visto que la sobreexpresión de *PIK3R2* en embriones de ratón mediante electroporación *in utero* causa un incremento en proliferación de células de glia radial (RGCs) y células progenitoras intermedias (IPCs), dando lugar a una mayor auto-amplificación. La migración de las neuronas corticales también está afectada, resultando en una acumulación de células en las capas corticales profundas en estadios embrionarios y postnatales. De forma similar, la sobreexpresión de *PIK3R2* en la corteza visual del hurón causa un retraso en la migración neuronal, un defecto observable en experimentos de largo tiempo de supervivencia en los que animales juveniles muestran un incremento en las células poblando las capas corticales V y VI respecto a los controles. En cambio, cuando la manipulación se realizó en la corteza parietal detectamos una mayor neurogénesis y una posición ectópica de las neuronas en estadios embrionarios, algo consolidado en hurones juveniles, presentando heterotopias periventriculares de neuronas y una sobremigración que altera la superficie cortical. Nuestros resultados desmuestran que el incremento de los niveles de *PIK3R2* altera la proliferación de progenitores corticales y la migración neuronal durante el desarrollo cortical de forma área-específica, lo que sugiere una diferente robustez de las áreas corticales.

La heterotopia nodular periventricular (PVNH) se ha asociado a mutaciones en el gen de *FLNA*. Aquí, analizamos el patrón de expresión de *FlnA* durante el desarrollo cortical de ratón y hurón, y observamos mayores niveles de expresión en las capas germinales de ambos modelos animales. Lo que es más importante, la expresión de *FlnA* en hurón es heterogénea a lo largo de la zona subventricular externa (OSVZ), siendo mayor en el giro prospectivo que en el surco. A continuación, realizamos experimentos de sobreexpresión y reducción de expresión mediante electroporación *in útero* de *FlnA* y *FlnA*-shRNA, respectivamente, en embriones de ratón. La sobreexpresión de *FlnA* causa un retraso en la migración neuronal a estadios embrionarios. Adicionalmente, esta retención se mantiene postnatalmente (P21), donde encontramos una

acumulación de neuronas que logran parecerse a nódulos periventriculares. Respecto a reducción de expresión de *FlnA*, encontramos que esas neuronas que escapan de la ectopia en la materia blanca fallan en adquirir su posición laminar correcta, acabando inapropiadamente localizadas en posiciones superficiales. Una alteración en la migración neuronal también es observable en hurones postnatales electroporados para la pérdida de función, pero no en condiciones de ganancia de función. Sin embargo, la sobreexpresión de *FlnA* causa disrupción de la lámina apical, lo que resulta en una prematura delaminación celular o muerte, aunque esto no causa la formación de nódulos periventriculares.

Finalmente, analizamos el patrón de expresión de un gen involucrado en la formación de bandas heterotópicas subcorticales, *Eml1*, durante el desarrollo corticald el hurón. Observamos que está expresado en las capas germinales y en la placa cortical, pero, adiferencia de los otros genes, la mayor expresión en las capas germinales empieza a estadios tardios del desarrollo, contrariamente a lo que se ha observado anteriormente en ratón. Experimentos de ganancia de función en la corteza visual del ratón muestran un leve defecto de migración neuronal en tempranos estadios postnatales que es corregido más tarde durante el desarrollo.

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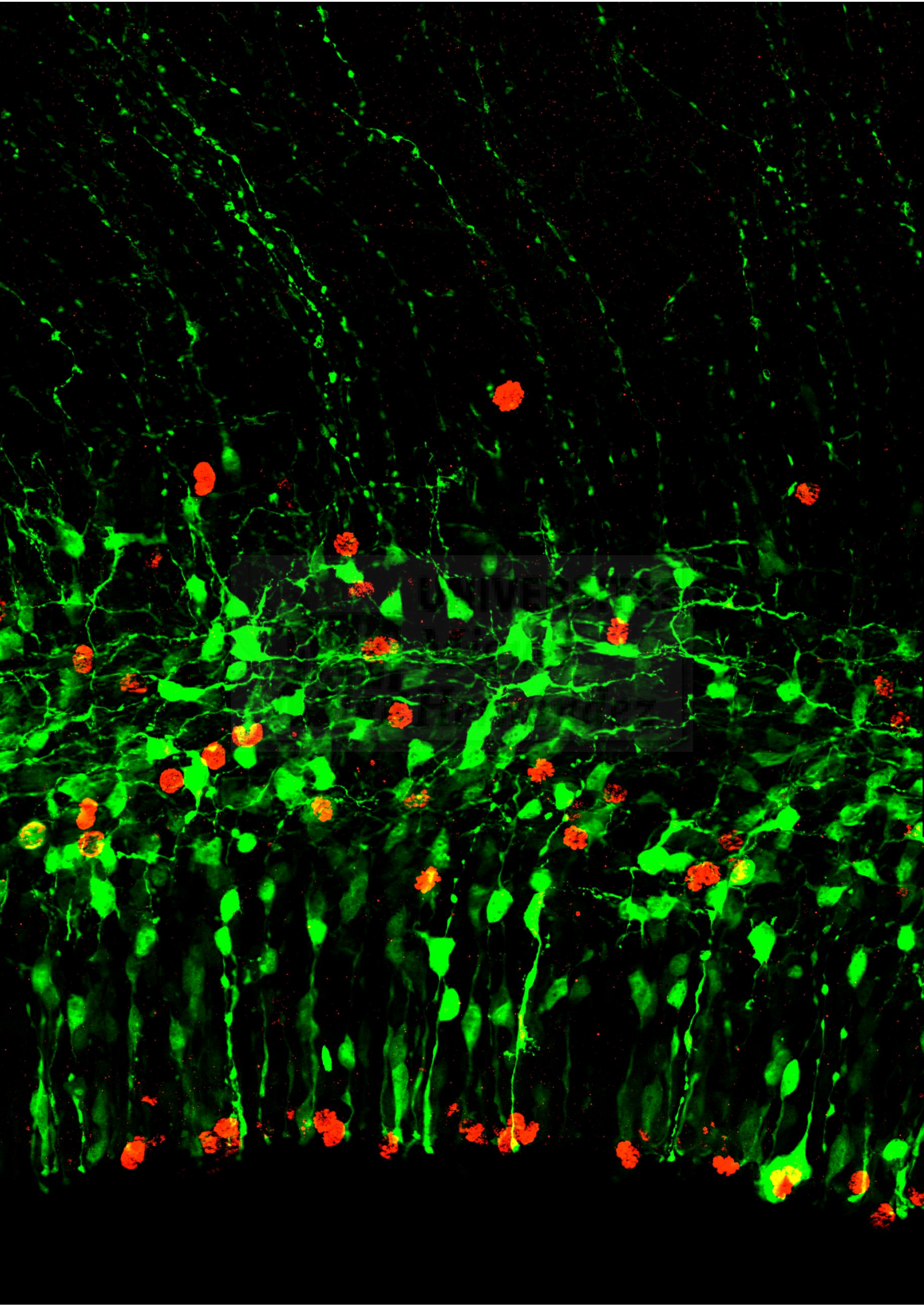

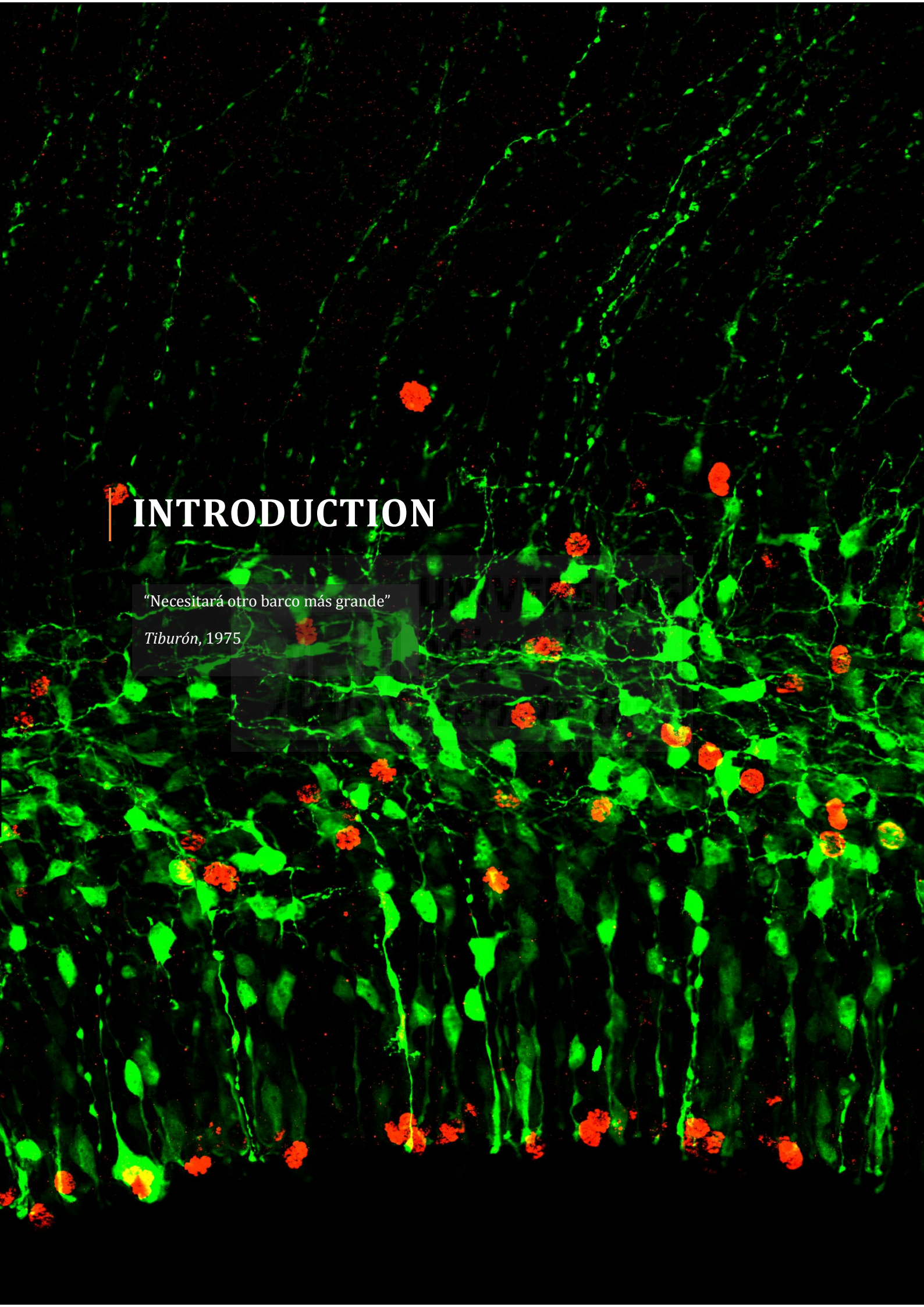

INTRODUCTION

"Necesitará otro barco más grande"

Tiburón, 1975

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INTRODUCTION

The brain is one of the most complex biological structures and constitutes the centre of control of each organism orchestrating both its own function and also that of all the organs in the body by integrating multiple inputs. It is constituted by a wide diversity of cells that conform a functional network able to receive and process information and to give a proper response through the systems it controls (Purves D, 2011). Brain size differs radically between mammals, mainly due to variations in cerebral cortex size (Gertz & Kriegstein, 2015).

In mammals, and particularly in humans, the cerebral cortex is the most evolved region of the brain and it is responsible for the processing of the inputs received such as hearing, vision and olfaction, and to control body movements. Most importantly, the cerebral cortex plays key roles in the higher brain functions that make us humans such as language, creativity and emotional response (Arai & Pierani, 2014; Dunbar, 1993; Fjell et al, 2015). The achievement of these extraordinary capabilities has an evolutionary origin underlied by a unique progress in the molecular and cellular pathways that govern species development (Rakic, 2009). For the proper accomplishment of its functions, the cerebral cortex counts with a particular and extremely organized composition of cells, regionalized and connected in different functional areas (Krubitzer, 2007; Purves D, 2011).

1. ORGANIZATION OF THE CEREBRAL CORTEX

The cerebral cortex (from latin, "bark of a tree") is the thin mantle of gray matter that recovers the surface of cerebral hemispheres which derives from the dorsal telencephalon. Cells conforming this structure are highly organized in both radial and tangential axes. Radially, different layers can be distinguished based on their cellular composition and connectivity. Tangentially, areas are defined by both their cytoarchitecture and the processes that they govern. Moreover, the cerebral cortex can be subdivided in: allocortex (composed by archicortex and paleocortex) and neocortex (Purves D, 2011).

1.1. Phylogenetic classification of the cortex

1.1.1. Archicortex

The archicortex (or archipallium) is, phylogenetically, the most ancient region of the cerebral cortex and is considered as the most basic type of cortex. It is composed by the entorhinal and retrosplenial cortex, the subiculum and the hippocampus, which is composed of with three cell layers (polymorphic, pyramidal and molecular). Importantly, this cortex is involved in the limbic system, which is associated with memory and emotions processing (Patestas MA, 2016). It also includes the mesocortex, a transitional zone between the older allocortex and the newer cortex

(see below), that consists of three to six layers located in the cingulate gyrus and the insula (Purves D, 2011).

1.1.2. Paleocortex

The paleocortex is formed after the archicortex. It consists of three to five cell layers. It is found in the primary olfactory cortex (the piriform cortex, which includes the lateral olfactory gyrus, anterior half of the uncus and periamygdaloid cortex) and the secondary olfactory cortex, formed by the entorhinal cortex of the parahipocampal gyrus. This particular cortex is involved in smelling and has also been associated with the process of emotions (Purves D, 2011).

1.1.3. Neocortex

The neocortex is a structure originated from the dorsal telencephalon and it is present only in mammals. In fact, the high evolution and expansion of this particular area has been linked to the higher order functions, including the most remarkable and unique of human beings (Arai & Pierani, 2014; Rakic, 2009). Importantly, variations in cortical size among species happens mainly in cortical surface instead of its thickness, and it involves the folding of this sheet of cells in some species. This fact allows us to distinguish between species with a smooth cortex (lissencephalic) and the ones presenting cortical folds and fissures (gyrencephalic), which will be discussed in further sections (Fernandez et al, 2016). In both cases, the neocortex is constituted by six cell layers named I-VI from more superficial to deeper positions. Generally, neurons in layers from I-IV are receptors of inputs, while those in layers V and VI are the main source of corticofugal projection fibers. Additionally, the neocortex is also subdivided in different functional areas that have a specific role such as sensory, motor and associative function (Patestas MA, 2016).

Neocortical circuits are composed by a wide range of different neurons that present characeristic propierties in terms of morphology and functionality (synaptic connections). Their relationship in the neocortex underlies the connexions and the proper activity of each cortical area. Interestingly, the circuits they form are robust but also can be modified depending on the context and the previous experience. These intrinsic and extrinsic signals affect the neuronal program and their participatiion inside a particular circuit. Alterations in the position of these neurons and their connectivity are causative for a wide range of developmental disorders (Jabaudon, 2017).

1.2. Radial organization of the neocortex

The cerebral cortex is organized radially in six main differentiated layers (or laminae) which present a particular composition and develop a specific role in connecting the cortex with other regions of the brain and within itself. These layers are produced following an inside-out manner,

where the early born neurons are positioned in the deeper cortical layers and the late-born populate the more exterior ones. In consequence, late-born neurons pass through the earlier generated to adquire their proper position in the upper layers (Patestas MA, 2016; Purves D, 2011).

Some variations are observed when different areas are compared, such as cellular composition, myielinization degree and also the presence of subdivision inside some of these layers, indicating higher complexity. Moreover, these cells are interconnected forming a cortical column, which consitutes the functional unit of the cerebral cortex (Mountcastle, 1997).

The cortical layers are described as follow (**Figure 1)**:

Layer I: molecular or plexiform layer. It is the most superficial, underlying the pia matter. It is basically composed by nerve cell processes: dendrites, that come from cells from other cortical layers; and axon terminals from the thalamus and from stellate and Martinotti cells (interneurons). There are also some cells, mainly horizontal Cajal-Retzius cells, which in the adult brain are almost absent.

Layer II: external granular layer. It is mainly formed by small granule cells (stellate) and small pyramidal cells which extend dendrites to layer I, while their axons connect to other cortical layers and subcortical white matter.

Layer III: external pyramidal layer. As its name indicates, the majority of cells populating this layer are pyramidal cells, although we can also find granule and Martinotti cells. They are diverse in size and specific location inside the layer, being the more superficial of medium size and the bigger ones in deeper positions. Their apical dendrites reach the molecular layer passing through the layer II. Their axons are the main cortico-cortical connexions and arrive to the subcortical white matter and project to other regions of the cortex and other parts of the CNS. Some thalamocortical fibers reach layer III.

Layer IV: internal granular layer. It is mainly populated by granule cells. It is the main receptor of thalamic inputs, although it also receives signaling from cortical layer VI. It is thicker in the primary sensory areas.

Layer V: internal pyramidal cell layer. The characteristic cells of this layer are pyramidal neurons, that present a bigger soma than those from layer III, and axons that connect with subcortical structures such as basal ganglia.

Layer VI: polymorphic or multiform layer. As layer V, is an infragranular layer but its composition is heterogeneous. It presents axons that arrive from other layers of the cortex and other that project to it as well as to subcortical areas as layer V.

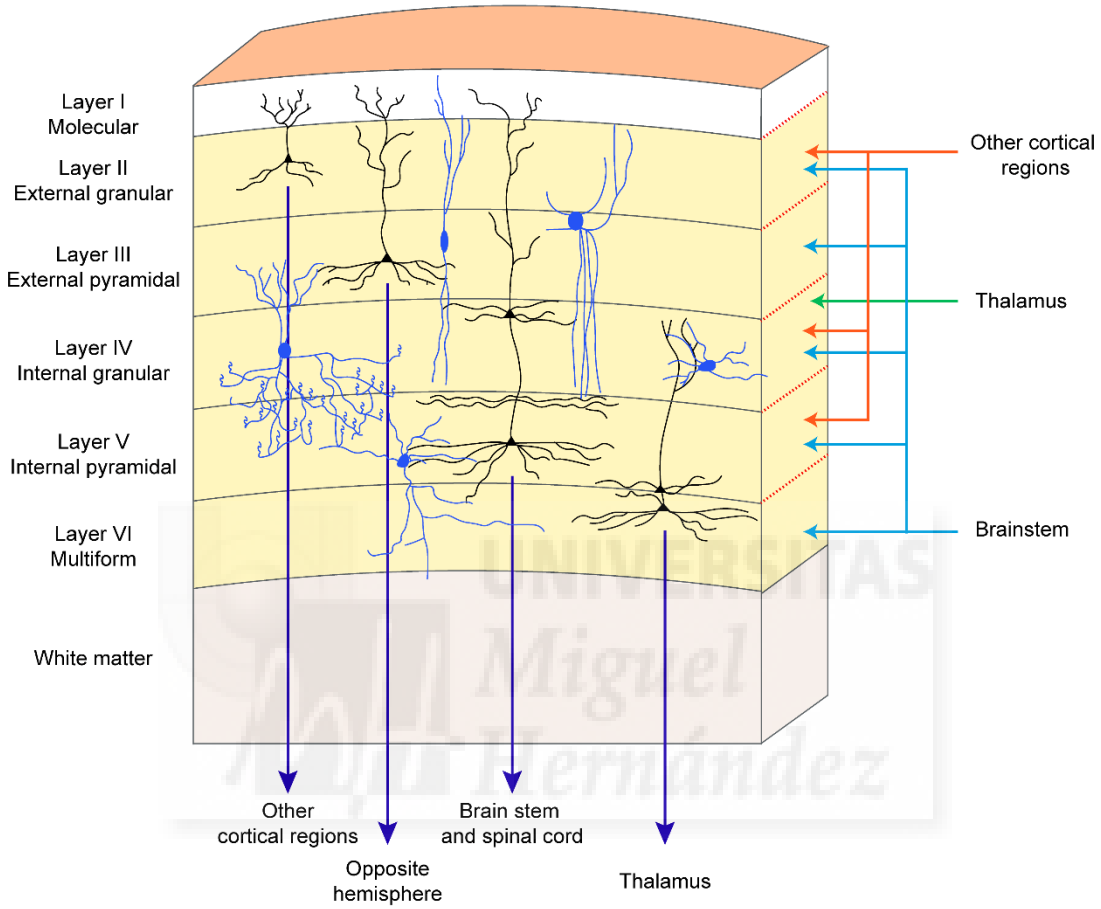


Figure 1. Cellular composition of the cortical layers in mammals. Illustration showing the layers conforming the cerebral cortex (from layer I to layer VI). Pyramidal neurons with characteristic morphologies of each layer are shown in black, while different subpopulations of interneurons appear in dark blue. Cortical layers project to other areas of the brain, as is illustrated by dark blue arrows. Similarly, cortex receives inputs form different structures (other cortical regions, in orange; thalamus, in green; and brainstem in blue). **(**Adapted from (Purves D, 2011).

1.3. Tangential organization of the neocortex

As mentioned, neocortex is not only organized radially but also tangentially regionalized in specific cortical areas with specific cellular, biochemical and physiological characteristics, as well as concrete types of connexions (Brodmann, 1909; Peters A, 1984; Rakic, 1988). Interestingly, the patterns that defined these cortical areas are preprogrammed and depend upon cortical gradients during development (Assimacopoulos et al, 2012; Shimogori & Grove, 2005), although they also are modified by afferent neurons and thalamic input, which are

essential for the proper structure and organization of the final cerebral cortex (Molnar et al, 2012; Molnar et al, 2014; Moreno-Juan et al, 2017).

We find two distinct types of areas in the neocortex that follow a hierarchical function: primary and secondary sensory areas. Primary are the first in receiving the sensory information from the sensory systems: taste (primary gustatory area), olfaction (olfactory cortex), touch (primary somatosensory cortex, S1), hearing (primary auditory cortex) and vision (primary visual cortex, V1). Exceptionally, there is the primary motor cortex, that is the last in processing the control movement orders (M1). This information that primary areas receive is transmitted to the secondary ones, that process the information and send it to the associative cortex, where it is finally integrated (Kandel ER, 2013; O'Leary et al, 2007).

1.4. Cellular components of the cortex

Cells populating the nervous system have an extraordinary complexity in terms of shape and branch processes. Two main different cell cathegories are defined: nerve cells, or neurons (that can be excitatory or inhibitory) and glial cells (Purves D, 2011).

1.4.1. Neurons

In the cortex, we find two types of neurons: excitatory and inhibitory neurons.

1.4.1.1. Excitatory neurons

Excitatory neurons (also known as glutamatergic neurons) comprehend those that release the neurotransmitter glutamate and we can distinguish two types: spiny stellate and pyramidal neurons neurons. Spiny stellate cells are star-shaped that have a great quantity of spines on their dendrites. They are predominantly located in layer IV of primary sensory areas, where their dendrites are commited, and they project to the local cortical column in layer II/III and to infragranular layers (Purves D, 2011).

Pyramidal neurons are the most common ones, representing about the 70% of all neuronal population in the mammalian cortex, and they are also present in other brain structures like the hippocampus and the amigdala. Due to its ubiquity and its main role in transmiting the neuronal impulse, aberrancy in their connections have been linked to neuronal disorders. One example is epilepsy, which in general terms its caused by an excessive neuronal excitation (Bromfield EB, 2006).

These particular type of neurons are generated during cortical development from the different populations of progenitors located in VZ (aRGCs) and SVZ (IPs, bRGCs) following the inside-out pattern of positioning mentioned before (Angevine & Sidman, 1961; Rakic, 1974). All pyramidal neurons share a characteristic morphology by which they receive its name: a

pyramidal shaped soma. They also present a long axon that starts from the base of the soma, that can travel long distances, has multiple branches and which makes synaptic contacts through all it. Also present apical and basal dendritic trees, covered with numerous dendritic spines, which are the postsynaptic site for most excitatory synapses. Importantly, the complexity of its dendritic arborization define the level of innervation that a particular neuron could have. They are units of synaptic integration by responding to exciting postsynaptic targets (Purves D, 2011). Even with the high resemblance between all pyramidal neurons, they present some variations, like genetical profile and size, when we compared them from different layers, cortical regions and species, which suggest a functional specialization (Guan et al, 2015; Molnar et al, 2012; Pietersen et al, 2014).

1.4.1.2. Inhibitory neurons

Inhibitory neurons (local circuit neurons or interneurons) express the neurotransmitter gamma-aminobutyric acid (GABA), which inhibitory action allows these cells to modulate closely located pyramidal neurons activity by targeting specific cellular domains. Interneurons do not present dendritic spines. They are found in lower proportion in comparation with excitatory ones, being around the 20% of neurons and they are very variable in morphology, electrophysiological properties and functionality. For their classification, genetical profile has been used determining three types by the expression of parvalbumin (PV), somatostatin (SST) and ionotropic serotonin receptor (5HT3a). However, morphology is the most used criteria to distinguish among them. In here we detail the three of them more known and abundant (basket, chandelier and martinotti cells) but there are other types of interneurons (Bartolini et al, 2013; Markram et al, 2004; Shipp, 2007).

- *Basket cells*: These interneurons are around the 50% of inhibitory neurons. They target perisomatic regions and are named by their axonal ramifications that form baskets around the soma. They are fast-spiking (very short duration of its action potential) and they have a role in reducing the chance of firing of their postsynaptic targets. Basket cells could be PV-negative or calbindin-positive and can be subclassified based on their axonal and dendritic morphologies.

- *Chandelier cells*: Chandelier cells make multiple contacts to their target cells by the axon, which make them to have a relevant role in cortical function. Their axon forms short vertically-oriented rows of buttons that give them their name. They can be multipolar or bitufted, are found in layers II-VI and can express PV or CB.

- *Martinotti cells*: They target the apical side and could affect both proximal and distal synapses. They express STT and are in layers II-VI, although project to layer I, or layer VI if they are infragranular. They have the most arborized dendrites and are the only ones that make a cross-columnar inhibition via layer I from layers II-VI.

1.4.2. Cajal-Retzius cells

Cajal-Retzius cells (CR cells) are the first glutamatergic neurons generated in the cortex, originated in discrete regions of the pallium (Bielle et al, 2005). After migrating tangentially promoted by factors released by the meninges, they locate in the marginal zone during development. They regulate the lamination of the cortex by secreting factors such as reelin, which relevance has been demonstrated in animal models that lack this protein (D'Arcangelo et al, 1995; Ogawa et al, 1995).

CR cells are one of the four transient populations in the neocortex, in addition to subplate neurons, cortical plate transient neurons and the first precursors of oligodendrocytes. They completely disasppear depending on a programmed cell death within the first two postnatal weeks. This process occurs in the entire CNS, probably as a mechanism of refinement. Also, their disappearance leaves extra space for the born neurons and their dendritic arborization. In the case of these group of cells, it has been suggested that their function may be just necessary in that particular time in terms of signaling and interaction with other members of the network. Interestingly, the presence of CR cells during postnatal life has been found in patients with temporal lobe epilepsy and polymicrogyria (Causeret et al, 2018; Eriksson et al, 2001).

1.4.3. Glial cells

Glial cells (glia, from greek “glue”, as they were thought to maintain nerve cells together) are much more abundant in brain than neurons, approximately tripplicating their number. These cells do not take part directly in synapsis or electrical signaling, on the contrary, they have a supportive role by modulating synaptic contacts and signaling properties of neurons through the uptake and metabolism of neurotransmitters (Purves D, 2011). Additionally, an implication in recovery from neuronal injury has been described (Xing et al, 2012). We can distinguish three main types of glial cells: astrocytes, oligodendrocytes and microglial cells. Glial cells maintain some properties characteristic of neural stem cells (Jakel & Dimou, 2017).

1.4.3.1. Astrocytes

These cells present a star-like appearance due to the abundance of their local processes. They are found only in the CNS and its principal function is to keep a proper chemical environment

which allows neuronal communication. Recent studies show that astrocytes properties are linked to layer specification (Lanjakornsiripan et al, 2018).

1.4.3.2. Oligodendrocytes

They are restricted to the CNS and are the productors of myelin, a lipidic structure that recovers partially some axons allowing a faster transmition of the electrical signals through this process. In the peripheral nervous system, cells providing myelin are called Schwann cells.

1.4.3.3. Microglial cells

These cells come mainly from hematopoietic precursor cells, and some from neural precursor cells. They eliminate the cellular debris produced normally after an injury, circumstance in which these cells accumulate in the site of disturbance. They are also able to modulate processes like inflammation and cell survival by the propagation of cytokines.

2. DEVELOPMENT OF THE CEREBRAL CORTEX

Cerebral cortex development comprises a group of complex events that are highly regulated and allows the generation of a high diversity of neuronal cell types. To explain this intriguing and thrilling process, we will address it from the early specification of telencephalic neuroepithelium, the germinal layers that are formed and describing progenitors populations, their proliferation and their generation of neurons (neurogenesis). We also will define the different types of neuronal migration and the elements that influence them, the final differentiation of cortical neurons and their integration in cortical networks. Additionally, we will analyse some of the main molecular and cellular mechanisms underlying these processes that ensure a proper cortical development (Fernandez et al, 2016; Rakic, 2000).

2.1. Origin of the Cerebral Cortex

In early embryogenesis, the proper formation of every organ in the body is subject to a correct establishment of foundational axes (anterior-posterior, dorso-ventral, medio-lateral). Specifically, in nervous system development, this process depends on gastrulation, the invagination of a subset of cells when embryo consist on a single sheet of cells. When it is complete, the embryo is formed by three layers of cells (germ layers): outer ectoderm (which will set the entire nervous system), middle mesoderm and inner endoderm.

Another key event at early development is the subsequent formation of the notochord, a transient cylindrical structure that defines embryo's topography. Notochord signals directed to the overlying ectoderm promote the differentiation of their cells in neuroectodermal precursor cells, a process known as neurulation, followed by the formation of the neural plate and, after its folding inward, the neural tube.

The neural tube acquires a first regionalization as a consequence of the morphological changes that this tube suffers, meaning its bending, folding and constriction. We can distinguish three subdivisions: prosencephalon (will generate the forebrain), the mesencephalon (or midbrain) and the rhombencephalon (hindbrain) (**Figure 2**). In the most caudal position, we find spinal cord precursors. Later, these areas are subregionalized. Concretely, rostral prosencephalon will form the telencephalon consisting on two symmetric vesicles including dorsal and ventral areas. Dorsal territory will generate cerebral cortex and hippocampus, while the ventral one gives rise to basal ganglia (ganglionic eminences), basal forebrain nuclei and olfactory bulb (Purves D, 2011).

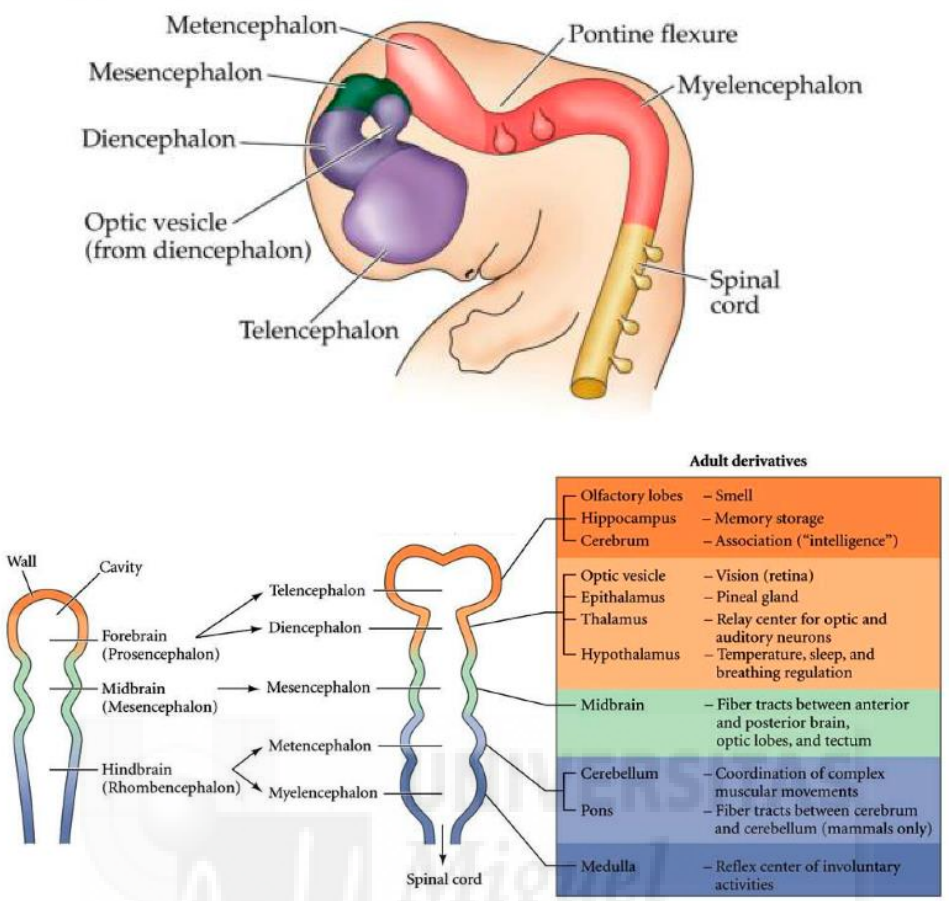


Fig. 2. Regional specification of the developing brain. Neural tube undergoes regionalization, distinguishing three areas: prosencephalon, mesencephalon and rhombencephalon. These primary vesicles will generate secondary ones, which will form the adult derivatives later in development (adapted from (Purves D, 2011).

2.2. Germinal layers and progenitor cells

In this section, I will describe the germinal layers formed during cortical development and the progenitor cells that populate them, as well as the main processes that occur during corticogenesis basically shared among individuals of different species (Namba & Huttner, 2017). Of note, neural progenitor cells come in a variety of morphologies and are distinguished by their polarity characteristics, their proliferation/differentiation potential and the place where their mitosis occurs. In this sense, we can make two general groups: the apical progenitors and basal. These progenitors will generate neurons directly or indirectly, that will form the mature cerebral cortex (Borrell & Gotz, 2014; Reillo et al, 2017; Taverna et al, 2014).

2.2.1. Telencephalic neuroepithelium and neuroepithelial cells

As mentioned above, the cerebral cortex forms from the dorsal telencephalon, which after the closure of the neural tube at early stages of embryogenesis appears as a monolayer of

neuroepithelial cells (NECs) known as neuroepithelium (Bayer & Altman, 1991). NECs present an apico-basal polarity and are attached to each other by adherent and tight junctions at the apical domain. These cells divide symmetrically producing an amplification of their own population. The size of the pool of these progenitor cells determines their progeny and, in consequence, the size of the final cerebral cortex. This process produces an expansion in lateral and radial dimensions (**Figure 3**).

During their divisions, NECs undergo a process called interkinetic nuclear migration (INM), which consists on the movement of the nucleus in the apico-basal axis according to the cell cycle phase. In this sense, nucleus is directed to basal positions at G1, acquires a basal location during S-phase, directs to apical region at G2 and undergoes phase M in the apical surface. This characteristic dynamic behaviour of apical progenitors is asynchronous, conferring the neuroepithelium a pseudostratified appearance (Borrell & Reillo, 2012; Miyata et al, 2010; Taverna & Huttner, 2010). This allows allocating a great number of progenitor cells in the VZ, and of apical mitoses (Fernandez et al, 2016; Taverna & Huttner, 2010).

Later, at the onset of neurogenesis (embryonic day E9.5 in mouse), these NECs start acquiring glial features, such as the expression of specific markers like GFAP and Pax6, becoming apical radial glial cells (aRGCs). Also, they start producing the first-born neurons, which form the preplate, where these founder cells are placed, named ventricular zone (VZ). (**Figure 3**). The timing for this transition has a relevant impact on aRGCs pool and, consequently, brain size (Borrell & Gotz, 2014; Uzquiano et al, 2018).

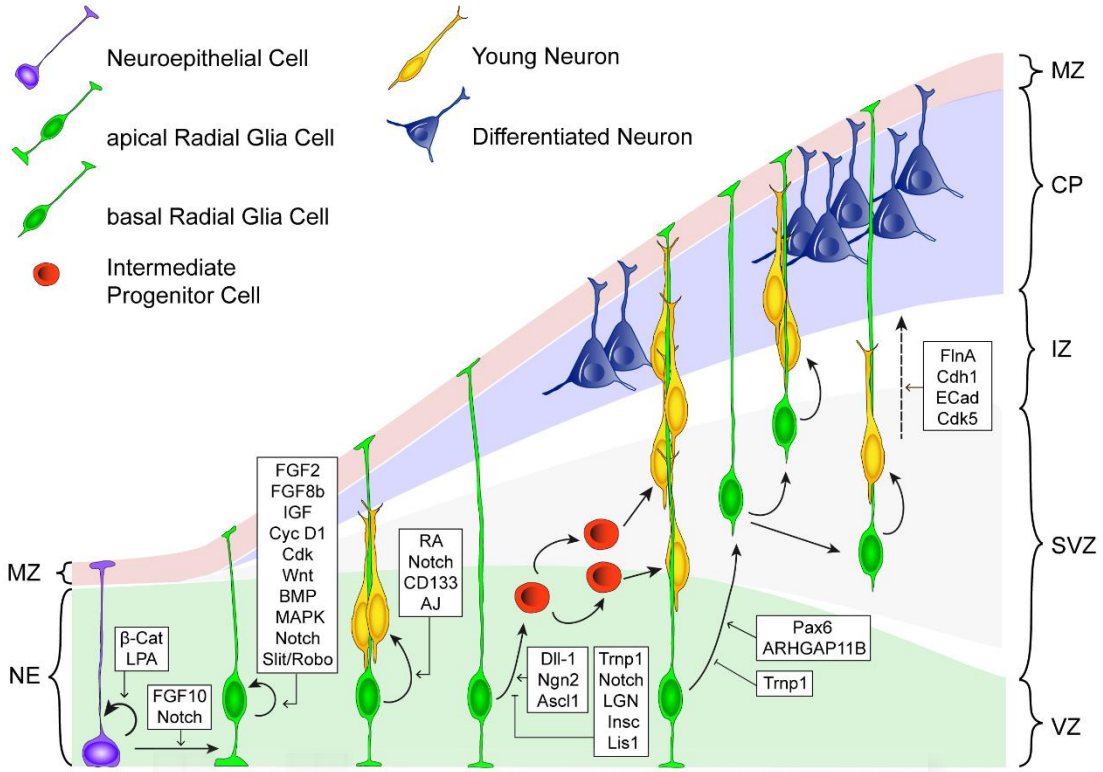


Figure 3. Progenitor cells progression during cortical development and their molecular regulation. At early embryogenesis, neuroepithelial cells (in purple) undergo symmetrical divisions amplifying their own population. Later, these cells become apical radial glial cells (aRCGs, in green), which also go through proliferative divisions. Then, in neurogenic phase, most aRGCs produce neurons (in yellow) directly (direct neurogenesis) or indirectly through the generation of other types of basal progenitors (intermediate progenitor cells, in red, and basal radial glial cells, in green). Some of the molecules or pathways implied in these processes are listed in squares. NE, neuroepithelium; MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone. Arrows indicate lineage relantionships (Modified from (Fernandez et al, 2016).

2.2.2. Ventricular Zone (VZ)

The ventricular zone (VZ) is the most apical germinal layer with a pseudo-stratified appearance that harbors the cell somas of the apical progenitor cells: NECs, aRGCs, short neural progenitors and subapical progenitors. This region is in contact with the lumen of the lateral ventricles containing cerebrospinal fluid, a source of signalling (growth factors, nutrients) received by cell's cilium (Arbeille et al, 2015; Lehtinen & Walsh, 2011; Lehtinen et al, 2011). These components are necessary for the proper functioning of these cells. Interestingly, this layer suffers a transformation of size during development. First, after appearing at the onset of neurogenesis, it starts its radial and lateral expansion as a consequence of the increase of neural progenitors population, arriving at maximum at the peak of neurogenesis Later, it starts to decline and, after neurogenesis, appears as a one-cell layer in the linning of the ventricles (Dehay & Kennedy, 2007).

2.2.2.1. Apical progenitors

- *aRGCs*

As mentioned, aRGCs come from NECs. In fact, they maintain some of the features of their predecesors. Their soma is located in the VZ and they have apico-basal polarity, contacting both apical and basal lamina with extended processes (Taverna et al, 2014). Like NECs, aRGCs exhibit the expression of some markers like Pax6 and nestin. However, they gradually begin to express other distinctive astroglial factors such as brain lipid-binding protein (BLBP), glutamate aspartate transporter (GLAST), vimentin and glial fibrillary acidic protein (GFAP) (Campbell & Gotz, 2002; Gotz & Huttner, 2005; Rakic, 2009; Reillo & Borrell, 2012). In case of the aRGCs, their basal (radial) process goes through all the cortical thickness achieving basal contact, which is maintained despite the radial increase of the cortical wall. This structure is of a high importance since cortical excitatory neurons generated through development will use this process as a guide to achieve their final position in the cortical plate (CP). Additionally, this basal process is highly dynamic and has a role in signalling and fate specification (Arai & Taverna, 2017).

During cortical development, aRGCs suffer changes in their proliferation-neurogenic nature through the regulation of intrinsic and extrinsic factors (**Figure 4**), that will be explained later in detail. Regarding their proliferative behaviour, aRGCs are both capable of divinding symmetrically producing two new progenitors or asymmetrically, by giving rise to one progenitor and one cortical neuron. In fact, this duality is not equally balanced. On the contrary aRGCs start with a greater number of self-amplifying divisions (**Figure 4a**) but, as neurogenesis proceeds, their divisions are more frequently asymmetric (Malatesta et al, 2003; Miyata et al, 2004; Noctor et al, 2001; Noctor et al, 2004). This deviation in the mode of division is an irreversible event (Campbell & Gotz, 2002; Delaunay et al, 2017; Gorski et al, 2002). They contribute to the formation of neurons directly (**Figure 4g**) and indirectly (**Figure 4h-i**) by producing new types of progenitors through self.renewal and self-consumption divisions (basal progenitor cells) (**Figure 3**; **Figure 4b-f)** (Kowalczyk et al, 2009; Pontious et al, 2008; Reillo et al, 2011; Shitamukai et al, 2011; Wang et al, 2011a; Wang et al, 2011b). These neurons will suffer a dynamic change of morphology during their migration process (**Figure 4j**).

- *Apical Intermediate Progenitors (AIPs)*

Apical Intermediate Progenitors (AIPs) or Short Neural Precursors (SNPs) derive from the aRGCs. This type of progenitor shares some of the features with aRGCs: they contact the apical surface, present AJs and express Pax6 (Florio & Huttner, 2014). Differently, they lose the expression of astroglial markers and its basal process do not reach the basal side, but its circumscribed in the VZ. Its self-renewing potential is decreased, meaning that when they are generated, they undergo a unique symmetric neurogenic division resulting in two cortical neurons (Stancik et al, 2010; Tyler et al, 2015). Interestingly, Nowakoski *et al.* observed that

during the transition from infragranular to supgragranular neuronal production in human, aRGCs become truncated, showing a basal process that stop reaching the basal surface, thus, presenting a discontinuous radial glia scaffold that could be implicated in neuronal type generation and their final migration (Nowakowski et al, 2016).

- *Subapical Progenitors (SAPs)*

Subapical Progenitors (SAPs) are progenitor cells that, as the other apical progenitors, are attached to the ventricular surface by its apical process. However, contrary to what is happening in the rest of them, mitosis occurs in subapical positions since the centrosome is anchored to the apical side. Therefore, it is free to move and to arrange elsewhere the mitotic spindle (Arai & Taverna, 2017; Pilz et al, 2013).

2.2.3. Subventricular zone (SVZ)

Subventricular zone (SVZ) is a second germinal layer found in the cerebral cortex which is located basally to the VZ. In this layer, we can find another type of progenitors derived from those populating the VZ. These progenitors lay their somas in the SVZ and generate the upper layer neurons (Dehay & Kennedy, 2007; Smart, 1961). They are known as basal progenitors, as they contact with the apical surface is absent (**Figure 3**). Interestingly, SVZ maintains its neurogenic potential beyond cortical development, being, in addition to the hippocampus, one of the two structures capable to generate neurons into adulthood.

Importantly, the basal progenitor pool experiments a huge variation among species, a direct consequence of the abundance of founder cells at earlier stages in corticogenesis. In consequence, the expansion of BPs goes with the enlargement of the SVZ, which splits in two morphologically different layers: inner SVZ (ISVZ), which conserves the appearance of the previous SVZ, and outer SVZ (OSVZ). The appearance of this extra germinal layer, the OSVZ has been described as a particular trait of gyrencephalic species, those presenting a folded cortex, suggesting a role of this layer and the progenitors which populate it in the process of cortical folding (Betizeau et al, 2013; Borrell & Gotz, 2014; Fernandez et al, 2016; Fietz et al, 2010; Hansen et al, 2010; Huttner et al, 2013; Lui et al, 2011; Reillo et al, 2011; Smart et al, 2002). However, some lissencephalic species do present OSVZ, which implies that the presence of this germinal layer is necessary but not sufficient for gyrification. Rather, it seems that the combination of both OSVZ generation and BP abundance are the key factors for cortical expansion and folding (Kelava et al, 2012; Reillo & Borrell, 2012; Reillo et al, 2011; Toda et al, 2016). Contrary to what is observed in ISVZ, which thickness is almost constant during neurogenesis, the OSVZ experiments an enlargement due to a huge increase in the basal protenitors production.

2.2.3.1. Basal progenitors

Basal progenitors undergo mitosis in the SVZ. They are generated by divisions of aRGCs and delaminate to move to a more basal position. We can discriminate two types: intermediate progenitors (IPs) and basal radial glia cells (bRGCs).

- *Intermediate Progenitor Cells (IPCs)*

Intermediate Progenitor Cells (IPCs) are in the SVZ, where their mitosis takes place, and represent the majority of BPs in rodents (Haubensak et al, 2004; Miyata et al, 2004; Noctor et al, 2004). Contrary to the other basal and apical progenitors, these are non-polar cells, meaning that they do not present apical nor basal processes, neither the AJs (Arai & Taverna, 2017). These progenitors are produced by apical ones and they undergo a process of delamination from the ventricular surface, losing their linking to the AJ belt and their attachment to the apical surface. Similarly, they also lack the basal polarity. Inside this group, we can differentiate between the high proliferative IPCs, which go through proliferative symmetric divisions and the neurogenic ones (**Figure 4**), which divisions are neurogenic (Betizeau et al, 2013). In rodents, they express Tbr2 but not Pax6, differently to what happens in gyrencephalic species, where IPCs maintain Pax6 expression (Englund et al, 2005).

- *bRGCs*

Basal radial glial cells (bRGCs) are the main BP in gyrenephalic species cortex, in contrast to the very scarce abundance in lissencephalic ones. bRGCs present heterogeneity in terms of morphology and transcriptional profile, although generally they express astroglial markers, phospho-vimentin and Pax6., some expressing Tbr2. Their somas are in the OSVZ and they are monopolar, presenting a basal process reaching the basal lamina but lacking the one that connects to the apical surface (Hansen et al, 2010; Reillo et al, 2011; Sauerland et al, 2018; Wang et al, 2011a). This basal process has been demonstrated to be key for their proliferative capacity (Fietz et al, 2010). In addition, the presence of the bRGCs confers the cortex not only an extra source of cortical neurons (**Figure 4h**) but also a large accessory radial glial fibers which neurons can use in their migration trip, a divergence achieved without increasing VZ surface area. In that sense, the abundance of this type of progenitor has been associated with cortical expansion and gyrification process (Borrell & Gotz, 2014; Dehay & Kennedy, 2007; Fernandez et al, 2016; Reillo et al, 2011). Interestingly, evidences in multiple species suggest that this progenitor is not a novel treat that appeared later in evolution but, on the contrary, it is present in the ancestor of all mammals (Garcia-Moreno et al, 2012; Kelava et al, 2012; Lui et al, 2011). Importantly, during the mitosis these cells show a nuclear movement known as mitotic somal translocation (MST) in which the cell soma is translocated to apical or basal directions before the generation of new cells (LaMonica et al, 2012).

2.3. Cell cycle and regulation of neurogenesis

During cortical development, progenitor cells divide themselves with different outcomes, which permits us to distinguish between two main types of divisions. The number and mode of these divisions will determine the total number of cells and the expansion of the cerebral cortex (Dehay & Kennedy, 2007; Gotz & Huttner, 2005; Taverna et al, 2014)

2.3.1. Modes of cell division

2.3.1.1. Asymmetric division

One progenitor give rise to two different cells, a mechanism common in the CNS to produce cellular diversity. It has been evidenced that this mode of division is consequence of unequal distribution of factors during mitosis, which has consequently an asymmetric inheritance of certain components (Dehay & Kennedy, 2007; Gotz & Huttner, 2005; Taverna et al, 2014).

a. *Asymmetric self-renewing*. One of the cells produced has the same identity as the mother cell, while the other has not. Eg. aRGC generating a new aRGC and a neuron.
b. *Asymmetric consumptive divisions.* The identity of the cells generated are different between them and respect to the mother cell. This is the case for an aRGCs generating a neuron and a BP.

2.3.1.2. Symmetric division

Whenever a progenitor generates two cells of the same type.

a. *Symmetric proliferative*. The two cells generated are progenitors, meaning that there is an amplification of the progenitors population. Eg. aRGC giving rise to two aRGCs (**Figure 4a**).
b. *Symmetric differentiative/consumptive.* The two cells generated are neurons, which is the double neurogenic potential than an asymmetric division. This is the case for an intermediate progenitor cell division generating two neurons (**Figure 4i**).

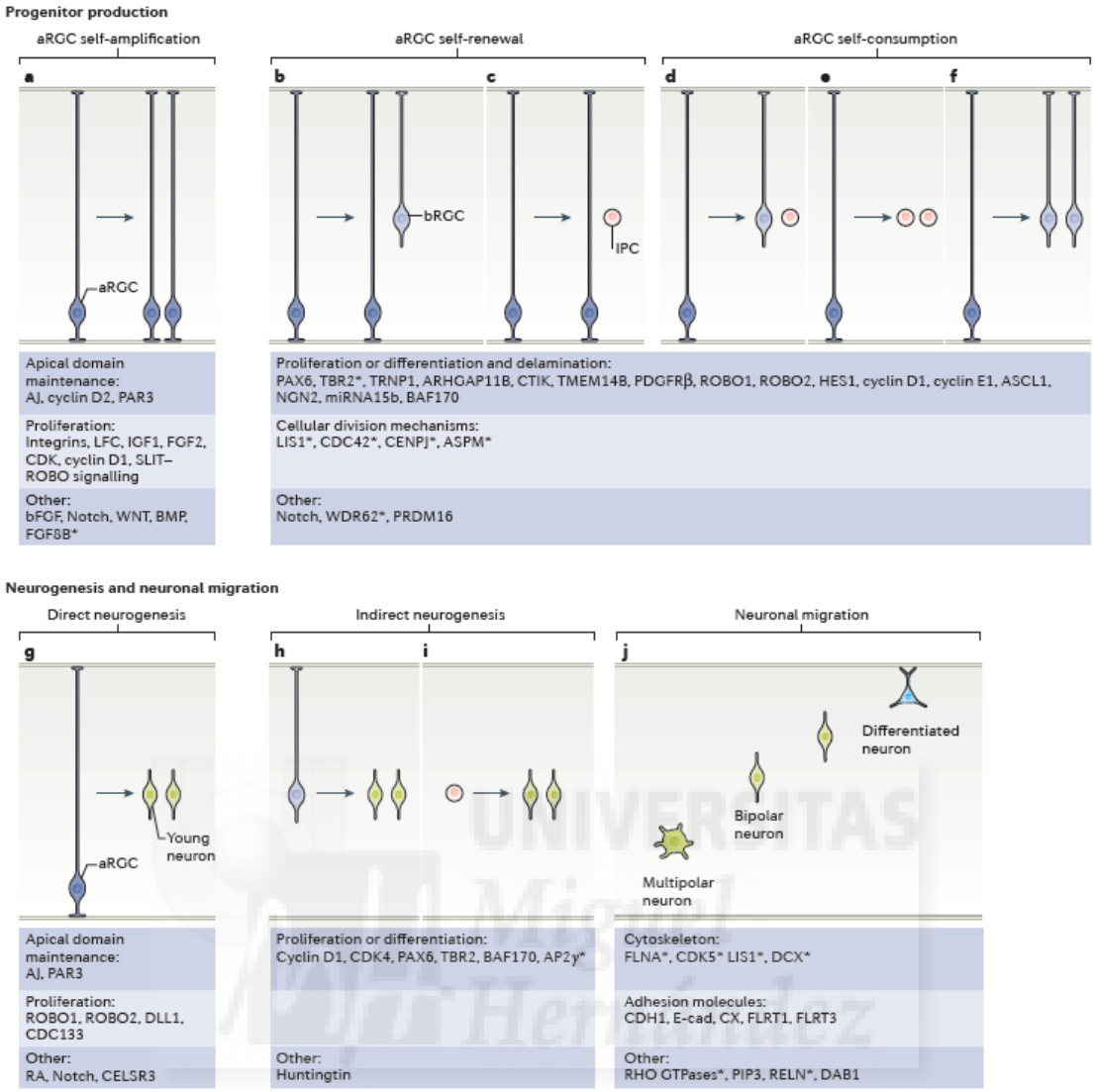


Figure 4 Genetic regulation of cortex folding: progenitor cells and neuronal migration. The division of cortical progenitor cells may result in different lineage outcomes. Regarding progenitor production, the main type of cortical progenitor cell, apical radial glial gells (aRGCs), may: self-amplify (a); self-renew (b,c), generating one aRGC plus either a bRGC or an IPC; or undergo self-consumption by producing two basal progenitor cells (in each combination; d–f). In addition, aRGCs may produce neurons via direct neurogenesis (g). Basal progenitors may also generate neurons (so-called indirect neurogenesis) (h,i). Newborn neurons change their morphology from multipolar to bipolar before starting their migration through the cortical thickness, to reach their final position in the cortical plate where they differentiate (j). Listed are some of the gene products that are critical for these lineage decisions and mechanisms. Cortical malformations in humans are associated with mutations in the genes encoding the products that are marked with an asterisk (from (Llinares-Benadero & Borrell, 2019)).

Progenitors cell cycle progression determine the proportion of progenitors and neurons generated, as well as neuronal heterogeneity (Delaunay et al, 2017). So that, in proliferative divisions, progenitors generated re-enter in the cell cycle, while in the neurogenic ones they are exiting cell cycle to undergo differentiation. Interestingly, although main specific mechanisms have not been elucidated for determining the preference of one of the two divisions, it has been

suggested that mode of division correlates with the regulation of the G1-phase length, being long in differentiative divisions and short in the proliferative ones (Uzquiano et al, 2018).

As corticogenesis proceeds, there is a gradual rise in cell cycle length and in the proportion of differentiative divisions ir order to increase the neurons generated (Borrell & Calegari, 2014). Additionally, a longer G1 phase and a lower cell-cycle progression occur. Also, the expression of a numerous factors temporally regulated influence the transition from symmetrical to asymmetrical divisions, although the mechanism determining the mode of division and the switching between modes are not completely understood. These factors also influence the radial migration of post-mitotic neurons (Frank & Tsai, 2009; Kawauchi et al, 2013; Nguyen et al, 2006).

2.3.2. Molecular mechanisms regulating cell cycle

Cell cycle refers to the process by which a single cell divides generating two daughter cells (Mitsuhashi & Takahashi, 2016). It can be subdivided in four consecutive phases: G1, S-phase, G2 and M-phase. During G1 phase, there is a control of the DNA integrity, growth factors and nutrients access, a checkpoint necessary to proceed to the next phase. It is considered as the most critical step, since at this point progenitor cells are receiving multiple signals that could determine if the cell goes on with the cycle to S-phase or, on the contrary, exits from the cell cycle (G0) starting cellular differentiation. If everything is correct, then cell starts S-phase, in which DNA is duplicated. When the replication is complete, G2 starts, a second restriction point in which genetic material stability and chromosomal segregation are evaluated before the division *per se* starts, named M-phase (Dehay & Kennedy, 2007; Zetterberg et al, 1995).

Although it has been demonstrated that the disruption of INM do not affect the cell cycle progression, this process has an influence in progenitor fate depending on the time spent in a specific position and, hence, the signals arriving to the progenitor cell (Delaunay et al, 2017; Taverna et al, 2014). In the same sense, also the total length of the cell cycle is key in determining the type of division that the cell will undergo.

2.3.2.1.Signalling pathways

Here, we briefly comment on the main different signalling pathways that modify transcription factors expression. This mechanism of differential expression establish identity for neuronal progenitors in the forebrain, prefigure morphogenesis and cellular differentiation in forebrain subdivisions, including the cortex. Moreover, these factors regulate cell cycle and cell fate specification. So that, the crosstalk among these cascades have an impact on corticogenesis (Paridaen & Huttner, 2014).

- *Notch*. Notch signalling is essential for neurogenesis during embryonic development but also in the adult brain (Pierfelice et al, 2011). Notch ligands Delta or jagged trigger Notch receptors, provoking the release of NICD and, consequently, promoting Hes genes transcription but bHLH proneural genes repression (Ngn and Ascl), mantaining the proliferative identity. Asymmetries in the inheritance of Delta-Notch signalling components have been linked to asymmetric aRGCs divisions (cell with higher Notch content stays as an aRGC, while the one with lower levels delaminates and differentiates to a neuron) (Dong et al, 2012; Ochiai et al, 2009; Shimojo et al, 2008).

- *Wnt/b-catenin***.** This pathway is determinant for genetic patterning of the developing brain and for the control of proliferation and differentiation processes (Harrison-Uy & Pleasure, 2012). The binding of Wnt ligands to their receptors (Frizzled/LRP5/6) produces the translocation of the β-catenin to the nucleus, which, in combination with LEF/TCF transcriptor factors, controls specific gene transcription. The role of Wnt changes during development. At early stages, this cascade enhances symmetric aRGCs divisions while at later ones its activity upregulates N-myc. This change in expression represses Notch signalling, resulting in an induction of IP formation and neuronal differentiation (Kuwahara et al, 2010; Munji et al, 2011).

- *Sonic hedgehog (Shh).* As Wnt signalling, Shh is key for the dorsoventral patterning of the CNS in vertebrates. Shh binds to Patched receptor, promoting gene transcription through Smootheened and Gli transcription factors activation. Shh activity trigger symmetric proliferative divisions of aRGCs mediating Hes1 (Notch transcription factor) (Paridaen & Huttner, 2014). Additionally, this cascade is downregulated during neurogenesis, being implied in the aRGCs cell cycle and in the generation of IPs and neuronal differentiation(Wang et al, 2011a).

- *Fibroblast Growth Factors (FGFs).* FGFs are essential in the establishment of anterior-posterior patterning of the brain. They are more than 20 different components differently implied in the self-amplification of aRGCs through symmetric divisions, which also is linked to the activation of Hes1 and the elements that this factor potentiates to be transcripted. They bind to their tyrosine kinase receptors (FGFR1-4), which mediates the activation of

Ras/mitogen activated protein kinase (MAPK) and PI3K/AKT pathways. At early development, the transient expression of Fgf10 has been shown to be essential for the transition from NEC to aRGC (Hatakeyama et al, 2004; Sahara & O'Leary, 2009). Interestingly, dysregulation of these factors leads to a change in the proportion of neuronal differentiation or proliferation due to the different role of each of the factors constituting this group (eg. Fgf15 promotes neuronal differentiation while Fgf8 induces proliferation), and even dysplasia appearance and the formation of ectopic masses (Fgf3) (Itoh & Ohta, 2013).

2.3.2.2. Intrinsic factors

- *Cyclins and cyclin-dependent kinases (Cdk)*

Cyclins and cyclin-dependent kinases (CDK) are protein complexes in charge of promote the transition from one phase of the cell cycle to the other and to ensure that this occurs correctly (Dehay & Kennedy, 2007). So that, these molecules and their inhibitors have been found to play relevant roles during cortical development such as the correlation between G1-phase lengthening and the identity of the cell generated. CDK inhibitors constitute two main families, the INK4 famliy, which has selectivity for CDK4 and CDK6; and the CIP/KIP family which inhibitory potential is wider. This proportion of these counterpats types of regulators determines the progression of the cell cycle (Zindy et al, 1997).

Experimental data manipulating the levels of cyclins and Cdks have been reported to influence in cell cycle and cell fate. Overexpression of CDK4 and cyclin D1, as well as cyclin D1 in combinination with E1 and CDK4 causes the shortening of the cell cycle, impulsing an increase in the proliferation degree and, consequently, in the progenitor pool. Another example is the overexpression of cyclin D1 and E1, which are essential for the progress between G1 and S-phase. Consistently, ablation of CDK4 and CDK2 promotes neurogenic cell division by lengthening the G1 phase (Borrell & Calegari, 2014; Lange et al, 2009; Nonaka-Kinoshita et al, 2013; Uzquiano et al, 2018).

- *Mitotic spindle orientation*

The mitotic spindle orchestrates the symmetry or asymmetry of cell division, meaning that its orientation plane may influence daughter cell fate. This model proposes that when the cleavage plane is oriented parallel to the apico-basal gradient of fate determinants, the repartition of these factors is not homogenous and results in asymmetric cell division. However, when the orientation is perpendicular to this gradient, both cells will receive the same proportion of fate determinats, acquiring the same cellular identity. However, this relation appears not to be so straight. The formation of a proper mitotic spindle involves multiple players such as

centrosomes, astral microtubules and other interacting proteins (Lancaster & Knoblich, 2012). Early aRGCs divisions present cleavage planes perpendicular to the ventricular surface, while the horizontal ones appear later in development, responsible for generating IPs and bRGCs, which relates mitotic spindle orientation with the cortical expansion produced by abundance of these type of progenitors (Kosodo et al, 2004; LaMonica et al, 2013; Postiglione et al, 2011; Shitamukai et al, 2011).

Additionally, the relevance of the finely regulation of a proper mitotic spindle orientation is reflected in cases in which the alteration of proteins involved in the spindle position have been mutated and give rise to MCDs such as microcephaly, as it is the case for Aspm, Lis1, Nde1and Lfc (Arhgef2) (Postiglione et al, 2011; Shitamukai et al, 2011; Yingling et al, 2008). In progenitors division, mitotic spindle orientation of aRGCs is known to have a great influence on the fate determination of daughter cells (Postiglione et al, 2011; Xie et al, 2013). Perpendicular orientations are associated with proliferative divisions while oblique ones are more abundant as neurogenesis proceeds (Mitchison & Kirschner, 1984; Yingling et al, 2008).

- *Segregation of cellular components and fate determinants*

Tighly associated with the mitotic spindle plane is the influence of the segregation of cellular components and factors with cellular fate, since it determines the distribution of the mother cell components to their progeny. Classically, it was proposed that the inheritance of the apical domain of the aRGC determines that the cell retaining it would maintain the same identity of the mother cell while the other will differentiate. aRGCs apical domain presents key factors relevant for their function such as the adherent junctions and associated actin regulated proteins (eg. Rho GTPases, RhoA, Cdc42 and Rac1) as well as polarity proteins (eg. Par3, Par6 and aPKC). However, studies suggest that other factors will be crucial for the daughter cell identity since equal division of the apical domain also occurs in asymmetric divisions (Asami et al, 2011; Konno et al, 2008; Shitamukai et al, 2011; Uzquiano et al, 2018) *5-9paradien2014*). Also, the adquisition of the basal process seems to have a role in cell identity such as integrins and cyclin D2 mRNA, which inheritance implies the maintance of the proliferative capacity (Fietz et al, 2010; Matsuzaki & Shitamukai, 2015; Radakovits et al, 2009; Tsunekawa et al, 2014). Cell receiving cyclin D2 will conserve its self-renewal potential, while the other will differentiate, a mechanism that may be conserved across higher mammals (Tsunekawa et al, 2014).

2.3.2.3. Extrinsic factors

Progenitor cells are influenced by extrinsic factors secreted to the extracellular space, a phenomenon that occurs dynamically based on the variations across time and space of cellular exposure to these elements. This hypothesis is known as Dynamic Extrinsic Code, a model that suggest that cortical progenitors may sense extrinsic signals through their soma and apical and

basal domains (Borrell & Gotz, 2014; Dehay & Kennedy, 2007; Noctor et al, 2004; Reillo et al, 2011; Wang & Kriegstein, 2009). These factors modulate progenitors behaviour and cellular fate.

The particular morphology of progenitors determines the signals they are exposed to. For instance, IPCs they may be only in contact for those cells close to them as their processes do not extend too much (Betizeau et al, 2013; Noctor et al, 2004). On the contrary, bRGCs receive inputs not only from the layer they are located but also from the territory in which the basal fiber is extended, so that, potentially, aRGCs could receive inputs from the total thickness of the cortex.

- *Afferent axons*

Major axon tracts from dorso-thalamic nuceli innervate the developing cortex. These afferent axons provide extrinsic factors, such as bFGF, that affect progenitor proliferation and differentiation processes (Dehay & Kennedy, 2007; Dehay et al, 2001). They represent a laminar arrangement of signals that would be read differently by each progenitor cell subtype depending on their spatial and temporal overlap. Developing thalamic axons exert significant and lamina-specific modulatory effects on cortical progenitor cells. This interaction between neurons and cortical progenitors may have contributed to cortical expansion and an increase in functional complexity in higher mammals. Communication between progenitor cells and axons has been suggested in descriptive data obtained by Reillo *et al.* (Reillo et al, 2017) in which basal processes of RGCs show contacts with calbindin-positive axons. Additionally, axons from different origins have a layer-specific distribution, clearly distinguishing between OSVZ and ISVZ, which suggest a specificity in axons impacting on progenitor cell populations.

- *Neurons*

Post-mitotic neurons produce molecules that influence RGCs (Haydar et al, 2000). This is the case for Cajal-Retzius cells, which mediate progenitor proliferation through reelin, that impulses Notch signalling and, consequently, the symmetric proliferative divisions. Other factors secreted by these cells have also an impact on neuronal migration, as was mentioned before when we described this type of cell. Additionally, late-born cortical neurons promote astrogenesis by the secretion of factors that switch RGCs program. Also, transient glutamatergic neurons that migrate tangentially into the dorsal telencephalon could impulse proliferative potential of RGCs since its ablation during development provoke a decrease in progenitor pool (Griveau et al, 2010; Lakoma et al, 2011; Seuntjens et al, 2009; Teissier et al, 2012).

- *Cerebrospinal Fluid (CSF)*

The lumen in direct contact to the apical progenitor cells, the ventricles, are filled with cerebrospinal fluid (CSF). CSF is a liquid produced by choroid plexus and is dynamically

regulated during corticogenesis (Taverna et al, 2014), It is composed by water and a complex combination of ions, proteins, lipids and molecules including IGFs, FGFs, Shh, BMPs and Wnts that impact in progenitors that sense these signals through the primary cilium and apical membrane domain. Interestingly, time and space asymmetries in ciliogenesis suggest a distinct exposure of the daughter cells to these molecules (Higginbotham et al, 2013; Lehtinen & Walsh, 2011; Lehtinen et al, 2011).

- *NPCs environment*

There are many other elements present in the niche of progenitors that influence the NPCs behaviour, since their domains are differently exposed to determinate factors. At the apical side, extracellular matrix components, like laminin, syndecan-1 and integrin b1 are involved in the maintaince of the apical adhesion and the proliferation of aRGCs (Arvanitis et al, 2013; Loulier et al, 2009; Wang et al, 2012). At the basal one, the contact with the basal lamina ECM has been linked to the self-renewing capacity of both aRGCs and bRGCs (Fietz et al, 2010). In this domain meninges generate several factors, including retinoic acid, that trigger the change of divisions from symmetric proliferative to asymmetric neurogenic (Siegenthaler et al, 2009; Taverna et al, 2014). The presence of other elements such as blood vessels, which provide nutrients and signalling elements; and microglia cells have also been shown to influence RGC population and their proliferative capacity (Antony et al, 2011; Cunningham et al, 2013; Javaherian & Kriegstein, 2009; Stubbs et al, 2009; Taverna et al, 2014). Additionally, neighbouring cells can communicate each other through gap junctions realising chemical signals that synchronize their behaviour in terms of nuclear migration and proliferation (Taverna et al, 2014). Besides, also physical signals affect cortical progenitors. This is illustrated in the case of an overcrowded apical surface, which promotes the left of progenitors from this area to basal positions (Okamoto et al, 2013).

2.4. Neuronal migration

Neuronal migration is a very complex process by which cells travel from their born place to their specific location. We can distinguish two modes of neuronal migration: radial, followed by excitatory cortical neurons; and tangential, which is followed by interneurons. Migrating cells depend on the coordination of intrinsic and extrinsic cues such as cytoskeletal dynamics, cellular adhesion and signalling inputs (Bielas & Gleeson, 2004; Fukuda & Yanagi, 2017; Gupta et al, 2003; Gupta & Tsai, 2003; LoTurco & Bai, 2006). Arriving to their proper position is essential for the correct functioning of the brain (Devreotes & Horwitz, 2015; Luhmann et al, 2015). Hence, disruption of molecular and/or cellular events controlling this process could alter neuronal positioning. These defects could result in malformations of cortical development, associated with developmental delay and epilepsy (Andres et al, 2005; Cepeda et al, 2005;

Chevassus-Au-Louis et al, 1999; Chevassus-au-Louis & Represa, 1999; Jacobs et al, 1999a; Jacobs et al, 1999b; Kriegstein, 2005; Najm et al, 2004).

2.4.1. Radial migration

Radial migration is the process by which newborn cortical neurons move from their layer of birth to the vicinity of the cortical surface, where they will form progressively the neuronal layers (**Figure 4j**; (Rakic, 1972)). Their cellular fate and their laminar position are determined by specific transcription factors (reviewed in (Kwan et al, 2012; Luhmann et al, 2015). Interestingly, this occurs in an inside-out manner, in which neurons belonging to the most upper layers will be form later in development and, therefore, pass through the earlier born ones, that are forming the deeper layers of the cortex. Neurons follow four different and consecutive stages during radial migration before their arrive to their final destination in the CP. These steps contemplate changes in cell morphology, polarity, migration pauses and modifications in their direction (LoTurco & Bai, 2006; Stouffer et al, 2016), which will be commented below.

2.4.1.1.Somal translocation

At the beginning of corticogenesis, the early born neurons are produced in the VZ and split the preplate in two layers located basally to the VZ: the marginal zone (MZ) and the deep subplate (SP). At this stage, the cortex is very thin, reason why neurons just need a somal translocation to be positioned between MZ and the subplate, generating the deep layers of the CP. This movement consist on the extension of a leading process to the MZ and then, by shortenning of this structure they shift their somas. This process is independent of the RGCs, unlike locomotion mode and they have some specific molecular mechanisms (Franco et al, 2011; Fukuda & Yanagi, 2017; Hirota & Nakajima, 2017; Marin & Rubenstein, 2003; Metin et al, 2008; Molnar et al, 2014; Rakic, 2003). After this early neuronal generation, cortex becomes thicker and neurons start migrating in diverse sequential modes (Hirota & Nakajima, 2017).

2.4.1.2.Multipolar migration

Once the cortex starts being thicker, newborn neurons stay in the VZ and then move out basally and acquire a multipolar morphology. For some periods, somas stay immobile, but they extend and retract numerous processes in dynamically, a phenomenon named multipolar migration (Kriegstein & Noctor, 2004; Noctor et al, 2004). This transient appearance will be transformed to bipolar morphology, an essential change for a locomotion migration, in which cells are directed to the IZ and CP (Fukuda & Yanagi, 2017; LoTurco & Bai, 2006; Stouffer et al, 2016).

Experiments in mouse show the implication of some molecules in the multipolar-bipolar transition, which in fact are responsible for neuronal migration disorders, pointing out the relevance of this shift for neuronal positioning. In this sense, FlnA, found mutated in patients showing ectopic accumulation of neurons (see malformations of cortical development section) has a role in multipolar stage exit. In fact, an overexpression of FlnA levels leads to a decrease in multipolar cells, while the opposite effect is achieved by diminishing FlnA levels. DCX manipulations in expression have similar effects, while just LIS1 knocking down result in the arrest of most cells in the multipolar shape (Bai et al, 2003; Hatten, 2005; Ramos et al, 2006; Tsai et al, 2005). Interestingly, although these players alter multipolar stage, they do it in a different step. FlnA manipulations affect cells closer to the VZ surface, within the VZ and SVZ, while DCX ones disturb migrating cells morphology in the IZ. This is consistent with where the final phenotype appears for each case: FlnA leads to ectopic nodules in the periventricular region while DCX or LIS1 mutations generate subcortical band heterotopia (Sicca et al, 2003a; Sicca et al, 2003b).

2.4.1.3. Locomotion migration

When neurons get the bipolar morphology, they can start the locomotion migration, which represents the largest part of the neuronal journey. These movements permit cortical neurons to travel through the cortical thickness to the CP using the radial glial fibers as a scaffold (Fukuda & Yanagi, 2017; Rakic, 1972; Rakic, 1974). In this modality of locomotion, they follow three coordinated and repeated steps: (1) elongation of the leading process, forming a cytoplasmic dilation directed towards the cortical surface; (2) movement of the centrosome to the nucleus and (3) flow of the nucleus close to the centrosome after the trailing process is removed (Ayala et al, 2007; Nadarajah et al, 2001; Schaar & McConnell, 2005; T, 2015).

In lissencephalic species, the radial glial fibers are perpendicular to the ventricular and pial surface and forms a network of paths that neurons use as guides during radial migration. So that, both neurons and progenitors establish an interaction highly regulated by many molecules. As glial fibers determine the trajectory of cortical neurons (Rakic, 1995), neurons coming from the same progenitor are expected to be located in close positions in the mature cerebral cortex (Fernandez et al, 2016; Gupta & Tsai, 2003; Gupta et al, 2002; O'Leary & Borngasser, 2006; Soriano et al, 1995).

In contrast, gyrencephalic ones present an increase in the cortical surface much greater than thickness and than the ventricular area, which result in the formation of characteristic folds and fissures. This enlargement of the pial surface is due to an increment of cortical neurons and basal progenitors, which are very scarce in lissencephalic animals. In this sense, bRGCs permit a

greater neurogenesis without expanding the ventricular surface, since, differently from aRGCs their soma is located in the OSVZ and they just present a basal polarity. Moreover, these basal processes are extra paths for migration, creating a greater divergence for neurons. Then, neurons can disperse laterally and locate at distant positions along the cortical surface (Fernandez et al, 2016; Fietz et al, 2010; Gertz & Kriegstein, 2015; Hansen et al, 2010; Reillo et al, 2011; Wang et al, 2011a). This role of bRGCs is supported by their higher abundance in prospective gyrus than prospective sulcus areas (Borrell & Reillo, 2012; Lui et al, 2011; Reillo et al, 2011). Additionally, experimental data producing an overproliferation of bRGCs leads to cortical expansion and folding in both gyrencephalic (Nonaka-Kinoshita et al, 2013) and lissencephalic models (Florio et al, 2015; Stahl et al, 2013), while partial reduction of their production results in a decrease in size of cortical folds (Reillo et al, 2011).

2.4.1.4.Terminal translocation

Finally, when cortical neurons are at the final phase of the migration, they transiently pause. Then they undergo a terminal translocation, a process similar to the somal translocation at early corticogenesis, in which the soma moves reaching its position beneath the MZ. This way, they migrate a short distance independently from the radial glial fiber and complete the process of migration (Hirota & Nakajima, 2017; Sekine et al, 2011).

2.4.2. Tangential migration

Inhibitory neurons born in the ventral pallium (medial, lateral and caudate ganglionic eminence (MGE, LGE and CGE, respectevely) and follow a tangential migration towards the cortex (Fukuda & Yanagi, 2017; Luhmann et al, 2015). Importantly, their different origin will determine also their final position. Then, interneurons originated from the MGE will be placed in the medio-lateral cortex, while those from LGE will be forming part of the rostral part of the cortex and the olfactory bulb. Finally, those generated in the CGE will arrive to the caudal areas. (Corbin et al, 2001; Nery et al, 2002; Parnavelas et al, 2000). Interestingly, in humans more than a half of the total interneurons number are form in the dorsal pallium, which implies that they born in the cortex and they migrate radially, not tangentially, to their predetermined position (Letinic et al, 2002).

At early corticogenesis, INs travel through the MZ, but later they are also able to go through IZ and SVZ. Finally, at the end of cortical development, tangential migration is done through the boundary between IZ and SVZ, the subplate and MZ. (Lavdas et al, 1999; Marin & Rubenstein, 2001; Marin & Rubenstein, 2003). Once they reach their cortical position, they follow radial migration to the cortical plate following a very dynamic behaviour with extension and branching of their processes (Stouffer et al, 2016) dependent on the cytoskeleton. This

process is susceptible to multiple molecules like semaphoring 3a, ephrin-As, mitogens, cytokines (Lysko et al, 2011; Lysko et al, 2014; Stouffer et al, 2016),

2.4.3. Molecular machinery underlying neuronal migration

We can distinguish two main players controlling neuronal migration. One is the cytoskeleton and its associated molecules, which rearrangements warrant the proper cellular movement. The second are the signalling molecules coming from the other cells: CRs, SP and CP cells.

2.4.3.1. Cytoskeleton

Cytoskeleton is composed by microtubules and actin filaments. Their dynamics control the continuous morphological changes that are necessary during neuronal migration, as well as axon and dendrite and synapse formation (LoTurco & Bai, 2006; Stouffer et al, 2016). Moreover, this structure is regulated by several signalling pathways (Cooper, 2013; Kawauchi & Hoshino, 2008; Kuijpers & Hoogenraad, 2011; Liu, 2011).

- *Microtubules cytoskeleton*

Microtubules (MTs) are long polymers of alpha-beta tubulin dimers, which are in constant reorganization responding to external and internal inputs. Some of the molecules affecting MTs cytoskeleton are stabilizing factors (eg. DCX) or destabilizing elements, such as motor proteins from kinesin and dynein superfamilies (Conde & Caceres, 2009; Stouffer et al, 2016). They are the main component of the centrosome, which movement is key during neuronal migration. They extend from the microtubule organization center (MTOC) of the centrosome, anchor it and raise it up inside the leading process. MTs are very dynamic structures and are stabilized when acetylated. Hence, acetylatilases determine the status of microtubules based on an equilibrium between acetyltransferases and deacetylases (Akella et al, 2010; Creppe et al, 2009; North et al, 2003; Shida et al, 2010).

- *Actin cytoskeleton*

Actin microfilaments are polymers composed by filamentous units of actin (F-actin). Its polymerization is essential for the formation of the traling and the leading processes, as well as growth cone (in reciprocal regulation with MTs). Importantly, the growth cone is susceptible to attracting or repeling signals that determine the direction of the migration.

During migration, adhesion to the substrate is essential as traction point and signalling center. This step is mediated by integrins, transmembrane receptors that bind actin by a diverse set of molecules. The affinity of integrins is facilitated by talin and kindlin, that join to its cytoplasmic domain, and PIP2, a player in the mTOR pathway. Adhesions regulate actin

polymerization and myosin II through Rho-family GTPases, such as Rac, RhoA and Cdc42. Other players involved are Ras proteins, calcium, cyclic nucleotides and kinases.

Some elements interact directly with the actin filaments influencing cell morphology and migration. FlnA is an actin-binding protein that, due to its homodimerized structure in Y shape, establish an orthogonal network of actin filaments. Other molecules, like Rac1 (a FlnA interactor) and POSH have been found to participate in the leading process extension. Additionally, PI3K signalling module is a relevant cascade that has a dynamic role by regulating the levels of phosphatidylinositol 3,4,5-triphosphate (PIP3) in the leading edge of the migrating cells and, consequently, modulating the cytoskeleton rearrangement. In fact, PIP3 is sufficient to impulse actin polymerization and generate cellular protusions, reason why variations in levels of this component affect cellular migration and cytokinesis, among other processes.

2.4.3.2. Signalling molecules

A wide variety of molecules can modulate neuronal migration secreted from other cells: CjRs cells, RG cells, SP cells and cells already placed in the cortical plate.

- **RGCs***:* Establish a communication with the migrating neurons. Their signals involve, among others, N-cadherin and connexins (Cx) adhesions mechanisms, that also regulates somal translocation and the multipolar-bipolar transition phase (Liu et al, 2012a; Liu et al, 2012b). Cx43 upregulates p27kip1, which mediates actin reorganization. Both Cx43 and Cx26, as well as integrin b1, are proteins able to stabilize the leading process on the glial fiber (Elias & Kriegstein, 2008; Kawauchi, 2012; Valiente et al, 2011).

- **CRs***:* Both Cajal-Retzius cells located in the MZ and early born neurons express immunoglobulin-like adhesion molecules (Nectin-1 and Nectin-3, respectively) that enhance the expression of N-cadherin and, thus, facilitates somal translocation. Another key molecule secreted by CjRs is reelin, which activates Dab1, PI3K, Notch and other intracellular cascades that result in variations in cytoskeleton (Bock et al, 2003; Hashimoto-Torii et al, 2008; Jossin et al, 2003; Kim & Kwon, 2015). The relevance of reelin has been point out in the KO mouse model which shows an inverted laminar organization (D'Arcangelo, 2005). Several studies suggest that reelin could be implicated in the stability and orientation of the

leading process through actin cytoskeleton stabliziation, as well as being a stop signal during neuronal migration, since neurons lacking reelin overmigrate into the MZ (Chai et al, 2009). It has also been proposed that reelin may cause the detachment of the migrating neuron from the glial fiber (Hirota & Nakajima, 2017; Santana & Marzolo, 2017).

- **SpNs/CP cells***:* In addition to CjRs, SpNs are the first generated in corticogenesis. SpNs establish connections between the thalamus and the cortex and demarcated the boundary between the upper and the lower cortical regions, where bipolar and multipolar neurons are distributed, respectevly; suggesting a role in the multipolar-bipolar transition. Recent work from Ohtaka et al. (Ohtaka-Maruyama et al, 2018) proposed that multipolar cells receive signals from SpNs, which induce the transition to bipolar morphology by glutamatergic synaptic transmission and calcium signalling. In case of the neurons already placed in the CP, they also release the neurotransmitter glutamate which act as a chemoattractant for migrating neurons through NMDARs receptors and calcium signalling.

2.5. Formation of cortical layers and neuronal differentiation

After the end of migration, neurons detach from the radial glial fiber and start to integrate their specific cortical layer according to their birthdates following an inside-out pattern, as mentioned in a previous section. So that, early born neurons form the deeper layers (V-VI) and late-born ones constitute the upper layers (II-IV). Then, these cells start their terminal differentiation according to their genetical program, which establish specific parameters of morphology and molecular markers. During this process, cells experiment a set of changes that have an impact on the final size of the cerebral cortex. Cell somas size is increase and so the volume of the neuropile. Neuronal apical and basal dendrites start growing and branching, as well as their axon. Moreover, it takes place the formation of spines and boutons for synaptic connectivity. Importantly, densitiy of cortical neurons, cell body size and their dendritic and axonal arborization are extraordinarly different among animals, which correlates with brain size differences and contribute to cortical expansion (Fernandez et al, 2016; Reillo et al, 2011).

3. EVOLUTION OF THE CEREBRAL CORTEX: EXPANSION AND FOLDING

The expansion of the cerebral cortex and the generation of the cortical folds and fissures is an intriguing issue that suscites multiple questions. For so many years, researchers have been trying to elucidate the origin of cortical folding, how it emerges during evolution, and which are the mechanisms that underlie its formation in terms of time, shape and specific location (Bayly et al, 2014; Chen & Walsh, 1993; Dehay et al, 1996; Fernandez et al, 2016; Kriegstein et al, 2006; Le Gros Clark WE, 1945; Reillo et al, 2011; Richman et al, 1975; Welker, 1990b; Xu et al, 2010).

3.1. Evolution of the cerebral cortex: lissencephaly and gyrencephaly

Brain size and shape differs dramatically among mammals through evolution, particularly due to a huge variation in the extension of the cerebral cortex maintaining the cortical thickness and resulting in cortical folding. When cortex is fold, the characteristic gyrus and sulcus are formed. A gyrus is the most expanded region and appears located between two sulci, which are the areas of fissures. Interestingly, the most pronounced total expansion is found in anthropoid primates, like humans (Hofman, 1985; Lewitus et al, 2013; Zilles et al, 1989). This expansion and modification of the cortical surface has been attributed to diverse factors comprehending genetic and molecular cues, modifications in cellular behaviour and cell populations and the time each species invest in all these processes. Also, theories about the implication of mechanical forces during cortical development have been discussed as an element implied in the formation of cortical folds (Bayly et al, 2014; Le Gros Clark WE, 1945; Toro & Burnod, 2005; Van Essen, 1997).

The classic idea of gyrencephaly as the most evolutioned manifestation of cortical expansion suggest that gyrencephaly comes from a lissencephalic ancestor that have been expanding its cortex through evolution. However, when we compared individual brains across phylogeny, we observed that folding is a trait that appeared numerous times in separated lineages, which suggest a convergent evolution (Lui et al, 2011). More to consider is the fact that the generation of gyrencephaly would be a very complex and energy consuming process and elements distinguishable in gyrencephalic animals from those with a smooth cortex are similar in phylogenetically separated animals (Kelava et al, 2012; Reillo & Borrell, 2012).

Based on those facts, the opposite theory was proposed: the common ancestor, rather than be lissencephalic, presented gyri and sulci in its surface. This hypothesis was supported by different evidences. First, as mentioned, gyrencephaly develops in species from across mammalian phylogeny. Second, one main difference between lissencephalic and gyrencephalic brains is the presence in the second ones of an extra germinal layer, the OSVZ, resulted from the split of the SVZ in ISVZ and OSVZ. Moreover, in gyrencephalic species there is a remarkably

abundance of basal progenitors (bRGCs and IPs), cells that have been found in lower abundance in lissencephalic species. So that, it seems more probable that these traits were shared in a common ancestor and they were loss or simplified, generating smooth corteces, or refined through evolution by genetic and molecular variations that trigger the potential of these cells and, eventually, result in different degrees of cortical folding.

3.2. Mechanisms underlying cortical folding

Folding of the cerebral cortex is characterized by the formation of folds (outward bending) and fissures (inward bending) of the cortical mantle (**Figure 5**). Cortical folding wavelength across species correlates with thickness of the grey matter (Mota & Herculano-Houzel, 2015; Welker, 1990a; Zilles et al, 2013), a correlation that emerges from the necessity of folds to contain the full complement of all six neuronal layers plus their descending axons, which varies between species. The thickness of the neuronal layers varies considerably along folds, being thickest at the gyral crown and thinnest at the sulcal fundus, whereas along the lateral walls the thickness remains relatively constant(Smart & McSherry, 1986; Welker, 1990a). The combination of these variations in shape and thickness of the neuronal layers along cortical folds correspond with variations in the morphology and arrangement of their constituent elements (**Figure 5a**).

In species with highly folded brains, as humans, the generation of the cortical fissures is hierarchic, starting from the primary ones (the deepest and first to be form during development). Then, a subdivision of the former generates the secondary, the tertiary fissures and so on. In animals where folding only involves the formation of primary fissures, their pattern is similarly well conserved(Welker, 1990a).

Many models have been proposed to explain and unravel the folding of the cerebral cortex, from mechanical forces to cellular and genetic mechanisms.

3.2.1. Biomechanics

Classically, it was thought that cortical folds appear randomly as a simple consequence of a cortical growth occurring inside a determined cranial volume, and then, the folding was considered as the solution for a space problem (Le Gros Clark WE, 1945). However, this idea was refused after experiments demonstrating that cortex folds in the absence of compressive constraint from the skull (Welker, 1990b). When cortex has extra space by the elimination of non-cortical structures, the folding occurs equally, while if this process would depend on skull pressure, one would expect to have a less folded cortex (Barron, 1950; Muckli et al, 2009). In fact, skull ossify after the brain growing is finished and the abnormal overgrowing of the brain can modify the skull expansion.

Another theory emerged at late nineties, when Van Essen proposed the axonal-tension hypothesis, in which he suggested that cortical folding occurs due to internal hydraulic pressure and cortico-cortical connections between different cortical areas. The distribution of these connections is not homogeneous, and the axonal tracks are under strong tension and exert pulling forces. Hence, the more connected two regions are, the bigger the tension and the closer the regions result, generating a greater folding (Van Essen, 1997), **Figure 5b**). Although this idea was consistent with tracing evidence in macaque (Hilgetag & Barbas, 2006), posterior experiments elegantly reveal that this hypothesis was not supported *ex vivo*. Xu *et al*. used living brain slices from developing ferrets at different stages and show that the direction of tension forces from cortical axons do not bring together the walls of developing gyri (Xu et al, 2010). In fact, axons are under tension, but this is along axon bundles in deep white matter tracks, not within the core of individual gyri. Moreover, computational models determine that folding could occur in absence of these forces, as a physical effect of buckling instability (Toro & Burnod, 2005).

Alternatively, another theory based on tissue mechanical properties and the growth progression of the cortex during development has been proposed, named differential tangential growth hypothesis (**Figure 5c**). This idea considers the cerebral cortex divided in two regions, an outer and an inner layer, which refers to the CP and transient embryonic layers, respectively. The outer one grows much faster than the other, experimenting a faster tangential expansion, which is predicted to induce folding of this layer. Interestingly, this concept was modelled using polymer gel preparations that, expose to a solvent, the outer shell would expand faster than the inner one. Working with different properties show that variations in its stiffness results in a different pattern of wrinkling. This idea could be extrapolated to cerebral cortex when the stiffness of both outer and inner layers is similar, since the resulting wrinkling remind us a folded cortex (Tallinen & Biggins, 2015; Tallinen et al, 2014). Moreover, these experiments also demonstrate that the size, the expansion and the thickness of specific areas of the brain are relevant players that dramatically influence the final pattern of cortical folds.

Underneath these mechanical forces, there are biological triggers. In this sense, differential tangential growth of the CP has been associated with neuronal differentiation. In this process, these cells generate and expand their dendritic tree, enlarge their cell body and consolidate their synapses (Wang et al, 2017). These processes concur temporally with the maturation and folding of ferret, macaque and human cortices, which support this idea (Borrell & Callaway, 2002; Callaway & Borrell, 2011; Kroenke & Bayly, 2018; Wang et al, 2017). However, differentiation of neurons from layer II/III and IV in ferret at P14, when a peak of folding is occurring, is still very poor. In fact, experimental reduction of cortical folding in ferret was driven by reducing the neuronal tangential expansion not by altering the cortical neurons differentiation, although modifying its compactation (Reillo et al, 2011).

Besides, cortical folds patterning was not randomly appearing but on the contrary are following a stereotypical and hierarchical fashion in which transcriptional protomaps have been described to concur with the particular location of cortical folds. These variations in gene expression and cellular composition could be defining mechanical properties and, hence, producing the effect of cortical folding mediated by these two forces: biological and mechanical (Borrell, 2018).

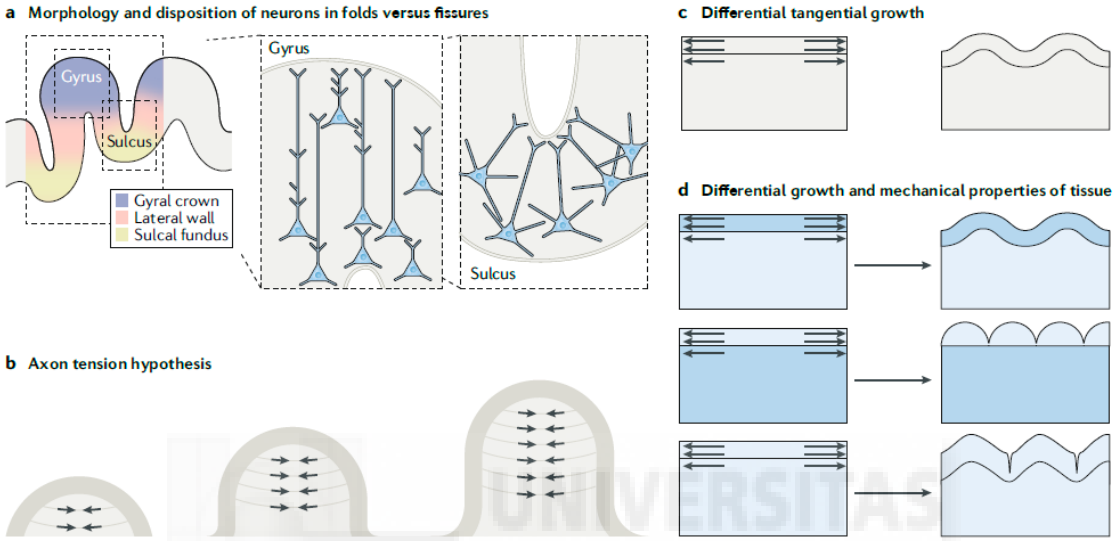


Figure 5. Anatomy and mechanics of cortical folding: push, not pull. Nomenclature of cortical folding and general morphology-disposition of cortical neurons within gyri (square) and sulci (square). Cells have a different orientation and a more extensive horizontal branching of apical and basal dendrites in sulci compared with gyri. b–d | Hypotheses postulated for biomechanical forces driving cortical folding. According to the axon tension hypothesis (b), axons connecting cortical areas are under tension, and the more connected these regions are (grey lines), the greater the tension (arrows) and the closer the regions become, resulting in increased folding. According to the differential tangential growth hypothesis (c), the cerebral cortex is divided into two regions, the outer layer growing faster than the inner core. This differential growth is predicted to induce folding of the outer layer. Another hypothesis of cortical folding is based on the notion that differential mechanical properties of the tissue (i.e. stiffness, elasticity) define its capacity to fold more or less (d). Dark bllue indicates the stiffer material; light blue indicates the softer material. The cerebral cortex is intrinsically connected with subcortical structures that have different mechanical properties. Variations in the difference of stiffness between superficial and deep regions influence the degree and mode of folding. Wrinkling occurs in a smooth, sinusoidal way (d1). Evagination of the grey matter forming cusped folds (d2). If both grey and white matter have similar properties, both will fold (from (Llinares-Benadero & Borrell, 2019)) .

3.2.2. Cellular mechanisms

Across mammalian evolution, the number of cortical neurons has been greatly amplified, specially the upper layer neurons (layer II/III) (Hill & Walsh, 2005). In fact, expansion and folding of the cortex has been linked to a substantial elevation of neurogenesis through the regulated proliferation of progenitors. Proliferation of NECs and aRGCS cortical progenitors

permits the tangential cortical spreading, while basal ones generate preferentially upper layer neurons, contributing to the radial cortical thickness. However, pathologies in which folding is altered like lissencephaly conserved broadly the number of cortical neurons, or microcephaly, which broadly conserves the gyrification patterning (mild cases) with lower number of neurons, suggesting that other mechanisms must be implied in gyrification. Other pathologies with an increased gyrus formation like polymicrogyria have been associated with the loss of deep layer neurons. These different phenotypes point out the relevance of the balance of upper and lower layers neurons for a proper folding (Friede et al, 1996; Goffinet, 2017). Moreover, many differentially expressed genes mutated in cortical malformations have a role in neuronal migration (i.e. FlnA, Tuba8, pafah1b1) rather than in proliferation, pointing out the relevance of divergence neuronal migration (Barkovich et al, 2012).

Studies in different folded species demonstrated that a prominent OSVZ is present in all gyrencephalic species, including primates, carnivores, ungulates and rodents. This trait appeared to be determinant for the formation of folds, but although is absent in almost all lissencephalic species like mouse and guinea pig (Fietz et al, 2010; Garcia-Moreno et al, 2012; Reillo et al, 2011), others presenting a smooth but large cortex do have an OSVZ, like marmoset monkey and rat. However, in these species the abundance of bRGCs is very scarce in comparation with the gyrencephalic ones (**Figure 6**) (Garcia-Moreno et al, 2012; Kelava et al, 2012; Martinez-Cerdeno et al, 2012). Moreover, the manipulation of bRGCs abundance in ferret increases cortical folding. These evidences suggest that OSVZ is necessary for the formation of cortical folds but must be concordant with a minimum abundance of bRGCs (de Juan Romero & Borrell, 2017; Nonaka-Kinoshita et al, 2013; Reillo et al, 2011).

The generation of the OSVZ and the bRGCs follows a different mechanism from the cell lineage known for mouse (and extensively to other lissencephalic species). In mouse, there is a linear lineage from aRGCs in VZ, IPCs in SVZ and neurons. In ferret, this occurs similarly in ISVZ, but not in OSVZ. Early in development, after aRGCs self-amplifying divisions, they start to generate some bRGCs that form the SVZ. Later, this production becomes massive through aRGCs consuming divisions, in which OSVZ starts to be generated during a critical period after which these bRGCs become independent from the other germinal layers, being able to populate and expand the OSVZ (Betizeau et al, 2013; Martinez-Cerdeno et al, 2012; Martinez-Martinez et al, 2016).

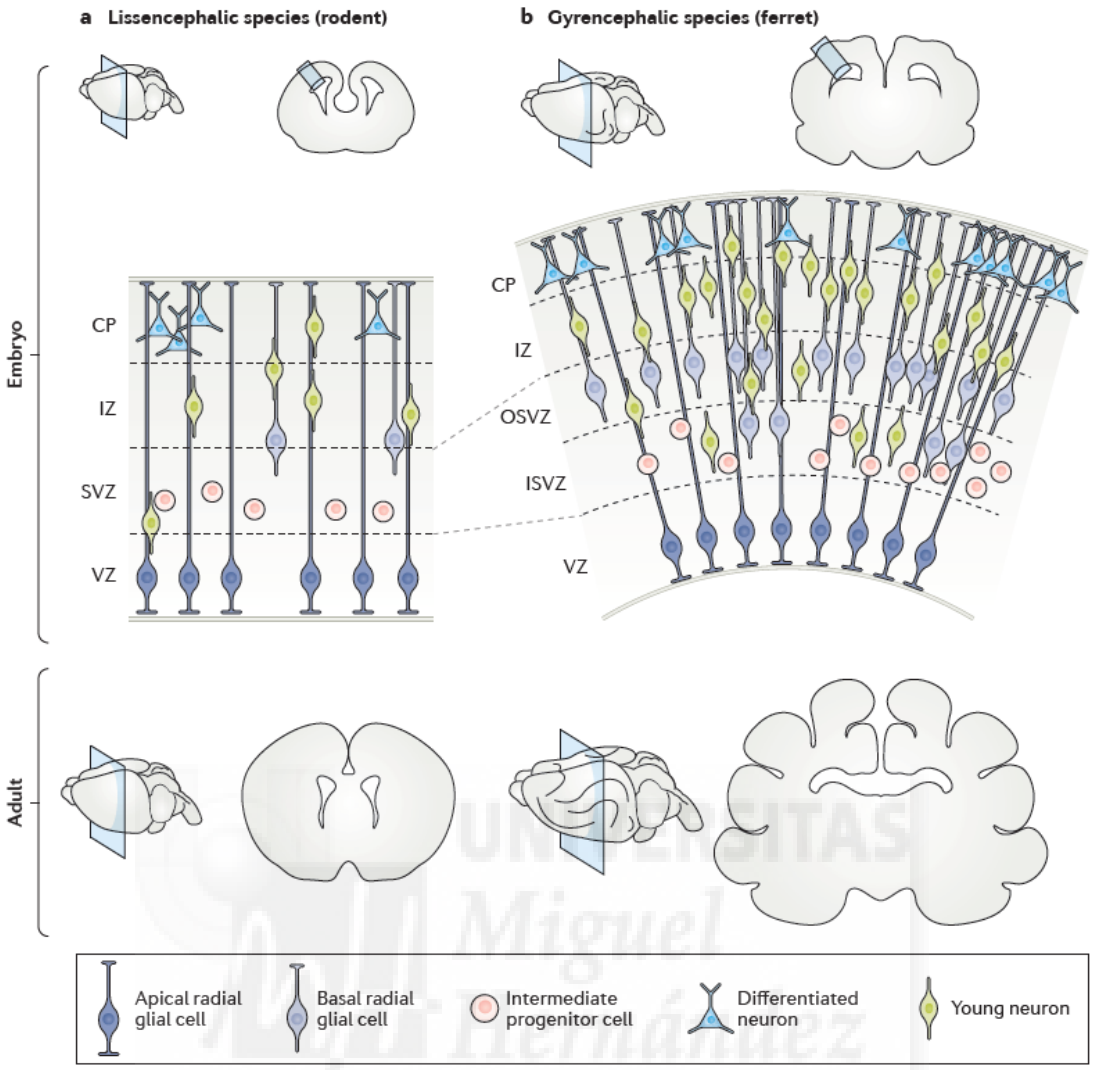


Figure 6. Cellular mechanisms of cortical growth and folding: progenitor cells. Different scenarios in lissencephalic (a) and gyrencephalic species (b) at both embryonic and adult stages. Row 1 a representation of a rodent (a) and ferret (b) brain at embryonic stages in external view and coronal section. Row 2 shows schematics of the cellular composition and organization of the developing cerebral cortex. In lissencephalic species, most cortical progenitors are aRGCs and IPCs, while bRGCs are scarce. After neurons are generated, they migrate intimately associated to radial glia fibers, which follow strictly parallel trajectories. In gyrencephalic species, the subventricular zone (SVZ) is greatly expanded and specialized in two germinal layers, the inner subventricular zone (ISVZ) and the outer subventricular zone (OSVZ). Both ISVZ and OSVZ are rich in bRGCs and IPCs. Species with a folded cortex present a much greater abundance of bRGCs than do lissencephalic species, particularly in OSVZ. Each bRGC extends its own basal fiber, which creates a dramatic divergence of the radial fiber scaffold. This leads to the tangential dispersion of radially migrating neurons, and hence to the tangential expansion and folding of the cortical surface. Row 3 shows a representation of the adult brains of both types of species in external view and in coronal section: a smooth cortex in lissencephalic species in contrast to an expanded and folded cortex in gyrencephalic species (from (Llinares-Benadero & Borrell, 2019)).

The abundance of bRGCs appeared to be key for the generation of a gyrated cortex. Moreover, the amount of this progenitor cell is heterogenic through the OSVZ, with higher number of cells in the formatted gyrus and lower in the sulcus, also coincident with an increased

level of mitosis and proliferation (Betizeau et al, 2013; Kriegstein et al, 2006; Martinez-Cerdeno et al, 2012; Martinez-Martinez et al, 2016; Reillo et al, 2011). This idea is supported by functional studies in which the modification of progenitor cell pool through genetic manipulation varies the cortical folding pattern (Masuda et al, 2015; Toda et al, 2016).

These evidences indicate that the formation of folds involves the OSVZ formation with the sufficient generation of bRGCs, increase in both neurogenesis and proliferation and a proper divergence neuronal migration. At the end of this migration, neurons must intercalate following reelin pathway promoted by Cajal Retzius cells (Goffinet, 2017; Hong et al, 2000). These particular traits are linked to a finely genetic regulation.

3.2.3. Genetic regulation of cortical folding

Cortical folding is subject to a strong genetic regulation. This idea is supported by several evidences observed in gyrencephalic species. First is the fact that the patterning of folding is stereotyped and highly conserved among individuals that belong to the same species. Second, that this patterning follows similar trends when we compared gyrencephalic species (Borrell & Reillo, 2012; Welker, 1990b). And third, the disposition and shape of folds and fissures in humans are conserved, specially in the cases of monozygotic twins which appears to be stronger around the frontal and temporal lobes, while other regions are more susceptible to environmental causes, like Broca's and perisylvian areas (Chang et al, 2004; Lohmann et al, 2008; Lohmann et al, 1999).

The time window for the OSVZ bRGCs seed is dependent on differential gene expression, as was evidenced by transcriptomic analysis in ferret. In those studies, variation in levels of expression of cell adhesion and differentiation genes match with a critical period for bRGCs generation, showing a precise temporal regulation of gene expression. This was also supported by functional experiments with some of these genes (Cdh1 and Trnp1), demonstrating that its downregulation during this particular time is necessary and sufficient for the great production of bRGs (De Juan Romero & Borrell, 2015; Martinez-Martinez et al, 2016).

Interestingly, transcriptomic analysis of the cortical germinal layers in ferret (VZ, ISVZ, OSVZ) comparing the prospective splenial gyrus and the adjacent lateral sulcus shows more than 2000 differentially expressed genes (DEGs). Mainly, differences in gene expression appear in the VZ and OSVZ, showing discrete modules of high or low expression levels. Some of them coinciding with high precision to prospective gyrus and sulcus, delineating a genetic protomap of cortical folding. Importantly, some of these DEGs show a similar pattern of modular expression in human, in which mutations in these genes are found in humans with malformations of cortical development. On the contrary, these differences in expression do not

occur in lissencephalic species, where gene expression is homogeneous or gradual (**Figure 7**; (De Juan Romero & Borrell, 2015; Elsen et al, 2013; Sansom & Livesey, 2009)). This fact could explain why some human cortical malformations fail to be reproduced in mouse models (as occurs with FlnA models (Feng et al, 2006; Hart et al, 2006).

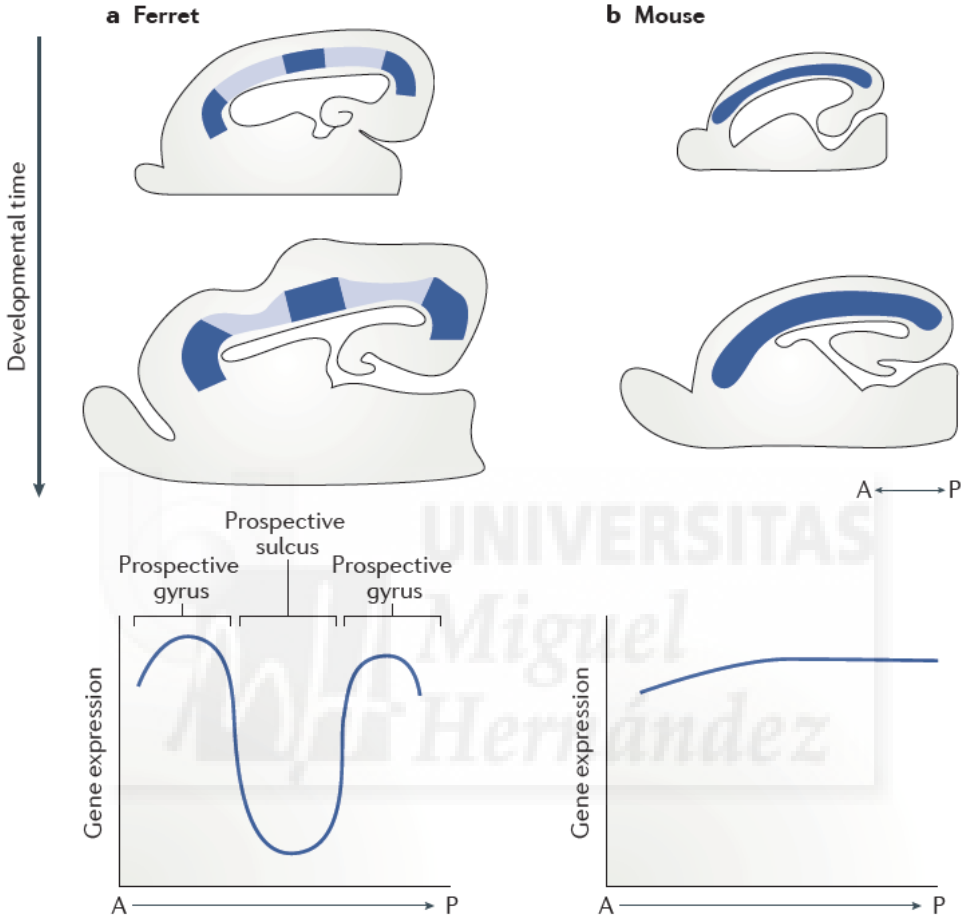


Figure 7. Genetic patterning of cortical folds. Genes are expressed in modular patterns, alternating high and low expression (grey tone), along germinal layers of gyrencephalic species like ferret **(a)**, whereas in lissencephalic species like mouse gene expression levels are quite homogenous **(b)**. Modules of expression levels concur with the prospective location of folds and fissures. Variations in expression are related to differences in proliferation and neuron migration and, eventually, to cortical expansion and folding. A, anterior; P, posterior (from Llinares-Benadero and Borrell, 2019)).

Differential gene expression is dependent on regulatory molecules, which have been suffered variations during evolution. Evolution of active genomic enhancers (*GPR56*, found mutated in polymicrogyria (Bahi-Buisson et al, 2010; Piao et al, 2002) and different pattern of expression among species are evidenced. Given that mouse and primates share a phylogenic ancestor more recent than with carnivores and cetaceans, the similar regulation of *GPR56* suggest convergent evolultion. Studies comparing genomes of human, chimpanzee, rat and

mouse found that genome is moderately conserved across these species (Bae et al, 2014). However, humans present some regions of high divergence, which, when compared to chimpanzee, show a rapidly nucleotide substitution in the immediate human lineage, named human accelerated regions (HARs). These HARs are mainly located in introns and intergenic regions, suggesting a role in gene regulation (Bejerano et al, 2006; Pollard et al, 2006a; Pollard et al, 2006b).

Other level of spatio-temporal pattern of gene expression is given by non-protein-coding RNAs, like miRNAs and lncRNAs (Dehay & Kennedy, 2007; Dehay et al, 2015; Lukaszewicz et al, 2005). For example, hundreds of miRNAs have been found expressed in apical and basal progenitors of macaque developing cortex but not in mouse, regulating cell cycle and neurogenesis, differential growth and complexication of germinal layers and cortical areas (Arcila et al, 2014).

Biological evolution also comes from variations in the sequence of protein-coding genes. Transcriptomic search for genes differentially expressed in radial glial cells between species identified 56 genes expressed in human but not in mouse RGCs. Among these, ARHGAP11B (Florio et al, 2015; Florio et al, 2016) and NOTCH2NL (Suzuki et al, 2018) arose from the partial and total duplication of an ancestor gene, respectively. of the ARHGAP11A gene on the human lineage after separation from the chimpanzees, and thus, it is hominid-specific gene. Experimental expression of ARHGAP11B in mouse induces the generation of self-reneweing bRGCs and results in the formation of cortical folds in mouse (Florio et al, 2015).

3.2.4. Radial Unit Hypothesis

A radial unit consist in the group of cells that born from different clones (RGCs) located in close positions in the VZ and then migrate through the glial fibers to be set in the same column in the CP. Radial Unit Hypothesis (RUH) postulates that the expansion of the cerebral cortex depends on the number of radial units, which is tightly related with the number of founder cells during cortical development. Then, horizontal location of neurons belonging to the same radial unit are determined by their precursor position, whereas its laminar location depends on the birthdate. In agreement with that, the size of the cortical surface is determined by the quantity of radial units, while the number of cells constitutuing the columns its associated with cortical thickness (**Figure 8**; (Rakic, 1988; Rakic, 2000)).

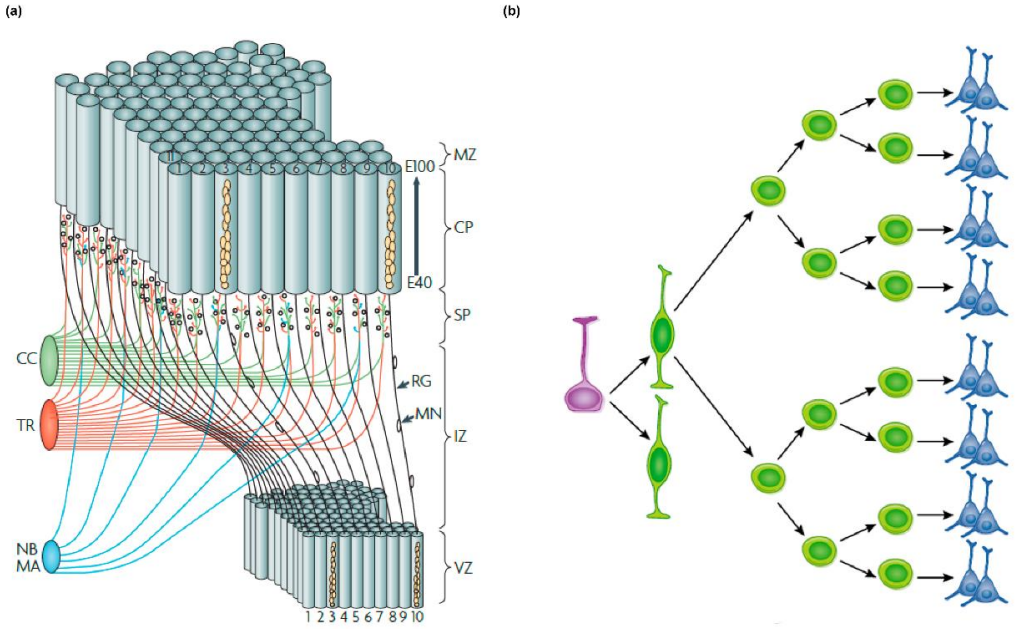


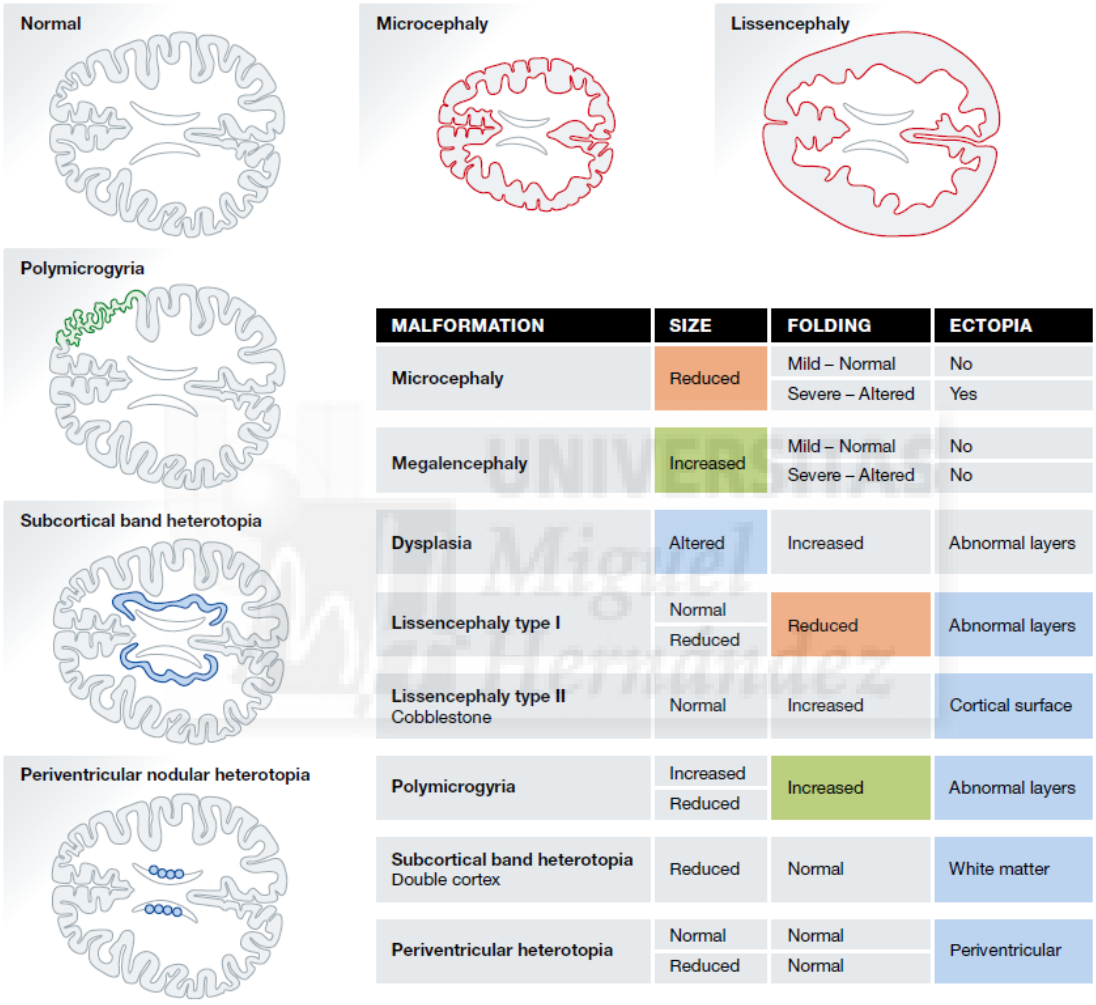
Figure 8. Radial Unit Hypothesis model. (a) RGCs proliferative potential establish the number of radial columns that will conform the cortex. Cortical thickness is defined by the level of neurogenesis. **(b)** Number of neurons generated through related progenitors will be forming part of the same layer. CC, cortico-cortical projections; TR, thalamic radiation; MN, migrating neuron; RG, radial glia; VZ, ventricular zone; IZ, intermediate zone; SP, subplate. (Adapted from (Fernandez et al, 2016; Rakic, 2000)).

In the same sense, number of founder cells (and then radial units) depends on their proliferative potential (asymmetric Vs. symmetric divisions), the duration of the cell cycle and the number of divisions and the total duration in which these processes take place. In fact, a slight prolongation of this phase could be responsible for a significant expansion of cortical surface, as exemplifies the differences in gestational time among species, eg. mouse (19 days, cortex being generated in about one week), macaque monkey (165 days, two months), ferret (42 days; two weeks), human (40 weeks; 14 weeks) (Rakic, 2000).

Although this idea was supported experimentally, we have to take in account that in gyrencephalic species, both neurogenesis and cortical surface are highly increase, which is independent of an expansion of the VZ region. On the contrary, it is possible because of the generation of extra basal progenitors, so that, this exemplifies that there are other contributors beneath cortical expansion process.

4. MALFORMATIONS OF CORTICAL DEVELOPMENT

Malformations of cortical development (MCDs) was a concept that appear to define a group of disorders resulted from the alteration of key steps during cerebral cortex development which could be affecting cortical structure. In consequence, brain function is perturbed reflected as distinct levels of intelectual disability, autism and epilepsy (Andrade, 2009; Guerrini et al, 2008; Hans J. ten Donkelaar, 2014).



MALFORMATION	SIZE	FOLDING	ECTOPIA
Microcephaly	Reduced	Mild – Normal Severe – Altered	No Yes
Megalencephaly	Increased	Mild – Normal Severe – Altered	No No
Dysplasia	Altered	Increased	Abnormal layers
Lissencephaly type I	Normal Reduced	Reduced	Abnormal layers
Lissencephaly type II Cobblestone	Normal	Increased	Cortical surface
Polymicrogyria	Increased Reduced	Increased	Abnormal layers
Subcortical band heterotopia Double cortex	Reduced	Normal	White matter
Periventricular heterotopia	Normal Reduced	Normal Normal	Periventricular

Figure 9. Human cortical malformations and their phenotypic manifestations. Schematic of horizontal sections through the cerebral cortex of a normal human brain compared to those of patients with cortical malformation: microcephaly, lissencephaly type I, polymicrogyria, subcortical band heterotopia (double cortex), and periventricular nodular heterotopia. The table summarizes the phenotypic manifestations associated with each malformation regarding brain size, cortical folding, and the formation of ectopias. The most representative effects are highlighted and color-coded: Features negatively affected by the pathology are in red, features augmented in green, and particularities are in blue. Uncolored cells indicate additional alterations that may be associated with the primary defect (From (Fernandez et al, 2016)).

Several classifications have been made during the last decades, first based on clinical observation and imaging techniques as MRI. Then, they were updated and combined with the constant advance in molecular biology of MCDs. An extensively accepted classification was

proposed by Barkovich *et al.* (Barkovich et al, 2015; Barkovich et al, 2012; Barkovich et al, 2001) in which the authors use the affected stage of development based on pathological description and genetical studies (Barkovich et al, 2012; Desikan & Barkovich, 2016). However, some diseases are difficult to classify since the specific mechanism is not well known or described yet. Moreover, due to the continuous research in this field, new syndromes and a more precise description of some already discovered come out frequently; so that, the list is in constant updating.

Another way to look at MCDs is focusing on the significant changes (excess or defect) in terms of cortex size, folding or the uncorrect location of cells (ectopia) due to the disruption of some cellular and molecular mechanisms during cortical development. So that, we can overview human malformations grouped according to the main affection: brain size, cortical folding or the formation of ectopias (Fernandez et al, 2016). Moreover, it is known that these pathologies could appear both isolated or as combined phenotypes (Barkovich et al, 2012) (**Figure 9**). Based on these terms, here we will describe some of the main diseases. Particularly, we will go deeper in two of the most common cortical malformations which are the focus of this work: periventricular nodular heterotopia, concerning ectopia formation; and polymicrogyria, an alteration of the folding pattern (**Figure 9**).

4.1. Group I. Brain size

During corticogenesis, both proliferation and apoptosis are fundamental processes that will define the size and expansion of the cortex. In consequence, variations in their equilibrium will result on alterations in proliferation and/or survival of neural progenitors leading to abnormal brain size, either excessive (megalencephaly), defective (microcephaly) or unbalanced (dysplasia) (Barkovich et al, 2012; Fernandez et al, 2016) (**Figure 10**).

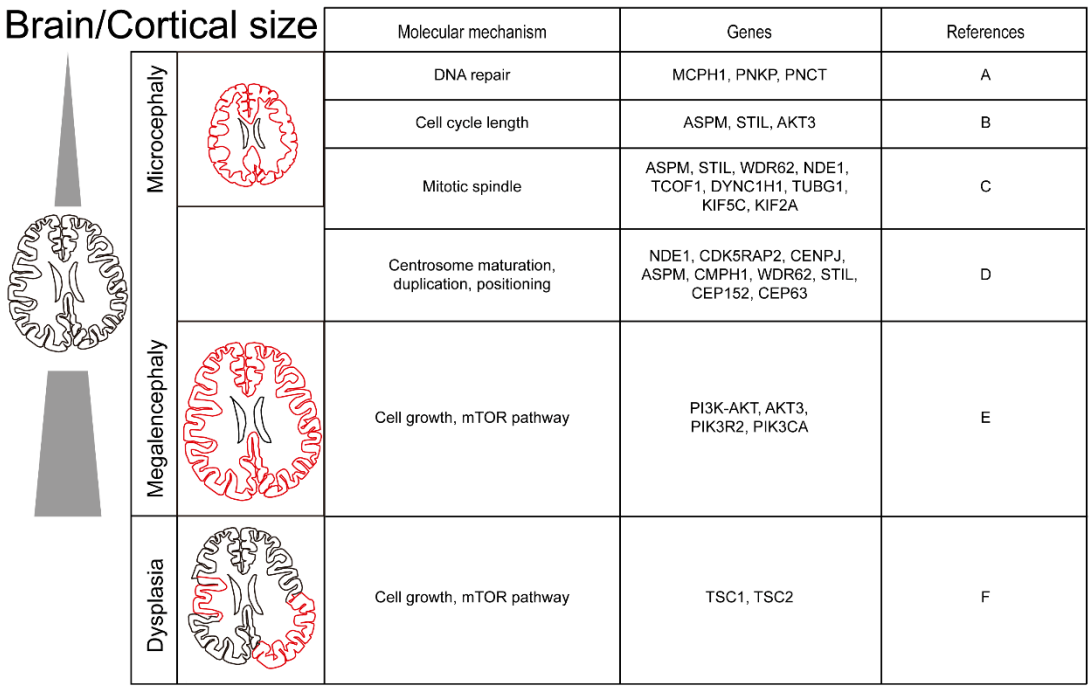


Figure 10. Human malformations of cortical size. Malformations influencing brain/cortical size, genes found mutated in patients with the corresponding pathologies and molecular mechanisms in which they are involved. References are group as followed: A: (Griffith et al, 2008; Gruber et al, 2011; Sheen et al, 2010; Woods et al, 2005). B: (Boland et al, 2007; Desir et al, 2008; Kumar et al, 2009; Passemard et al, 2009). C: (Bilguvar et al, 2010; Feng & Walsh, 2004b; Nicholas et al, 2010; Poirier et al, 2013a; Poirier et al, 2013b; Yu et al, 2010);. D: (Abrieu et al, 2000; Alkuraya et al, 2011; Bakircioglu et al, 2011; Bhat et al, 2011; Bond et al, 2005; Graser et al, 2007; Mirzaa et al, 2014; Nicholas et al, 2010; Sir et al, 2011; Thornton & Woods, 2009; Yao et al, 2000). E: (DiLiberti, 1998; Lee et al, 2012; Mirzaa et al, 2013; Poduri et al, 2013; Riviere et al, 2012) F: (Barkovich et al, 2012; Crino et al, 2006).

4.1.1. Microcephaly

Microcephaly is a developmental disorder characterized by a reduction of head circumference compared to control individuals (Bond et al, 2002; Desikan & Barkovich, 2016; Gilmore & Walsh, 2013). This condition may be mild, when only brain size is affected, or severe, when also the cortical folding is altered. We can distinguish between primary microcephaly (true microcephaly or *microcephaly vera*) and secondary or postmigrational microcephaly (Adachi et al, 2011; Barkovich et al, 2012; Bilguvar et al, 2010; Desikan & Barkovich, 2016; Faheem et al, 2015; Yu et al, 2010). Primary microcephaly is present at birth and is a static anomaly. Differently, postmigrational microcephalies refer to a progressive neurodegenerative condition in which patients develop a severe microcephaly during the first 2 years of life.

Generally, genes found mutated in patients suffering from this disease are related with neurogenesis, cell proliferation and apoptosis rate, particularly in cell-cycle progression and checkpoint regultaion, mitotic spindle formation, centrosome duplication and maturation and also some related with microtubules cytoskeleton (**Figure 10**). The most common cases are due

to mutations in microcephalin (MCPH1) (Gruber et al, 2011; Jackson et al, 2002; Woods et al, 2005) and in ASPM (Fish et al, 2006; Gul et al, 2006; Kumar et al, 2004; Shen et al, 2005).

4.1.2. Megalencephaly

Megalencephaly is a condition defined as an abnormal enlargement of the brain. As ocurring in microcephaly, it is related with proliferation, neurogenesis and apoptosis. However, as being the opposite phenotype, these processes are altered in the contrary way, meaning an excessive production of progenitors and neurons due to a lower rate of apoposis or a shortening of cell cycle and increased cell cycle re-entry (Barkovich et al, 2012; Dehay & Kennedy, 2007; Hansen et al, 2010; Wang et al, 2011b).

Some cases of megalencephaly appear combined with alterated patterns of cortical folding, as the 6% of patients with polymicrogyria present megalencephaly. These combined syndromes are known as macrocephaly, polymicrogyria, polydactyy hydrocephalus (MPPH), N-CMTC (macrocephaly cutis marmorata telangiectasia congenita) and MCAP (macrocephaly capillary malformation) (Conway et al, 2007; Mirzaa et al, 2004; Tore et al, 2009). In the last years, several studies shed light to the genetic causes underlying these pathologies. Concretely, it has been shown that components of the phosphatidylinositol 3-kinase (PI3K)-AKT signaling has a key role in the control of celular growth, and, consequently, in brain size (Desikan & Barkovich, 2016; DiLiberti, 1998; Lee et al, 2012; Mirzaa et al, 2013; Riviere et al, 2012).

4.1.3. Dysplasia

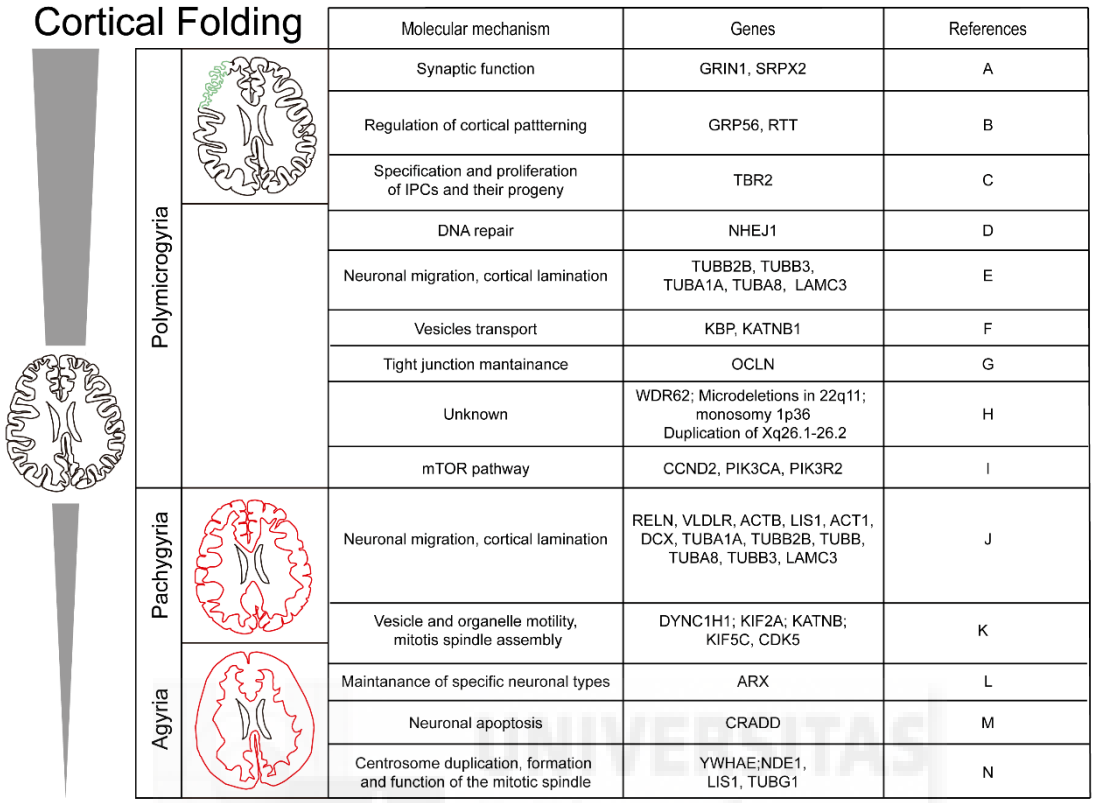
Focal cortical dysplasia (FCD) is a common and heterogeneous group of disorders of localized histologic alterations in any part of the cortex that are epileptogenic. These deffects include: disturbed layering, polymicrogyria, heterotopia, abnormal cell morphology (balloon cells, cytomegaly) (Barkovich et al, 2012; Palmini et al, 2004; Tassi et al, 2002) *Barkovivch2005*. Briefly, three types can be distinguished (Blumcke et al, 2013):

(I) Isolated, with disrupted cortical lamination that can be vertical (Ia) or horizontal (Ib). There is not a clear related gene mutation associated with neither of them.

(II) Isolated, present dysmorphic neurons (IIa) or with balloon cells (IIb). Suggested as a malformation due to abnormal proliferation, somatic and germline mutations in the mTOR pathway.

(III) Associated with a main injury, as hipocampal sclerosis (IIIa), glial or glio-neuronal tumor (IIIb), vascular malformation (IIIc) and others (truama, ischemic injury, encephalitis) (IIId).

4.2. Group II. Cortical folding



Molecular mechanism	Genes	References
Synaptic function	GRIN1, SRPX2	A
Regulation of cortical pattterning	GRP56, RTT	B
Specification and proliferation of IPCs and their progeny	TBR2	C
DNA repair	NHEJ1	D
Neuronal migration, cortical lamination	TUBB2B, TUBB3, TUBA1A, TUBA8, LAMC3	E
Vesicles transport	KBP, KATNB1	F
Tight junction mantainance	OCLN	G
Unknown	WDR62; Microdeletions in 22q11; monosomy 1p36 Duplication of Xq26.1-26.2	H
mTOR pathway	CCND2, PIK3CA, PIK3R2	I
Neuronal migration, cortical lamination	RELN, VLDLR, ACTB, LIS1, ACT1, DCX, TUBA1A, TUBB2B, TUBB, TUBA8, TUBB3, LAMC3	J
Vesicle and organelle motility, mitotis spindle assembly	DYNC1H1; KIF2A; KATNB; KIF5C, CDK5	K
Maintanance of specific neuronal types	ARX	L
Neuronal apoptosis	CRADD	M
Centrosome duplication, formation and function of the mitotic spindle	YWHAE;NDE1, LIS1, TUBG1	N

Figure 11. Human malformations of cortical folding. Malformations affecting cortical folding, genes found mutated in patients with the corresponding pathologies and molecular mechanisms in which they are involved. References are group as followed: A: (Fry et al, 2018; Roll et al, 2006). B: (Bae et al, 2014; Bahi-Buisson et al, 2010; Faisst et al, 2002; Kheradmand K, 2012; Piao et al, 2002; Piao et al, 2005). C: (Baala et al, 2007). D: (Cantagrel et al, 2007). E: (Barak et al, 2011; Poirier et al, 2013a; Poirier et al, 2013b; Squier & Jansen, 2014; Valence et al, 2013). F: (Mishra-Gorur et al, 2014; Valence et al, 2013). G: (O'Driscoll et al, 2007). H: (Battaglia et al, 2008; Bingham et al, 1998; Jansen & Andermann, 2005; Shiba et al, 2013; Yu et al, 2010). I: (Mirzaa et al, 2004; Riviere et al, 2012). J: (Bahi-Buisson & Cavallin, 1993; D'Arcangelo et al, 1995; Hong et al, 2000; Pilz et al, 1998; Riviere et al, 2012). K:(Hertecant et al, 2016; Mishra-Gorur et al, 2014; Parrini et al, 2016; Poirier et al, 2013b; Tian et al, 2016). L: (Kitamura et al, 2002; Uyanik et al, 2003). M: (Di Donato et al, 2018). N:(Derewenda et al, 2007; Di Donato et al, 2018; Parrini et al, 2016).

4.2.1. Lissencephaly

Lissencephaly (from greek, "smooth brain") includes a variety of disorders characterized by the simplification of the folding pattern such as agyria (absence of folds), pachygyria (simplified pattern of folds) and subcortical band heterotopia (gyral pattern is either normal or simplified with broad convolutions and a thickened cortex) (**Figure 11**). Interestingly, both phenotypes could concur in the same patient, presenting a gradient of affected cortical pattern (antero-posterior, posterior-anterior) (Di Donato et al, 2018; Guerrini & Marini, 2006).

We can distinguish between two main types of lissencephaly: type I (or classic) and type II (or cobblestone). Type I is due to mutations in genes related to the cytoskeleton and altered neuronal migration. Most cases of type I lissencephaly are due to mutations in LIS1 or DCX (Pilz et al, 1998). These are proteins that interact with the tubulin cytoskeleton allowing its polymerization and stability (Caspi et al, 2000; Sapir et al, 1997). In consequence, give rise to an accumulation of neurons below the preplate and resulted in a largely disorganized and thickened cortex (Golden & Harding, 2010). Type II or cobblestone, caused by alterations in radial glia and pial surface interactions, which result in the disruption of the cortical surface and the overflow of neurons above the meninges (mainly related with heterotopia formation, so that, further details in section ectopia) (Bizzotto & Francis, 2015).

4.2.2. Polymicrogyria

Polymicrogyria is a spectrum of disorders characterized by the formation of abnormally abundant and small cortical folds (**Figure 11**), which could have a macro or microscopic appearance (Barkovich, 2010; Judkins et al, 2011; Leventer et al, 2010). This excessive folding could be affecting all or only the upper layers of the cortex (Barkovich, 2010; Squier & Jansen, 2014; Stutterd & Leventer, 2014). It has been suggested that this phenotype come from an abnormal cortical development, that could be affecting earlier stages, changing cortical organization; or later ones, disturbing neuronal migration (Barkovich, 2010) or postmigrational processes (Williams et al, 1976) In consequence, the resultant cortex appears thinner, sometimes with the interdigitation of white matter, causing abnormal lamination, specially altering layer V due to a loss of neurons in the deep cortical layers (Barkovich et al, 1999; Budday et al, 2015).

This cortical malformation is highly heterogeneous, which difficult its understanding and classification. It could appear in several forms and areas (focal, multifocal or diffuse; unilateral, bilateral asymmetrical or symmetrical), being the one involving the sylvian fissure the most common case (perisylvian polymicrogyria) (Barkovich, 2010; Kuzniecky et al, 1993) (**Figure 12a-d**). Patients affected by this pathology could present a wide range of different clinical presentations with different level of severity depending on the cortical area affected, but generally they share epilepsy and cognitive deficits (from mild to severe) (Barkovich, 2010; Kuzniecky et al, 1993).

Layering could be perturbed in several ways, depending on the deffect during development: simplification (with four layaers similarly to lissencephaly type I, due to postmigrational insults) or complete disruption and disorganization (unlayered, due to early neuronal migration disorder) (Barth, 1987)(**Figure 12e, f**), thought to be due to an early neuronal migration deffect. In fact, five subtypes are defined; 1-unlayered festooned, 2-four-layered cortex with a sinous upper layer, 3-parallel four-layered cortex, 4-miniature gyri wich are fused and 5-poorly laminated (Norman MG, 1995). In brief, despite of the wide range of

histologic apperance that polymicrogyric patients could present they have a dearrangement of six-layered cortex and of sulcation due to a fusion of molecular layer across sulci (Barkovich et al, 1992; Barth, 1987; Englund et al, 2005; Evrard P, 1989; Kuzniecky et al, 1993).

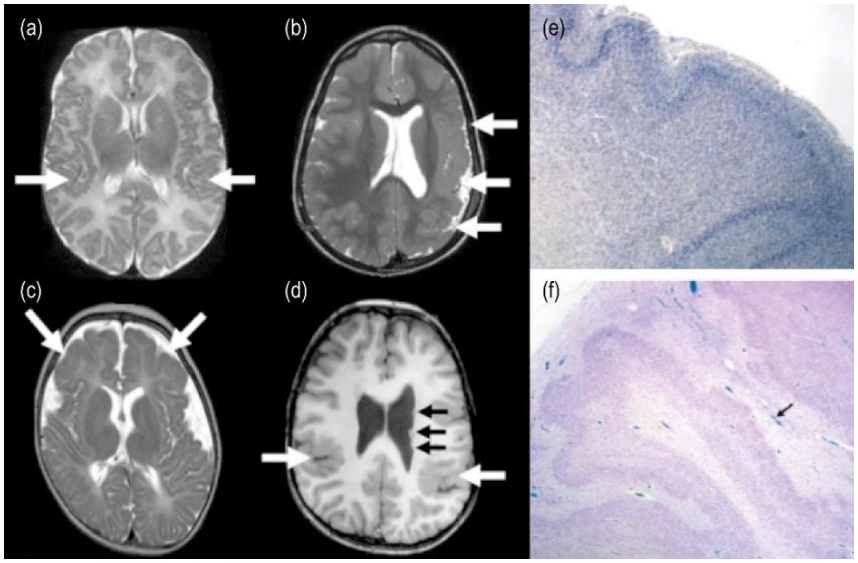


Figure 12. Polymicrogyria heterogeneity. (a-d) MRI of polymicrogyric patients showing the variability of this malformation appearing bilaterally (a, c, d), unilaterally (b), in perisylvian areas (a, b) or frontal territories (c). Also, it could appear in combination with other malformations like PVNH (d). (e. f) Sections of polymicrogyric brains, showing an alteration of cortical layering. (Adapted from (Jansen et al, 2016; Stutterd & Leventer, 2014).

Although polymicrogyria could appear isolated, it is frequent to find it in combination with other alterations of cortical development such as microcephaly, periventricular heterotopia or subcortical band heterotopia (Barkovich, 2010; Bilguvar et al, 2010; Wieck et al, 2005; Yu et al, 2010) **Figure 9, Figure 12d**), as a part of multiple congenital syndromes associated with megalencephaly: MPPH (macrocephaly, polymicrogyria, polydactyly, hydrocephalus), M-CMTC (macrocephaly cutis marmorata telangiectasia congenita), and MCAP (macrocephaly capillary malformation)(Conway et al, 2007; Mirzaa et al, 2004; Tore et al, 2009); or as a feature of specific syndromes like Aicardi syndrome (Aicardi, 2005); Delleman syndrome (Moog et al, 2005; Pascual-Castroviejo et al, 2005); DiGeorge syndrome (Robin et al, 2006) and Warburg Micro syndrome (Graham et al, 2004; Warburg et al, 1993); D-bifunctionalproteindeficiency (Ferdinandusse et al, 2006a; Ferdinandusse et al, 2006b).

Polymicrogyria could have a genetic origin (**Figure 11**) or be the consequence of insults during embryogenesis such as hipoxia, hypoperfusion and congenital infections (eg. Cytomegalovirus)(Jacobs et al, 1999a; Jacobs et al, 1999b; Squier & Jansen, 2014). Due to the heterogenity among this group and the little understanding of the different patterns and molecular causes underlying this pathology, Barkovich *et al.* consider four groups based on phenotypic appearance and suggested etiology: A, schizencephalic clefts or calcifications, due to infection or vascular cases; B, without clefts or calcifications, genetic origin; C, feature inside a

multiple congenital syndrome; D, in conjunction with inborn errors of metabolism. Moreover, different grades of severity could be distinguished, from lower to upper severity (1-4) (Squier & Jansen, 2014).

Considering the different examples of polymicrogyric patients in the literature, different mechanisms giving rise to this phenotype have been proposed (Squier & Jansen, 2014):

a. Disorders of the brain surface: altering leptomeningeal cells, basement membrane and anchoring of radial glial cells
b. Abnormal surface fusion
c. Premature cortical folding, starting before the normal gyrification process occurs
d. Temporospatial patterning: incomplete migration or loss of signalling functions of leptomeninges
e. Physical and mechanical constraints: surface abnormalities and increased collagen deposition alter mechanical properties and increase stiffness of the brain surface, disrupting the normal properties for an appropiate folding
f. Vascular impairment could be underlying the formation of microgyri(Robin et al, 2006).

It is important to point out that some authors consider the disruption of pial membrane and the consequent gaps as polymicrogyria (Jaglin & Chelly, 2009), but others consider that these cases are better considered as cobblestone malformations (Jansen & Andermann, 2005; Judkins et al, 2011; Leventer et al, 2010).

Among the multiple genetical causes that could give rise to polymicrogyria (**Figure 11**), the PI3K AKT-mTOR pathway acquired relevance in the last years. As mentioned before, polymicrogyria has been found as a feature in MCAP and MPPH syndromes, which are overtgrowth disorders associated with a dysfunction of the mTOR pathway. Both pathologies share features of megalencephaly, growth dysregulation with asymmetry, cortical malformation and mild connective tissue dysplasia. However, MCAP patients also have aberrant vascularity and syndactyly (Gripp et al, 2009; Mirzaa et al, 2012). The mutated genes found mutated associated with these pathologies are regulatory elements in different levels of the pathway: PTEN, PIK3CA and PIK3R2 (upstream), AKT3, TSC1, TSC2 (central) and CCND2 (downstream).

4.3. Group III. Ectopia

As commented in previous sections, when neurons are generated, they must migrate through the cortical thickness to arrive to their position in the cerebral cortex, which come defined by its time and place of generation. Then, disturbances in this process could lead to the appearance of cells in ectopic positions (**Figure 13**).

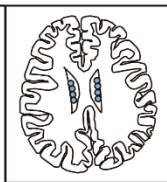
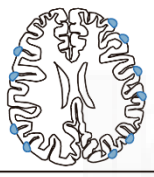
Ectopias		Molecular mechanism	Genes	References
PVNH		Neuronal migration, radial glial scaffold	FlnA	A
		Vesicles transport, synapse	ARFGEF2, TMTC3/SMILE	B
		Neuronal migration	C6orf70/ERMARD	C
		Molecular adhesion	FAT4, DCHS1	D
		Ubiquitination, mTOR pathway	NEDD4L	E
		Unknown	Deletions of: 5q14.3; 7q11;6q-qter monosomy 1p36 duplications of: 5p15;2p16.1p23,,	F
SBH		Neuronal migration, cortical lamination	DCX, LIS1, TUBA1A, TUBG1, EML1	G
Cobblestone		Pial surface stability	POMT1, POMT2, FKTN, FKRP, LARGE, POMGNT1, LAMB1	H
		Vesicle transport, synapse	TMTC3/SMILE	I

Figure 13. Human malformations by the aberrantly located cells (ectopias). Genes found mutated in patients with periventricular nodular heterotopia (PVNH), subcortical band heterotopia (SBH) and cobblestone (lissencephaly type II) and molecular mechanisms in which they are involved. References are group as followed: A: (Ferland et al, 2009; Fox et al, 1998; Parrini et al, 2006; Sheen et al, 2001). B: (Farhan et al, 2017; Ferland et al, 2009; Sheen et al, 2004b). C: (Conti et al, 2013). D:(Cappello et al, 2013). E: (Broix et al, 2016). F: (Cardoso et al, 2009; Descartes et al, 2011; Ferland et al, 2006; Neal et al, 2006; Saito et al, 2008; Sheen et al, 2003). G: (Francis et al, 1999; Gleeson et al, 1998; Keays et al, 2007; Kielar et al, 2014; Mineyko et al, 2010; Sicca et al, 2003a; Sicca et al, 2003b). H: (Beltran-Valero de Bernabe et al, 2002; Brockington et al, 2001; Longman et al, 2003; Roscioli et al, 2012; van Reeuwijk et al, 2005; van Reeuwijk et al, 2006; Willer et al, 2012; Yoshida et al, 2001). I: (Farhan et al, 2017).

4.3.1. Periventricular Nodular Heterotopia

Periventricular nodular heterotopia (PVNH) is one of the most common cortical malformation in patients with epilepsy (Gonzalez et al, 2013). It is a condition characterized by the ectopic accumulation of cells in the vicinity of the telencephalic ventricles forming nodules, that act as epileptic foci (Fallil et al, 2015; Fox et al, 1998; Huttenlocher et al, 1994; Parrini et al, 2006). This phenotype has been associated to a defect in the migration of cortical neurons, which, due to altered cytoskeleton remodeling, vesicle trafficking, molecular adhesion and other processes, are unable to follow radial migration (Andrade, 2009; Guerrini, 2005; Parrini et al, 2006). It was also been proposed that the formation of excessive neurons are not able to migrate properly or

even the alteration of radial glial cell proliferation and scaffold defect could be altering neuronal migration (Carabalona et al, 2012; Eksioglu et al, 1996).

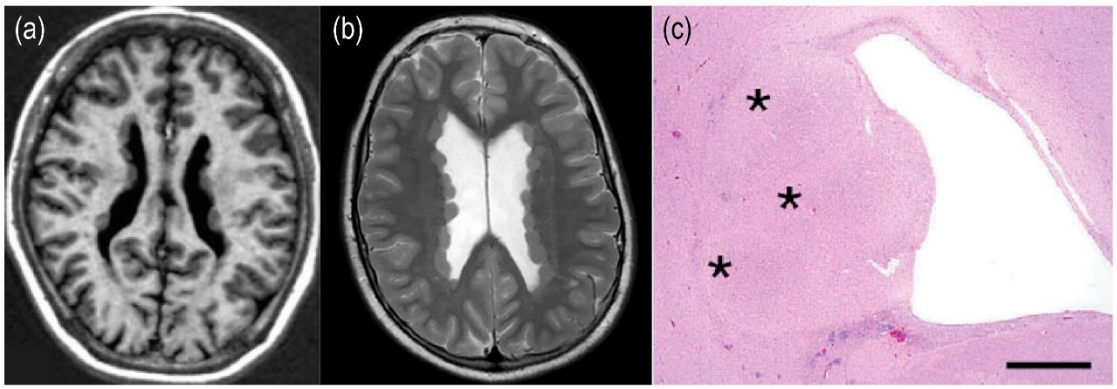


Figure 14. Periventricular nodular heterotopia. (a, b) MRI from patients with PVNH, showing a different distribution of periventricular nodules. (c) Histologic appearance of nodules (asterisks). Adapted (Ferland et al, 2009; Kakita et al, 2002; Lange et al, 2015).

These nodules consist of a combination of some glial cells (astrocytes and oligodendroglia) and neurons, which show a normal morphology but with multiple orientations (Eksioglu et al, 1996). Ectopias could present a wide variability in their location and conformation: bilateral, unilateral, laminar, sub-ependymal, and subcortical white matter (Andrade, 2009; Ferland et al, 2009) (**Figure 14**). Radial glial scaffold is disupted around the heteorotopic nodules, which are surrounded by astrocytes (Santi & Golden, 2001). Ectopic neurons are not isolated from the cortex, but on the contrary, seizures can be originated either from the ectopic nodules or from the normocortex in interaction with them (Battaglia et al, 2006; Battaglia et al, 2005). Also, functional imaging demonstrated the heterotopia integration in motor circuits (Lange et al, 2004). Moreover, they are richly innervated, although the origin of the synaptic input is unknown and exist fibers originated in the nodules going through the whitte matter to reach the overlying cortex (Eksioglu et al, 1996).

Types of epilepsy associated to PVNH include temporal lobe epilepsy, generalized tonic-clonic epiplepsy and mixed seizure (Eksioglu et al, 1996) and absence epilepsy (de Wit et al, 2010). Also, some asymptomatic cases have been described(Huttenlocher et al, 1994; Kamuro & Tenokuchi, 1993). Due to the variability in the clinical manifestation and the phenotype outcome, several studies tried to find correlations using many variables in different cohort of patients as the location of the ectopia, the number of nodules, their volumen, gender of the patient, developmental delay, age at seizure onset, epilepsy duration, ventricular and cerebelar abnormalities and laterality of the seizure onset (Battaglia et al, 2005; Fallil et al, 2015; Lange et al, 2004). In these studies, they conclude that the epilepsy outcome was dependent on the location and distribution of PVNH but its extent does not correlate with seizure phenotype (Battaglia et al, 2006). Also, Fallil et al. found a correlation between high heterotopia burden

with female gender and trigonal location. However, total heterotopia volumen did not reveal any correlation with any of the other criteria (Fallil et al, 2015).

Although the majority of PVNH cases present the nodules as a unique feature, this cortical malformation could appear as a part of complex syndromes (eg. Ehlers-Danlos); (Bernstein et al, 2011; Hehr et al, 2006; Lord et al, 2014; Oegema et al, 2013; Reinstein et al, 2013; Robertson, 2005) and other congenital malformations, such as microcephaly (Parrini et al, 2006; Sheen et al, 2003), ventriculomegaly, cerebelar abnormalities or abnormalities of the corpus callosum (Fallil et al, 2015).

There is a huge variability among patients in terms of intelligence outcome, who could present from normal to borderline levels, while others have seriuos developmental delay (particularly those related with *ARFGEF2* mutations) or even dyslexia (Chang et al, 2007). Additionally, cardiac, skeletal (oto-palato-digital syndrome, OPD spectrum skeletal phenotypes (Bernstein et al, 2011; Hehr et al, 2006; Lord et al, 2014; Oegema et al, 2013; Reinstein et al, 2013; Robertson, 2005; Robertson et al, 2003) or even respiratory deficiencies could also be found in some of these patients, particularly in those related with mutations in FlnA (Berg et al, 2010).

PVNH can have an external origin as irradiation (Ferrer, 1993), infection (Sheen et al, 2004a) and injury (Montenegro et al, 2002); but more frequently, it is related with genetical mutations. Different genes have been associated with PVNH, being the most frequent those in *FLNA* and *ARFGEF2* (Ferland et al, 2009; Fox et al, 1998; Parrini et al, 2006; Sheen et al, 2004a; Sheen et al, 2001).

FLNA gene encondes for X-linked Filamin A, a 280-kD an actin-binding phosphoprotein that regulates the reorganization of the actin cytoskeleton in ortogonal networks in the cytoplasm. Moreover, it has a wide spectrum of interacting proteins such as integrins, transmembrane proteins, receptors and second messengers. All these communications mediate the cellular response in terms of shape and motility (Fox et al, 1998) . As cytoskeleton is crucial for these processes, the difficulties in regulation of cytoskeleton remodeling has been proposed as causative for this malformation in patients carrying FlnA mutations (Fox et al, 1998; Maestrini et al, 1993). The majority of patients with this malformation are female with bilateral periventricular heterotopia (BPVNH, also known as classical). Most males carrying these mutations die prematurly, being survival cases rare which may present mental retardation and additional congenital anomalies (Parrini et al, 2006; Sheen et al, 2001).

ARFGEF2 (adenosine diphosphate-ribosylation factor guanine exchange factor 2) encodes brefeldin inhibited GEF2 (BIG2), a protein implicated in the guanine diphosphate (GDP) to guanine triphosphate (GTP) exchange for ADP ribosylation proteins (ARPs). These groups of proteins are part of the trans-Golgi network and are involved in the intracelular membrane and

vesicular trafficking of adhesion molecules, which is necessary for neuronal precursors departure from the ventricular zone (Sheen et al, 1999). Missense and truncation mutations in *ARFGEF2* are responsible for an autosomal recessive form of PVNH combined with microcephaly and severe developmental delay. Although their functions appear to be very different, similarities in pattern of expression and radiographic features of the nodules in both cases, suggest that ARFGEF2 and FLNA share a common mechanism (Ferland et al, 2009; Sheen et al, 2004b). Deregulation of vesicle trafficking as a consequence of alterations of the cytoskeleton machinery could affect cell adhesion and adherent junctions, which, eventually, could give rise to the periventricular nodules (Ferland et al, 2009).

4.3.2. Subcortical Band Heterotopia

Subcortical band heterotopia is a malformation consisting of the accumulation of neurons in the cortical white matter (Barkovich et al, 2001; Ross & Walsh, 2001) forming a thick band of cells below a normal cortical gray matter (Francis et al, 1999; Gleeson et al, 1998). This double cortex results also in a reduction in the cerebral cortex size due to the loss of neurons from the normocortex, affecting cortical surface area and thickness. Eventually, it could cause microgyria (Barkovich et al, 2012). Generally, this malformation has been linked to mutations in genes related or interacting with the cytoskeleton (**Figure 13**).

4.3.3. Cobblestone/Lissencephaly type II

Contrary to what it is described in most cases of heterotopia, cobblestone (type II lissencephaly) is not due to a deficient neuronal migration but to an excess of this process. In normal conditions, cortical basement membrane and the RGCs attachment to it is the finish line for radially migrating neurons, reason why, when this pial surface is disrupted, overmigration of cortical neurons occurs (Luo et al, 2011b; Yamamoto et al, 2004). These cells continue their forward movement to the meningeal space, thus resembling cobblestones on the cortical surface (van Reeuwijk et al, 2005; van Reeuwijk et al, 2006). In consequence, mutations in genes involved in the attachment of the radial glial fiber to the pial surface (Li et al, 2008; Luo et al, 2011a) or associated with reduced glycosylation of Alpha dystroglycan, fundamental to anchor the dystrophin complex to the extracelular matrix (Buysse et al, 2013; Roscioli et al, 2012; van Reeuwijk et al, 2005) have been associated with this malformation. In addition, several complex syndromes also present cobblestone lissencephaly: Fukuyama congenital muscular dystrophy (FCMD), muscle-eye-brain disease (MEB), and Walker-Warburg syndrome (WWS).

5. PIK3R2

5.1. The PI3K-AKT mTOR pathway

The mammalian target of rapamycin (mTOR) pathway is a complex combination of proteins that integrates multiple intracelular and extracelular signals controlling cell metabolism, proliferation, cell growth and survival (LiCausi & Hartman, 2018). mTOR, a serine-threonine kinase of PI3K-related kinase family, is higly conserved among species and is part of two complexes, mTORC1 and mTORC2. mTORC1 is composed by five proteins (mTOR, Raptor, MLST8, PRAS40 and Deptor) while mTORC2 contains six (mTOR, Rictor, Protor-1; mLST8, mSIN1 and Deptor). In both complexes, mTOR is the catalytic subunit, being the other regulators and estabilizators of the complex. These complexes also differ in the processes in which they are implied (Peterson et al, 2009). A visual schema of the multiple participants in this pathway is found below (**Figure 15**).

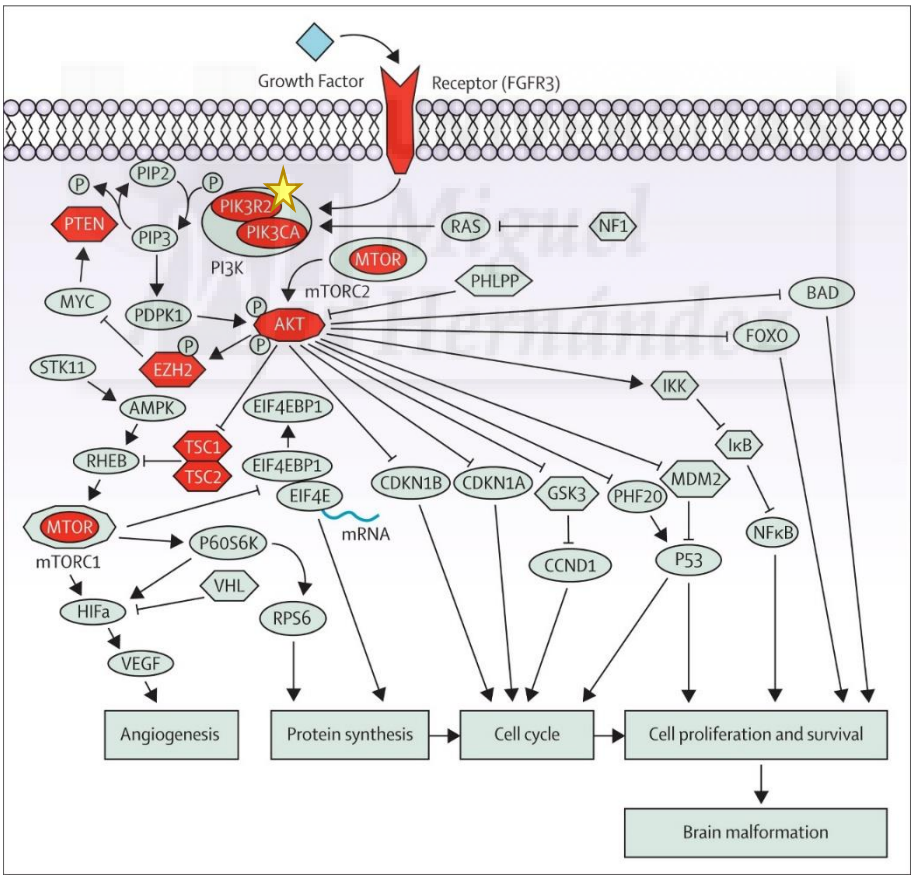


Figure 15. mTOR pathway and its main players. The complex cascade depending on mTOR interactors influence angiogenesis, protein synthesis, cell cycle, cell proliferation and cell survival. PIK3R2 is depicted with a yellow star (Adapted from (Mirzaa et al, 2013).

mTORC1 is mainly a regulator of cellular growth and metabolism through the promotion of mitocondrial metabolism and biogenesis, synthesis of proteins (through 4E binding protein 1 and p70 ribosomal S6 kinase1), lipids and organelles and the limitation of autophagy. For impulsing these processes, mTORC1 senses different signals: growth factors (through the activation of the canonical insulin and Ras signaling pathways), energy status (AMP-activated protein kinase, AMPK levels), oxygen and amino acids. In addition, other changes in cellular conditions as genotoxic stress or inflammation have an effect in mTOR activity.

mTORC2 is less known than mTORC1, but it has a crucial implication in cell survival, metabolism and proliferation through AKT phosphorilation, that promotes these processes, (Sarbassov et al, 2005a; Sarbassov et al, 2005b) reviewed by (Manning & Cantley, 2003). Inhibition of AKT produces the activation of forkhead box protein (FoxO1) and FoxO3a, that regulate the gene expresion of elements of stress, metabolism, cell-cycle retention and apoptosis (reviewed by (Calnan & Brunet, 2008)). Moreover, mTORC2 inhibition alters actin polymerization and, in consequence, cellular morphology (Jacinto et al, 2004; Sarbassov et al, 2004). It has been suggested that this control goes through protein kinase Cα (PKCα), which phosphorylates paxilin, relocating it to focal adhesions, and the GTP loading of RhoA and Rac1 (Laplante & Sabatini, 2009).

In mTOR pathway, PI3Ks are implicated in signal transduction. They are classified in three cathegories (I-III) depending on their sequence and their preferent substrate. Class I is subdivided in two families according to the receptors to which they interact. Whereas Class IA PI3K are activated by growth factor receptor tyrosine kinases (RTKs), class IB is associated with G-protein-coupled receptors (GPCRs).

Class IA are heterodimers form of p85 (PIK3R2, regulatory subunit) and p110 (PIK3CA, catalytic subunit), both presenting diverse isoforms. The p85 isoforms share a core structure which binds the catalytic subunit (inter-SH2 domain), flanked by two Src-homology 2 (SH2) domains. In the same way, p110 family count with a p85-binding domain, among others that mediate its activation. Moreover, some isoforms, such as p110β, could be regulated, apart from p85, for subunits of heterotrimeric G proteins, which suggest an integration of signals from GPCRs and RTKs. On the contrary, Class IB do not present p85, reason why is not regulated by RTKs and are activated only by GPCRs, although they have another regulatory subunit (p101). It is important to point out that class I PI3K are involved in glucose homeostasis, cell migration, growth and proliferation mediated by growth factors (Engelman et al, 2006).

PIK3R2, the regulatory subunit of PI3KCA, is a 728aa lenght (in human, 722aa in mouse) which sequence is highly conserved. It is expressed in the nucleus and in the cytosol and it has four domains: SH3, Rho-GAP and two Src-homology domains 2 (SH2). SH3 function is not well defined, although it has been proposed that it mediates the assembly of protein complexes using

proline-rich peptides. Rho-GAP is an GTPase-activating proteins domain. And SH2 acts as regulatory modules of intracelular signaling. They interact with high affinity to phosphotyrosine containing target peptides in a phosphorylation-dependent manner.

Relatively little is known about class II and class III functions. Class II PI3K only have a catalytic subunit similar to p110 of the other clases, which phosphorylate PIP and PIP4. They are activated by RTKs, cytokine receptors and integrines. Moreover, they have been suggested to have a role in membrane trafficking since they bind clathrin and are in coated pits. Class III, in yeast, has been identified as an interactor of mTOR in response to amino-acid availability, which suggest a possible implication in controlling cell growth (Byfield et al, 2005; Nobukuni et al, 2005).

5.2. Mutations in PI3K-AKT-mTOR pathway

Mutations of genes within the phosphatidylinositol-3-kinase (PI3K)-AKT-mTOR pathway are known to cause a wide range of developmental brain and body disorders, also known as mTORopathies (Szepetowski, 2018). Mutations of PIK3CA, PIK3R2, PTEN, AKT3, CCND2, TSC1 and TSC2 have been associated with focal, segmental (multifocal), or generalized megalencephaly with variable other features (Lee et al, 2012; Mirzaa et al, 2014; Poduri et al, 2012; Poduri et al, 2013; Riviere et al, 2012). More concretely, class IA PI3K, PIK3CA (p110α) and PIK3R2 (p85β) with MCAP and MPPH, respectevely (Cheung et al, 2011; Engelman et al, 2006) (**Table 1**).

Focusing on PIK3R2, patients with mutations in this gene present bilateral perisylvian polymicrogyria (BPP) in presence or absence of megalencephaly, in addition to other features such as ventriculomegaly, cutaneous capillary malformations, oromotor weakness (eg. language or speech delay), intelectual disability and epilepsy. Although no association with specific epilepsy syndromes was apparent, patients with constitutional mutations show an earlier age onset and higher severity than those with mosaic ones. Moreover, the polymicrogyria is only affecting the perisylvian area, with different severity depending on the region and extension involved. Normally, it is present bilaterally and mildy asymmetric in most individuals (Mirzaa et al, 2015; Mirzaa et al, 2013).

Table 1. Mutations in PI3K-AKT-mTOR pathway genes. The main mutations found in genes implied in PI3K-AKT-mTOR cascade regarding AKT3, PIK3R2 and PIK3CA, modifications caused by the mutations, mode of inheritance and the disorder resulting from these alterations.

GENE	DNA CHANGE	AA CHANGE	INHERITANCE	DISORDER
AKT3	c.1393>T	ARG465TRP	*De novo*	MPPH/MCAP
	c.686A>G	Asn229Ser	*De novo*	MPPH
PIK3R2	c.1117G>A	Gly373Arg	*De novo,* mosaic or germline	MPPH
	c.1202T>C	Leu401Pro	*De novo*, germline	MPPH
	c.1669G>C	Asp557His	germline	
	c.1126A>G	K376E		
PIK3CA	c.241 G>A	Glu81Lys	N/A	MCAP
	c.263G>A	Arg88Gln	*De novo*	MCAP
	c.1090G>A	Gly364Arg	*De novo*	MCAP
	c.1093G>A	Glu365Lys	*De novo*	MCAP
	c.1133G>A	Cys378Tyr	*De novo*	MCAP
	c.1359_1361del	Glu453del	*De novo*	MPPH
	c.1633G>A	Glu545Lys	N/A	MCAP
	c.2176G>A	Glu726Lys	*De novo*	MCAP
	c.2740G>A	Gly914Arg	*De novo*	MCAP
	c.3062A>G	Tyr1021Cys	*De novo*	MCAP
	c.3073A>G	Thr1025Ala	*De novo*	MCAP
	c.3104C>T	Ala1035Val	*De novo*	MCAP
	c.3129G>T	Met1043Ille	*De novo*	MCAP
	c.3139C>T	His1047Tyr	*De novo*	MCAP
	c.3145G>A	Gly1049Ser	*De novo*	MCAP

Four different mutations have been described in PIK3R2 associated to these pathologies, including *de novo* constitutitional mutations, germline mosaic mutations and somatic mutations (Terrone et al, 2016), **Figure 16**): Three out of four (G373R, K376E and L401P) are located in the SH2 domain, indicating the relevance of the domain in this disorder ((Liu et al, 2006; Nakamura et al, 2014; Riviere et al, 2012). In this site occurs the binding to phospho-tyrosine residues on activated RTKs adaptor molecules (e.g. IRS1), recruiting the p85-p110 heterodimer to its substrate and promoting the activation of the PI3K-AKT pathway (Cantley, 2002).

The other mutation described, c1669G>C (D557H) on the contrary, is located between the two subunits of the kinase. This residue, in combination with N561, generate H-bonds with the side-chain nitrogen atom of the N345 residue of PIK3CA. Although it is predicted that this variant would fit and that the loss of negative charge would not disturb the interaction, it has been speculated that could result in a smaller groovee and altered hydrogen bonds with an adaptor molecule (Terrone et al, 2016). Patients harbouring this mutation present, besides MPPH, FCD, an alteration also observed in other megalencephaly cases, illustrating the overlapping between PI3K-AKT-mTOR pathway pathologies.

Among the mutations in PIK3R2 mentioned, G373R has been found as the most recurrent, which is consistent with the fact of being a CpG position. Levels of mosaicism partly explains the variability in severity, but it is not known why perisylvian region is predominantly affected. It has been suggested that perisylvian cortex is more vulnerable to disruptions depending on PIK3R2 mutations, although this could occur in a limited amount of randomly distributed cells (Mirzaa et al, 2015; Mirzaa et al, 2013).

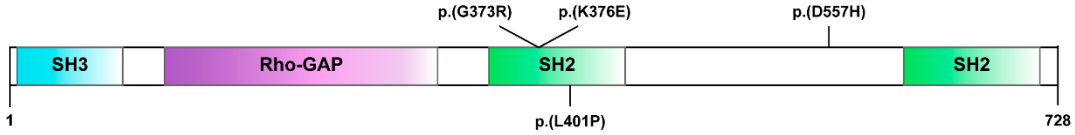


Figure 16. PIK3R2 strucutre and mutations identified in patients with polymicrogyria. Domains conforming PIK3R2 are illustrated: SH3 (blue), Rho-GAP (in purple) and two SH2 domains (in green). The four mutations identified until now as causative for polymicrogyria are indicated in their location (Adapted from (Terrone et al, 2016)).

To elucidate the implication of the mutations in these genes of the mTOR pathway, functional studies were developed in patient cells. The activation of this pathway results in a greater amount of its readout. This is the case for PIP3, S6 ribosomal protein and the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). Rivière *et al.* compare the levels of PIP3 in lymphoblastoid cell lines derived from control and patient cells, observing an increase in this product (Riviere et al, 2012). Other studies observed also a higher amount of phospho-S6 (Ser240/244) in comparation with cells from control individuals, corroborating the activation of the pathway (Negishi et al, 2017). Moreover, when cells are treated with a PI3K inhibitor (PI-103), a reduction of PIP3 is observed in mutant cell lines. These results indicate that mutation in PIK3R2 (G373R) and in PIK3CA (E453del) potentiate an elevated activity of PI3K. This is in agreement with conformational studies in cancer cells considering mutations in PIK3R2, that suggest that the catalytic subunit remain in a high-activity state, impossibiliting the inactive conformation (Cheung et al, 2011), thus, maintaining a constant activation of the pathway which eventually will result in an overgrowth of the brain and other affected tissues (Riviere et al, 2012).

5.3. Animal models

Diverse animal models have been generated to study the roles of mTOR pathway effectors during cortical development. Mainly they are developed in mouse, focusing on different genes such as *Tsc1* (Abs et al, 2013; Bateup et al, 2011; Chevere-Torres et al, 2012; Goorden et al, 2007), *Tsc2* (Auerbach et al, 2011; Ehninger et al, 2008), *PTEN* (Kwon et al, 2006; Sperow et al, 2012), *PIK3CA* (Roy et al, 2015), among others (some reviewed in (Borrie et al, 2017).

One interesting example is a model realized by Roy et al. for PIK3CA, the catalytic subunit of PI3K. In their work, they conditionally activate two common hotspot mutations in this gene (E545K and H1047R) in two developing times of mouse brain. They reproduce the brain overgrowth and the seizures observed in patients harbouring these modifications. These experiements also revealed common mechanisms affected by both mutations, although with different severity in the terms of cell proliferation, cell cycle exit and neuronal migration, with the consequent alteration of cortical lamination (Roy et al, 2015).

In the case of PIK3R2, a conditional mouse was generated affecting T cells, in which Oak et al. model the autoimmune syndrome called Sjogren. Although this study exhibits the relevance of class IA PI3K signaling in immune response, there is not a model for studying its role in brain development (Oak et al, 2006).

6. FLNA

6.1. FLNA, a member of a family

Filamins are highly conserved proteins that, by crosslinking F-actin proteins, organize the actin cytoskeleton and link it to the extracelular matrix, detecting and impulsing response to the forces and changes of the environment. In vertebrates, they conform a family of three members, large homodimers (240-280 kDa) that join by the carboxy termini: FlnA, FlnB and FlnC. In humans and mice, *FlnA* gene is located in X chromosome, while *FlnB* and *FLnC* are located in ausotomal chromosomes (3 and 7, in humans; 14 and 6 in mice, respectevely). They have both common and different aspects in terms of functionality and structure (Feng & Walsh, 2004a; Nakamura et al, 2007).

The different filamins in vertebrates share basically the same structure: an actin-binding domain (ABD) located in the N-terminal, 24 repetitions of IgFLN with two hinge regions located between the IgFLN15 and 16 (H1) and the IgFLNA23 and 24 (H2), before the last IgFLNA repetition in the C-terminal. Moreover, FlnC present an exclusive region of 82 aminoacids between IgFln19-20. In other groups of animals filamins are also present although the number of IgFln repetitions is different ((Zhou et al, 2010), **Figure 17**).

The ABD contains two calponin homology domains (CH1 and CH2) formed by three actin-binding sites (two and one, respectevely) and mediate the actin-binding properties of multiple proteins such as dystrophin, utrophin, Alpha-actinin and b-spectin. Respect to hinge units, they confer flexibility to the whole structure, allowing its dimerization in Y-shape, and divide filamin in two domains: rod domain 1 (from 1-15) and rod domain 2 (from 16-23). Interestingly, while H2 is in the three filamin isoforms, H1 is lacking in the chicken's and in some variants of FlnB and FlnC (Nakamura et al, 2007; Nakamura et al, 2011). These regions have been proposed as possible domain of filamins regulation. Regarding the Fln subunits, 1-8 flexibility allow 9 to 15, which present a F-actin binding domain crucial for F-actin migration, the proper alignment of the actin monomers. From 16-24 do not link F-actin, making it available for interactions with other proteins. Last filamin repeat (IgFln24) is the dimerization domain, were two monomers of filamin interact forming a homodimer (Nakamura et al, 2007).

Based on the similar structure and the overlapping expression in some areas, the formation of the heterodimers between different types of filamins has been proposed (*sheen2002*). Some studies have shown the interaction between FlnA and FlnB and transient heterodimerization of FlnA-FlnC in myofibrils. However, other studies show that just FlnB and C formed heterodimers *in vitro*. Interestingly, if the heterodimerization could occur *in vivo*, this could explain a partially compensation process in mutants of any filamin (Himmel et al, 2003; Zhou et al, 2010).

6.2. Expression

In terms of expression, it is important to point out that FlnA and FlnB are ubiquitously expressed, including vasculature, while FlnC is restricted to cardiac and skeletal muscles, with some exceptions. So that, only FlnA and FlnB are express in the central nervous system (Stossel et al, 2001).

Focusing on the cortex, *FlnA* and *FlnB* present some differences and some overlapping in the expression pattern during mouse development. At early stages (E12.5), both *FlnA* and *FlnB* are expressed in the germinal layers (VZ, SVZ). Later, at midgestation and late neurogenesis, *FlnA* is found across the cortex, including the CP, while *FlnB* is expressed in the periventricular region and forebrain. Moreover, protein levels are higher in neuronal precursons and postmitotic migrating neurons, although they have *FlnA* expression in their final position. The wide distributed expression of *FlnA* in comparation with the restricted expression of *FlnB* suggest that *FlnA* is implied in a greater number of processes during corticogenesis, being the function of *FlnB* more restricted. *FlnA* expression is finely regulated temporally and spacially during corticogenesis. It has been suggested that neuronal migration rate depends on the proportion of FlnA:actin (LoTurco & Bai, 2006). Interestingly, expression of both isoforms decreases considerably in postnatal stages, implying the relevance of these proteins during cortical development (Sheen et al, 2002).

Filamins are mainly cytoplasmatic. They colocalize with F-actin in the stress fibers and in the leading edge. Additionally, a little fraction of full-lenght FlnA and its C-terminal fragment is found in the nucleus, where it interacts with BRCA2 and androgen receptor, which suggest a possible role in DNA damage response and transcriptional regulation by the distribution of transcription factors in the cytoplasm or in the nucleus (Feng & Walsh, 2004a; Nakamura et al, 2007; Zhou et al, 2010).

6.3. FLNA interactors and related cellular processes

Besides their function in organizating actin filaments. Filamins family interacts with more than one hundred partners, which points out the relevance of these proteins in a wide number of processes and in the normal cellular functioning (**Figure 17**). The majority of filamin interactions are through IgFLN domains, particularly on rod domain 2. The wide spectrum of partners that interact with FlnA implies its participation in multiple processes that eventually could be affecting cortical development in several ways (Modarres & Mofradt, 2014; Robertson, 2005; Zhou et al, 2010).

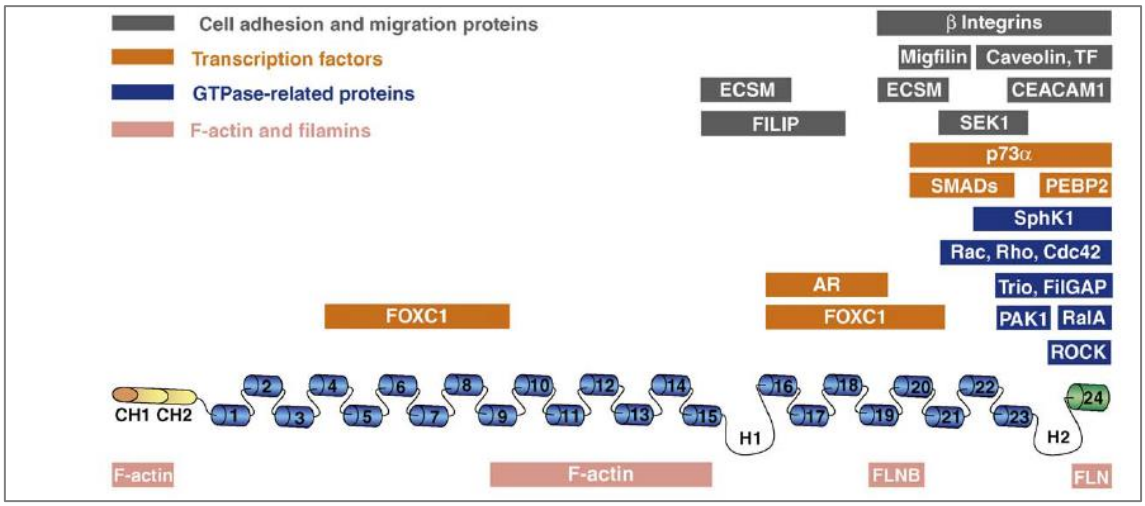


Figure 17. Monomeric structure of FlnA and its main interactors. FlnA structure consist on an actin binding domain (two calponin-homology domains (CH1 and CH2)), followed by 24 FlnA repeats interrupted by two hinge structures (H1 and H2) that confer flexibility to the protein. The majority of proteins interact with the C-terminus (From (Zhou et al, 2010)).

FlnA establishes a mechanical link between ECM, actin cytoskeleton and the plasma membrane through transmembrane receptors and adhesion molecules such as cadherins and integrins. These associations permit the signal transduction of growth factors and secreted products. Interestingly, alteration in these proteins, as an elevated interaction between FlnA and b1-integrin, have been shown to produce abnormalities in neuronal migration (Calderwood et al, 2001; Pfaff et al, 1998).

Interaction of FlnA with integrins activates small GTPases, that modulate the actin polymerization. Some of them are Rho, Cdc42, Rac and RalA. FlnA also could control this process by its relationship with both upstream or downstream effectors as Pak1 or ROCK (Cappello, 2013; Ohta et al, 1999). Importantly, all these factors and interactions must be well equilibrated for a proper control of the cellular motility and morphology.

Activity and expression of FlnA could be modulated by interacting with signaling transduction molecules such as adaptor molecules, kinases and phosphatases, due to it is a phosphoprotein. Some of them are RSK, MEKK4 and Pak1; the last two phosphorilating Ser2152, a residue associated with actin mobilization. In addition, this mechanism has been described in tumors in which this phosphorilation mediated by AKT is implicated in vascularization and in promoting breast cancer cell migration (Ravid et al, 2008).

Another role attributed to this protein is the transcription regulation. Some studies suggest that it is able to modulate it in a negative way maintaining transcription factors in the cytoplasm, as does with PEPB2-b or CBFB, for example; or on the contrary, allowing its accumulation in the nucleus, like SMAD2 and SMAD5. Additionally, its interaction with BRCA2 suggest an implication in DNA damage response (Yuan & Shen, 2001).

As mentioned in previous section, other frequent cause of PVNH is the occurrence of mutations in ARGEF2, a gene codifing for Big2 protein, implicated in vesicular trafficking, which could suggest partially shared pathway. Interestingly, FlnA colocalize with Big2 and it has been suggested that this interaction may mediate the trafficking processes since actin cytoskeleton controls the movement of vesicles (Sheen, 2014a).

Filamins Isoforms A and B have been proposed to interact by the C-terminal forming a heterodimer due to its overlapping in temporal and spatial expression as its higher sequence identity. In that case, it could be a feasible compensatory mechanism by which the phenotype of ectopic cells just affects a little proportion of cells in case of dysfunctional FlnA homodimers. However, the data obtained is contradictory (Himmel et al, 2003; Sheen et al, 2002; Zhou et al, 2010).

Proteins such as FlnA- interacting protein (FILIP) might have a central role in mediating this effect by directly regulating FlnA levels, since its expression levels are complementary to the FlnA ones. This fine regulation seems to be directly connected with the initiation of neuronal migration (Nagano et al, 2004; Nagano et al, 2002; Sato & Nagano, 2005).

6.4. Mutations in filamins

Mutations in filamins have an important impact in cellular processes and they have a different outcome depending not only in the filamin isoform affected but also in the particular modification in the resultant protein. So that, alteration of filamins could result in developmental malformations of the brain, skeleton, limbs and heart.

Human FLNA mutations are placed all along the gene (**Figure 18**), which position would affect differently a specific domain and, in consequence, the resultant phenotype. The most common disease associated with FLNA mutations is X-linked PVNH (previously described in detail) (Fox et al, 1998), which can be nonsense, missense or frameshift or missense mutation. The majority of patients are females, since males embryonic or prental death occur in FlnA-null mutated cases (Moro et al, 2002; Robertson, 2005; Sheen et al, 2001).

Classically, the accepted model of this pathology considers that FlnA deficient neurons cannot properly remodel its actin cytoskeleton, reason why the migration initiation results to be impaired. Then, these neurons would accumulate and form the characteristic nodules in the lateral ventricles (Sarkisian et al, 2008). However, clinical and experimental evidences show that this pathology emerge from a much more complex scenario for several reasons. One is the fact that, being an X-linked pathology, one would expect that, for random X-inactivation phenomenon, approximately the 50% of cells would be affected, but the proportion of cells forming these nodules is much lower (Battaglia & Granata, 2008). Second, autosomal mutations

that are not related with FlnA but still give rise to this phenotype (Parrini et al, 2006). Third the diversity in males containing FLNA mutations and PVNH. Four, mutations are distributed all along the gene and are not exclusive for the ABD, suggesting that other domains and, consequently, other processes are implicated in the formation of the nodules. And finally, the fact that the animal moddels of FlnA dysfunction did not show netiher migration defects or PVNH (Robertson, 2005).

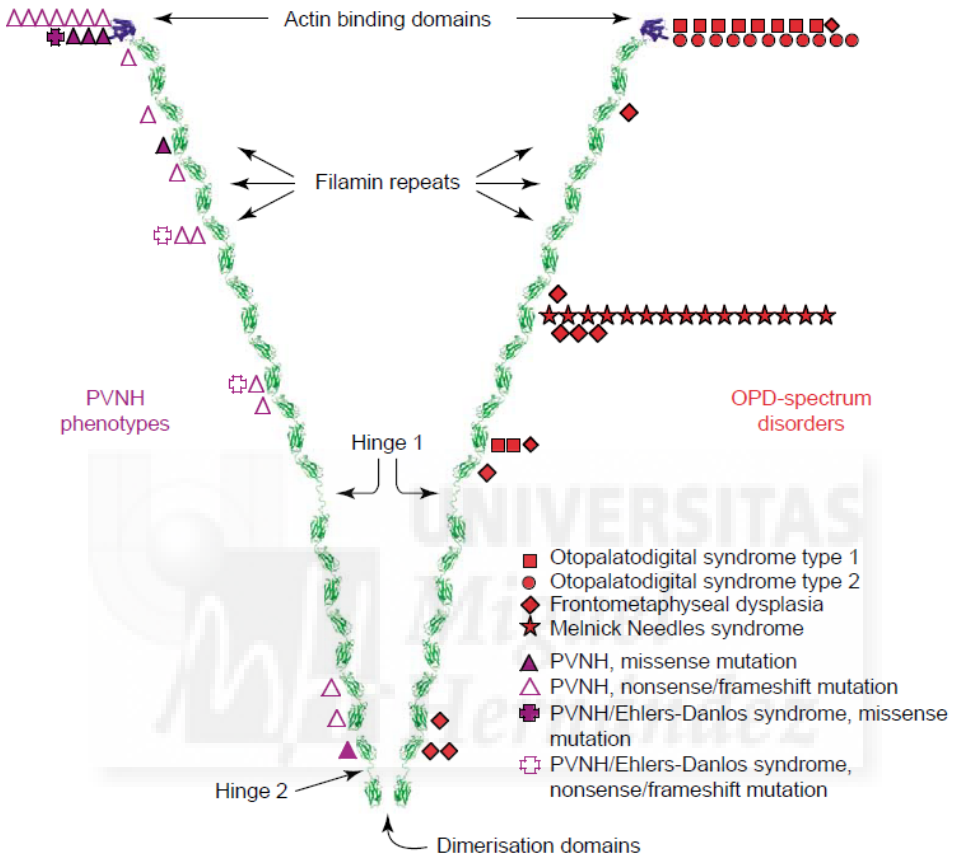


Figure 18. Model of FlnA dimerization and mutations reported. Some of the mutations identified for both periventricular nodular heterotopia and the otopalatodigital syndrome spectrum are indicated. Mutations corresponding to the OPD spectrum are all missense or small in-frame deletions (in red). Mutations linked to PVNH are mainly nonsense or frameshift and could occur all over the gene (in purple). (From (Robertson, 2005)).

Alterations of other systems are found in patients with FlnA mutations, especially cardiovascular alterations in combination with PVNH, such as Ehlers Danlos syndrome, aortic aneurysm, cardiac malformations, premature strokes (Fox et al, 1998; Kakita et al, 2002) or alone, as myoxid valvular dystrophy (XMVD)(Kyndt et al, 2007). Lethal vascular defects and intractable hemorrage has been found in males with PVNH (Feng et al, 2006; Feng & Walsh, 2004a).

Some missense mutations and in-frame deletions, different from the ones causing PVNH, with a particular location in the FlnA gene have been found otopalatodigital syndrome (OPD), frontometaphysea dysplasia (FMD) and Melnick-Needles syndrome (MNS) (Robertson et al,

2003), phenotypes that appear in combination with general dysplasia affecting craniofacial structures, digits and bones. It has been proposed that concerning mutations in CH2 have a gain-of-function effect since the the resultant protein is predicted to have a higher actin binding, while others link to OPD spectrum are not well characterized (Feng & Walsh, 2004a; Robertson, 2005). These observations suggest a distinct mechanism for the different phenotypes associated with FlnA mutations (**Table 2**).

Regarding *FLNB* mutations, all of them are vinculated to skeleton malformations, although, as happen with *FLNA*, the phenotype depends on the nature and location of the mutation. They contemplate from spondylocarpotarsal syndrome (FLNB loss), which result in short stature, severe vertebral alterations and defectuous retinal vessels (Krakow et al, 2004; Steiner et al, 2000); boomerang dysplasia (BD), characterized by hypoossification of limb bones; Larsen syndrome (LS), which presents multiple joint dislocations (Bicknell et al, 2005); and autosomal dominant-lethal skeletal dysplasias (AOI and AOIII), that results in skeletal dysplasias and vertebral abnormalities (**Table 2**;(Krakow et al, 2004)).

Finally, *FLNC* mutations only affect skeletal and muscular development due to its pattern of expression. This is the case for myofibrillar myopathy (MMEM), which mutation in the dimerization domain unable the protein to form homodimers. Consequently, patients present a progressive skeletal muscle weakness due to the great accumulation of FlnC molecules and sarcolemmal proteins in their fibers (**Table 2**; (Vorgerd et al, 2005)).

Table 2. Gene-Phenotype relationships in filamins mutations related with the system affected. Blue: brain; orange combination of brain, muscular and others; red: muscular; grey intestinal; Green: skeleton.

Gene	Phenotype	OMIM number	Inheritance	References
FLNA (Xq28)	PVNH	300049	XLD	(Fox et al, 1998)
	FG syndrome	300321	XL	(Hehr et al, 2006)
	Cardiac valvular dysplasia X-linked	314400	XLR	(Kyndt et al, 2007)
	Congenital short bowel syndrome	300048	XLR	(Gargiulo et al, 2007; van der Werf et al, 2013)
	Intestinal pseudoobstruction, neuronal	300048	XLR	
	Frontometaphyseal dysplasia 1	305620	XLR	(Robertson et al, 2003)
	Melnick-Needles syndrome	309350	XLD	(Robertson et al, 2003)
	OPD type I	311300	XLD	(Robertson, 2007)
	OPD type II	304120	XLD	(Robertson, 2007)
	Terminal osseous dysplasia	300244	XLD	(Sun et al, 2010)
FLNB (3p14.3)	Atelosteogenesis, type I	108720	AD	(Krakow et al, 2004)
	Atelosteogenesis type III	108721	AD	(Krakow et al, 2004)
	Boomerang dysplasia	112310	AD	(Bicknell et al, 2005)
	Larsen syndrome	150250	AD	(Bicknell et al, 2007)
	Spondylocarpotarsal synostosis syndrome	272460	AR	(Brunetti-Pierri et al, 2008)
FLNC (7q32.1)	Cardiomyopathy, familial hypertrophic, 26	617047	-	(Brodehl et al, 2016; Brodehl et al, 2017)
	Cardiomyopathy, familial restrictive 5	617047	AD	
	Myopathy, dystal 4	614065	AD	(Duff et al, 2011)
	Myopathy myofibrillar, 5	609524	AD	(Vorgerd et al, 2005)

6.5. Models of Filamin A dysfunction

Different models have been done in order to unravel the role of FlnA and the mechanisms by which PVNH appeared. Here we will go over the most relevant ones highlighting the main important findings that different groups have described (**Figure 19**).

6.5.1. FlnA *null mouse*

A *FlnA* null mouse model was generated using a loxP strategy in which exons from 3 to 7 were remove. In this model, some null females appear to develop normally although around the 20% died in the firs trimestre with many abnormalities affecting lungs, liver and heart. Males die embryonically (E14.5) with severe hemorraghe and vasculature defects (defectuous angiogenesis). Although F-actin distribution and migration appears normal, entothelial cells organization and adherent junctions in vascular cells are disrupted in null animals (Feng et al, 2006) (**Figure 19a**). A thinner cortical plate was observed, but no neurons were arrested in the ventricular zone. However, the disruption of cadherin and the ventricular lining could be an underlying mechanism for explaning the formation of heterotopic nodules (Feng et al, 2006). Posterior studies show that loss-of-function of FlnA produces an increment of Cdk1-b as a consequence of an impairment in its degradation. As a result, there is a delay in progression through mitosis, resulting in a longer cell cycle (Lian et al, 2012).

6.5.2. Dilp2 mouse

The *Dilp2* mouse is a model which contains a nonsense mutation in the exon 44 of *FlnA* that was induced by the mutagen N-ethyl-N-nitrosourea (ENU). Consequently, the protein lacks the last two IgFlnA repetitions that conform the dimerization domain. As occurs in FlnA null mice, not F-actin distribution nor cellular morphology and migration were affected in fibroblasts obtained from this model. Similarly, these animals also show huge hemorrhages and die prematurly. In addition, skeletal defects were registered in Dilp2 mutants such as incomplete fusion of the sternum, cleft palate, consistent with patients with OPD and MNS syndromes. However ectopic nodules are not present, neither other signs of neuronal migration defects (Hart et al, 2006).

6.5.3. Mekk4 KO mouse

Mitogen-activated protein kinases (MAPks) are signal transduction proteins that regulate cellullar responses to external inputs. They configurate an hierarchical network of kinases named MAPKs, MAP2Ks and MAP3Ks. One of the main groups of kinases, JNKs mediate cytoskeletal arrangement and cellular migration through its interaction with microtubules. Upstream of JNK is MKK4, an interactor of FLNA which activity is sensitive to growth factors, cytokines and stress. A high proportion of *Mekk4* KO mice develop ectopic nodules of postmitotic neurons invading the ventricle and a complete diruption of the ventricular linnig. These animals

present a higher expression of *FlnA* and its phosphorylation form implicated in arrangement of actin dynamics, thus, preventing FlnA cleavage or degradation and affecting actin dynamics for FlnA accumulation, and, in consequence, neuronal migration, as demonstrate other studies (Sarkisian et al, 2006). Additionally, the overexpresion of *FlnA* by *in utero* electroporation in mice corroborate that an imbalance in levels of *FlnA* has an impact in neuronal migration suggesting an implication of the interaction of MEKK4 and FLNA in the regulation of these pathways (**Figure 19b**).

6.5.4. FlnA/FlnB *KO*

As mentioned, based on the random X-inactivation, one should expect that approximately the fifty percent of cells would be defectuous in migratory capability and, therefore, being part of the ectopias. However, the percentage is much lower. Based on overlapping expression and high identity of *FlnA* and *FlnB*, a possible compensatory mechanism has been proposed to explain both the absence of nodules in *FlnA* models and for the low proportion of cells forming the nodules in patients (LoTurco & Bai, 2006; Sarkisian et al, 2008; Sheen et al, 2004a).

For that reason, Houlihan *et al.* decided to develop a combined KO model of both *FlnA* and *FlnB*, having this last in complete absence or in heterozygosis. They first observed that all the animals with double KO genotype present periventricular nodules consisting of an accumulation of neurons, mainly differentiated upper layer neurons but also some early-born neurons, and glial cells (**Figure 19c**). In the case of the $FlnA^{-/-}FlnB^{+/-}$, just the 20% present this feature. Interestingly, birthdating studies revealed that the formation of this ectopias came from an increase in regional neurogenesis instead of a failure of neuronal migration. They also observed a defecuous adherent junctions, integrity of apical surface and mislocated IPs, which divided proximal to the ventricular lining and gave rise to ectopic neurons. Moreover, this phenotype seems to be associated with an increased local angiogenesis and altered vascularity. As happens in patients, this does not affect neither the cortical thickness or the layering (Houlihan et al, 2016).

6.5.5. In utero *electroporation studies*

There are some functional works done performing *in utero* electroporation modifying the expression of a population of cells inside the whole cortex. In one of them, Nagano *et al*. show how an increase in FlnA due to a reduction of filamin interacting protein (FILIP) results in alterations in cell polarity and shape of cortical neurons, as well as when they express a FLNA lacking the actin binding domain, demonstrating their relevance in cell morphology and neuronal migration (Nagano et al, 2004) (**Figure 19d1**).

In another study realized by Carabalona *et al.* in rats, they electroporated a siRNA against *FlnA* at embryonical stages. These animals showed deficiencies in ventricular lining, radial glia fibers and neuronal migration, which eventually gave rise to periventricular and subependymal nodules. Interestingly, these animals had a greater susceptibility to inducible epileptic seizures with pentylenetetrazol (PTZ) (Carabalona et al, 2012), **Figure 19d2**). However, in their experiments, the overexpression of *FlnA* did not generate alteration in radial migration, which was in contradiction with what others found in previous models (Sarkisian et al, 2006).

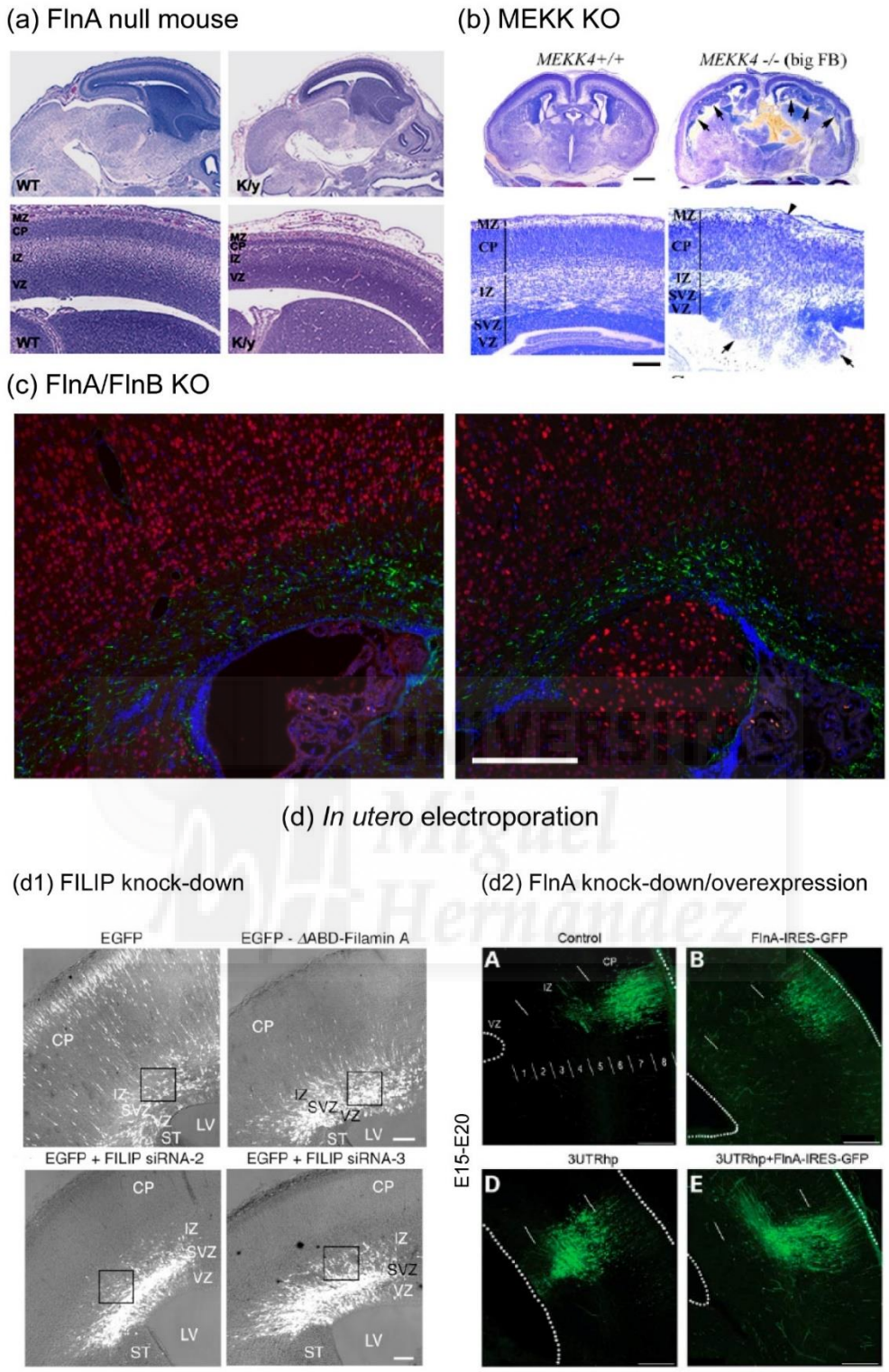


Figure 19. Different experimental models trying to elucidate FlnA role in cortical development. (a) FlnA Knock-Out model shows a reduction in the cortical plate thickness, but there are not periventricular nodules formed (Feng et al, 2006). (b) MEKK KO model developes ectopias through an indirect pathway (Sarkisian et al, 2006). (c) Doble KO for *FlnA*/*FlnB* recapitulates the heterotopias mainly formed by differentiated neurons (in red) at postnatal stages (Houlihan et al, 2016). (d) *In utero* electroporation experiments with (d1) FlnA lacking the actin binding domain and the knock-down of *FILIP* or *FlnA* (d2) (Nagano et al, 2004) produce a delay in neuronal migration (Carabalona et al, 2012).

7. EML1

Different genes have been found mutated in patients with SBH, such as *PAFAH1B1* and *TUBA1A* (please refer to ectopia malformation section, **Figure 13**), mainly encoding proteins associated with microtubules (Jaglin & Chelly, 2009). The identification of new genes and appropriate mouse models of this pathology give rise the opportunity to study the mechanism for the formation of these heterotopias of the corresponding human malformation (Kielar et al, 2014). This is the case for Eml1, a gene responsible for SBH that is mutated in a spontaneous model of SBH, the HeCo mouse (Croquelois et al, 2009; Kielar et al, 2014).

7.1. Function and expression

Eml1 (Echinoderm microtubule-associated protein-like 1) is a microtubule associated protein which specific role in cortical development has been study for the last years (Bizzotto et al, 2017; Kielar et al, 2014). This protein interacts with the MT cytoskeleton and It has a cell-cycle dependent localization, which higher expression is found in midzone areas of mitotic progenitors. Other members of its family, EML2, EML3 and EML4 has also been linked to MT regulation (Eichenmuller et al, 2002; Hamill et al, 1998; Houtman et al, 2007; Tegha-Dunghu et al, 2008; Vasquez et al, 1994).

Previous work has been shown that, in mouse, *Eml1* is expressed mostly in progenitor proliferative zones at early and mid-corticogensis and in the CP. At later stages (E17), this expression is restricted to postmitotic neurons (**Figure 20a-d**, (Kielar et al, 2014). Interestingly, in ferret, a comparative study of proliferative zones between prospective gyri and sulci reveal hundreds of DEGs, among which *Eml1* was found to be higher expressed in the splenial gyrus than in the lateral sulcus (de Juan Romero et al, 2015). Considering the higher complexity of progenitors in gyrencephalic species than in lissencephalic ones, to study Eml1 in ferret could contribute to the understanding of the pathogenic mechanisms that lead to the formation of SBH.

7.2. HeCo mouse and functional experiments

HeCo mouse (Heterotopic Cortex) is a model of SBH that exhibits a misplacement of cortical progenitors from early corticogenesis in the IZ and the CP (Croquelois et al, 2009; Kielar et al, 2014). These ectopic progenitors give rise to abnormal neuronal positioning in the white matter beneath a normotopic cortex, leading to a double-cortex appearance (**Figure 20e-l**). On the contrary, neuronal locomotion is not being altered, possibly due to a compensation mechanism of other EML isoforms (EML2, EML4, EML5). This suggest that the wrongly place of borning is the origin of these heterotopias (Kielar et al, 2014).

Remarkably, Eml1 has been found mutated in these mice. In this model, apical progenitors have a greater number of oblique mitotic spindle orientations, associated with

asymmetric inheritance of apical membrane (Kielar et al, 2014; Konno et al, 2008). Additionally, functional studies perform by *in utero* electroporation in wild type mice determine how the knocking down of Eml1 is causative for the ectopic placement of progenitors and cortical neurons (Kielar et al, 2014).

In mutant cells, mitotic spindle length, cell shape and MT dynamics of these cells are altered, as well as centrosomes and primary cilia. Although apical junction proteins are not affected in HeCo mice, it has been suggested that pathological features of the VZ apical progenitors could lead to the delamitation of some of these, causing their ectopic positioning (Bizzotto et al, 2017; Kielar et al, 2014), a mechanism also described in humans (Betizeau et al, 2013; Hansen et al, 2010).

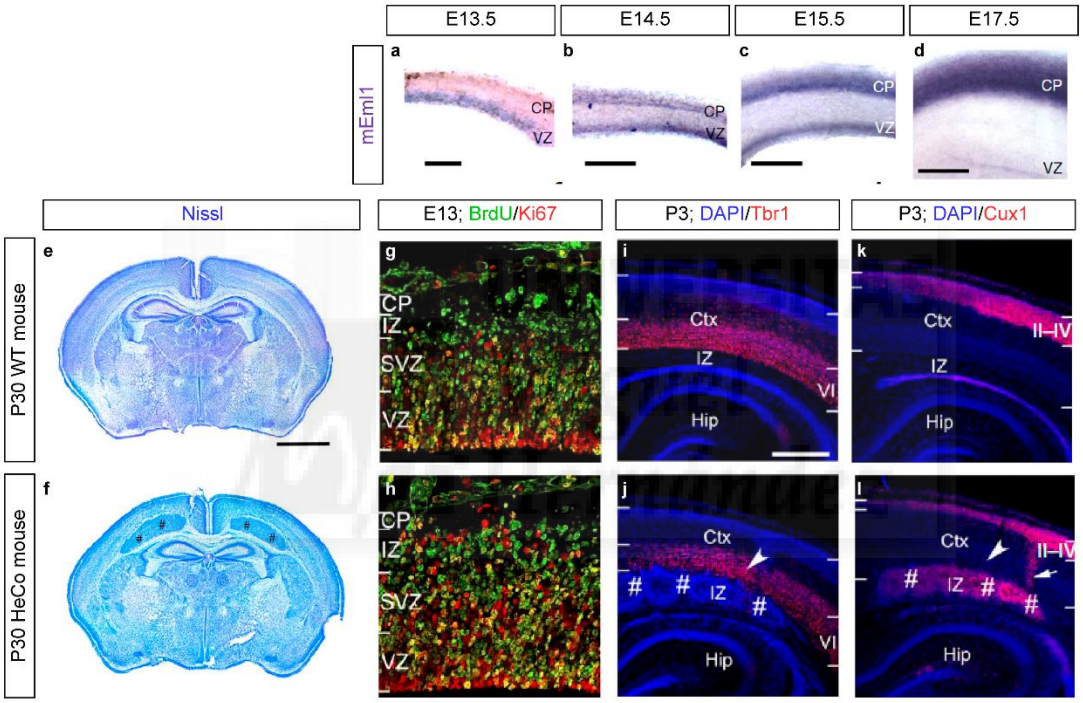


Figure 20. Eml1 expression and HeCo mouse model features. (a-d) Expression of mRNA at different time-points during mouse cortical development. Highest expression of *Eml1* is located in germinal layers at early corticogenesis (a, E13.5). At mid-corticogenesis, transcription levels of *Eml1* are also evident in the CP (b, c, E14.5 and E15.5). At later stages, it is only found in the CP (d, E17.5). (e, f) Nissl staining showing the different appearance of HeCo mouse cortex (f) compared to a WT animal (e). (g, h) Cycling progenitors are misplaced in the IZ and CP in HeCo mouse (g), something that do not appear in control animals (h). (i-l) Ectopic positioning of cortical neurons of both deeper (i, j) and upper (k, l) layers. Adapted from (Kielar et al, 2014).

7.3. Human mutations

EML1, map in 14q32, has been previously associated to oncogenic chromosomal rearrangements and telomeric deletions that lead to lissencephaly and intellectual defects (Ravnan et al, 2006; van Karnebeek et al, 2002). Later studies identified patients which mutations lead to bilateral periventricular and ribbon-like subcortical heterotopia, in presence of polymicrogyria and corpus callosum defects (Barkovich et al, 2012; Kielar et al, 2014), specially in the frontal areas. Among these, they found both heterozygous and homozygous mutations, as well as missense and nonsense modifications, that are predicted to be deleterious, damaging or benign depending on each case (Kielar et al, 2014).

Identified human missense mutations affected a highly conserved domain named HELP (Hydrophobic Echinodrm-Like Protein), which has been suggested to participate in tubulin binding (Eichenmuller et al, 2002; Richards et al, 2014; Tegha-Dunghu et al, 2008). In fact, the assotiation of Eml1 to microtubules has been demonstrated to be affected in presence of this mutations, which point out the relevance of this protein in the cytoskeleton, a major player in progenitor divisions and neuronal migration (Kielar et al, 2014).

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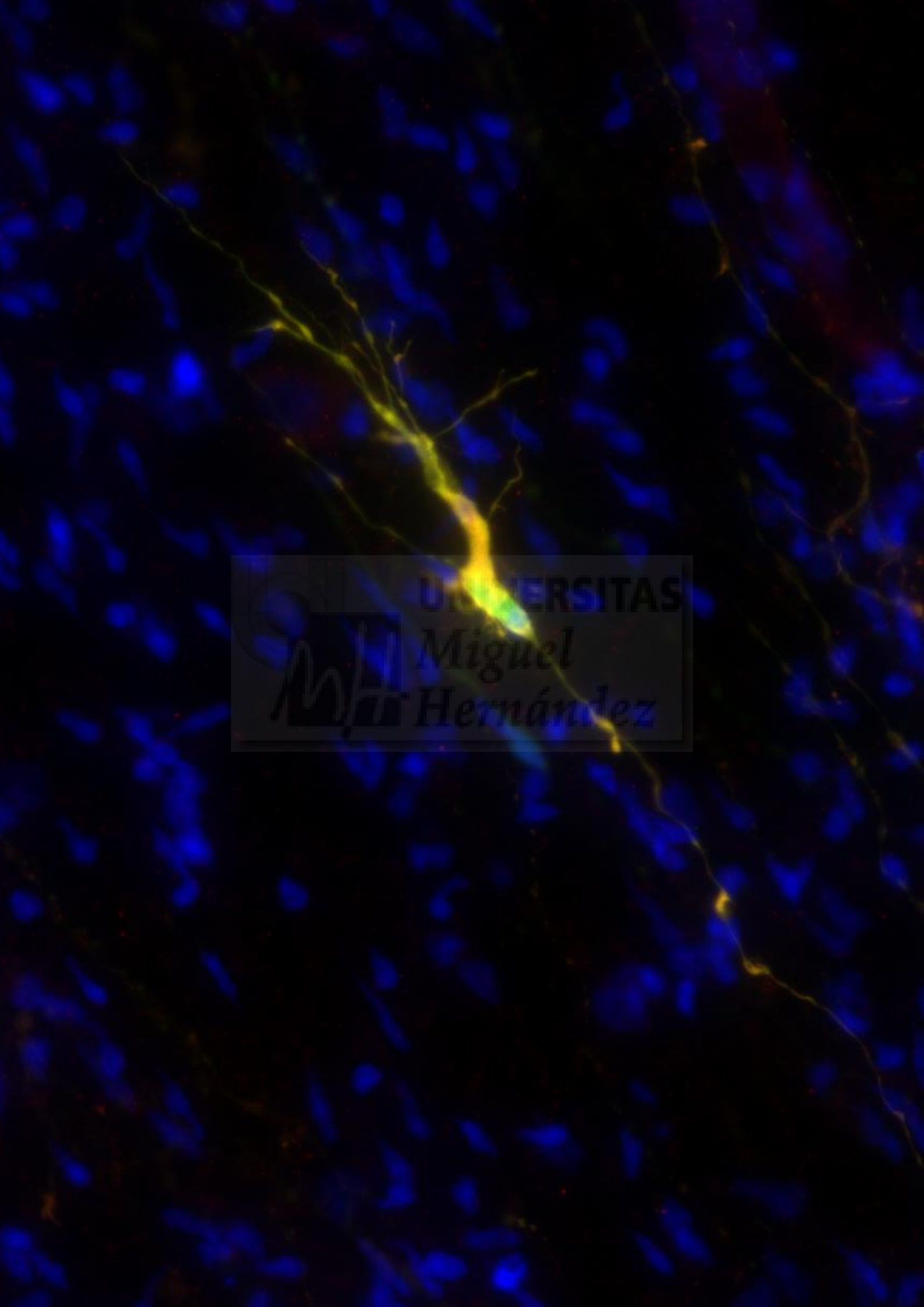

OBJECTIVES

"No va a ser fácil la misión, los monos retrasan la expedición"

Jumanji, 1995.

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The main goal of this work is to unravel the pathomechanisms that underlie some of the most common malformations of cortical development, specifically periventricular nodular heterotopia (PVNH) and polymicrogyria, due to mutations in specific causative genes: *FLNA* and *PIK3R2*, respectively. We are interested in understanding how alteration of the function of these genes impacts corticogenesis in a lissencephalic (mouse) and gyrencephalic (ferret) models. For that purpose, we defined the following specific objectives:

1. To determine the gene expression pattern of *Pik3r2* and *FlnA* during mouse and ferret cortical development.
2. To determine how manipulating the expression of *Pik3r2* and *FlnA* in mouse and ferret corticogenesis impact on progenitors and neuronal migration.
3. To describe how embryonic manipulation of *Pik3r2* and *FlnA* in mouse and ferret alters the mature cortex formation.

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MATERIAL AND METHODS

"La estadística es la ciencia que dice que, si mi vecino tiene dos coches y yo ninguno, los dos tenemos uno."

George Bernard Shaw

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MATERIAL AND METHODS

Animals

Mice

Wild type mice (*Mus musculus*) maintained in an ICR backround were used for both short (embryonic-embryonic) and long-time experiments (embryonic-postnatal). The day of vaginal plug was considered as embryonic day (E). Mice were kept at the Instituto de Neurociencias de Alicante in accordance with Spanish and European Union regulations.

Ferrets

Pigmented ferrets (*Mustela putorius furo*) were obtained from Marshall Bioresources (North Rose, New York) and Euroferret (Copenhagen, Denmark). They were maintened in a 16:8 h light: dark cycle at the Experimental Animal Service (SEA) following Spanish and European regulations. Experimental protocols were approved by the Universidad Miguel Hernández IACUC.

Constructs

For electroporations, *Gfp* was subcloned into a pCAG promoter-containing vector. Overexpression was developed using the coding sequence of rat *FlnA* (*Rattus novergicus*) clonned with IRES-*Gfp* under a CAG promoter. Knock down of *FlnA* was done using a shRNA targeting the coding sequence of both *Rattus novergicus* (nucleotides 5963-5983) and *Mus musculus* (nucleotides 6411-6430) and subcloned into an mU6pro vector. Both plasmids were a gift from Dr. Alfonso Represa and Dr. Carlos Cardoso. Loss of function of *FlnA* in ferret was performed using specific CRISPR guides against ferret *FlnA* that were designed as follows: Crispr-f*FlnA* fw2: caccgAGTAGCTCCCACTCCCGGGC Crispr-f*FlnA* rv2: aaacGCCCGGGAGTGGGAGCTACTc Crispr fw6: caccGAGTAGCTCCCACTCCCGGG Crispr rv6:. aaacCCCGGGAGTGGGAGCTACTCThey were clonned in a plasmid containing both the nuclease Cas9 and Gfp (Addgene, #458).

Related to *Pik3r2* experiments, *PIK3R2* human sequence wild type and containing the human mutation Gly373Arg (*Homo sapiens*) were clonned with IRES-*Gfp* under a CAG promoter. These constructs were a gift from Dr. Renzo Guerrini and Dr. Valerio Conti.

Related to *Eml1* experiments, a vector containing Eml1 depending on a CAG promotor was used. This was co-electroporated with pCAG-*Gfp* as a reporter. This construct was a gift from Dr. Fiona Francis.

The sharing of these plasmds for functional experiments of the three genes (*FlnA*, *Pik3r2* and *Eml1*) is inside the European Consortium DESIRE (FP7, 602531), of which this project is part of.

Mouse and ferret *in utero* electroporation

For electroporation, the constructs described previously were used.

Timed-pregant females were deeply anesthetized with isoflurane and maintened in 2% Isoflurane during the surgery. The abdominal cavity was opened, and the uterine horns were exposed. Then, DNA solution was injected into the lateral ventricle of the embryos through the uterus wall using pulled capillaries (1B120F; World Precision Instruments). Embryos were electroporated by applying electric pulses (35-45V/50ms (mouse); 80V (ferret); on/950ms off/5 pulses) with an electric stimulator (CUY21/CUY650P3 Bex C., LTD) using round electrodes (CUY650P5 (mouse), CUY650P7 (ferret); Nepa Gene). DNA was diluted in PBS and coloured with 0,5% of Fast Green (Sigma-Aldrich) with the concentration of 1 μg/μl for mice and 2 μg/μl for ferret. After appropiate survival times, mouse pregnant females were killed using cervical dislocation and embryos were fixed using PFA 4%. In case of ferret pregnant females, they were sacrified using Dolethal (Proyma Ganadera, S.L.) injections previous to the perfusion of the litter.

Ferret postnatal electroporation

For postnatal electroporation, P1.5 ferret kits were deeply anesthetized and maintained with 2% Isoflurane during surgery. Injections of DNA solution with pulled capillaries were performed in the telencephalic lateral ventricle using stereotaxic coordinates: 22,5º from bregma -0.5mm Anterio-Posterior; 2.0mm Latero-Medial; -2.0mm Dorso-Ventral. Electroporation was performed using electric stimulator (CUY21/CUY650P3 Bex C., LTD) applying electric pulses (75V/50ms on/950 off/5 pulses) using round electrodes (CUY650P7, Nepa Gene) directed to the area of interest. After the desired survival time, ferrets were sacrified with Dolethal (Proyma Ganadera, S.L.) and perfused with PFA 4%. Further details of this procedure are described in Borrell, 2010).

Inmnohistochemistry and *in situ* hybridization

Mouse embryos were obtained after cervical dislocation of the pregnant female and were perfused transcardially with paraformaldehyde 4% (PFA) in PB pH 7.3. In case of ferrets, they were injected with Dolethal previous to transcardiac perfusión with PFA 4%. Brains were then extracted and fixed at 4°C overnight.

For immunohistochemistry, brains were sectioned at 60 μm using a vibratome (VT1000S, Leica) or 50 μm on a criotom (Leica). Sections were wash several times with PBS and PBS-0,25% Triton previous to their incubation with primary antibodies overnight followed by appropriate fluorescently-conjugated secondary antibodies and counterstained with DAPI (Sigma). For anti-BrdU staining, sections were pretreated with 4N HCl for 30 minutes. Antibodies list is placed int he following table.

Primary antibody	Dilution	Host	Source
β-catenin	1:2000	Rabbit	Sigma
BrdU	1:200	Rabbit	Abcam
Cux1	1:250	Rabbit	Santa Cruz
GFP	1:2000	Chicken	Aves Labs
Ki67	1:1000	Rabbit	Abcam
NeuN	1:250	Mouse	Millipore
NeuroD2	1:400	Rabbit	Abcam
Par3	1:500	Rabbit	Millipore
Pax6	1:500	Rabbit	Millipore
Phosphohistone H3	1:1000	Rabbit	Upstate
Tbr2	1:250	Rabbit	Abcam
Sox2	1:400	Goat	R&D systems

Secondary Antibody	Dilution	Host	Source
Alexa 488 anti-chicken	1:200	Donkey	Jackson Immunoresearch
Alexa 555 anti-mouse	1:200	Donkey	Invitrogen
Alexa 555 anti-rabbit	1:200	Donkey	Invitrogen
Biotinylated anti-goat IgG	1:200	Horse	Vector Laboratories
Biotinylated anti-mouse IgG (H+L)	1:200	Goat	Vector Laboratories
Biotinylated anti-rabbit (H+L)	1:200	Goat	Vecor Laboratories
Biotinylated Fab anti-rabbit IgG	1:200	Donkey	Jackson Immunoresearch
Cy5 Streptavidine	1:200		Jackson Immunoresearch

For *in situ* hybridization (ISH), sense and anti-sense cRNA probes were synthesized and labeled with digoxigenin (DIG; Roche Diagnostics) according to the manufacter's instructions. Briefly, brain sections were hybridized with DIG-labeled cRNA probes overnight in hybridization solution (50% formamide (Ambion), 10% dextran sulfate, 0,2% tRNA (Invitrogen), 1x Denhardt solution (from 50x stock, Sigma), 1x salt solution [0,2M NaCl, 0,01 M Tris, 5mM NaH2PO4, 5 mM Na2HPO4, 5mM EDTA, pH 7,5]). After sections were washed, alkaline phosphatase.coupled anti-digoxigenin Fab fragments were applied. For visualization of the labeled cRNAs, sections were incubated in nitroblue etrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution (3,4ug/ml from NBT stock and 3,5 ug/ml from BCIP stock in reaction buffer (100 mg/ml NBT stock in 7.% dimethylformamide; 50 mg/ml BCIP stock in 100% dimethylformamide; Roche).

In situs mFlnA, fFlnA, mPik3r2, fPik3r2, hPIK3R2

For ISH probes, specific primers were used in each case. PCR was performed using Go Taq Flexi DNA polymerase (Promega), and the resulting amplicons were purfied from the gel using GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and cloned into pGEM-t Easy Vector System (Promega).

Probe	Fw	rv	
m*FlnA*	CCGAAAAAGACCTAGCAGAAGA	TGGGAAACTGAGACAGGTAGGT	Allen Brain Institute
f*FlnA*	GCTAGAAGGTGGCATCGTTG	TTGGCATTGACCGTGAAGTG	Designed
m*Pik3r2*	Probe designed by Biomatik		
f*Pik3R2*	CGAGTTCCTGGGTCCTGTAG	AGCATTTCCTCAGCCTGTGT	Designed
h*PIK3R2*	Probe designed by Genome Cube		
f*Eml1*	GGTCACTGTGATTTCTG	AAGGATACATACAAACAG	Designed, Camino de Juan

Bromodeoxyuridine labeling experiments

BrdU was diluted at 10 mg/ml in 0,9% NaCl and always administered at 50 mg/kg body weight. For cell cycle re-entry, a single intraperitoneal injection of BrdU was administrated at E15.5, one day after the electroporation. Embryos were fixed 24h later and the percentage of Ki67+ cells labeled with BrdU was determined.

Time-lapse imaging

Mouse embryos were electroporated at E14.5. At E17.5, brains were dissected out, embedded in Sea Plague Agarose 3% and vibratome sliced at 250 µm in ice-cold DMEM-F12 (Sigma) bubbled with carbogen (5% CO2, 95% O2). Slices were covered in collagen matrix (Nitta gelatin) on a filter membrane (Millipore) and cultured in DMEM-F12 (Sigma), 5% Fetal Bovine Serum (FBS), 5% Normal Horse Serum (NHS), 1:100 N2 (Invitrogen), 1:50 B27 (Invitrogen), Penicillin/Streptomycin (100u/ml), glucose (0,7g/l) and sodium bicarbonate (0,3 g/l) (Pilz et al., 2013; Shitamukai et al., 2011). Imaging was performed under a multiphoton imaging system compound by a Laser Femtosecond Mai Tai HP: sapphire modelocked laser system (SpectraPhysics, Mountain View, CA, USA), at 880 nm, a Laser Scanning Spectral Inverted Confocal Microscope Leica TCS SP2 AOBS (Leica Microsystems GmbH, Manheim, Germany). We used a 40x immersion objective and a 5% CO_2/37°C atmosphere (Martinez-Martinez et al., 2016). Stacks of frames separated 5 µm were captured every 30 min for 24h. Digital images were acquired, contrast-enhanced and analyzed with Imaris software 9.2 version (Bitplane AG Zurich, Switzerland). Before the analysis, a correction drift was applied using a reference point on the 2D orthogonal projection of the movie. For each condition, at least 100 cells were followed in each experiment and two different experiments were performed for each condition. For neuronal migration analysis, only the neurons appearing in at least 16 frames (out of 49) were considered for speed studies. In the case of the path straightness, all the neurons followed, independently on the caption of their migration during the film, were taken in account.

Image adquisition and quantification

Images were acquired using a florescence microscope (Zeiss Axio Imager Z2) with Apotome.2 and coupled to two different digital cameras: AxioCam MRm for fluorescence images and

AxioCam ICc for bright field images. For colocalization quantifications, single planes adquired with Apotome.2. All the images were analysed by ImageJ (Fiji),

Statistical analyses

Statistical analysis was carried out in GraphPad Software. P values below 0.05 were considered statically significant. Data are presented as mean ± standard error of the mean (SEM) throughout the Thesis. Normality and variance tests were fort applied to all experimental data. When data follows a normal distribution, paired comparisons were analysed with t-test, while multiple comparisons were analysed using either ANOVA with post-hoc Bonferroni correction (equal variances) or the Welch test with post-hoc Games-Howell (different variances).

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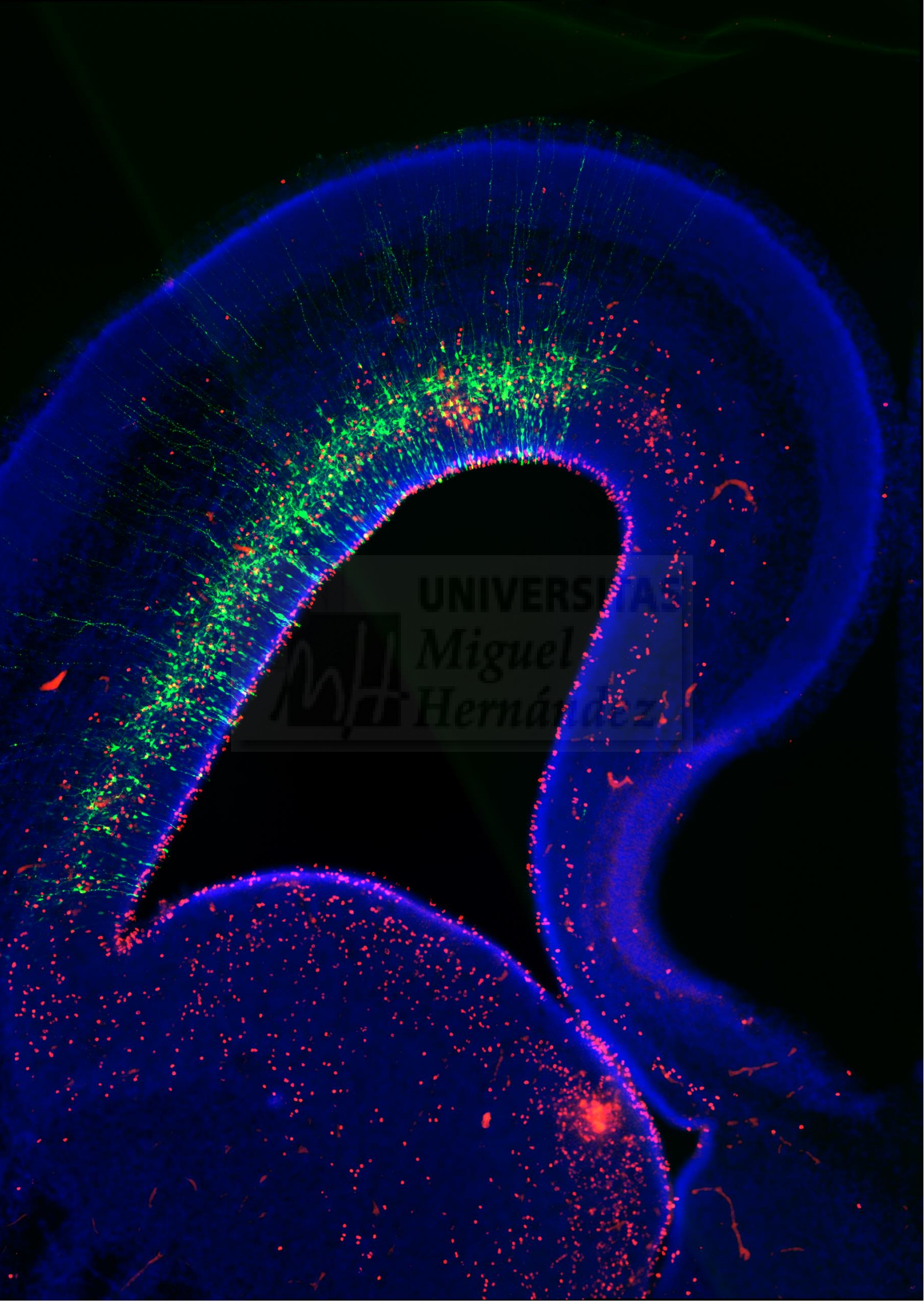

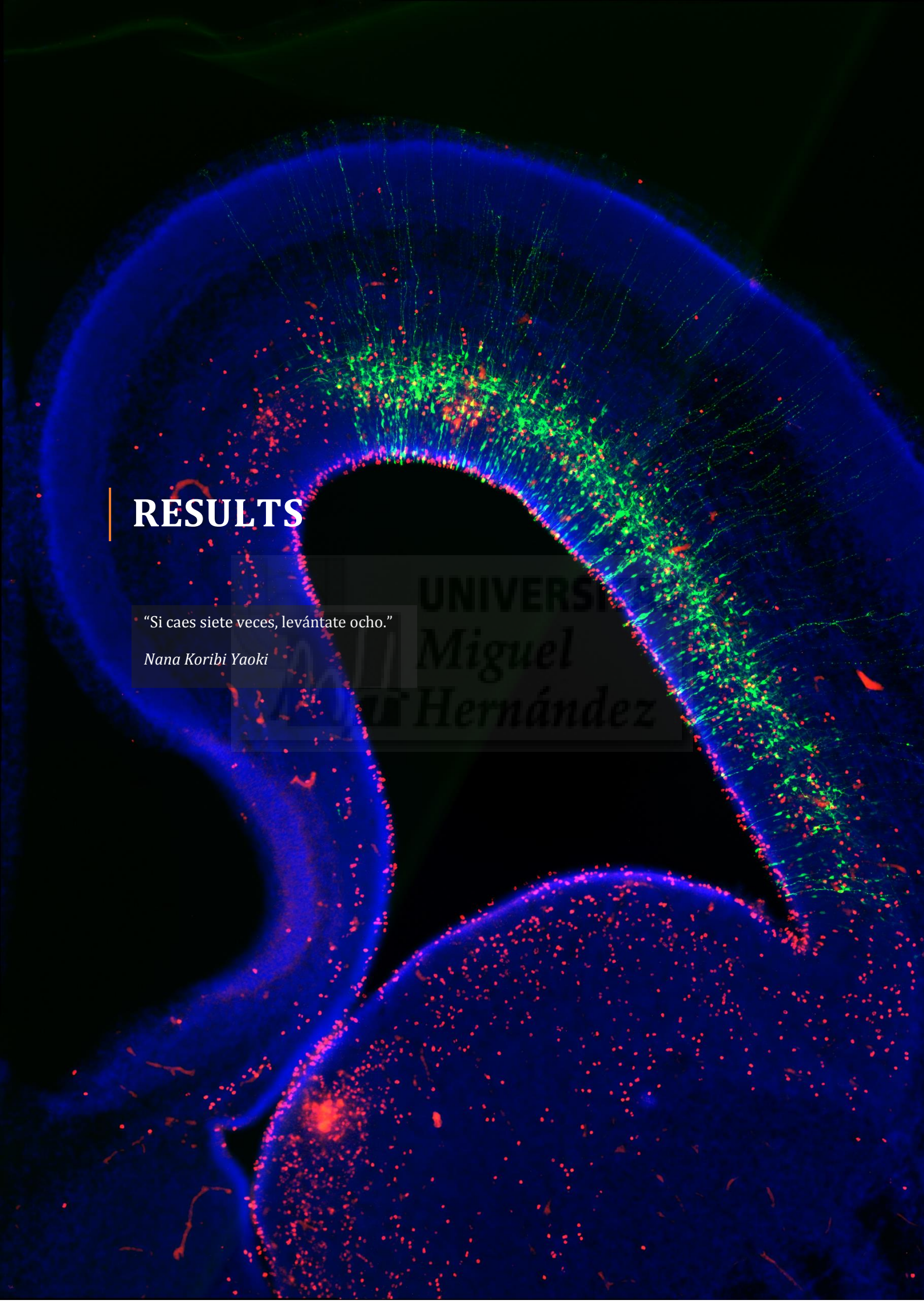

RESULTS

"Si caes siete veces, levántate ocho."

Nana Koribi Yaoki

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1. Novel mutations of *PIK3R2* in perisylvian polymicrogyria and their impact on neurogenesis and neuronal migration

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1.1. *PIK3R2* is expressed in germinal layers and cortical plate during human and mouse cortical development

Several genes belonging to the mTOR pathway are found mutated in patients with polymicrogyria, including *PIK3R2*. Mirzaa *et al.* characterized mutations in this gene using next-generation sequencing method, which allow them to distinguish between germline and somatic mutations. They identified both germline and somatic mutations in patients with bilateral perisylvian polymicrogyria isolated and also in cases of polymicrogyria in association with MPPH syndrome, being the most recurrent the missense mutation G373R (Mirzaa et al, 2015), that has been suggested to be a gain-of-function mutation (Riviere et al, 2012). Interestingly, while in germline cases all the cells in the organism (and, consequently, also in the brain) have the mutation, somatic ones imply that just a proportion of cells carry the mutation, but this is sufficient to cause the developmental malformation.

In order to elucidate the role of PIK3R2 during cortical development, we first decided to study its pattern of expression in human tissue. For that purpose, we performed *in situ* hybridization experiments at two key points in human development: gestational week 16 (16gw) and 21gw, in which neurogenesis and neuronal migration are the most important events occurring (**Figure 21**).

When we analysed the pattern of expression of *PIK3R2* in human, we observed that at both gestational points 16gw and 21gw, the highest levels of expression are located in the germinal layers (VZ, ISVZ and OSVZ) and in the cortical plate (CP) (**Figure 21**). This indicates the relevance of this gene for the cells populating these areas, meaning the cortical progenitor cells and the differentiated neurons, respectively.

Moreover, the intensity of *PIK3R2* expression along the same layer was different between areas of the cortex, in that sense, we noticed that there are regions with higher expression than others (**Figure 21a', a''**; **Figure 21b', b''**). This modular expression has been previously reported in other genes (DEGs), including some genes vinculated with MCDs (De Juan Romero & Borrell, 2015). Taking together, those result indicates that this gene may play a more important role in some areas than in others.

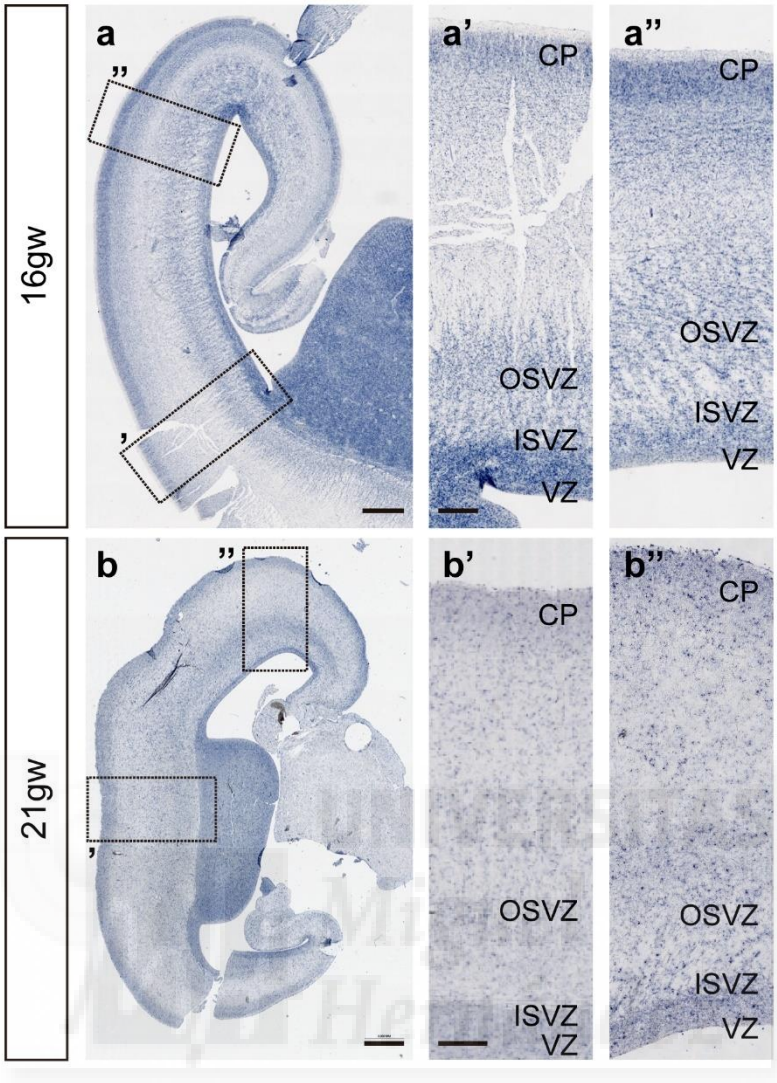

Figure 21. Expression of PIK3R2 in germinal layers and CP through human cortical development. *In situ* hybridization of PIK3R2 showing the pattern of PIK3R2 gene expression at 16gw (a) and 21gw (b). a', a''; b', b'' show a magnification of the squared areas in a and b, respectively. CP, Cortical plate; OSVZ, outer subventricular zone; ISVZ, inner subventricular zone; VZ, ventricular zone. Scale bars, 1 cm (a), 500 µm (a', a'', b', b''), 2 cm (b).

When we analysed the expression of *Pik3r2* in mouse, we found that this is comparable to what we found in human. The highest expression was also restricted to the germinal layers (VZ, SVZ) and the CP through corticogenesis (**Figure 22**), suggesting a conserved role of PIK3R2 during development. This fact prompts us to start performing functional experiments in mouse model in order to unravel the role of PIK3R2 in cortical development.

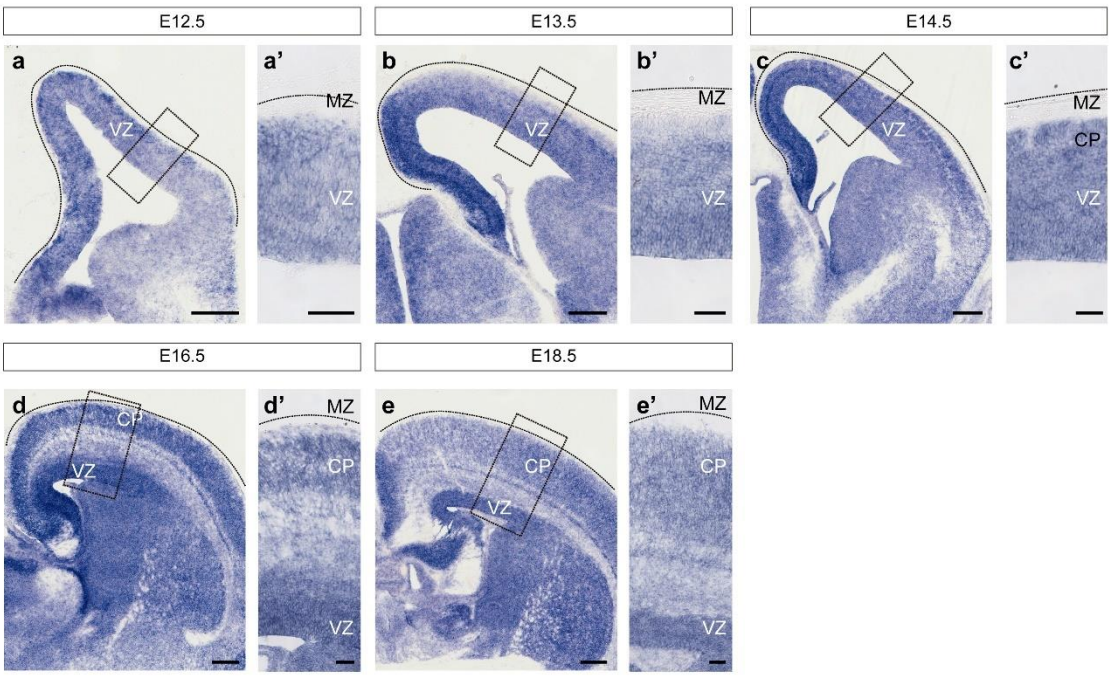

Figure 22. Expression of *Pik3r2* in germinal layers and CP through mouse corticogenesis. *In situ* hybridization of Pik3r2 showing the pattern of Pik3r2 gene expression at several stages during corticogenesis: E12,5 (a, a'), E13,5 (b, b'), E14,5 (c, c'), E16,5 (d, d'), E18,5 (e, e'). MZ, marginal zone; CP, cortical plate; VZ, ventricular zone. Scale bars, 200 µm (a, b, c, d, e), 50 µm (a', b'. c', d'. e').

1.2. *PIK3R2* overexpression increases the number of progenitor cells and its proliferation rate

We were interested in understanding the role of PIK3R2 in cortical development. To approach this question, we decided to use the technique of *in utero* electroporation, since it allows us to change the expression of a proportion of cells in the brain, similarly to the situation in humans patients with somatic mutations in *PIK3R2*. We used two different constructs, the wild type form of the gene (*PIK3R2*-WT) and other containing the recurrent human mutation (*PIK3R2*-G373R), both containing a GFP reporter which allowed us to visualize the electrorated cells and their lineage. In order to study the impact of PIK3R2 during mouse corticogenesis, we have overexpressed these forms at E14.5, when a peak of neurogenesis takes place.

First, we analysed the effects of our manipulation two days after the electroporation, at E16.5 (**Figure 23**). At this timepoint, we did not observed differences in the distribution of GFP^+ cells in the cortical layers (**Figure 23a-d**). Then, we decided to study the progenitors population by immunostaining with specific markers for IPCs (Tbr2) and for aRGCs (Pax6). We have seen that in both, overexpression of wild type and mutated form, there was a significant increase in the proportion of IPCs (**Figure 23e-h**). Regarding aRGCs, there was a significant increment in the apical progenitors as a cause of the wild-type form overexpression (**Figure 23i-j, l**).

However, the presence of the *PIK3R2*-G373R form result in a clear tendency toward increase of apical progenitor numbers, that turns out not to be significant (**Figure 23i, k-l**).

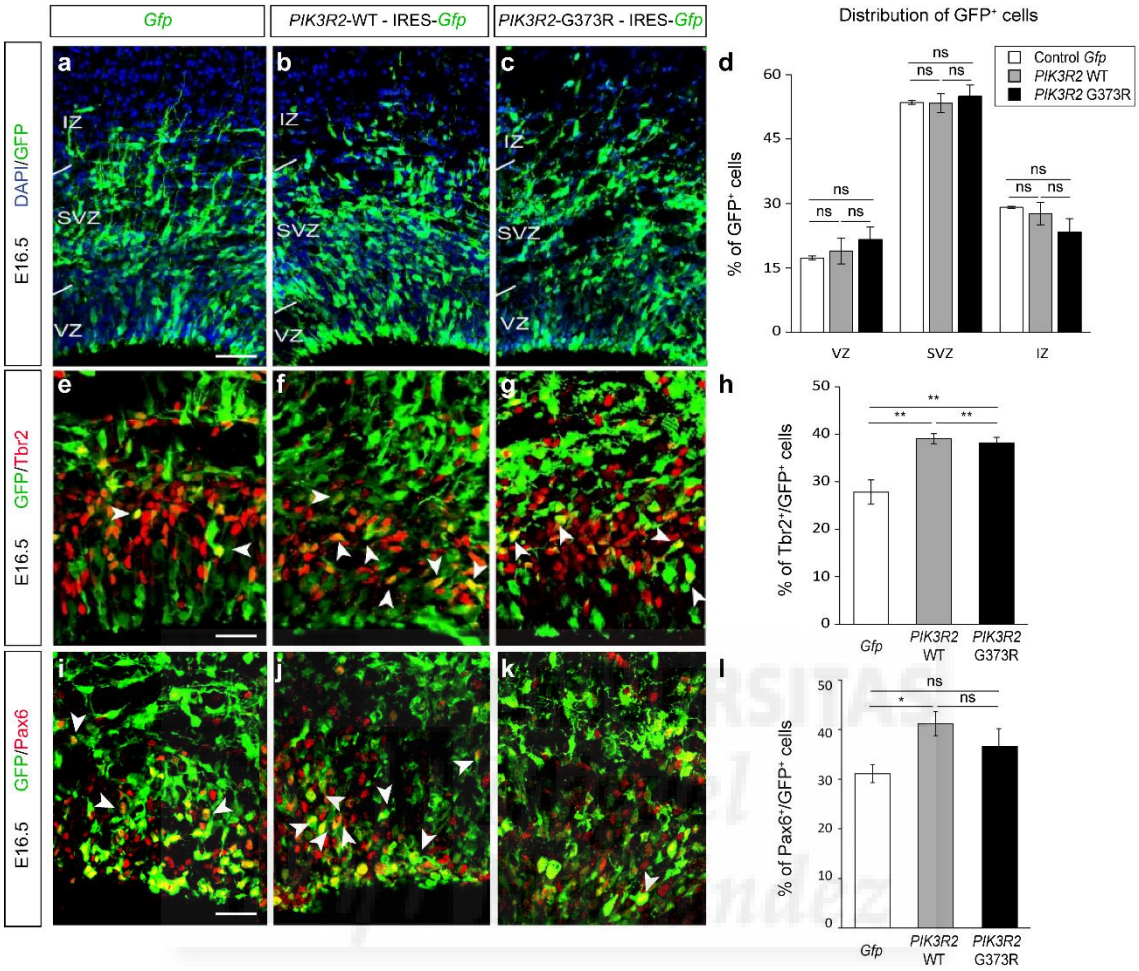

Figure 23. PIK3R2 increases the number of aRGCs and IPCs at midcorticogenesis in mouse. Functional experiments overexpressing GFP, PIK3R2-WT or PIK3R2-G373R at E14.5 and analized after two days. (a-d) Quantification of the GFP⁺ cells distribution (e-h), the proportion of Tbr2⁺-GFP⁺ cells, IPCs and (i-l) of the Pax6⁺-GFP⁺ cells, aRGCs. Values correspond to ≥ 3 embryos for each condition; *p<0,05; **p<0,01. IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. Scale bar, 50 µm.

We wonder if the increase in the amount of progenitor cells was due to a higher production of these cells or to a decrease in the cell cycle progression (having more progenitor cells but in quiescent state). For that reason, we decided to study the mitotic behaviour of the progenitors and their cell cycle by analysing their mitosis and the cell cycle re-entry (**Figure 24**).

For studying the mitoses, we have performed immunostaining against phosphohistone-3 (PH3), a specific marker of the core histone protein and an indicator of cellular M-phase. Additionally, we distinguish between apical (those located in the apical surface) and basal mitosis (those located at least 50µm away from the apical side), corresponding to aRGCs and IPCs divisions, respectively. We have observed that upon the overexpression of the wild-type form of *PIK3R2*, both apical and basal divisions were raised. On the contrary, the mutated form

overexpression lead to a selective increment in the basal mitosis (**Figure 24a-c, m-n**). Overall these findings were in accordance with the different proportion of progenitors that we have previously reported, since the PIK3R2-G737R form did not seem to have such a great effect on the amount of aRGCs neither causing a greater division rate (**Figure 24a-c, m, n**).

Additionally, we performed a cell-cycle re-entry analysis. For that purpose, we injected BrdU 24 hours before the fixation of the tissue. With this procedure, we labelled the cells that are in S-phase at the moment of the injection and during this time. Then, we immunostained with an antibody against BrdU and Ki67 for labelling both the cells in S-phase and cells in the cell cycle, respectively. We quantified the cells that expressed the two markers ($BrdU^{+}Ki67^{+}$), since these cells are the ones that were in S-phase when we inject the BrdU, and are still progenitors ($Ki67^{+}$), meaning that they have re-enter in the cell-cycle. From this assay, we determine that an excess of the *PIK3R2*-WT led to a greater rate of the cell cycle re-entry (**Figure 24d-l, o**). In summary, overexpression of both forms, especially *PIK3R2*-WT, results in the generation of a higher proportion of progenitors that also undergo mitosis more. In the case of the *PIK3R2*-WT overexpression, these cells preferentially generate new progenitors instead of producing neurons.

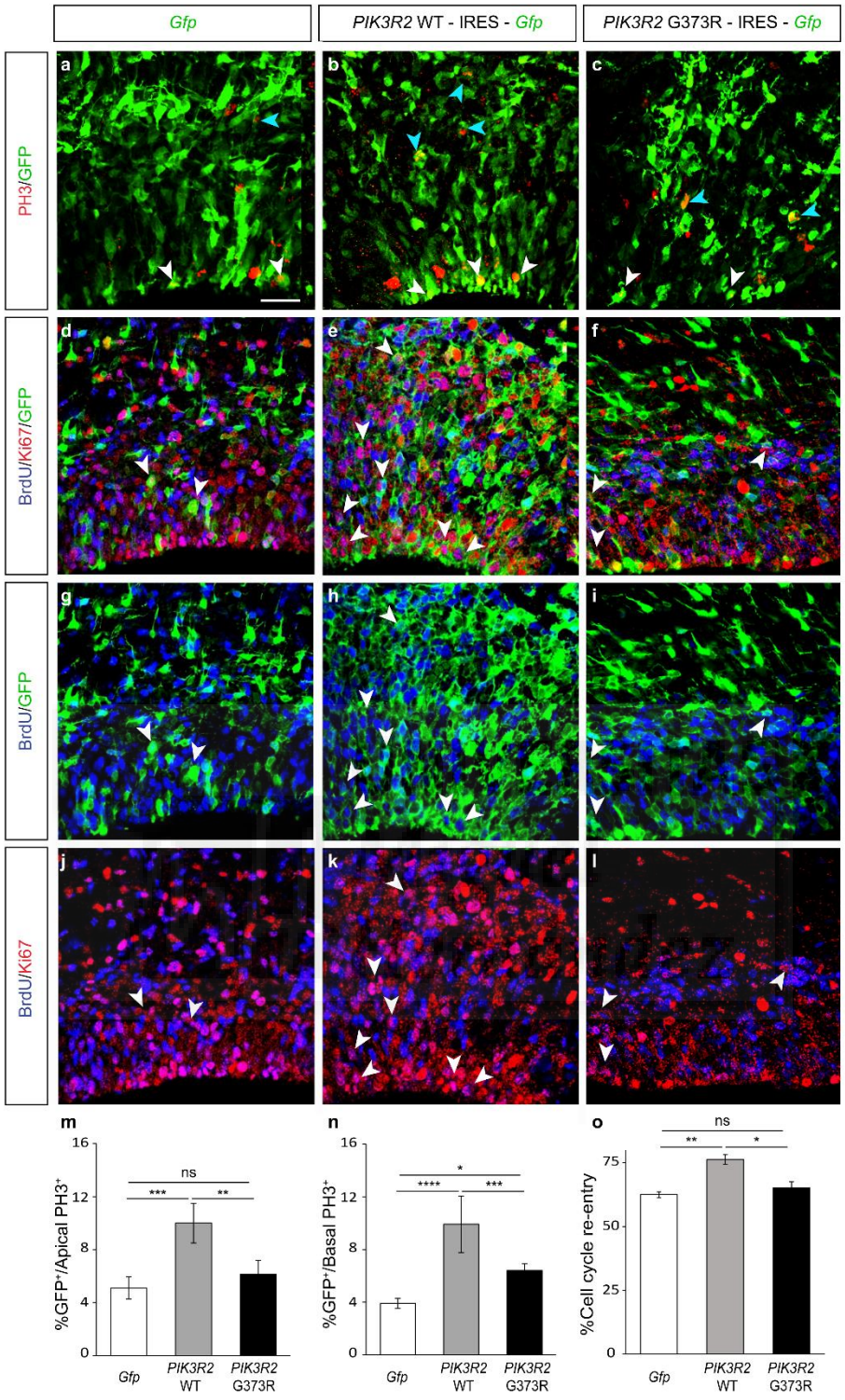

Figure 24. Higher number of divisions and cell-cycle re-enter rate under the overexpression of *PIK3R2*. (a-c, m, n) Quantification of apical and basal mitosis at E16.5 after *in utero* electroporation at E14.5. We observe a higher number of cells undergoing mitosis, specially the basal ones (m, n). (d-l) Cell cycle re-entry study shows an increase in the rate of cell cycle re-entry in the case of the overexpression of the *PIK3R2*-WT form, in comparation with the controls (o). Values correspond to ≥ 3 embryos for each condition; *p<0,05; **p<0,01. Scale bar, 50 µm.

1.3. PIK3R2 gain-of-function impairs radial neuronal migration causing the formation of ectopias in mouse

Although the etiology of polymicrogyria is not well defined, some authors have proposed that a defective neuronal migration could underlie the irregular formation of the cortical folds that we observed in this malformation (Barkovich, 2010; Squier & Jansen, 2014). For this reason, we decided to study the effects of *PIK3R2* in radial neuronal migration. For that purpose, we electroporated at E14.5, but this time we waited for four days in total after our manipulation (until E18.5) (**Figure 25**) since at this time point the majority of the neurons should have arrived to the CP. Then, we wanted to evaluate the positioning of these cells by quantifying the distribution of the GFP^+ cells through the cortex divided in bins. In control animals, at E18.5, a great proportion of neurons have reached the CP as expected. In contrast, wild-type or the mutant form overexpression lead to a clear delay in neuronal migration as was evident by the accumulation of cells in lower cortical layers and in the IZ (**Figure 25a-c**). We could see this defect at rostral and caudal levels, being more dramatic caudally (**Figure 25a''-c''**). The distribution of GFP+ cells was plotted considering the percentage of cells occupying each bin in which the cortex was subdivided for this quantification. In there we could observe that animals with either *PIK3R2* or *PIK3R2*-G373R overexpression showed a significant decrease in the proportion of cells that have arrived to the upper positions of the CP (bin1) in comparation with the control animals (bin1) (**Figure 25d**).

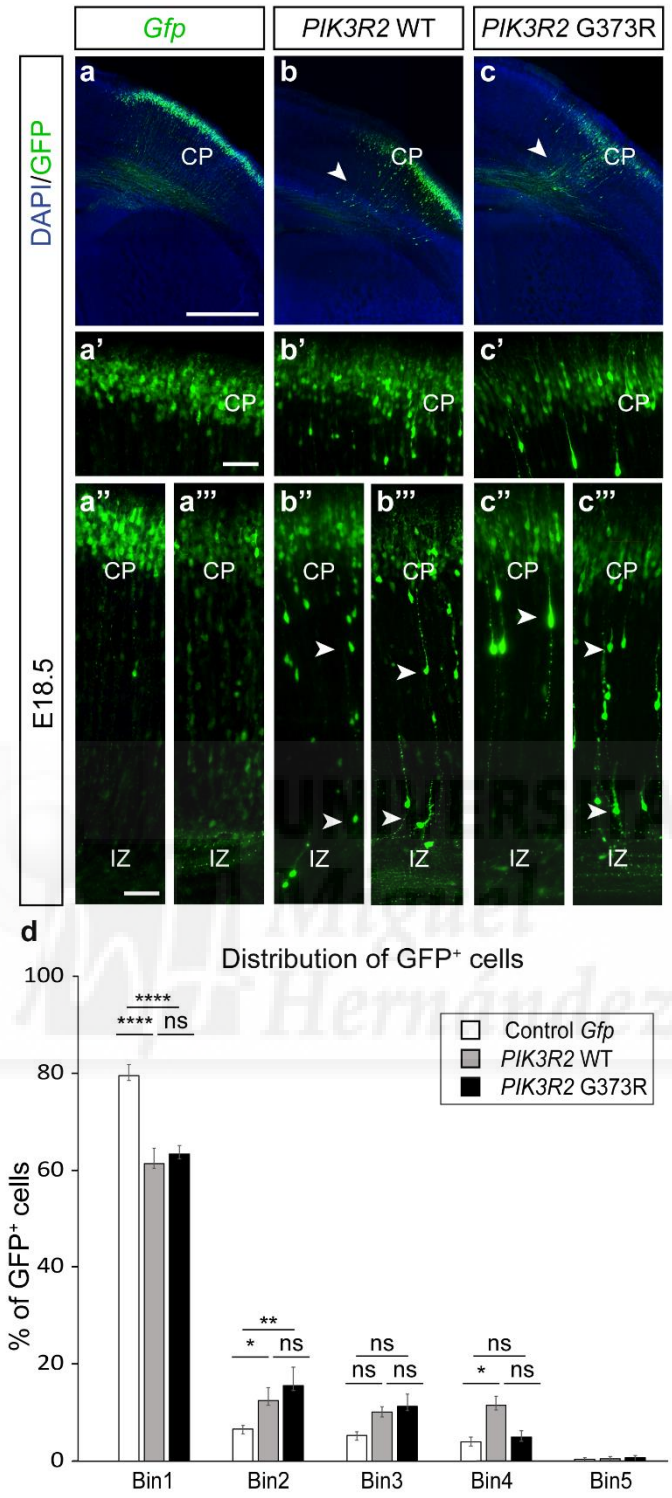

Figure 25. *PIK3R2* overexpression causes neuronal migration defects at E18.5. (a-c) Images showing the distribution of cells after the electroporation at E14.5 in coronal sections of E18.5 embryos. (a', a''; b', b''; c', c'') High magnifications of the cortical thickness at rostral and caudal levels. (a''', b''', c''') High magnifications of the CP, showing that in *PIK3R2*-WT and *PIK3R2*-G373R conditions some cells have not reached the CP, although those who did seem to have a normal morphology in contrast to control. (d) Quantification of the distribution of GFP⁺ cells. Values correspond to ≥ 3 embryos for each condition; *p<0,05; **p<0,01. CP, cortical plate; IZ, intermediate zone. Scale bars, 500 µm (a, b, c), 50 µm (a', b', c'; a'', b'', c'').

It is known that radial migration defects can be compensated or rescued later in development. To evaluate this possibility, we decided to analyse the effect of overexpressing both forms of *PIK3R2* several days after being born, at P5. We observed that, although a great quantity of neurons has arrived at the CP, there was a considerable number of pyramidal neurons placed in lower cortical layers and in subcortical positions (**Figure 26a-c**). This result confirms that, at least at this stage and for a proportion of neurons, the defect is still present. Consequently, we wanted to look how this aberrant radial migration could affect juvenile P21 animals. In this case, the majority of cells overexpressing *PIK3R2*-WT or *PIK3R2*-G373R reached the CP (**Figure 26d-f**). Although there was a low proportion of cells retained throughout the thickness of the cortex and even in the white matter, something that we never observed in control animals (**Figure 26d'-f'**). Importantly, these cells were identified as upper layer neurons, as indicates its colabelling with the specific upper-layer marker, Cux1. The severity of the ectopia formation was variable among individuals but it was always present under these conditions.

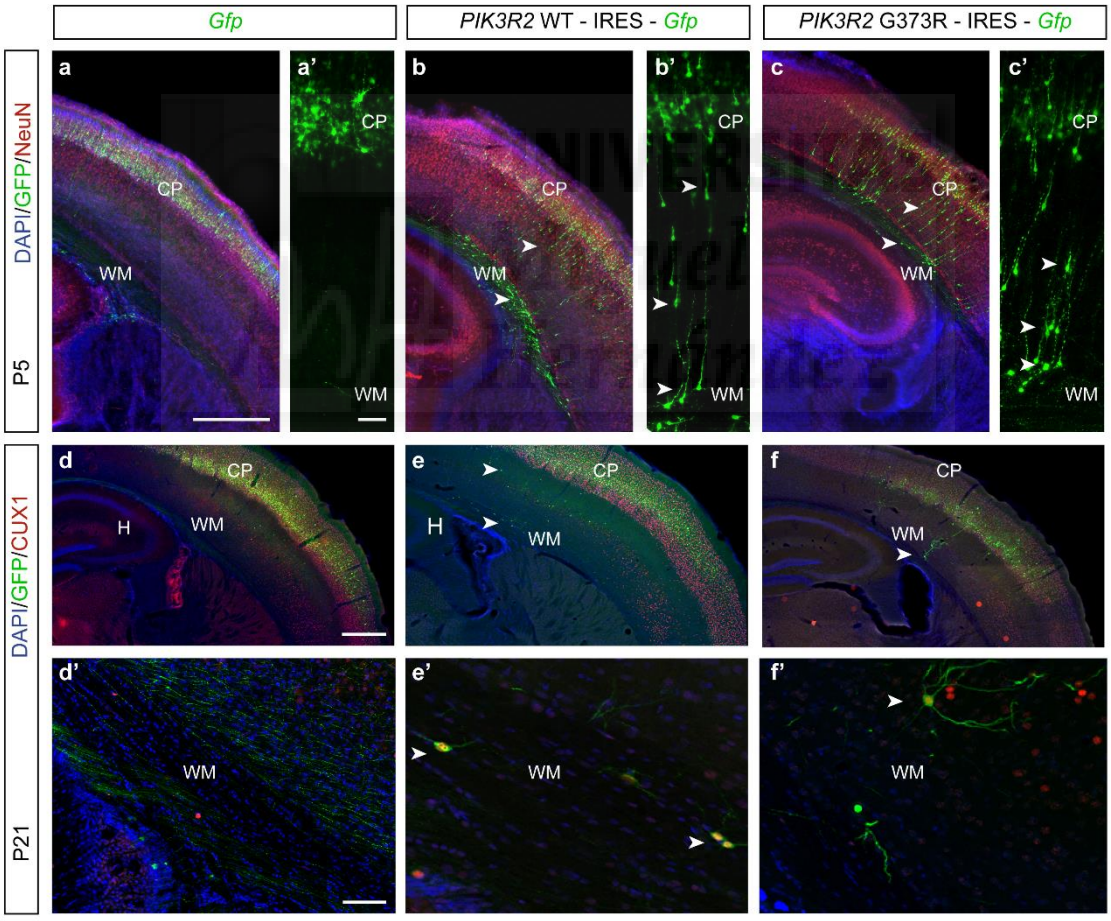

Figure 26. *PIK3R2* overexpression led to postnatally consolidated neuronal ectopias. (a-c) Coronal sections of P5 pups showing the distribution of GFP+ cells with high magnifications of the cortical thickness (a'-c'), showing cells retained in lower cortical layers and in the WM in (b, b') and (c, c'). (d-f) Coronal sections of P21 animals showing the majority of cells placed in the CP, but a relevant proportion is present in deep cortical layers and WM. (d'-f') High magnification exhibiting the ectopic cells, differentiated as upper layer neurons. CP, cortical plate; WM, white matter; H, hippocampus. Scale bars, 500 µm (a, b, c, d, e, f), 50 µm (a', b', c', d', e', f').

These data gave as a consistent phenotype in mouse. However, we were not able to reproduce the polymicrogyria found in patients with *PIK3R2* mutations. Considering that this malformation is manifested as an overfolding of the cerebral cortex, we wonder if maybe a pre-folded cortex is needed to induce the generation of an aberrantly folded phenotype. We based this hypothesis on the different subpopulation of progenitors in gyrencephalic species compared to lissencepphalic (Betizeau et al, 2013; Dehay et al, 2015; Florio et al, 2017; Florio & Huttner, 2014; Reillo & Borrell, 2012; Reillo et al, 2017), as well a differential gene expression of some genes as was previously demonstrated in both human and ferret (De Juan Romero & Borrell, 2015). For these reasons, we decided to move to a gyrencephalic model: the ferret.

1.4. *Pik3r2* is expressed in germinal layers and cortical plate during ferret corticogenesis

We first analysed the pattern of expression of *Pik3r2* in the developing ferret cortex considering different points through ferret corticogenesis (**Figure 27**). At early stages, a E32, we observed that *Pik3r2* is expressed in germinal layers (VZ, SVZ), particularly higher in the VZ. Also, levels of expression were detected in the CP (**Figure 27a**). Then, at E34 this pattern of expression is maintained. Interestingly, we showed that although *Pik3r2* mRNA is detected in VZ, SVZ and CP, we noticed that the expression was not homogenous along the SVZ (**Figure 27b**). Rather, we detected caudal regions with higher Pik3r2 expression (visual cortex) (**Figure 27b'**) while more rostral ones show lower levels (**Figure 27b''**). Later, at P0, we still detected the highest expression at germinal layers (VZ, ISVZ, OSVZ) and CP. As was reported at E34 in the SVZ, a differential expression was detected, in the OSVZ at this stage (**Figure 27c**). Finally, at ferret mid-corticogenesis (P6), the expression in the germinal layers is practically restricted to the VZ and ISVZ, as well as to the CP.

Interestingly, as happen in mouse, the expression pattern of *Pik3r2* was comparable to human's: the highest expression was found in the germinal layers (VZ, ISVZ, OSVZ) and in the CP. Moreover, as we previously have shown in human, also this gyrencephalic species presented some regions of differential expression, as we observed at E34 (**Figure 27b**) and P0 (**Figure 27c**) along the SVZ and OSVZ, respectively (**Figure 27**).

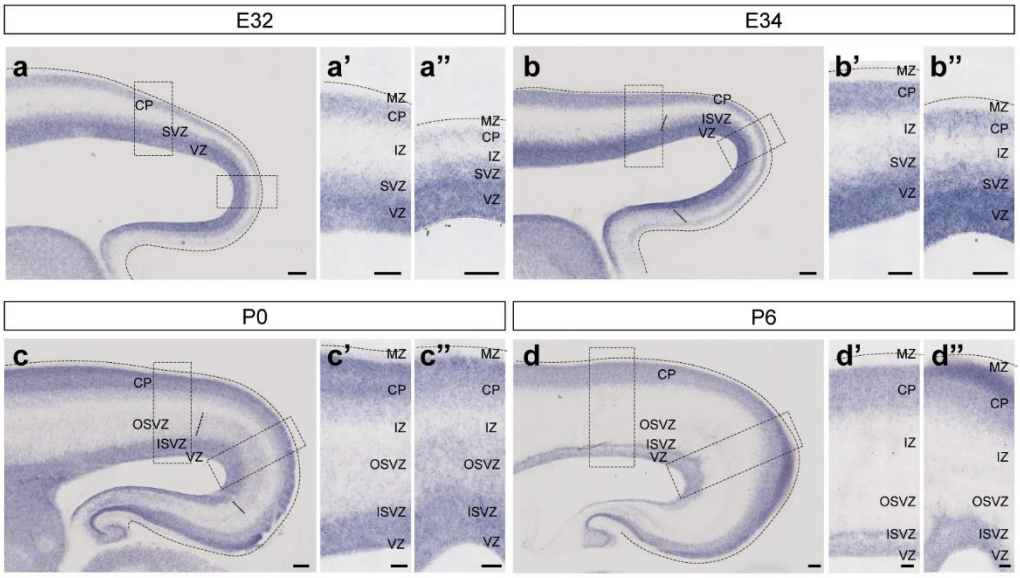

Figure 27. Highest levels of *Pik3r2* gene expression are found in germinal layers and CP in ferret corticogenesis. (a-d) *In situ* hybridization of Pik3r2 in the developing ferret cortex showing the pattern of expression of *Pik3r2* shows higher levels in the germinal layers (VZ, ISVZ and OSVZ) through development, Additionally, we found regions with differential expression along the ISVZ and OSVZ at E34 and P0, respectively (black lines). MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; OSVZ, outer subventricular zone; ISVZ, inner subventricular zone; VZ, ventricular zone. (a'-d') High magnifications of the areas squared in the respective (rostro-medial positions). (a''-d'') High magnifications of the areas squared in the respective images (visual cortex). Scale bars, 200 μm (a, b, c, d), 100 μm (a', a''; b', b''; c', c''; d', d'').

1.5. *PIK3R2* overexpression did not alter neuronal migration at mid corticogenesis in ferret

Based on the previous results of defective migration that we found in mouse, we determined to evaluate the effect of PIK3R2 in neuronal migration as a first approach. We electroporated postnatal pups at P1.5 and waited until P14 for the analyses, because those are comparable ages to the migration study in mouse (E14,5→E18,5). Similarly to the mice experiments, we have evaluated the distribution of GFP^+ cells across ferret cortical layers. Although the amount of GFP^+ cells was lower when we overexpressed PIK3R2-WT than in control animals, when we quantified the proportion of cells per layer, we did not find any significant difference. Altogheter those results suggest that the excess of PIK3R2 did not cause a defect in neuronal migration at this stage (**Figure 28**).

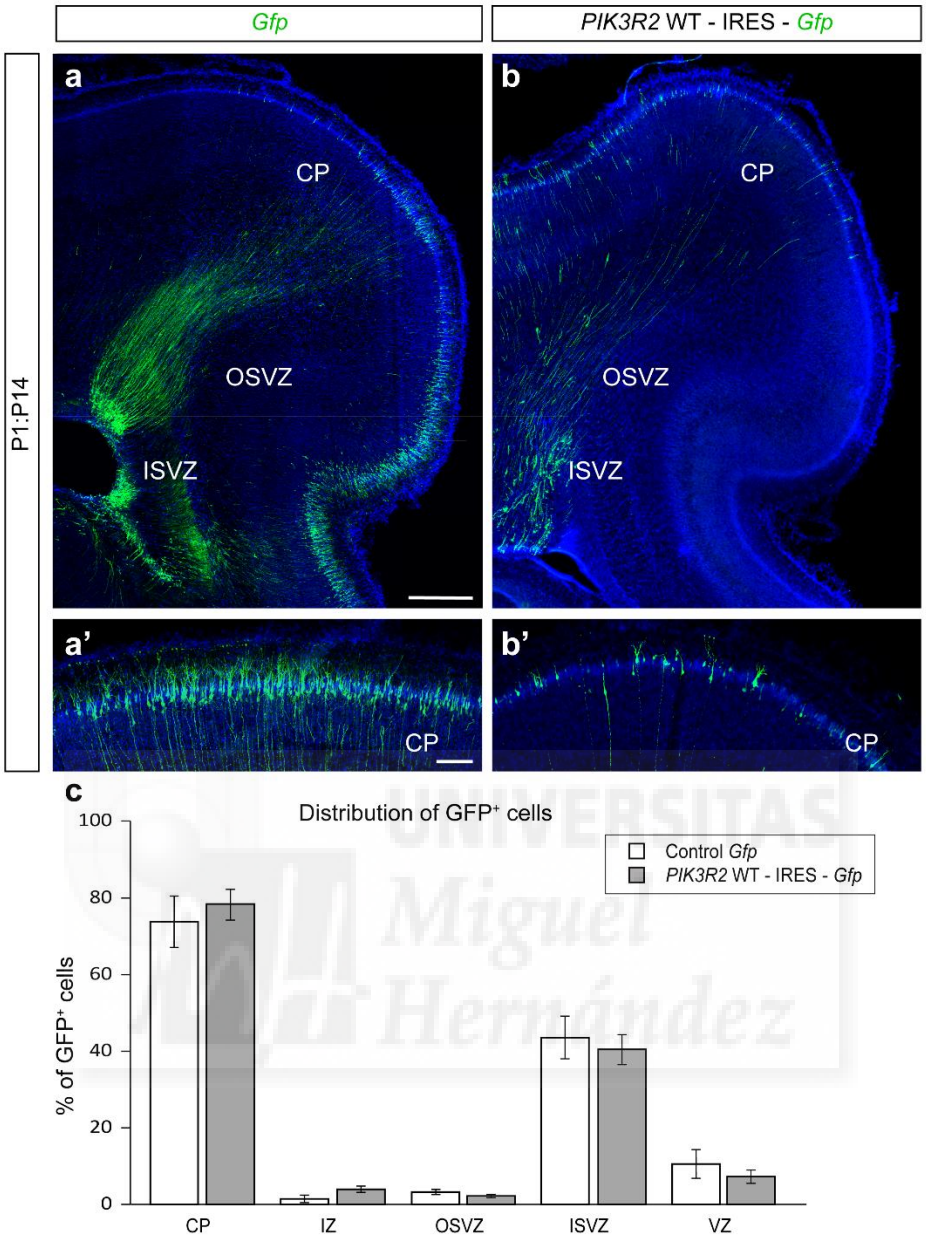

Figure 28. Overexpression of *PIK3R2* did not cause a cellular distribution defect on postnatal ferrets. (a-b) Sagital sections of P14 ferrets after being electroporated at P1.5 in the visual cortex. (c) Quantification of the distribution of the GFP+ cells through the cortex. The data plotted here corresponds to more than 3 animals for each condition. CP, cortical plate; IZ, intermediate zone; OSVZ, outer subventricular zone; ISVZ, inner subventricular zone; VZ, ventricular zone. Scale bars, 500 µm (a, b), 100 µm (a', b').

1.6. Early *PIK3R2* overexpression led to neuronal migration impairment in ferret visual cortex

In our previous experiment, when we electroporated at P1.5 we were modifying the expression of the aRGCs populating the VZ and the lineage derivated from those, meaning basal progenitors of the ISVZ and cortical neurons. With those experiments, we were able to affect mainly aRGCs located in the VZ. In consequence, the cells affected were those derived from these progenitors

(directly or indirectly): neurons, that would migrate to the CP, and progenitors that would be populating either the VZ or the ISVZ, but not the OSVZ, since this layer becomes independent after the bRGCs massive production at E34-E38 (Martinez-Martinez et al, 2016). Consequently, we chose an earlier stage of the development to perform our functional experiments in order to influence in a greater number of progenitors.

As mentioned before, E34 is a key point in which there is a time-window of great production of the bRGCs that will populate the OSVZ, a distinctive layer for gyrencephalic species (De Juan Romero & Borrell, 2015; Martinez-Martinez et al, 2016; Reillo et al, 2011). We have performed our electroporation experiments at E34 to see if we could affect the production of basal progenitors, just as happen in mouse experiments. We have hypothesized that if we can increase the amount of bRGCs, neurons would have more radial fibers to migrate, eventually generating a local increase in the gyrification.

We started electroporating visual cortex at E34 and evaluating first the distribution of GFP^+ cells two days later, at E36 (**Figure 29c-e, f**). We observed a clear alteration in the positioning of these cells, particularly in the case of overexpression of *PIK3R2*-WT in which there was an increase in the proportion of cells in ISVZ, in consonance with a decrease in those in the OSVZ and the VZ. When we quantified the total number of neurons generated after the electroporation using the neuronal marker NeuroD2, we noticed that this proportion did not change (**Fig.29h**). However, the distribution of these neurons was altered, indicating a delay in neuronal migration caused by the excess of *Pik3r2* and a premature detachment of some cells from the VZ, justifying the lower proportion of cells in this layer in presence of *PIK3R2*-WT (**Figure 29g**).

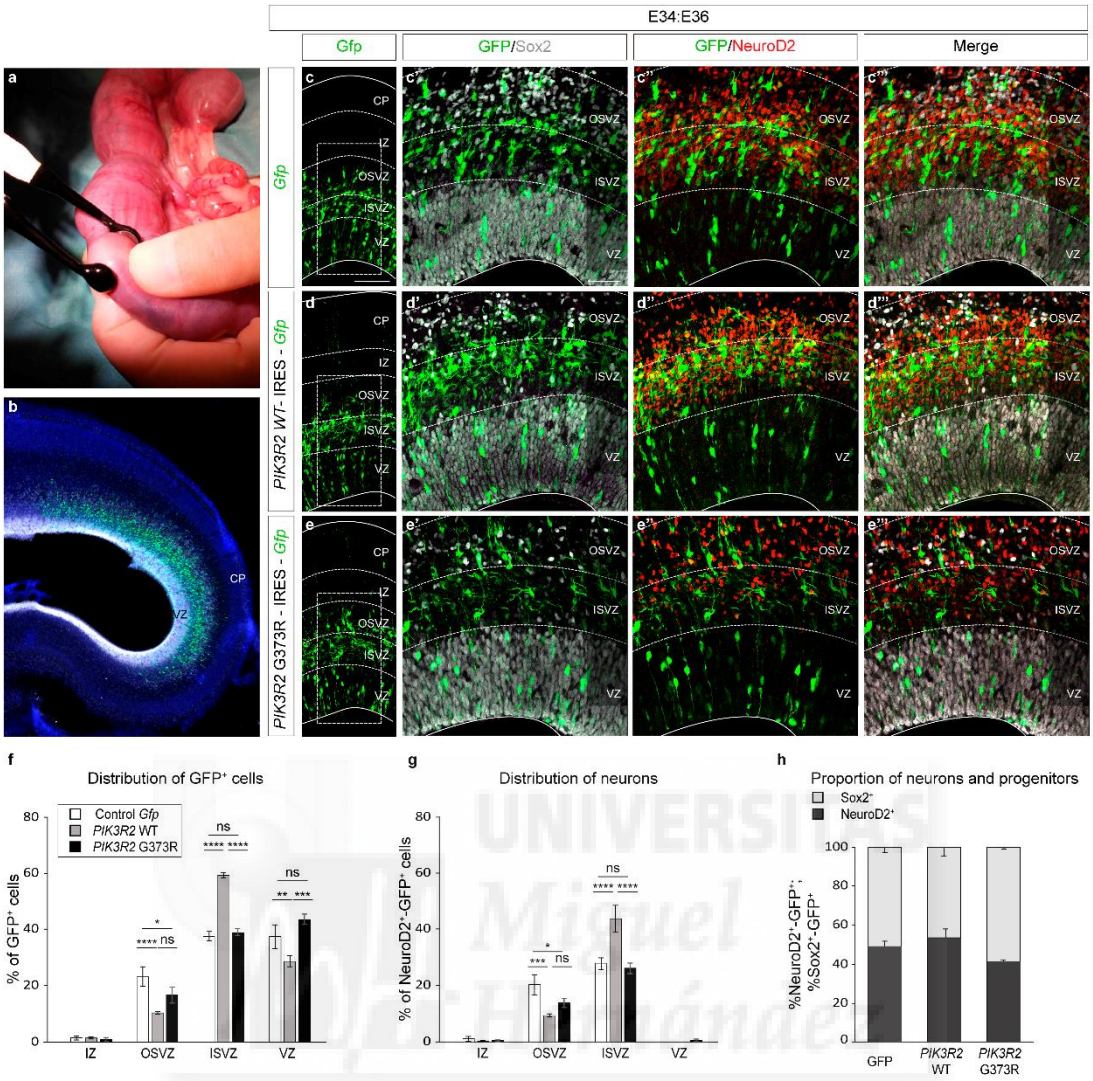

Figure 29. Early overexpression of *PIK3R2* alters neuronal positioning. (a) Technique of *in utero* electroporation, directing the paddles to the visual cortex. (b) Sagital section of E36 embryo, two days after electroporation at E34. (c-e) Analysis of the distribution of GFP⁺ cells and their identity as progenitors (Sox2⁺) or neurons (NeuroD2⁺). (f) Quantification of the GFP+ distribution cells. (g) Quantification of the distribution of neurons, NeuroD2⁺. (h) Proportion of neurons and progenitors generated after the electroporation of GFP, *PIK3R2*-WT or *PIK3R2*-G373R. Values correspond to ≥ 3 embryos for each condition; *p<0,05; **p<0,01; ***p<0,005; ****p<0,001. CP; cortical plate; OSVZ, outer subventricular zone, ISVZ, inner subventricular zone; VZ, ventricular zone. Scale bars, 100 µm (c, d, e), 50 µm (c', c'', c'''; d', d'', d''', e', e'', e''').

Given this defect in neuronal migration at early stages in ferret development, we sought to know the long-term effects of this phenotype in juvenile animals after overexpressing *PIK3R2*-WT at E34. At P30, when cortical folding is almost complete in ferret, we noticed that the location of cells in cortical layers was modified (**Figure 30c, d**). Cells overexpressing *PIK3R2* were preferentially located in layers V and VI, in contrast to those just expressing GFP, in which they were mainly populating layer IV (**Figure 30e**). Similar to what happens in mouse experiments, these results at short and long term illustrate that the overexpression of *PIK3R2*-WT causes a neuronal migration defect that is consolidated postnatally.

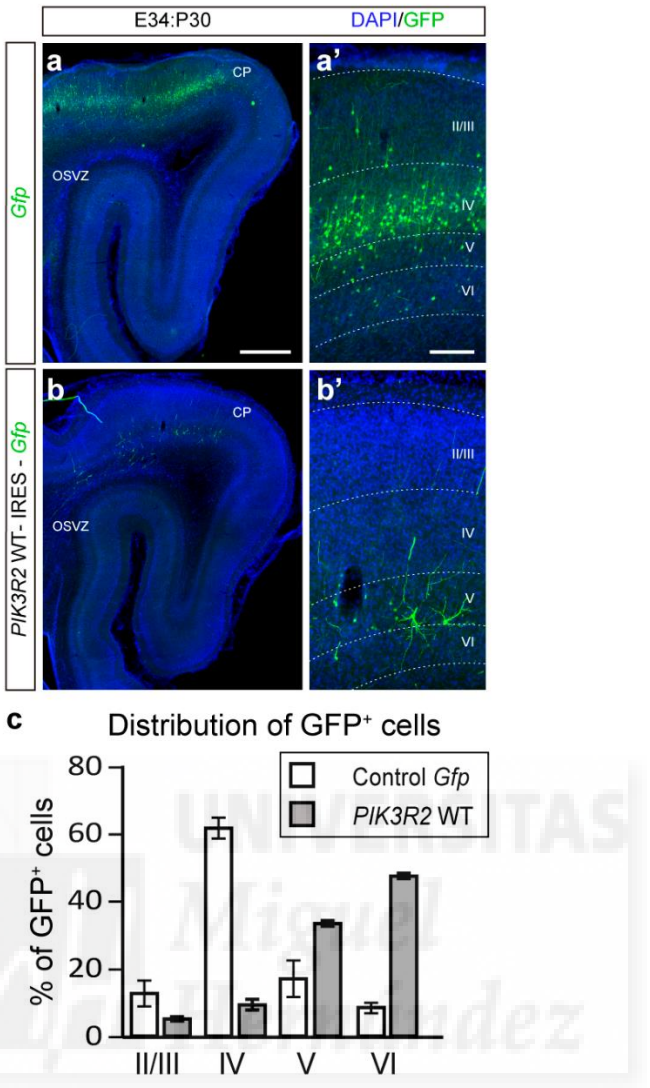

Figure 30. Delay in neuronal migration after early overexpression of *PIK3R2* is consolidated postnatally. (a, b) Sagital views of a P30 ferret after being electroporated at E34 with GFP (a, a') or PIK3R2-WT (b, b'). (b') and (b') show the cortical layers of the control GFP and the overexpression of PIK3R2-WT, respectively. (c) Quantification of the distribution of GFP⁺ cells in the visual cortex. CP; cortical plate; OSVZ, outer subventricular zone; cortical layers indicated as II/III; IV, V, VI. Scale bars, 1 cm (a, b), 200 µm (a', b').

1.7. OE of *PIK3R2* in parietal area produces an aberrant number and positioning of neurons, resulting in polymicrogyria and nodular heterotopia

In human patients, mutations in *PIK3R2* lead to polymicrogyria. Our experiments, although consistent with the mouse phenotype, did not result in modifications in the gyri formation. As mentioned in the introduction, polymicrogyria occurs preferentially in the perisylvian area in humans (Squier and Jansen, 2014). Accordingly, we decided to electroporate the ferret parietal cortex, which is the equivalent to the perisylvian region in humans (**Figure 31**).

Similar to our previous study in the visual cortex, we analysed the distribution of GFP$^+$ cells two days after the electroporation of the three different constructs (*Gfp*, *PIK3R2*-WT and *PIK3R2*-G373R) at E34 (**Figure 31a-d**). We found that, in the case of overexpressing the mutated form of PIK3R2, there were not differences in the cellular positioning considered by layers (**Figuew 31a, c-d**). On the contrary, when we overexpressed the *PIK3R2*-WT, we observed that a higher proportion of cells is placed in the VZ and lower in the ISVZ, indicating a change in the cellular distribution caused by the excess of PIK3R2 (**Figure, 31a-b, d**).

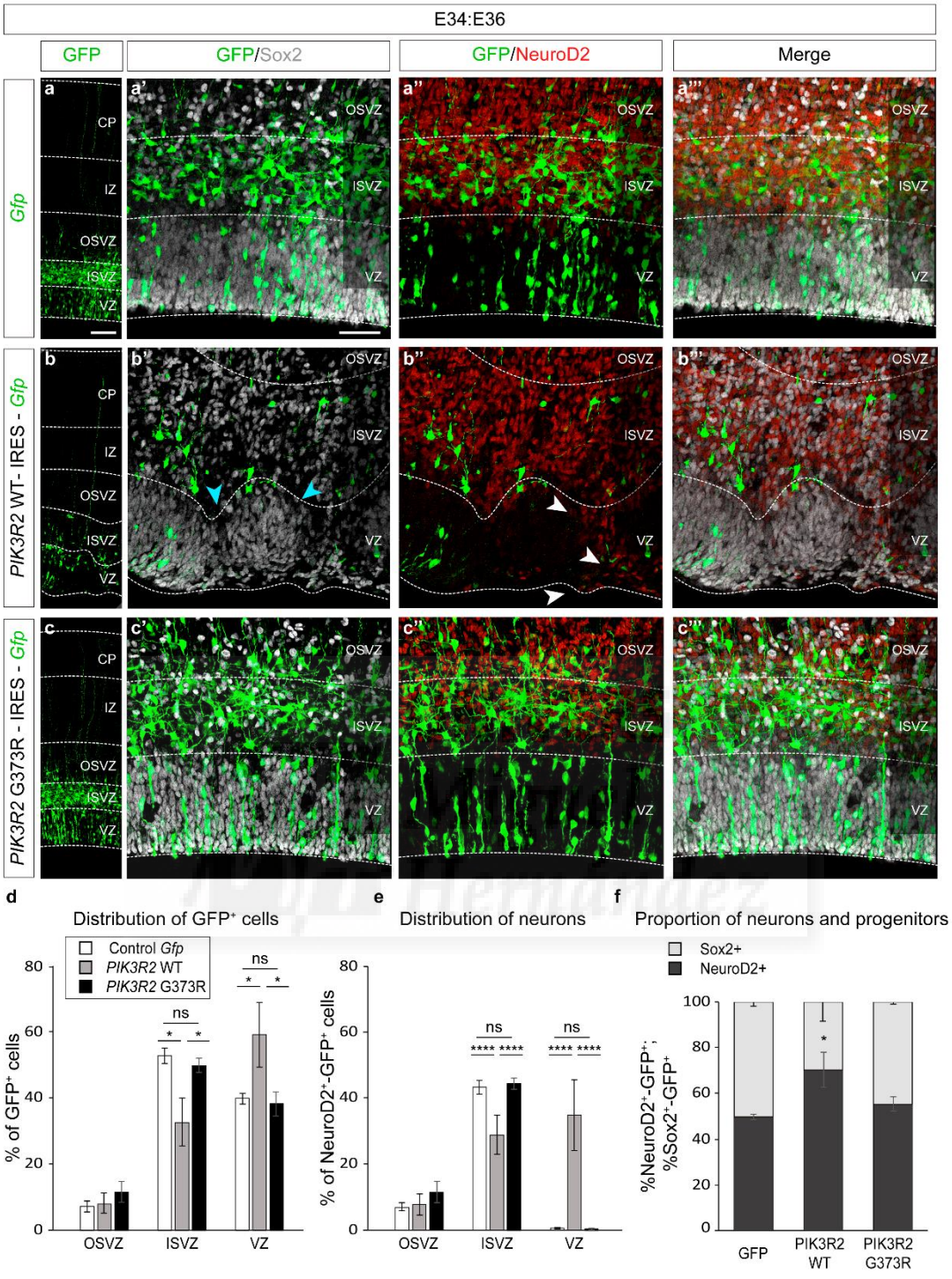

Figure 31. Early overexpression of *PIK3R2* in the parietal cortex increases the neuronal production, that locate aberrantly. (a-c) Coronal sections of E36 embryos, two days after electroporation at E34. Analysis of the distribution of GFP⁺ cells and their identity as progenitors (Sox2⁺) or neurons (NeuroD2⁺). (d) Quantification of the GFP⁺ distribution cells. (e) Quantification of the distribution of neurons, NeuroD2⁺. (f) Proportion of neurons and progenitors generated after the electroporation of GFP, PIK3R2-WT or PIK3R2-G373R. Blue arrows point to the circular structures found in the VZ form by Sox2⁺ cells, surrounded by NeuroD2⁺ cells. White arrows mark NeuroD2⁺ cells intruding in the VZ. Values correspond to ≥ 3 embryos for each condition; *p<0,05; **p<0,01;

***p<0,005. CP; cortical plate; OSVZ, outer subventricular zone, ISVZ, inner subventricular zone; VZ, ventricular zone. Scale bars, 100 µm (a, b, c), 50 µm (a', b', c').

Contrarily to what we observed in visual cortex electroporations, we detected an increase in the total number of neurons generated two days after the electroporation of *PIK3R2*-WT (but not with the mutated form), indicating a higher rate of neurogenesis (**Figure 31f**). Of note, these were mainly located in the VZ, something that never happened in the control situation neither in the *PIK3R2*-G373R condition (**Figure 31e**). These neurons were intruding the VZ, suggesting that this higher neurogenesis also produce an aberrant location of these cells (**Figure 31a''-b''**). Not all the ectopic neurons were GFP⁺, which suggest a non-cell autonomous effect (**Figure 31b''**). Moreover, surrounded by these ectopic neurons, we could notice a clear alteration of the VZ in *PIK3R2*-WT overexpression condition, forming circular structures differently from the laminar appearance in control animals and in presence of *PIK3R2*-G373R (**Figure 31a'-c'**).

Next, we analyzed the divisions of ferret progenitors by PH3 labelling, distinguishing between apical and basal mitosis in the electroporations performed in both visual and parietal cortex, following the same criteria (**Figure 32**). Differently to what we have previously reported in mouse, the mitoses in ferret with overexpression of PIK3R2 were either reduced (apical) or unaffected (basal). More concretely, in the case of the visual cortex, the overexpression of both forms of *PIK3R2* caused a decrease on the apical mitoses (**Figure 32a-d**), while the basal ones remained the same in both conditions (**Figure 32a-c, e**), which could be a consequence of a premature detachment of cells (decreased number of cells in the VZ) that we have found. In the case of the parietal cortex, also the apical mitoses were negatively affected, but just in presence of the *PIK3R2*-WT, although a clear tendency was observed in the *PIK3R2*-G373R (**Fig32f-j**). This reduction could fit with the increase in the neuronal production in the apical side.

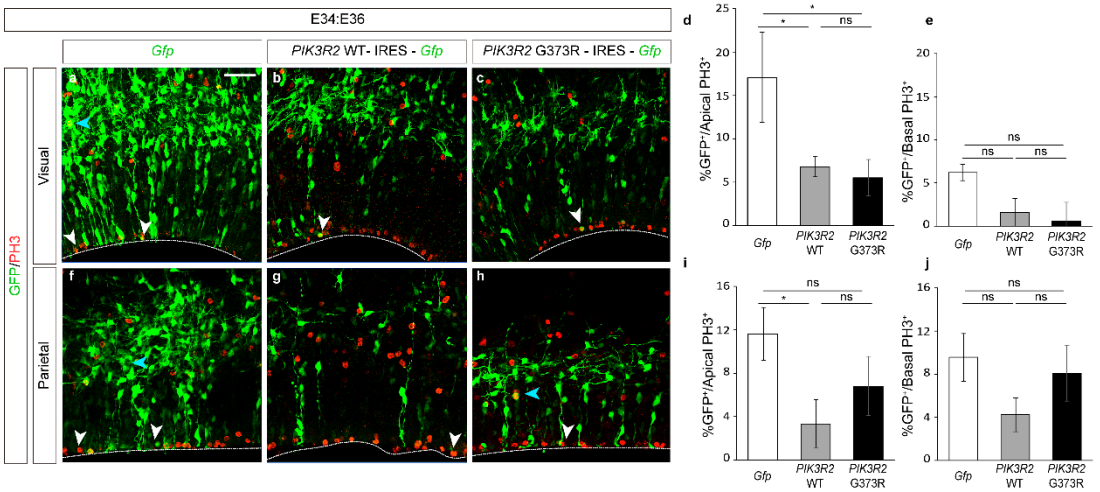

Figure 32. Apical mitoses are reduced in excess of PIK3R2 in visual and parietal cortex. (a-c) Sagittal sections of E36 embryos after being electropoted in the visual cortex at E34 with *Gfp*, *PIK3R2*-WT and *PIK3R2*-G373R. (d) Quantification of the apical mitoses. (e) Quantification of the basal mitoses. (f-h) Coronal sections of

E36 embryos after bein electroporated in the parietal cortex at E34 with GFP, *PIK3R2*-WT and *PIK3R2*-G373R. (i) Quantification of the apical mitoses. (j) Quantification of the basal mitoses. Values correspond to ≥ 3 embryos for each condition; *p<0,05; **p<0,01; ***p<0,005. Scale bar, 50 µm.

Next, we performed long-term experiments (E34::P30) in order to see if the radial migration defect were maintained later in development (**Figure33**). In this case, we found that the ectopic neurons that we had observed at E36, led to the formation of periventricular nodules (**Figure 33c, f**). These structures were mainly composed by differentiated neurons (NeuN⁺). Importantly, these nodules almost lacked GFP⁺ cells, while some of them were around the nodule, corroborating the non-cell autonomous effect shown embryonically.

Another destacable feature that we found in these animals with an excess of *PIK3R2*-WT was the overmigration of neurons, which formed an alteration of the cortical surface (**Fig33b, e**). Here, we could observe the formation of small additional folds in the parietal cortex, that reminded the histological appearance of polymicrogyria patients. This phenotype could appear single or multiple. Taking together, these results show how an imbalance of the normal number of neurons generated in a specific area could drive the formation of heterotopias and extra gyri.

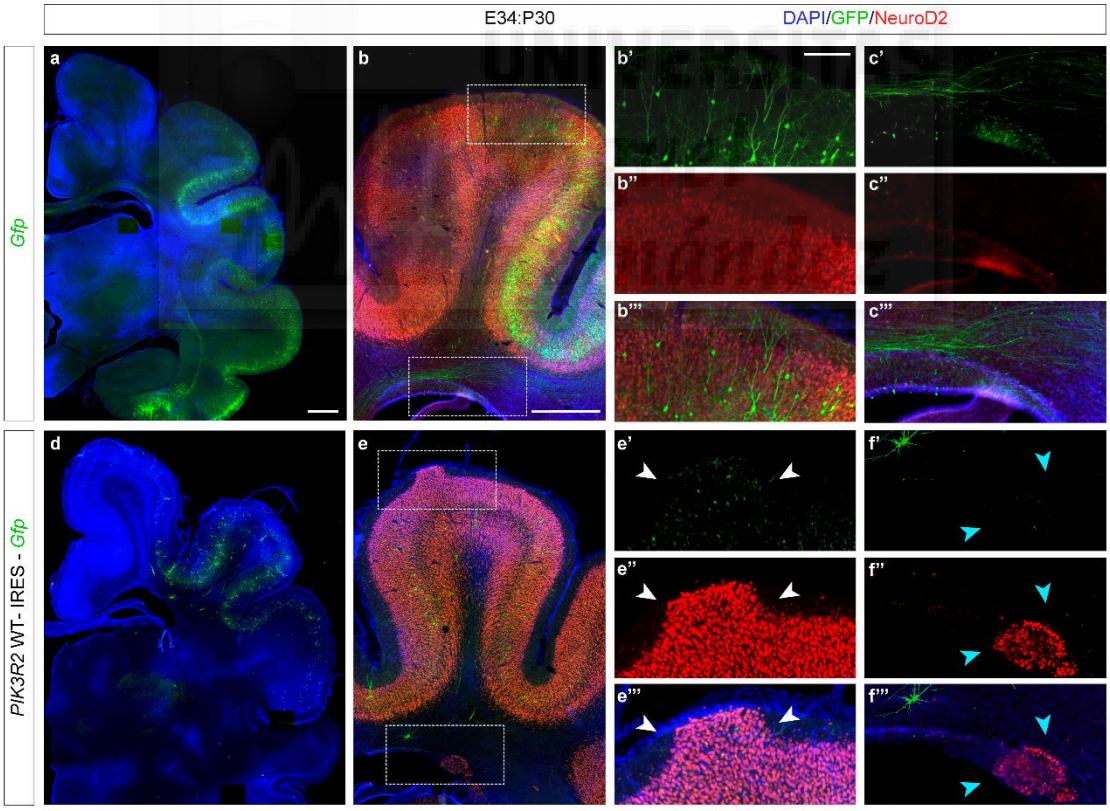

Figure 33. Overexpression of *PIK3R2* at early stages in ferret development causes the formation of ectopias and extra-folding. (a, d) Coronal sections of juvenile ferrets at P30 showing the extension of the electroporation performed at E34 in the parietal cortex of *Gfp* (a) and *PIK3R2*- WT - IRES-*Gfp* (d). (b, e) Section comprending the thickness of the entire cortex, from the ventricle to the cortical surface. Squared areas of (b) and (e) are magnified in b'; b''; b''' and c', c'', c'''; and e', e'', e''' and f', f'', f'''; respectevly. (e) shows the perturbation of the cortical surface appearing with extra folding, that could be single or multiple. (f) High magnification of the

ectopic nodules located in the periventricular area, mainly populated by differentiated neurons (NeuN). These experiments contemplate ≥ 3 animals. MZ, marginal zone; CP, cortical plate; VZ, ventricular zone; LV, lateral ventricle. Scale bars, 1 cm (a, d), 1 cm (b, e), 200 µm (b', b''; c', c''; d', d''; e', e'', f', f'').

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2. Role of FLNA in neuronal migration

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2.1. *FlnA* is expressed in germinal layers and cortical plate during mouse corticogenesis

FLNA mutations have been identified in patients with PVNH or OPD in which defects in neuronal migration and vascularity are evidenced, respectively. In order to understand what could be the role of FLNA in cortical development, we first analysed its expression pattern in mouse corticogenesis by *in situ* hybridization. We observed that at early stages, E12.5, *FlnA* was homogeneously expressed through the cortex, mainly formed by the VZ at this stage. Later, when CP starts to consolidate, the expression was higher in the germinal layers (VZ, SVZ) and also in the CP, a pattern that was maintained during development (**Figure 34**). These results indicated the relevance of *FlnA* in cortical progenitors that populate the germinal layers, but also in neurons and neuronal migration. Those results were in concordance to what happen with other genes associated with migration where neurons expressed those genes when they arrive to their final position.

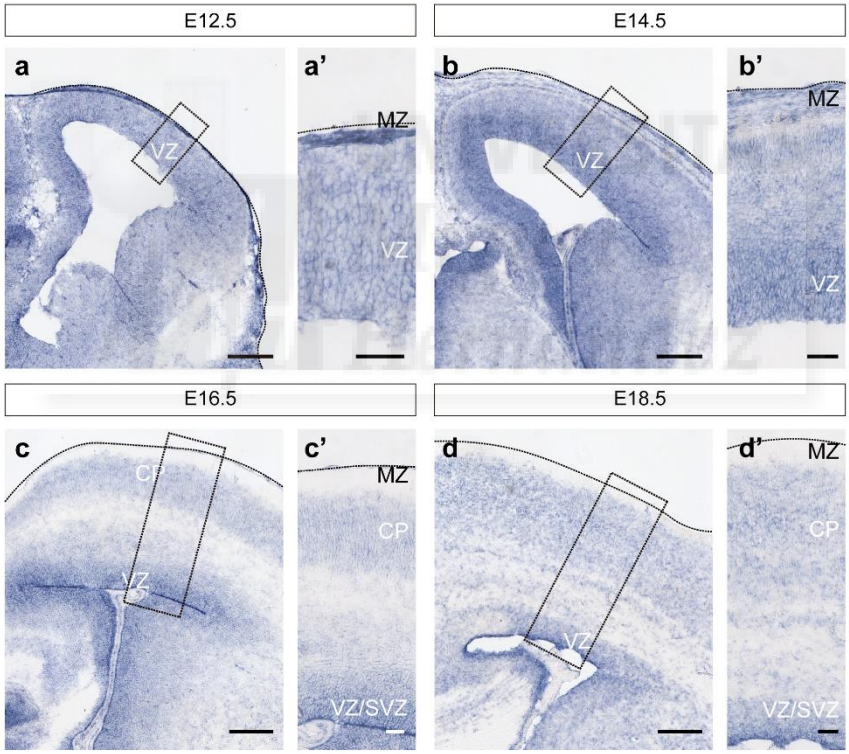

Figure 34. Highest levels of *FlnA* expression in germinal layers and CP in mouse corticogenesis. (a-d) *In situ* hybridization of mRNA *FlnA* in mouse cortical development showing the pattern of expression at several points from early (E12.5) to late corticogenesis (E18.5) with higher levels in the germinal layers (VZ, SVZ) and in the CP. MZ, marginal zone; CP, cortical plate; VZ/SVZ; ventricular/subventricular zone. Scale bars, 200 μm (a, b, c, d), 50 μm (a', b', c', d').

2.2. *FlnA* dysregulation causes a delay in neuronal migration during mouse cortical development

Previous models knocking out *FlnA* have fail to reproduce the heterotopia phenotype in mouse (Feng et al, 2006; Hart et al, 2006). Given the high degree of homology among the different members of the filamin family, it has been proposed that when all cells in the brain are affected by lack of FLNA, a mechanism of compensation could be avoiding the formation of ectopias. For that reason, we decided to approach this issue using *in utero* electroporation in order to manipulate a proportion of progenitors and, consequently, their lineage (new progenitors and neurons). For performing overexpression and knocking down functional experiments, we have chosen E14.5. At this stage, the peak of neurogenesis occurs and also it is the time when *FlnA* was highly expressed in the VZ and SVZ and also in neurons populating the CP (**Figure 34b**).

We considered three different conditions. First, the control situation in which we electroporated a plasmid containing GFP. Second, the *FlnA* knocking down condition, using a previously validated shRNA against the coding sequence of mouse *FlnA* to knock-down its expression coelectroporated with the reporter GFP. Third, the overexpression (OE) case by electroporating the rat full-length *FlnA*-IRES-GFP. After electroporating at E14.5, we allow the cells to migrate for four days and then we analysed how this change in *FlnA* levels could be affecting neuronal migration (**Figure 35**). For that purpose, we quantify the distribution of GFP^+ cells through the cortex in the different layers: germinal layers (VZ/SVZ), IZ and CP (**Figure 35a-d**). We observed that in the control animals, around a 70% of cells have achieved the CP at E18.5. However, we detected a significant decrease in the percentage of *FlnA*-KD and -OE electroporated cells with respect to those electroporated with just GFP. In the case of KD, a proportion of cells expressing the shRNA against *FlnA* were mainly located in the germinal layers and also in the IZ. Finally, an excess of *FlnA* lead to problems in radial neuronal migration and cells that failed to complete their migration were retained ectopically in the IZ (**Figure 35d**).

In order to elucidate whether or not the different distribution of cells was due to a defect in neuronal migration speed, time-lapse experiments were performed. With these experiments we can follow *in vivo* the neuronal migration of cells in their transition towards the CP. Based on our previous observation of defective neuronal migration at E18.5, we electroporate at E14.5 and start recording the *ex vivo* slice at E17.5 during 24h, for finishing at the time-point we detected the main defect (**Figure 35e**). Using a specialized software, we were able to follow the movement of the neurons that have changed their shape from multipolar to bipolar to migrate to their final position in the cortical plate. The tracking of these cells allows us to analyse different parameters such as the straightness of their path during migration and the mean speed of the cells in each condition (**Figure 35f-i**).

The straightness of the trajectory was automatically evaluated by the software we used for all the analysis (see material and methods), by considering the first and the last position associated to a particular neuron. When we compared this parameter between neurons that overexpress *FlnA* and neurons that just express GFP, we observed a clear reduction in those neurons with higher levels of FlnA, indicating a different structured path in that condition. Differently, in the case of the *FlnA*-shRNA, neurons do not present significative differences in their straightness, indicating that the neuronal movement follows a similar direction than control neurons.

On the other hand, we considered the mean speed of migration of the neurons that could be followed by at least 16 frames (out of 49) of the film. We observed that only neurons with an excess of *FlnA* have a decrease in their migration speed (in average terms), while neurons defective for *FlnA* present a speed comparable to neurons expressing GFP. In addition, we evaluate the proportion of cells for different ranges of speed (fast, intermediate or slow) (**Figure 35i**) or even more accurate by classifying them in intervals of mean speed (0-5μm/h, 5-10μm/h, 10-15μm/h, 15-20μm) (**Figure 35h**). In those cases, we detected a divergent proportion of cells, particularly for the lower speeds (0-5μm/; 5-10μm/h), in which an increase in the proportion of cells with speeds under 5μm/h were increase in the case of *FlnA* OE. This result could justify the decrease in the mean speed, where all speeds are considered together for each condition.

Taken together, all these results indicate that in the case of *FlnA* OE, neurons have a defective neuronal migration produced by an alteration on their direction path and a lower migratory speed. These defficiences are probably the cause why a significative proportion of neurons were still remaining in lower positions in the CP and in the IZ at E18.5 (**Figure 35b, d**). On the contrary, in the case of neurons in which *FlnA* was knock down, there were no differences in any of these parameters, suggesting that the migration process could be undergone normally. These analyses suggest that the different distribution of cells at E18.5 (**Figure 35a-d**) could be due to another factor, such as a different birthdating of the neurons, which would justify the higher presence of GFP^+ cells in the germinal layers (**Figure35a, c-d**).

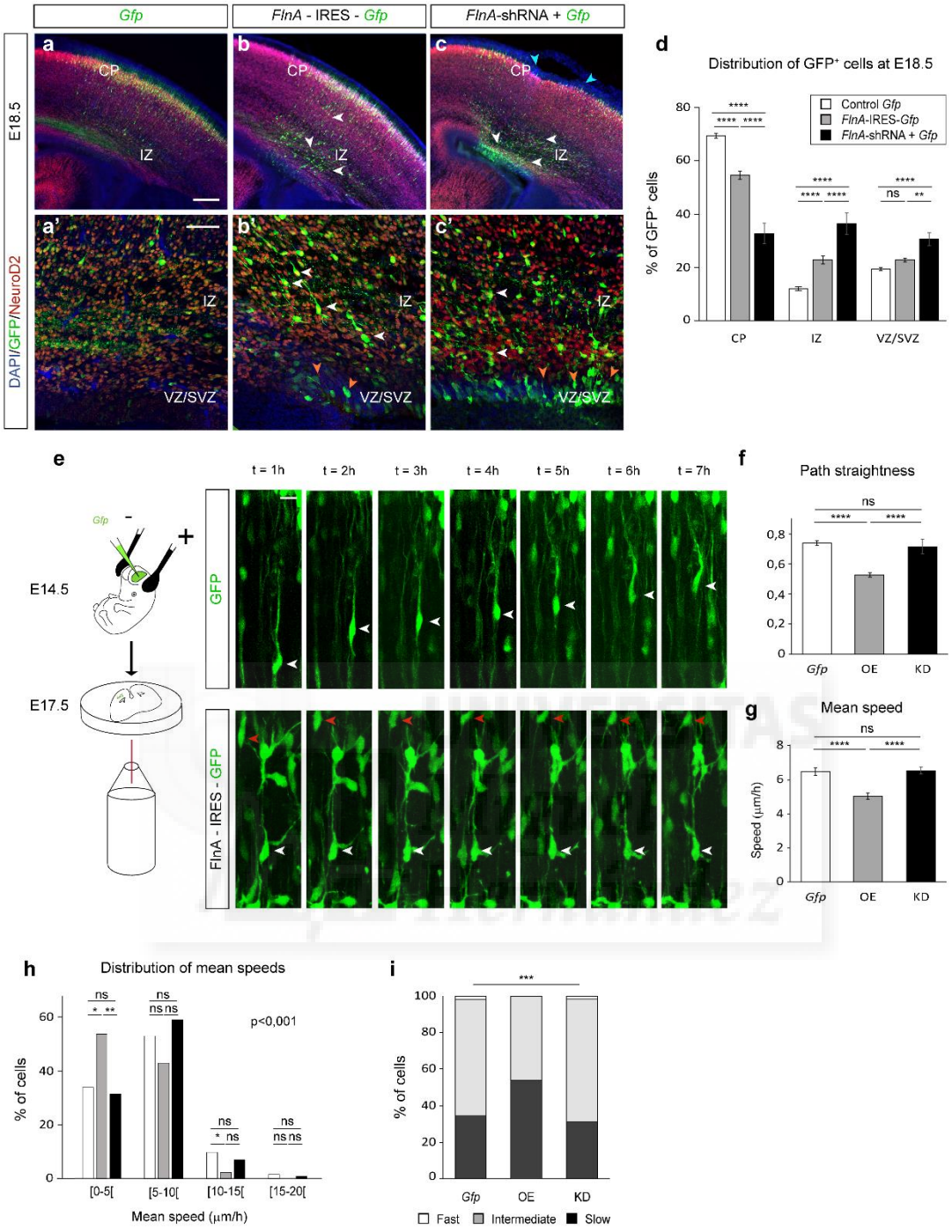

Figure 35. Alteration of *FlnA* levels led to neuronal migration defects. (a-c) Coronal sections of E18.5 mice after electroporating GFP, *FlnA*-IRES-GFP and *FlnA* shRNA+GFP at E14.5. (a', b', c') High magnifications of IZ amd germinal layers (VZ, SVZ), showing co-labeling of GFP+ cells with the neuronal marker, NeuroD2+. White arrows point the abnormal position of neurons in comparation with the control situation, which are identified as neurons. Blue arrows indicate the lack of cells in the CP in the KD condition. Orange arrows point the cells located in the VZ/SVZ in the KD condition, which are negative for NeuroD2 marker. (d) Quantification of the distribution of GFP+ cells at E18.5 through the layers (e) Time-lapse experiments schema: three days after electroporating E14.5 embryos, the neuronal migration of cortical neurons is recorded using 2-photon videomicroscopy during 24h. Migrating neurons are followed through the film, as it is indicated in the different frames, highlighted by white and red arrows. (f) Quantification of the path straightness followed by the neurons in each condition, considering at least 100 cells per experiment, and two experiments per condition (GFP: 200

cells; OE: 263 cells; KD: 227 cells). (g) Quantification of the mean speed of the neurons for each condition, considering cells that are at least in 16 frames of the film, and two experiments per condition (GFP: 177 cells; OE: 195 cells; KD: 170 cells). (h) Distribution of neurons in groups of speeds for each condition, same considerations as in (g). (i) Distribution of neurons for each condition, grouped as fast, intermediate speed and low speed migrating neurons, same considerations as (g) and (h). Chi-square tests performed in (h) and (i) show abnormal distribution of cells ($p<0,001$); *$p<0,05$; **$p<0,01$; ***$p<0,005$. CP; cortical plate; IZ, intermediate zone; VZ/SVZ, ventricular/subventricular zone. Scale bars, 200 μm (a, b, c), 50 μm (a', b', c'), 20 μm (frames in e).

2.3. Focal disruption of *FlnA* expression levels causes an ectopic positioning of neurons

After the neuronal migration defects that we have observed in our short-term experiments at embryonical stages, we wondered if the defect shown would be recovered postnatally. To answer this question, we repeated the previous manipulation experiments under the three conditions at E14.5 and we waited five days after the animals were born for evaluating its effects, at P5 (**Figure 36**). At this time-point, we detected that the majority of cells have correctly concluded their neuronal migration and have reached the CP in both KD and OE conditions. However, we noticed that a significant proportion of cells is still ectopically placed (**Figure 36a-c**).

In *FlnA* OE, the ectopic cells were distributed in germinal layers, white matter and lower subcortical positions. Contrarily, in the knock-down situation, the cells were mainly retain in the germinal layers and only a small proportion of them were located in the white matter in accordance with what we have observed in previous experiments. In order to characterize the ectopic cells, we immunostained them with the neuronal marker NeuroD2^{+}. When we increased levels of *FlnA*, we could identify the cells located in the germinal layers mainly as neurons whereas in *FlnA* KD just a small proportion was identified as NeuroD2^{+}, suggesting that the 10% of cells located in the white matter are not neurons (**Figure 36a-c, g-h**).

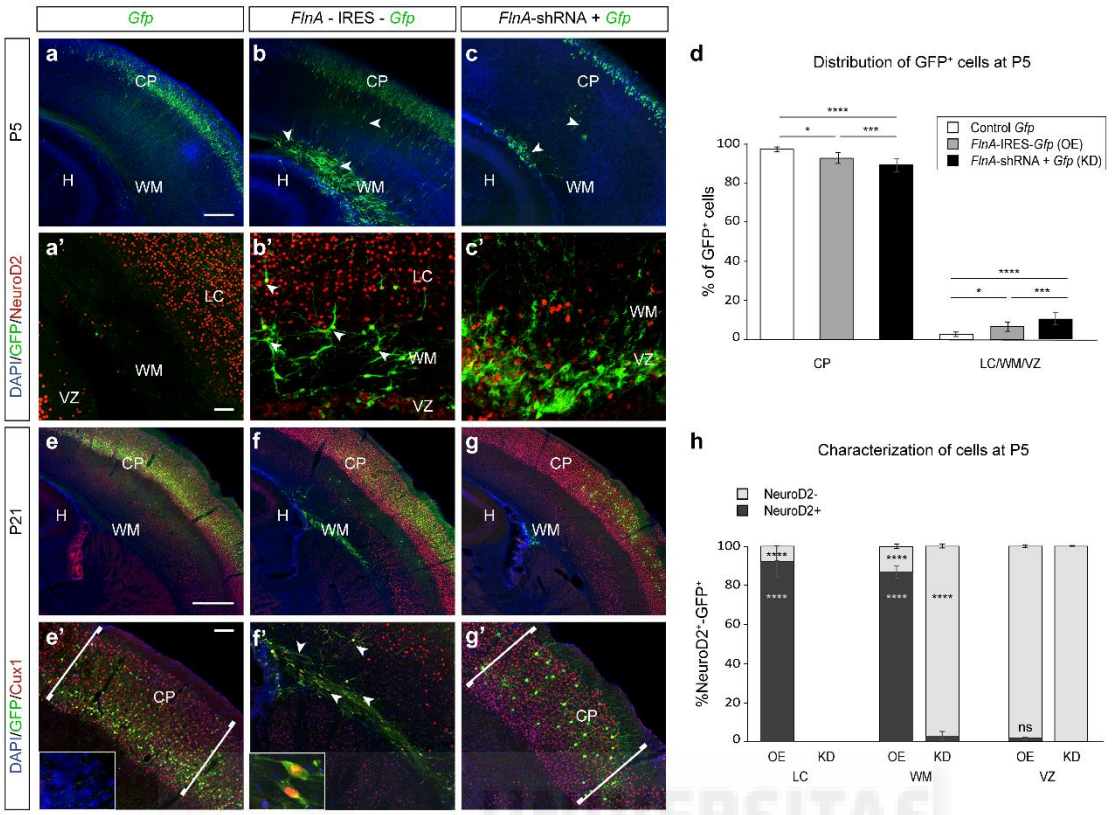

Figure 36. Alteration of *FlnA* levels led to abnormal neuronal positioning postnatally. (a-c) Coronal sections of P5 mice after electroporating GFP, *FlnA*-IRES-GFP and *FlnA* shRNA+GFP at E14.5. (a', b', c') High magnifications of lower cortical areas and WM, showing co-labeling of GFP+ cells with the neuronal marker, NeuroD2+. (d-f) Coronal sections of P21 mice after electroporating GFP, *FlnA*-IRES-GFP and FlnA shRNA+GFP at E14.5. (d'-f') High magnifications of the CP (d', f') show an altered distribution of neurons in FlnA KD condition. (e') High magnification around the lateral ventricle and WM identified the ectopic cells as differentiated neurons, Cux1+ (square). (g) Quantification of the distribution of ectopic cells at P5. (h) Characterization of the ectopic cells at P5. Values correspond to ≥ 3 animals for each condition; *p<0,05; **p<0,01; ***p<0,005. CP; cortical plate; WM, white matter; H, hippocampus. Scale bars, 200 µm (a, b, c), 50 µm (a', b', c'), 500 µm (e, f, g), 100 µm (e', f', g').

In order to understand what will happen with these ectopic cells, we have studyied the effect of our electroporation in juvenile animals (P21) (**Figure 36d-f)**. In the case of *FlnA* OE, we confirmed that the ectopia is consolidated postnatally, as was evidence by the remaining proportion of cells in the white matter and in deeper layers, something that never happens in the control situation (**Figure 36d-e**). Importantly, these cells are differentiated as upper layer neurons, as indicates the colabelling with the Cux1 marker. These results showed as that although all GFP+ cells have an artificially higher level of *FlnA*, just a slight number of neurons are finally defective in their positioning. Similarly, in patients with PVNH, although the proportion of cells affected are between 50% to 100%, just a few are constituting the periventricular nodules. Interestingly, the ectopic positioning occurs even though these cells were correctly differentiated for their specific layer, as it corresponds to neurons born from

E14.5, when the electroporation was performed. In agreement, also the ectopias found in patients are majoritarily formed by upper layer neurons.

In contrast, in the *FlnA* KD condition, the ectopic cells in the white matter were no longer present, which could be due to later migration compensation or due to the elimination of these ectopic cells by cell death. Importantly, although all GFP^+ cells were located in the upper layers, the knock-down of *FlnA* produced an upper location of the neurons in the CP in comparation with control animals (**Figure 36d, f**).

2.4. *FlnA* is expressed in ferret germinal layers and cortical plate during cortical development

We have been able to reproduce the ectopias found in PVNH patients in the *FlnA* overexpression conditions. However, the most common cause for this disorder is the loss-of-function of *FlnA*. By knocking down FlnA in mice we could see a transient effect and an alteration in neuronal positioning without the formation of nodules. We hypothezed that this could be due to a delay in neuronal migration that caused that neurons arrived later to the CP, which eventually would cause their different location in the CP. As this manipulation did not produce the formation of the periventricular nodules in mouse, we decided to move to a more proximal to human model, the ferret. We expected that similarities shared by these two gyrencephalic species gave us the opportunity to model this malformation in this ferret. In this sense, could be, as was previously shown in human, that FlnA presents areas of different expression that could underlie the reason why the formation of nodules occur in particular location in patients. Moreover, as the formation of ectopias has been suggested to have a cortical progenitors component, the complexity of the gyrencephalic progenitors could also give us a more feasible scenario in which altering their FlnA expression has greater consequences in terms of ectopia formation.

Our first approach was to analyse the pattern of gene expression of *FlnA* in ferret during cortical development by *in situ* hybridization (**Figure 37**). At embryonical stages E34 and E42 we have detected *FlnA* expression in germinal layers at the the VZ, and also in the CP. This pattern was maintained later at postnatal stages, when ferret corticogenesis is still on going. Interestingly, this distribution of expression was similar to what we found in mouse development, which suggest that FLNA could have a similar role in progenitor and neurons. Moreover, it is important to point out that within the OSVZ we found different levels of expression across different areas (prospective gyrus/prospective sulcus), as has been published in human cortical development (De Juan Romero & Borrell, 2015). This suggested a distinct requirement of *FlnA* along the OSVZ, and, consequently, along different cortical areas (**Figure 37**).

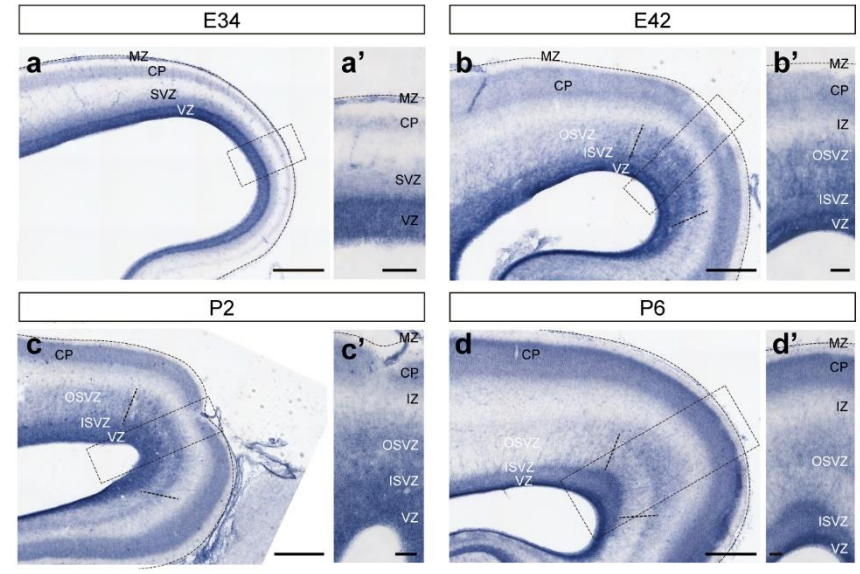

Figure 37. Highest levels of *FlnA* gene expression are found in germinal layers and CP in ferret corticogenesis. (a-d) *In situ* hybridization of *FlnA* in the developing ferret cortex showing higher levels in the germinal layers (VZ, ISVZ and OSVZ) through development. Additionally, we found regions with differential expression along the ISVZ and OSVZ at E42, P2 and P6 (black lines). MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; OSVZ, outer subventricular zone; ISVZ, inner subventricular zone; VZ, ventricular zone. (a'-d') High magnifications of the areas squared in the respectively images. Scale bars, 500 µm (a, b, c, d), 100 µm (a', b', c', d').

2.5. *FlnA* OE and KD in ferret reduce the number of cells in VZ and KD alters migration of cortical neurons

We have seen that in mouse, *FlnA* manipulations lead to neuronal migration defects. In order to evaluate whether or not that is also the case in ferret, we performed postnatal electroporations in ferret pups at P1.5 (**Figure 38**). We have used a plasmid containing GFP as a control and a plasmid containing the full-length *FlnA* for the overexpression condition. In the case of the KD, we took advantage of the CRISPR-Cas9 system that allow us to knock-out the gene in the electroporated cells as well as in their lineage. For that purpose, we have designed two specific guides against ferret FlnA and cloned them in a plasmid with the CRISPR-Cas9 system.

We have electroporated the ferret visual cortex at P1 and then we have analysed the impact of the manipulation at P14, which is an equivalent stage of mouse E18.5. First, we have quantified the distribution of GFP+ cells though the layers (**Figure 38a-e**). When we looked at P14, we noticed that In the OE condition, the number of GFP^+ cells were in all the animals clearly disminished in comparation with the control. However, the proportion of cells able to arrive to the CP was not changed compared to the control situation. Contrary, in KD cases, a clear defect in neuronal location was observed, as it is indicated by the lower proportion of cells reaching the CP (**Figure 38d-e**).

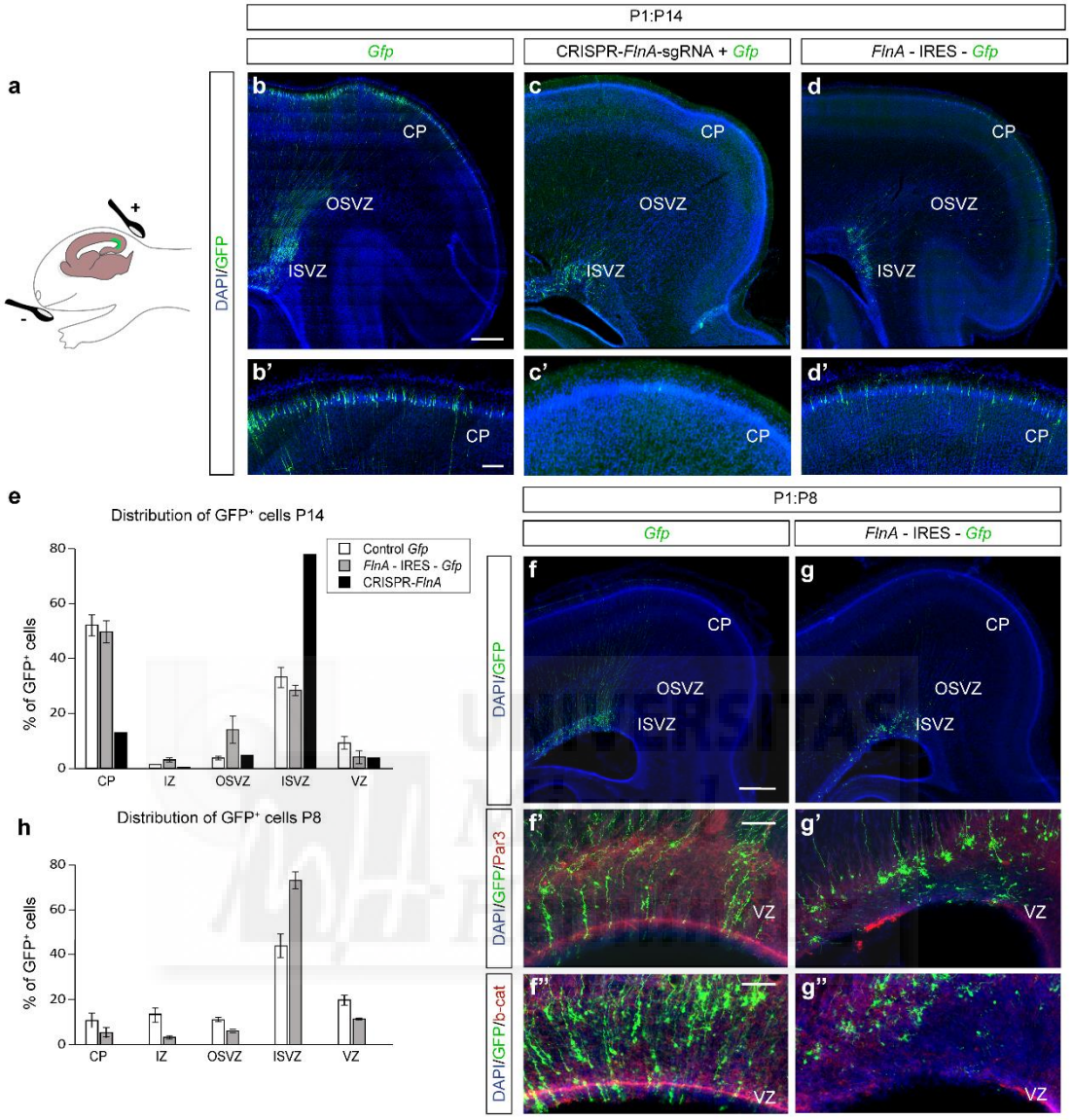

Figure 38. *FlnA* OE and KD reduces the number of cells in VZ, while KD alters migration of cortical neurons. on postnatal ferrets. (a) Postnatal electroporation of ferret visual cortex. (b-d) Sagittal sections of P14 ferrets after being electroporated at P1.5 in the visual cortex. (e) Quantification of the distribution of the GFP⁺ cells through the cortex. (f-g) Sagittal sections of P8 ferrets after being eletroporated at P1.5. Animals with an overexpression of *FlnA* show an alteration of progenitors morphology and a defect in adherent junctions Par3 (f', g') and β-cat (f'', g''). (h) Quantification of GFP⁺ cells distribution at P8. The data plotted here corresponds to 2 animals for each condition. CP, cortical plate; IZ, intermediate zone; OSVZ, outer subventricular zone; ISVZ, inner subventricular zone; VZ, ventricular zone. Scale bars, 500 µm (b, c, d, f, g), 100 µm (b', c', d', f', g').

2.6. *FlnA* OE produces a loss of adherent junctions in the ventricular linning

We have detected a considerable reduction in the number of GFP⁺ cells in *FlnA* OE condition. Moreover, some progenitors showed an altered morphology. To have a better insight in the effects of overexpressing *FlnA* earlier in development, we have decided to have a look to what

happen in an intermediate stage between the electroporation at P1 and the analysis performed at P14. For this reason, we have tested the effects of FlnA at P8 (**Figure 38f-h**).

First, we have found by quantifying the distribution of GFP^+ cells, a decreased proportion of GFP+ cells in the VZ that comes together with an increase of GFP^+ cells in the ISVZ (**Figure 38h**). Moreover, we could observe a dramatic lack of most apical contact of aRGCs, which could suggest that these cells have detach from the ventricular lining and displaced to more basal position in the ISVZ. To attempt this idea, we decided to perform immunostaining against Partitioning defective 3 (Par3) and β-catenin (β-cat), which are proteins of the apical adherent junctions (**Figure 38f, g**). In both cases, we found a clear disruption of these fundamental proteins which maintain the integrity of the apical side. Importantly, alterations in the ventricular lining have been reported in models of PVNH (Carabalona et al, 2012; Houlihan et al, 2016), suggesting that this defect could be part of the mechanism required for the formation of nodules. However, our experiments at P14 showing that ferrets with increased levels of *FlnA* do not present periventricular nodules indicates that this mechanism could be necessary but not sufficient to produce the periventricular nodules phenotype in this species.

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3. Role of EML1 in ferret development

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3.1. *Eml1* is highly expressed in CP during ferret corticogenesis, with a slight expression in the germinal layers

Eml1, a microtubule associated protein, has achieved interest in the last years since mutations in its gene were identified as causative for SBH (Kielar et al, 2014), as others genes related with microtubules (Jaglin & Chelly, 2009). In order to unravel its role in cortical development, studies in mouse were performed recently describing its endogenous gene expression in mouse and the effects of its mancance in the spontaneous model of SBH related to *Eml1* in the HeCo mouse (Bizzotto et al, 2017; Kielar et al, 2014).

Previous work on *Eml1* mouse gene expression has shown that it was highly expressed in the CP and the germinal layers during early and mid-corticogenesis, while at later stages this expression was not longer found in VZ and SVZ but restricted to the CP (Kielar et al, 2014). In order to examine progenitor zones that resembles the features of the ones that we find in human brains, we have used a gyrencephalic species, the ferret, as experimental model. First, we performed *in situ* hybridization at different time points during development (**Figure 39**). At early stages (E30), a slight expression started to appear in the CP and in the germinal layers, specially in the ISVZ (**Figure 39a**), which was more prominient at E34 (**Figure 39b**). As corticogenesis proceed, this pattern was maintained until P6 (a developmental stage comparable to E17 in mouse) when we observed that levels of *Eml1* were higher in germinal layers than at previous stages (**Figure 39d**).

These results were consistent with the relevance of *Eml1* in cortical progenitors and in postmitotic neurons in a lissencephalic model, the mouse. In addition, these data also suggested a different regulation of *Eml1* during ferret development, since there was expression at comparable time-points, although much more reduced than in mouse. Additionally, *Eml1* expression in ferret started to be more pronounced in germinal layers at P6 (**Figure 39d**), a moment when in mouse was not observed in the VZ (Kielar et al, 2014). Taken altogether, these data suggest a possible diverse regulatiton or necessity of *Eml1* during corticogenesis of lissencephalic and gyrencephalic species.

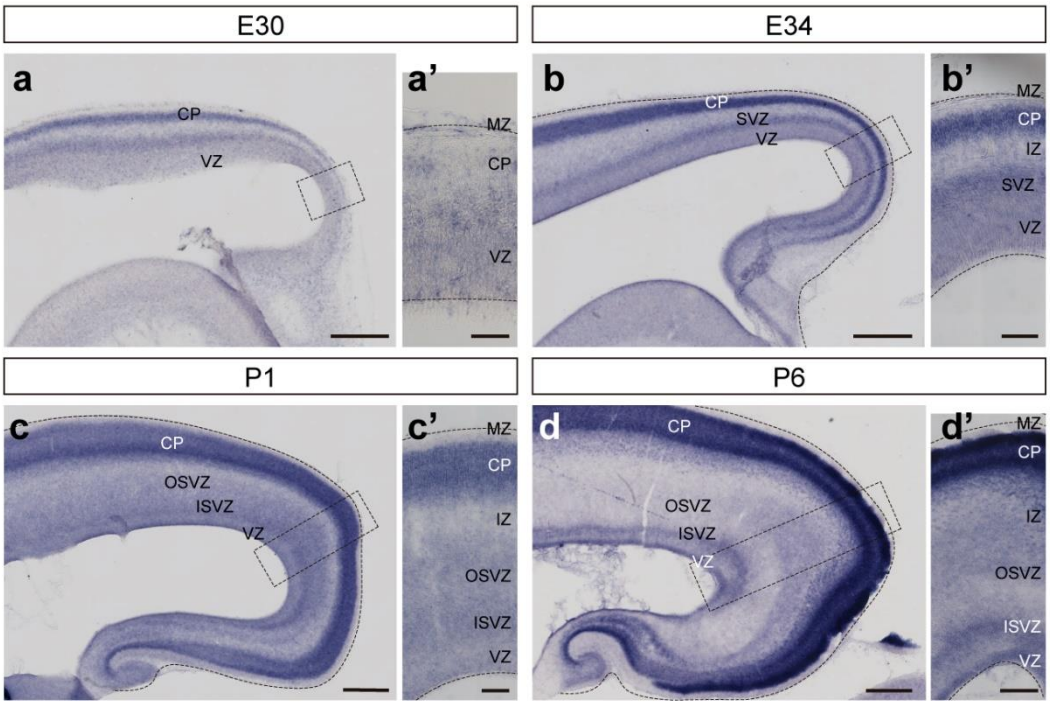

Figure 39. Levels of *Eml1* gene expression are detected slightly in germinal layers and highly in CP CP in ferret corticogenesis. (a-d) *In situ* hybridization of *Eml1* in the developing ferret cortex showing a slight expression at early corticogenesis (E30, a), starting to be more prominenet in the VZ and CP in early and late stages of cortical development (E34, b; P1, c) and (P6, d). MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; OSVZ, outer subventricular zone; ISVZ, inner subventricular zone; VZ, ventricular zone. (a'-d') High magnifications of the areas squared in the respectively images. Scale bars, 500 µm (a, b, c, d), 50 µm (a'), 100 µm (b', c', d').

3.2. *Eml1* impact on neuronal migration in ferret

As mentioned, Kielar *et al.* shown higher levels of expression in the VZ at early and mid-corticogenesis in mouse. Consistent with this expression pattern, they knocked-down *Eml1* in mouse progenitors at mid-corticogenesis (E15). Differently, in ferret cortical developpment we have found that *Eml1* expression is much lower in the VZ at comparable stages. For that reason, we decided to perform the complementary experiment by overexpressing Eml1 at P1.5, a similar time-point to E15 in mouse, in the ferret visual cortex. We analysed the distribution of GFP^+ cells after one week, observing a minor but significant proportion of cells in the OSVZ, compared to the control animals. (**Figure 40a-b, f**). To evaluate a possible migratory defect, we decided to wait until P14 when the majority of neurons have reached the CP (**Figure 40d-e, g**). However, the distribution of cells at this stage was not altered since the same proportion of cells was detected in each layer (**Figure 40g**). Moreover, cortical neurons seemed to present a normal morphology (**Figure 40d', e'**). These results suggested that it may be a possible migratory defect at early stages that get compensated later in development.

Further analysis will be necessary to elucidate the mechanisms of these results. These studies at different time points show the cells could suggest that a previous migratory defect is later compensated, reason why it was no longer observed at P14. Another possibility will match the corollary that *Eml1* is critical for the normal distribution of RGCs (Kielar et al, 2014), and that the extra cells observed at P8 were ectopic progenitors that were later eliminated and no longer part of the cortex at P14, reason why the distribution of GFP⁺ cells appeared normal at this stage.

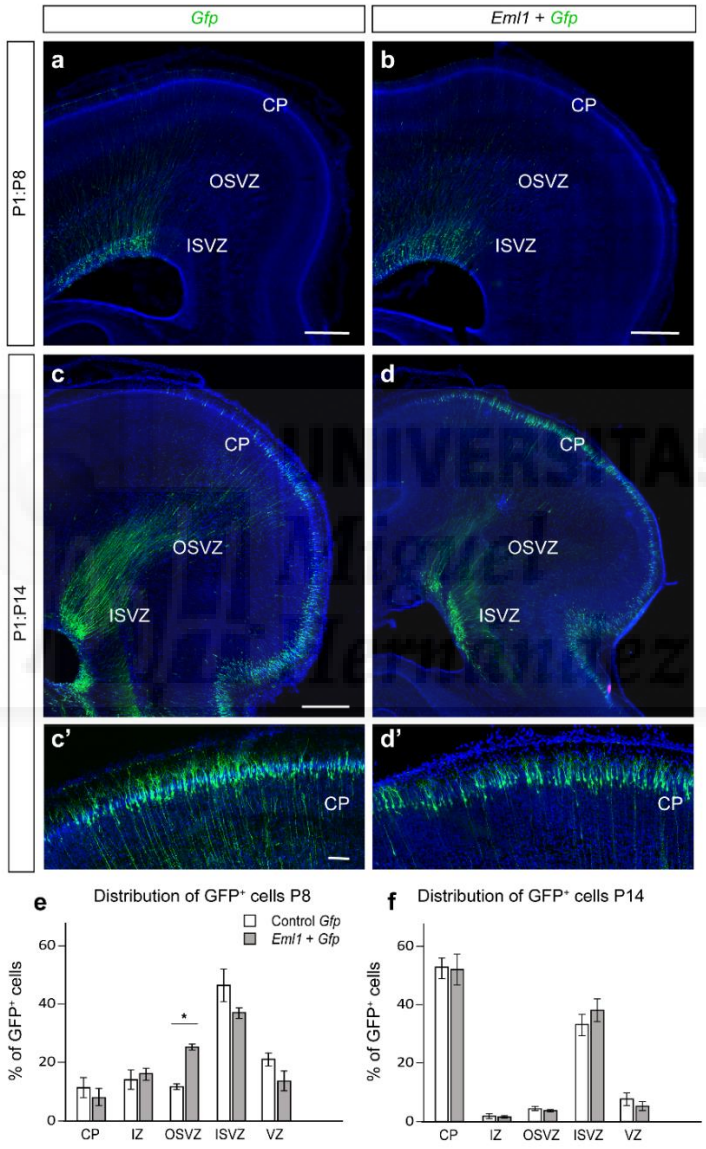

Figure 40. Overexpression of *Eml1* in postnatal ferrets slightly impair cellular distribution, which is corrected later in development. (a-b) Sagittal sections of P8 ferrets after being electroporated at P1.5 in the visual cortex. (c-d) Sagittal sections of P14 ferrets after being electroporated at P1.5 in the visual cortex. (c', d') Higher magnifications show a normal morphology of neurons that reach the CP. (e) Quantification of the distribution of the GFP⁺ cells through the cortex at P8. (f) Quantification of the distribution of the GFP⁺ cells through the cortex at P14. Values correspond to ≥ 3 animals for each condition; *p<0,05. CP, cortical plate; IZ, intermediate zone; OSVZ, outer subventricular zone; ISVZ, inner subventricular zone; VZ, ventricular zone. Scale bars, 500 µm (a, b, c, d), 100 µm (c', d').

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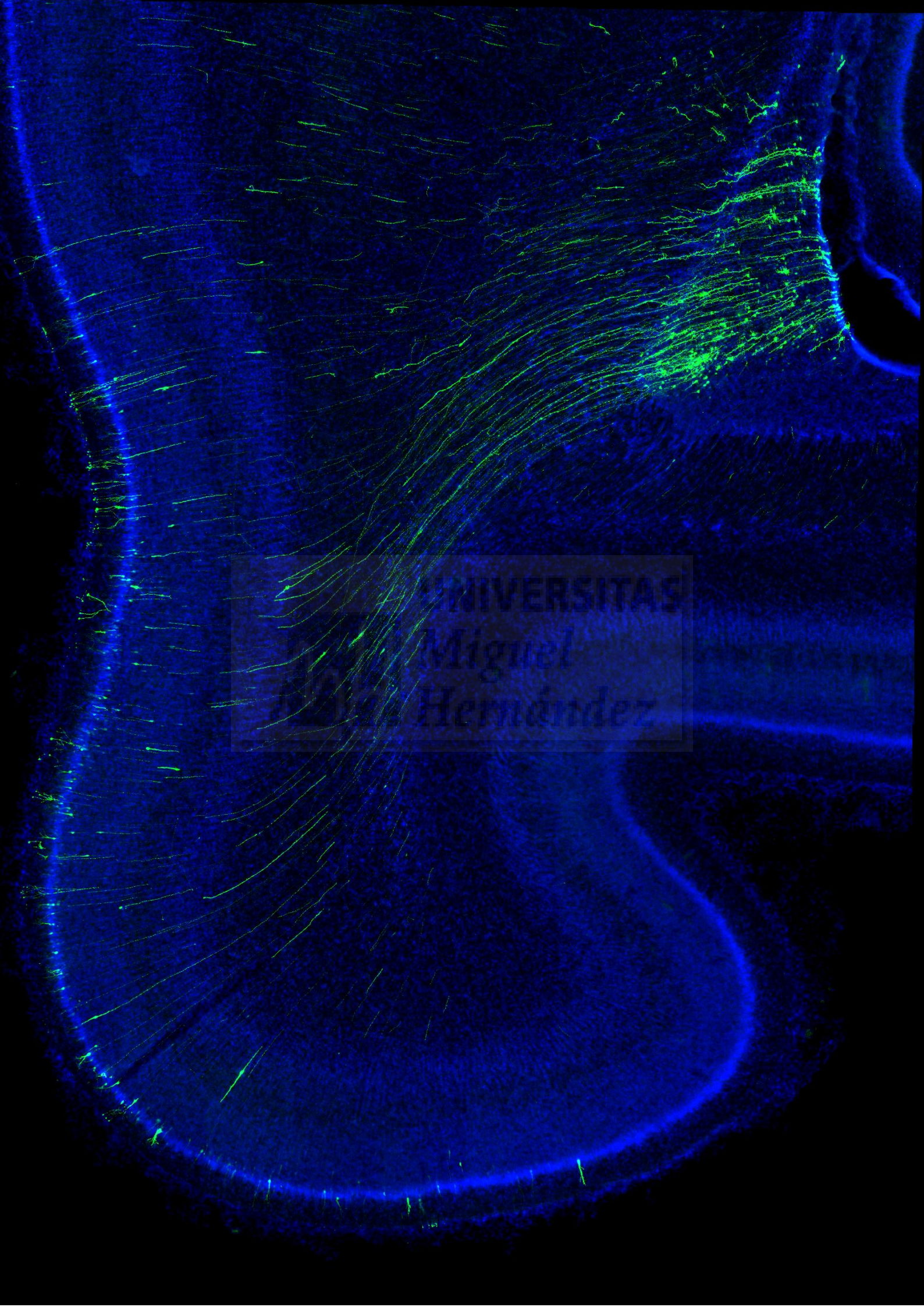

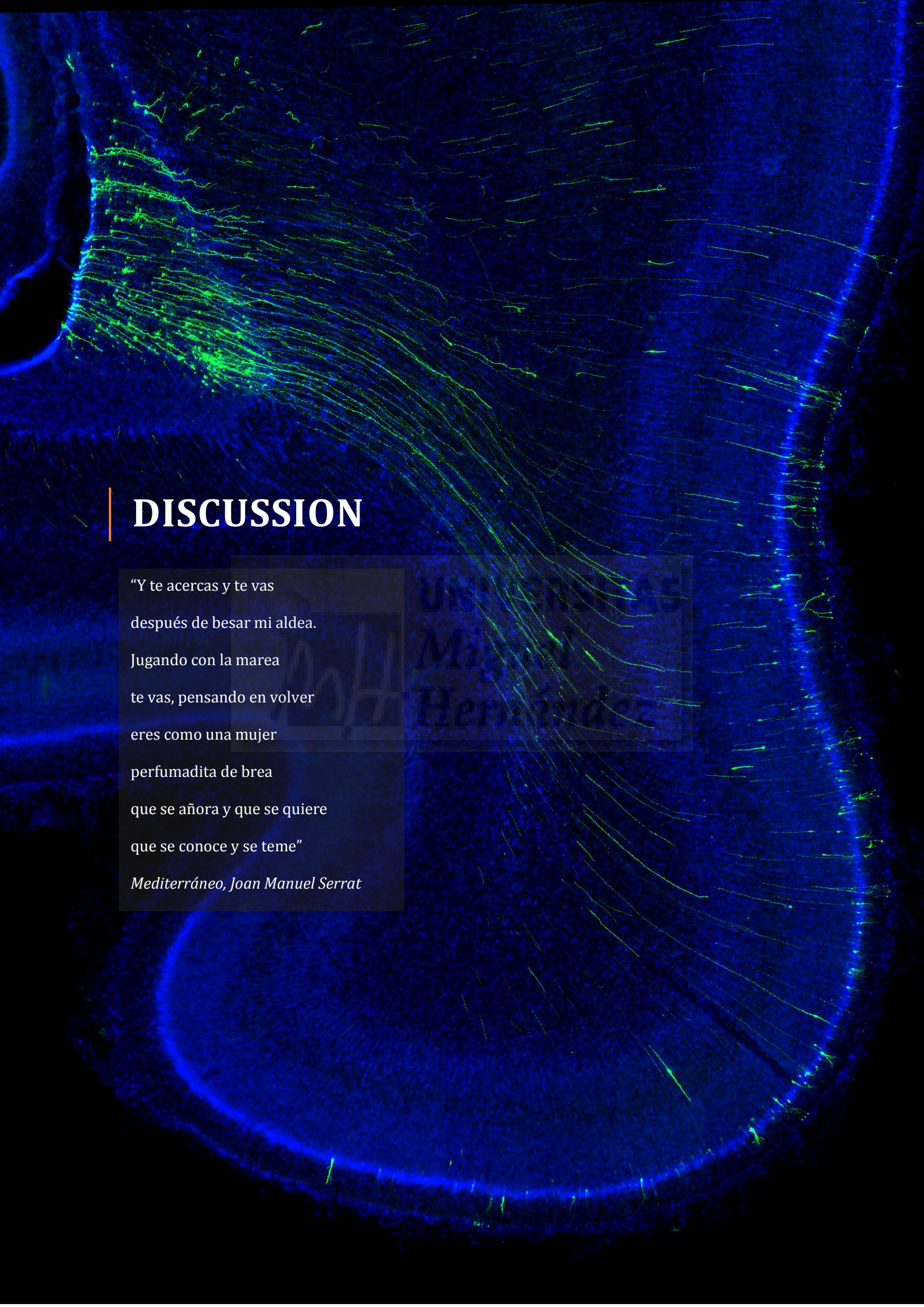

DISCUSSION

"Y te acercas y te vas
después de besar mi aldea.
Jugando con la marea
te vas, pensando en volver
eres como una mujer
perfumadita de brea
que se añora y que se quiere
que se conoce y se teme"
Mediterráneo, Joan Manuel Serrat

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Cortical development is a complex process that involves the spatio-temporal coordination of multiple events that include cellular proliferation, migration and cellular differentiation. All these processes are rigorously regulated by many molecules. Consequently, mutations in genes controlling these processes, could give rise to malformations of cortical development (Barkovich et al, 2012; Caviness et al, 1995; Fernandez et al, 2016; Rakic, 1995). MCDs are disorders in which cortical structure and function are altered, resulting in intellectual disability, autism and epilepsy (Andrade, 2009; Guerrini et al, 2008; Hans J. ten Donkelaar, 2014).

MCDs may be classified either by the developmental stage when the disruption gives rise to the pathology, or by the main effect observable in the cortical structure. Following the second criterion, we distinguish between alterations in brain size (excess or defect), cortical folding or the aberrant cellular location (ectopia). These alterations may appear isolated or combined (Barkovich et al, 2012). The heterogeneity of type of disorder and even inside the ones considered as the same pathology, makes the understanding of these malformations a very hard path.

This thesis was developed as a part of the DESIRE consortium, in which a continuity from human mutation to clinical studies was followed. This work has tried to shed light in the pathomechanisms that underlie some of the most common MCDs, including polymicrogyria and periventricular heterotopia. For that purpose, we have performed gene expression studies and functional experiments in two animal models, mouse and ferret, studying the role of genes that are known to be mutated in patients with these pathologies, *PIK3R2* (Mirzaa et al, 2013) and *FLNA* (Fox et al, 1998), respectively. Additionally, some preliminary data were presented also here, regarding *EML1*, a gene recently related to subcortical band heterotopia (Kielar et al, 2014). Interestingly, these three pathologies have been associated to migratory defects, although the implication of progenitor's behaviour has also been suggested (Bizzotto & Francis, 2015; Carabalona et al, 2012; Santi & Golden, 2001). In the present section, we will try to discuss how our results could fit (or not) to what others previously have found and to what human patients present.

For both *FlnA* and *Pik3r2*, we observed that the pattern of expression found in mouse and ferret was comparable to the one in human, in which the highest mRNA levels of these genes are located in the germinal layers and in the CP through corticogenesis. The fact that those genes are being expressed by the same cells in both species propose a similar need of those genes for cortical progentiros and neurons, consistently with the highly conserved protein structure among species. However, it is important to point out the differences in complexity of each organism, which definitively influence the divergence of some of the results that we obtained.

Pik3r2 is a regulatory gene of the mTOR pathway, a very complex cascade that has been related to protein synthesis, metabolism, cell cycle, growth and proliferation (Engelman et al,

2006; Vanhaesebroeck et al, 2012). In fact, multiple genes involved in this pathway are mutated in patients with overgrowth syndromes and polymicrogyria. Also, non-genetic causes as vascular insults have also been identified as causative for this pathology (Harvey et al, 2008). The human most recurrent mutation in *PIK3R2*, G373R, has been found both as germline and as somatic mutation, suggesting that a small proportion of cells affected is sufficient to produce the malformation in humans. Also, functional experiments in cells derived from patients suggest that this is a gain-of-function mutation in which the mTOR pathway is overactivated, which fits with the phenotypic consequences found in patients (i.e. macrocephaly syndromes) (Riviere et al, 2012).

In order to evaluate the effect of PIK3R2 during cortical development, first we have performed *in utero* electroporation in mouse embryos at the peak of neurogenesis (E14.5). Interestingly, we found that this manipulation resulted in a significant increase on cortical progenitors, particularly the basal ones (IPCs), which also divided at a greater rate, as it was shown by the increase in the number of mitoses. This increment could be a consequence of an increased cell-cycle re-entry, meaning, that when we looked at these cells at a particular time-point, we observed more mitoses and more progenitors because they stayed longer in this stage. However, normally when the cell cycle is extended, it is due to a longer G1 or S-phase, not to a lengthening in the M-phase. A longer cell-cycle is associated with the cell-cycle length. To discriminate between the two possibilities, we performed a cell-cycle re-entry analysis that confirmed that the greater number of progenitors that we have observed was due to a higher cell-cycle re-entry. Thus, when these progenitors divided, they preferentially gave rise to new progenitors instead of neurons. Interestingly, somatic gain-of-function mutations in *PIK3R2* have been found in sporadic tumors, in which overproliferation is expected (Cheung et al, 2011).

PI3K is a complex formed by the catalytic subunit PI3KCA and the regulatory subunit PIK3R2, which promotes the formation of PIP3, an initial substrate that activates the mTOR pathway. The increase in *PIK3R2* levels and the overexpression of the mutated form were expected to cause an overactivation of the mTOR pathway, since G373R has been suggested as a gain-of-function mutation (Riviere et al, 2012). Surprisingly, the effects we obtained in progenitors were not observed in mouse models with conditional *PI3KCA* activated (H1047R, E545K). These animals presented megalencephaly when modified alleles are expressed early in development, while neonatal activation of E545K did not produce an alteration in brain size, which suggests that causing brain overgrowth requires overactivating PIK3CA during embryogenesis (Roy et al, 2015).

In our case, a visible increase in brain volume was not expected as the proportion of cells affected by electroporation are much less than in these conditional mice models. Interestingly, in H1047R mutants, cortical expansion seemed to be due to a reduced cell density and increased

cell size instead of greater proliferation or reduced apoptosis. In E545K mice, the cell somas were also longer with respect to the controls. Moreover a greater cell-cycle exit was detected (Roy et al, 2015), contrary to what we observed in our OE E16.5 mice.

Different possibilities could explain this discrepancy. First, the complexity of the pathway. Although the three mutations are activating the mTOR pathway (Kinross et al, 2012; Lee et al, 2012; Riviere et al, 2012; Yuan et al, 2013) this cascade has multiple interplayers that could be counteracting in higher or lower level the effects of the mutated forms (reviewed in (Saxton & Sabatini, 2017). Additionally, our experiments were based on the regulatory subunit, while Roy *et al*. work was focus on its partner PI3KCA, an even with that, the two mutations were also causing different phenotypes. Moreover, we do not know how these mutations could be affecting the conformation of these proteins and the interactions with the other parts of the complex and to other cascade partners. So that, the implication of the consequences could be variable. Finally, our experiments were affecting a subset of cells in the brain, while the conditional models were modyfying the majority of brain cells in those mice. Consequently, mutant neurons and progenitor cells are in a mutant environment, contrarily from ours, that are coexisting with wild-type cells that influence them and interact with them differently. In addition, a non-cell autonomous effect cannot be discarded, especially regarding what we found in functional experiments in ferret.

When we decided to evaluate the effect of overexpressing *PIK3R2* in neuronal migration, we noticed that increased levels of *PIK3R2* cause a neuronal migration defect that was consolidated postnatally. These neurons that were found ectopically placed at embryonic stages were reduced in number later in juvenile animals (P21), which suggests that this defect could be either compensated or that these cells were eliminated by the system through programmed cell death. These neurons were correctly differentiated and present a normal morphology, expressing a specific marker of upper layers, as corresponds to neurons born from E14.5, when the electroporation was performed. In agreement, conditional mouse models of *Pi3kca* also presented a problem of neuronal migration, although in this case was reflected as a laminar disorganization of both early and late-born neurons, despite their correct specification (Roy et al, 2015).

Nevertheless, considering the higher cell-cycle re-entry that we found, another explanation for this defect on neuronal migration would be the late stage of birth of these cells. In other words, considering the relevance of the space and time for the positioning of migratory neurons, a delayed birth could cause the ectopic positioning of these cells. However, one would expect that these would affect to all GFP^+ neurons, not just a subset. It may be that some mechanism of compensation avoided mostly the defect, but some neurons scaped to this. The fact that the number of cells showing the phenotype is smaller than those that were genetically

affected is something that we found here but that is also common in human pathologies of neuronal migration, like PVNH, in which although around 50% of cells are affected in females, the number of neurons forming the ectopias are much less than expected (Sarkisian et al, 2008).

Although mouse overexpressing *PIK3R2* did not develop polymicrogyria, the neuronal migration defect was a consistent phenotype. Moreover, although neuronal migration was suggested as causative for polymicrogyria, other authors support that either during migration, this pathology could develop after this process is completed (Squier & Jansen, 2014) Additionally, as mentioned, polymicrogyria could appear in combination with ectopias, particularly periventricular ones, and also with overgrowth (Barkovich et al, 2012), something that we possibly could obtain if we affected a greater number of progenitors, for example, by electroporating earlier in development. However, we could not exclude the possibility that this rise in progenitors was a time-specific event that could be counterbalanced later, rise to a normo-sized cortex for these mice. Moreover, the percentage of cells affected by electroporation might not be sufficient to cause the phenotype.

Previous models using *AKT3*-activating (Lee et al, 2012; Poduri et al, 2012; Poduri et al, 2013) and *PTEN*-disrupting mutations in mice (Groszer et al, 2001; Kwon et al, 2006) also were able to reproduce a greater signalling activity and brain size, but with no consequences in gyrification. Considering our results and the possible inherent species specificity in signalling and cellular regulation, we decided to move to a gyrencephalic model: the ferret. We hypothetized that by producing similar effects to those found in mouse and with the higher complexity in their progenitors could give us the possibility to emulate the human disorder. Moreover, as we described in human, we observed a clear regional expression of *Pik3r2* in ferret cortical development, particularly in the OSVZ, where the highest proportion of basal progenitors is placed.

Our first attempt was to evaluate the possible effect on neuronal migration postnatally, but contrary to what we expected, the overexpression of *PIK3R2* did not affect the distribution of GFP^+ cells. This experiment was performed following developmental stages comparable to those we have previously used in mouse studies. However, although the pattern of expression was similar, we could not avoid the fact that in other species the mechanism of compensation could be greater and sufficient to contrarest the effect of gain-*PIK3R2*. In fact, other studies in cancer cells show how the increase of mTOR activation by PIK3R2 resulted in a higher production of PTEN, which counteracted the activation of the pathway, and consequently, could reduced the expected effects of a higher PIK3R2 (Cheung et al, 2011). Moreover, with this experiment we were not able to affect the OSVZ cells, the lineage of which becomes independent from VZ at late embryonic stages. This is in agreement with mouse models of *Pik3ca*, in which a

different timing in overactivation of PIK3CA influences greatly in the generation of the megalencephaly (Roy et al, 2015).

We then reasoned that an interesting experiment would be to manipulate *Pik3r2* expression earlier in ferret development. For that, we chosed E34 for performing our experiments, as a key point in which basal progenitors are massively produced (Martinez-Martinez et al, 2016). Interestingly, as we have observed previously in mouse, the overexpression of *PIK3R2* resulted in an accumulation of neurons in the ISVZ, showing a neuronal migration defect. This different effect respect to our previous postnatal experiment reflects the relevance of temporal gene regulation. However, differently from what we have found in mouse embryos, the proportion of progenitors was not affected. This suggesst that, at least in ferret, it is less likely that the migration delay was due to the later generation of these neurons, although a neuronal birth-dating study should be performed to confirm this hypothesis in both species.

As we did in mouse, we wondered what would be the long-term effect of altering neuronal migration. For that, we performed electroporations at E34 and waited until P30, when cortical folding in ferret is almost complete. We observed that neuronal migration was not compensated later. These neurons overexpressing *PIK3R2* were able to arrive to the CP, but their positioning in the cortical layers was altered, preferentially located in layer V, differently to what happened in control animals, where the majority of cells are in layer IV. This result indicates that neurons probably migrate slower, or simply are stack during more time in one particular step of migration. Several genes involved in neuronal migration disorders have been associated with difficulties in escaping the multipolar stage (Bai et al, 2003; Bielas & Gleeson, 2004; Hirotsune et al, 1998; LoTurco & Bai, 2006; Nagano et al, 2002; Tabata & Nakajima, 2003; Tsai et al, 2005). Then, although they finally reached the CP, the positioning is not correct, pointing out again the relevance of timing in developmental processes.

Although our data in ferret were consistent to what we have observed in mouse in terms of neuronal migration, this impairment did not result polymicrogyria. Based on the brain region where this pathology appears most frequently, we decided to replicate our experiments in the ferret parietal cortex. Similarly, to what we have detected in visual cortex, we also observed a neuronal migration defect after increasing the levels of *PIK3R2*, evidenced by the different distribution of these neurons, that were highly accumulated in the VZ, where no neurons were found in the other conditions (control, neither OE of *PIK3R2* mutated form). Moreover, also neurogenesis was increased in *PIK3R2* - WT OE, with respect to the controls. So, these ectopically located neurons could be the result from of greater production, in addition to the difficulties in the radial migration. Additionally, not all ectopic neurons are GFP^+, suggesting a non-cell autonomous effect. This increment in neuronal generation that we observed in *PIK3R2*-WT OE

matches with the results obtained in the conditional *Pi3kca* mutant mice, in which a greater cell cycle exit was describe (Roy et al, 2015).

We have observed that in *PIK3R2*-WT OE animals there was the alteration of the germinal layers structure, particularly the VZ, not only by the presence of intruding neurons but also by the formation of the abnormal circular structures that the progenitors were forming. In agreement with cells in the VZ in *PIK3R2*-WT overexpression, there was a lower number of apical mitosis in this condition. Contrary to what happens in mouse, where the number of progenitors was increased, in here we have obtained even less amount of these type of cells. However, we cannot exclude the possibility that this aberrant organization could be due to a massive production of progenitors non-cell autonomously, since this analysis was considering GFP^+ cells only. Alternativelly, if a greater ratio of proliferation occurs very fast, the plasmid could be diluted at some point and as a result not green cells would be able to be perceived anymore.

In order to elucidate the long-term effects of this manipulation, we analysed our animals at P30, after being electroporated at E34. We found that the ectopic neurons found embryonically, at this stage were appearing as periventricular nodules conformed mainly by differentiated neurons, although the majority were not GFP^+. This suggests that there is a non-cell autonomous effect of PIK3R2 and the cells forming the final heterotopia derived from the non-cell autonomous ectopic neurons that we had observed at E36, while the GFP^+ either die or arrived to the CP.

Nonetheless, the positioning of a subset of these cells was also perturbed, since they overmigrated and stayed located in the meninges. This altered migration caused a disturbance in the cortical surface, with a different level of severity, reminiscent of an extra micro-fold. In more extreme cases, the OE of *PIK3R2*-WT is followed by the formation of multiple micro-folds. The combined phenotype of polymicrogyria and periventricular heterotopia has been previously found in the work perform by Masuda *et. al.* where they have overexpressed a thanatophoric dysplasia linked gene, the fibroblast growth factor-8 (*Ffg8*) (Masuda et al, 2015). Interestingly, this molecule activates FGFR3 and is activates the mTOR pathway by promoting the production of PIP3. Taking altogether, these results indicated that different manipulations of this cascade in the same area, the somatosensory cortex, caused both the alteration of cortical folding and the ectopic accumulation of neurons.

Gain-of-function mutations in PIK3R2

An important aspect to discuss here in order to understand the role of PIK3R2 is the impact of the G373R mutation in cerebral cortex development. Previous studies in cells from human

patients carrying this alteration reveal an increase in pS6, the readout of the mTOR pathway, which suggest that G373R is a gain-of-function mutation (Riviere et al, 2012). With this idea in mind, one would expect that the effects obtained with the overexpression of both *PIK3R2*-WT and *PIK3R2*-G373R would be similar. However, we systematically observed that the consequences of electroporating the mutated form were much less dramatic than the wild type. In mouse experiments, the effect of *PIK3R2*-G373R on aRGCs abundance was mild, while the overexpression of *PIK3R2*-WT produced a clear increase in this type of progenitors, as well as in cell-cycle re-entry, which was only evident in this last case. Neuronal migration defects observed both embryonically and postnatally were found in both conditions. Regarding ferret experiments, *in utero* electroporations of *PIK3R2*-G373R just produced a slight neuronal migration defect on visual cortex, while manipulations performed in the parietal area did not differ from control cellular distribution, neuronal positioning nor neuronal production.

Different possibilities could explain the lighter phenotype found in the overexpression cases of the *PIK3R2*-G373R in comparison with the *PIK3R2*-WT. 1) It is important toconsider that we were electroporating the human-*PIK3R2* and, although this is protein is highly conserved among species, we could not be sure of the effects of this mutation in other organisms like mouse and ferret. This fact could partly explain also the differences that we have found in our experiments between mouse and ferret, but this will be discussed later. 2) Another aspect to consider is the fact that, when we electroporate, the cell receiving the mutant form has also the endogenous wild type form. So, one possible scenario is that in presence of both, although we introduced the mutant form in excess, the system preferentially conducted the pathway with the endogenous, wild-type form. On the contrary, in the case of the WT overexpression, all the versions of *PIK3R2* are normal, which would potentiate the system correctly but abnormally higher. 3) PIK3R2 is one of the multiple isoforms of the regulatory subunit of PI3K. In that sense, in presence of mutated forms, we cannot rule out the possibility that in the presence of mutated forms, PIK3R2 may be partially substituted by another isoform.

How can we put together our results in mouse and ferret and link them to human pathology?

We have done all these manipulations I n mouse and ferret to understand the role of PIK3R2 in cortical development and have an insight in the pathogenesis of polymicrogyria in humans with a mutated form of this gene. Recapitulating the results presented in this thesis regarding the experiments in mouse and ferret, we observed that the excess of *PIK3R2* has a deleterious effect on neuronal migration. As mentioned, PI3K (conformed by PIK3CA and PIK3R2) regulate the generation of PIP3 (Cantley, 2002). Interestingly, levels of PIP3 in the leading edge of migrating neurons modulate the actin cytoskeleton organization, a key element in the process of radial migration. Thus, variations in *PIK3R2* levels or the expression of an

altered form of *PIK3R2*, potentially increases PIP3 levels, and, consequently, modifies the cytoskeleton rearrangement (Cantley, 2002; Jacinto et al, 2004), affecting the process of neuronal remodelling for its migration.

Why we did not get an alteration of the cortical surface in mouse? In here, we showed that in mouse we had an increase in the proportion of progenitors and also a neuronal migration defect that it was maintained postnatally. In ferret, the proportion of progenitors GFP^+ is diminished and a higher neurogenesis is detected. However, in our quantifications we were not distinguishing between basal and apical progenitors, so even if the number of progenitors is lower at least self-autonomously, an effect that could be happening is a switch in the balance in which the proportion of bRGCs is greater than aRGCs, that would generate a greater amount of basal radial fibers, and, together with with the increased number of neurons, could result in the cortical folding and the overmigration of neurons.

Higher levels of PI3K signalling are suggested to be epileptogenic although the underlying mechanisms are not known. Some aspects have been proposed as alteration in development, cellular size, growth proliferation and circuitry disturbances (Zhang & Wong, 2012). A possible experiment to check if our manipulations are able to produce this effect is using pentylenetetrazol (PTZ), a drug is capable to induce epileptic crisis (De Deyn et al, 1992). One would expect that performing this experiment, the susceptibility to suffer these crises would be greater in animals with excess of *PIK3R2*, even the mutated form.

Another interesting finding was the cortex alteration and the formation of ectopias when manipulations were developed in the parietal cortex, but not in the visual cortex. This difference may be based on several aspects. One is different expression pattern of *Pik3r2* when we compare the visual and the parietal cortex. We have shown in our results that, particularly in the OSVZ at E34 and at early postnatal stages (P0), *Pik3r2* was highly expressed in the prospective gyrus corresponding to the visual cortex, but the level of expression was much lower in areas corresponding to the somatosensory cortex. These differences in expression could explain the different effect that our manipulations are producing in one area or the other. Accordingly, our *PIK3R2* OE experiments at P1 in the visual cortex did not show any effect in neuronal migration.

Regarding this issue it is important to mention the different susceptibility that the perisylvian region (located in the somatosensory area) has demonstrated to polymicrogyria. Although the cause is not clear, it is known to be the area most commonly affected by this malformation in human patients by far (Barkovich, 2010; Squier & Jansen, 2014; Stutterd & Leventer, 2014). In fact, it has been suggested that differences in gene expression, the specific timing for the gyrification of this region and particularities on the vascularity of this region could be implicated. Importantly, other aspects that have been linked to polymicrogyria are affecting

vascularity, as vascular insults are one of the non-genetic causes for this disease (Barkovich, 2010; de Juan Romero et al, 2015; Squier & Jansen, 2014). This idea is in consonance with the differences in expression found when we compare mouse, ferret and human. While in mouse we observed a homogeneous expression, areas of differential expression were detected in both human and ferret, as happen with other genes related with MCDs (De Juan Romero & Borrell, 2015). In fact, PIK3R2 mutations generated a regional brain malformation, as happen with PIK3CA variations (Riviere et al, 2012), which support the resistance to manipulations (robustness) of some areas respect to others, that could be based on genetic expression profiles, progenitors subpopulations, complexity in molecular requirement and/or system higher redundancy.

Because all those differences regarding the biology of these experimental species, an attempt to model cortical folding involved the use of human cerebral organoids (Li et al, 2017). The mTOR pathway has been activated in human cerebral organoids by deleting *PTEN*, which catalyzes the hydrolysis of PIP3 to PIP2. As a result, an increase in progenitor proliferation was detected, as we observed in mouse. Although this increase in proliferation resulted in folding in these organoids, this involved the germinal layers but not the neuronal layers, in contrast to what happens in physiological folding. Indeed, similar manipulations in mouse organoids did not cause the folding whereas the overexpression of a human-specific gene ARHGAP11B did result in the generation of cortical folds in mouse (Florio et al, 2015). These results point out the relevance of the species differences, such as progenitors identity and genetical profile, proportion of progenitors (basal/apical) or timing for progenitors amplification (Li et al, 2017).

The relevance of finely tuned **FlnA** *levels*

Mutations in *FlnA* has been associated with the formation of periventricular nodules constituting the cortical malformation of PVNH. Due to the high incidence of this pathology, multiple functional studies have been done in order to elucidate the role of FLNA in cortical development. As mentioned before, FLNA is a ubiquitous protein with capacity to interact with multiple and diverse partners, what makes very challenging to unravel its function, specially if we consider that the formation of ectopias could have numerous and different origins during development. Thus, the implication of FLNA in several of key developmental processes offers multiple explanations for the origin of PVNH, as others established in their studies and we did in this work.

Similar to previous works (Sheen et al, 2002), we detected the highest expression levels of FlnA in germinal layers and CP during mouse corticogenesis, pointing out the relevance of the gene in the cells populating these areas, cortical progenitors and pyramidal neurons, respectively. It has been suggested that the balance of *FlnA* levels during corticogenesis is key

for a correct development (LoTurco & Bai, 2006; Sarkisian et al, 2008), reason why we decided to manipulate its expression both highly increasing (by overexpression) or decreasing (by knocking-down) their levels.

Therefore, we observed that in both conditions there was an impairment in neuronal migration, resulting in an ectopic accumulation of cells in the germinal layers and the IZ at embryonic stages. Interestingly, it has been previously reported in a rat *FlnA* knock-down model a neuronal migration delay (Carabalona et al, 2012). However, in the case of overexpression, different results have been reported. In some cases, the increase of *FlnA* was found innocuous (Carabalona et al, 2012), while in others both the direct (Sarkisian et al, 2006) or the indirect (Nagano et al, 2004; Sarkisian et al, 2006) ways of producing higher FlnA cellular levels resulted in an impairment of neuronal positioning. These differences could be based in the different models used, since Carabalona *et al*. studies were performed in rat by using OE, while Nagano *et al*. and Zhang *et al*., as ours, were done in mouse through FLNA-associated proteins, FILIP and MEKK4, respectevly.

Another important aspect is the fact that the KO models for *FlnA* did not produce the formation of ectopic nodules (Feng et al, 2006). This phenomenon has been attributed to a possible compensatory mechanism with FlnB, another member of the family which shares a high homology with FLNA and that presents a very similar expression pattern (Sheen et al, 2002). Moreover, heterodimer formation between FLNA:FLNB has been suggested based on their overlapping expression in cortex (Sheen et al, 2002) although this idea was contradicted by other authors (Himmel et al, 2003; Hu et al, 2017). In any case, there are two different models where this phenotype concurs with this possible compensation: the *Ptbb1* KO (Shibasaki et al, 2013; Zhang X, 2016) and the *FlnA*/*FlnB* KO mouse models (Houlihan et al, 2016), in which both *FlnA* and *FlnB* were disrupted, generating the formation of neuronal ectopias in periventricular areas.

Particularly interesting is the fact that this combined loss of *FlnA* and *FlnB* caused the alteration of the ventricular lining and the detachment of the RGCs. In our electroporated mice we did not find this disruption in the lining of the ventricle that has been shown previously in other models related to *FlnA* deficiency (Carabalona et al, 2012; Feng et al, 2006; Ferland et al, 2009; Sarkisian et al, 2006). In our experiments, we have also generated an ectopia, but its position was located in the white matter and in subcortical areas instead of periventricularly, which suggest that the disruption of the apical surface is necessary for the formation of this type of heterotopia.

When we performed our experiments in stage-matched ferrets by postnatal electroporation, we also observed the disruption of the ventricular lining by an alteration of the apical junction proteins (β -catenin and Par3). The defect on β-catenin was also reported in the

model of a FLNA lacking the actin binding domain, in which neuronal migration defects were also present(Zhang J, 2013). However, in this model the periventricular nodules were not formed, which suggest that this condition may be necessary but not sufficient to cause the PVNH.

Whether or not *FlnA* is expressed at RGCs endfeet and impacts the integrity of the VZ surface is not clear (Sarkisian et al, 2006), but our results showed a highest expression in this germinal layer and how the manipulation of its levels do influence the apical surface stability. In relation with apical surface disruption, we found that the overexpression of *FlnA* in ferret did result in the detachment of cortical progenitors, as indicated by the decrease in the number of cells in the VZ and the subsequent increase in ISVZ cells. This fact is in consonance with two previous hypothesis: One is that the disruption of the neuroepithelium may alter radial glia endfeet and even be caused by the localized death of RGCs (Sheen et al, 2005), although in our case did this not cause the impairment of migrating neurons at later stages. The other is that defects on adhesion may alter progenitor behaviour and neurogenesis leading to the formation of ectopic nodules in patients with PVNH (Feng et al, 2006). We were not able to demonstrate if these progenitors are actually dying, although their morphology appeared to be altered, displaying multiple processes. (Sarkisian et al, 2008). In rat, decreasing levels of FlnA also caused an alteration of the radial glial fibers integrity as was observed by Carabalona *et al*., suggesting that, in addition to the negative effect of FlnA levels in neurons, this defective neuronal migration may also have a component originated from RGCs (Carabalona et al, 2012).

In relation with *FlnA* expression during ferret development, we observed that the highest levels of expression are, as in mouse, located in germinal layers and in the cortical plate. We have identified areas of differential gene expression, as was previously observed in humans (De Juan Romero & Borrell, 2015), particularly in the OSVZ from E42 and at postnatal stages. These variations in FlnA amount correlates with to the phenotypic variability observed in patients, as was previously suggested (Parrini et al, 2006). Similar to what we have found with PIK3R2 manipulations, the modification of FlnA levels in other areas of the ferret brain could result in the neuronal migration defects. In fact, others found the the formation of nodules in ferret by manipulating the expression of FGF8 (Matsumoto et al, 2017a; Matsumoto et al, 2017b), a gene mutated in association with bilateral PVNH in combination with limb abnormalities (Parrini et al, 2006). However, this was not the case in our postnatal experiments when we overexpressed FlnA in the parietal cortex, which does not discard the possibility that an earlier embryonic overexpression manipulation could cause it. Other proteins associated with molecular transport, as ARFGEF2 and BIG2 have been described to have an important role in the endfeet of the RGCs. In one side, those proteins interact with FlnA and its alteration causes PVNH. In the other hand, they are also associated with adhesion molecules and with the actin filaments, which have been related to the integrity o f the apical side of the VZ. In addition, BIG2

might direct FLNA to leading end traling process (Ferland et al, 2009; Sheen, 2014b; Sheen et al, 2004b).

Classically, *FLNA* alterations were associated with a defective radial migration. In our hands, both OE and KD conditions led to a neuronal migration delay with the consequent accumulation of cells in the germinal layers and in the IZ. This is in agreement with the suggested influence of fine determined levels of *FlnA* in cellular behaviour in terms of movement and morphology, although the origin of this could be different.

Having in mind the main role described for FLNA as an organizer of the actin-cytoskeleton network, an increased amount of *FlnA* levels could cause that migrating neurons become more rigid with difficulties to undergo the specific morphological changes characteristic for radial migration process (LoTurco & Bai, 2006; Sarkisian et al, 2008; Zhang et al, 2012). Importantly, for these remodelling events also FlnA partners have been found to be relevant, as are SEK1/MEKK4 pathway and FILIP, which explains the migratory defect found in some models (Nagano et al, 2002; Sarkisian et al, 2006). In this sense, one could purpose that a greater amount of FLNA conferred greater rigidity to cells, while lower levels of FlnA impide the cell to modify its cytoskeleton and then, its morphology. Differently, when FlnA levels were decreased, the cell potentially could count on a partial compensation by FLNB, which could explain the different phenotype found in these conditions (Hu et al, 2017; Nakamura et al, 2011; Sheen et al, 2002).

In order to elucidate how these two conditions could be affecting the neuronal migration process, we performed time-lapse experiments in which we evaluated the speed of pyramidal neurons in their journey to the CP. We have found that neurons overexpressing *FlnA* have a lower mean speed of migration and a higher proportion of cells grouped as slow migrating neurons. Additionally, these neurons follow a less straight path than those that were expressing just GFP^+. These two factors could be underlyng the ectopic position that these neurons present at embryonic stages, unable to arrive to their correct place in the CP as fast as the control ones. Interestingly, these two parameters were not found altered in *FlnA*-KD neurons, contrary to what we expected based on previous data and hypothesis. In terms of the straightness, we were surprised this factor was not affected, since Carabalona *et al.* have described a glial disturbance in rats embryos were *FlnA* was knock down (Carabalona et al, 2012; LoTurco & Bai, 2006). For that reason, we speculated that if glial fibers were altered with our manipulations, the path straightness was lower than that observed in control neurons. Moreover, the mean speed did not differ from neurons expressing GFP, which suggest that *FlnA*-KD neurons should migrate normally. In that case, regarding the role of FlnA in the cytoskeleton, a compensatory mechanism could explain that these neurons maintain a normal migration. Moreover, considering the higher proportion of cells negative for NeuroD2 that are located in the germinal layers, one could speculate that in the case of reduced levels of *FlnA*, these progenitors stays longer in the cell

cycle and neurons born later. Another possible explanation resides in the transition from multipolar to bipolar morphology. In our experiments, we considered the cells which had adquired a bipolar morphology. However, under the dysregulation of *FlnA* levels is conceivable that this step was affected, delaying the initiation of migration. Then, although they started their trajectory later, they could do it normally. In both hypothetical scenarios, the migration parameters would not be altered, while the cellular positioning would be clearly different from a control situation and in the case of *FlnA* OE, as we found.

Another aspect that we have to take in account for considering these results are aspects relative to this particular type of experiment, the time lapse. In our results, we have two experiments per condition (around 200 cells each), which would be interesting to raise in order to have a more trustable result. As an experiment *in vivo* performed with two-photon videomicroscopy, its visibility depends on the impact on a laser that affect the resistance of the slice. Ideally, this aspect should be maintained through different experiments, but of course the the tissue is exposed to multiple variables. Moreover, during the experiment, the slice undergoes several changes, specially in its consistency, which produces a displacement of this slice. Although these factors are artificially corrected by the software, one could not discard a partial effect of this phenomenon, which would be disminished by increasing the number of experiments.

Considering that *FLNA* is coded in the X chromosome, one would expect that, by random X-chromosome inactivation in females, around fifty percent of cells should be forming the ectopic nodules in the periventricular areas. However, this is not the case, and just a slow proportion of neurons are found in ectopic positions. Similarly, in our *in utero* electroporation experiments, we observed that just a proportion of the GFP^+ cells present the migratory defect. This was confirmed postnatally, when the OE of *FlnA* caused the ectopic positioning of some upper layer differentiated neurons, while the majority were correctly located in the CP. Interestingly, in the case of the KD condition, the neurons reached more upper positions within the CP as compared to the control animals, suggesting also an impaired neuronal migration but affected in a different way.

A previous study has found that the loss of FLNA caused the arrest of the cells in the M phase cell cycle (Lian et al, 2012) altering the neural progenitor proliferation and differentiation (Lian et al, 2018). Another work done in mouse showed that, with loss of both *FlnA* and *FlnB*, there was a greater formation of IPCs, which generated neurons ectopically (Houlihan et al, 2016). In absence of FLNA, the cell cycle may be arrested, and the formation of neurons is not taking place at the expected time. Having in mind the relevance of the time when and place where neurons are born for their proper location in the cortex, this alteration could lead to an altered neuronal positioning. If a proportion of neurons is born later, those would migrate

differently, as late-born neurons, which would explain its presence in upper positions in the CP. In fact, when we analysed the cellular identity of ectopic cells at earlier postnatal stages (P5), we showed that the proportion of GFP^+ cells that were neurons is much lower than in the case of the overexpression of *FlnA*. To test this idea, it would be interesting to perform a birthdating study at embrionary stages to determine the date of birth of neurons in KD and OE conditions.

The majority of cases of PVNH are due to loss-of-function mutations, while in our case we observed the consolidation of ectopias in OE, not in KD. These ectopias in mouse were formed by upper-layer neurons, the main component of ectopic nodules in patients. This suggests that late-born neurons are more susceptible for alterations in neuronal migration, either because they have less time to correct their location, or because the unproperly located early-born ones are eliminated from the system (Sheen et al, 2002). In contrast, when we moved to a gyrencephalic model, the ferret, we did observe that only the KD, but not the OE, resulted in an altered cellular positioning. This could suggest different robustness and compensatory mechanisms taking place on ferret experimental model, but not in mouse. In fact, something similar occurs in heterotopia models of *Dcx*. In those experiments, the authors were able to develop the formation of the heterotopia in rat but not in mouse by knocking down this gene (Ramos et al, 2006).

As mentioned, experiments done in ferret that overexpress *FGF8* showed the formation of neuronal ectopias in their periventricular area (Matsumoto et al, 2017a; Matsumoto et al, 2017b) and polymicrogyria (Masuda et al, 2015). Intriguingly, FGF8 causes an activation of the mTOR pathway, leading to migratory heterotopias and abnormal neuronal shape (Assimacopoulos et al, 2012; Borello et al, 2008). Similarly, *NEDD4L* mutations that have been found mutated in patients with PVNH, were also associated with the mTOR cascade (Broix et al, 2016). These two examples, in combination with our results of PIK3R2 overexpression of a connexion between the mTOR signalling and the formation of ectopias that resemble those due to *FLNA* mutations. This could explain the combined phenotypes that appear in patients in which we can find both alterations in folding (polymicrogyria) and the presence of ectopias, either periventricular (PVNH) or subcortical (SBH).

Eml1

The relevance of the cytoskeleton in cortical development, and more specifically in progenitors behaviour and neuronal migration was also evidenced in other models related to other proteins of both actin and microtubule cytoskeleton (Cappello, 2013; Francis et al, 1999; Kuijpers & Hoogenraad, 2011; Stouffer et al, 2016). This is the case, for instance, for Eml1, which mutations have been identified in patients with SBH. The sporadic model of *Eml1* KO in mouse, the HeCo mouse (Croquelois et al, 2009), reflects how the defect of this protein has a tremendous impact on the progenitors positioning and the ectopic positioning of neurons (Bizzotto et al, 2017;

Kielar et al, 2014). In here, we showed that the relevant role of Eml1 in progenitors in a lissencephlic model as mouse could also be shared in a gyrencephalic species, since in our model, the ferret, we also observed an Eml1 expression in germinal layers. Additionally, our functional experiments also revealed the importance of finely regulated levels of this cytoskeleton associated genes, when artificially increasing the Eml1 amount, we observed a. significant accumulation of cells in the OSVZ in early postnatal ferrets (P8). This defect was not observed at P14, suggesting that it was corrected later, at least in terms of GFP^+ cellular positioning.

In summary, cortical development involves a complex combination of events in which the variation of any of their players may have critical consequences in the final structure, organization and function of the brain, as evidenced by MCDs. The combination of alterations in folding, size and the formation of ectopias give us the idea that for this final phenotype a common path is followed. In fact, even in cases where the genes related to the malformation seem to be so different, they are somehow connected in key cellular cascades and processes as cytoskeleton arrangement and mTOR pathway. Understanding how this equilibrium is maintained during corticogenesis and how some changes impact on the normal development is a long way that started several decades ago. We have tried with this work to shed some light to some of the mechanisms that underlie the generation of some of the most common MCDs.

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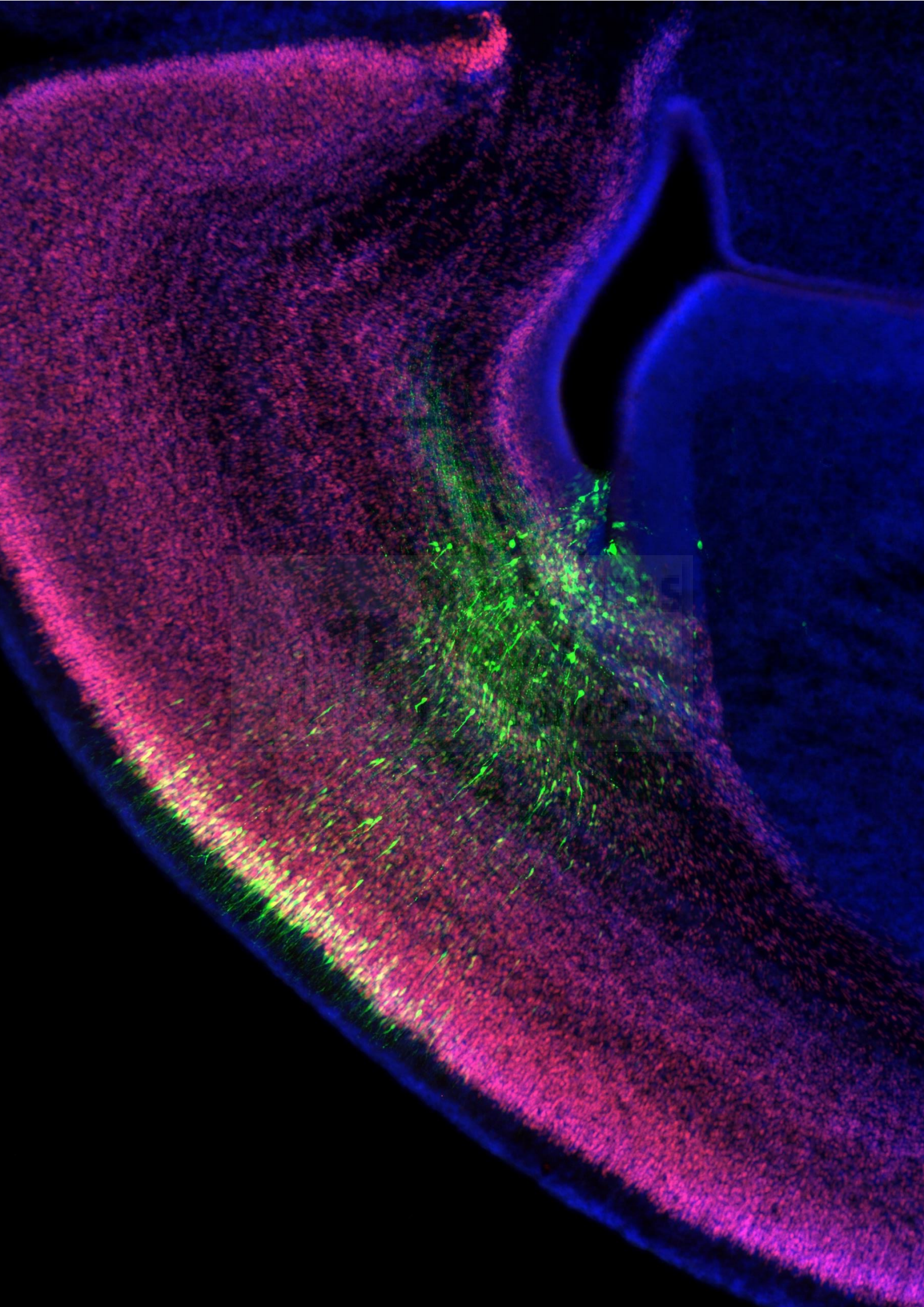

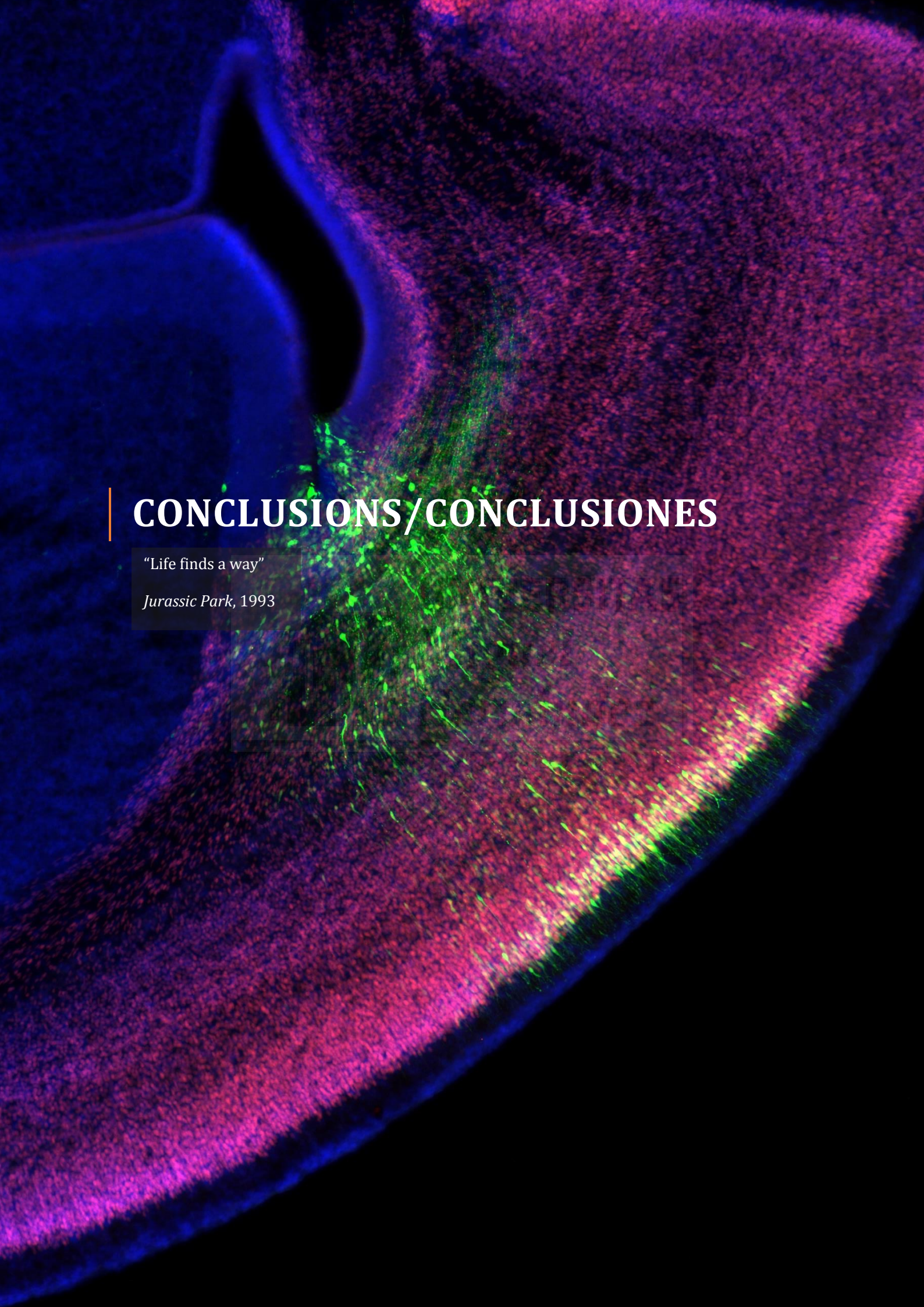

CONCLUSIONS/CONCLUSIONES

"Life finds a way"

Jurassic Park, 1993

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CONCLUSIONS

The conclusions of the work developed in this thesis are the following:

1. *Pik3r2* is highly expressed in germinal layers of the developing mouse, ferret and human cerebral cortex, with areas of differential expression along germinal layers in gyrencephalic species.
2. Increased levels of *PIK3R2* during mouse corticogenesis causes an increase in aRGCs and IPCs elevated cell cycle re-entry, and a delay in neuronal migration resulting in the formation of neuronal ectopias.
3. The effects of overexpressing *PIK3R2* in the early ferret cerebral cortex are highly region-dependent. In the visual cortex, it causes migration defects and abnormal lamination of neurons, without altering neurogenesis. In the parietal cortex, it increases neurogenesis and produce the mispositioning of newborn neurons, forming periventricular nodular heterotopias. Radial neuron migration is also impaired, with the overmigration of neurons forming subpial ectopias.
4. *FlnA* is highly expressed in germinal layers and CP of the developing mouse and ferret cortex, with highly regionalized expression in ferret.
5. Overexpression and knock down of *FlnA* in the developing mouse cortex cause a delay in neuronal migration. In the former, this results in subcortical ectopias of upper layer neurons, while in the latter it produces a mild defect in neuron lamination.
6. Loss-of-function of *FlnA* in ferret alters neuronal lamination in the mature cortex. *FlnA* overexpression disrupts the ventricular apical adherent junction belt, altering Par3 and β-catenin, but this is not sufficient to cause periventricular nodules.
7. *Eml1* is highly expressed in the CP during ferret corticogenesis. Expression in germinal layers is much lower, but it increases at later developmental stages.
8. Overexpressison of *Eml1* causes a mild defect in the positioning of cortical neurons at early stages, which is corrected later in development.
9. Malformations of cortical development emerge with different severity on distinct cortical regions, indicating that cortical areas have very diverse robustness to genetic insults during early development.

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CONCLUSIONES

Las conclusions del trabajo desarrollado en esta tesis son las siguientes:

1. *Pik3r2* está altamente expresado en las capas germinales durante el desarrollo cortical de ratón, hurón y humano, con áreas de expresión diferencial a lo largo de las capas germinales en especies girencéfalas.
2. Niveles incrementados de *PIK3R2* durante la corticogenesis de ratón causan un incremento en la cantidad de células de glia radial apical y células progenitoras intermedias y en su reentrada en ciclo, así como un retraso en la migración que resulta en la formación de ectopias neuronales.
3. Los efectos de sobreexpresión de *Pik3r2* en la corteza cerebral de hurón en estadios tempranos son altamente dependientes de la región. En la corteza visual, causa defectos de migración y una laminación anormal de las neuronas, sin alterar la neurogénesis. En la corteza parietal, incrementa la neurogénesis y produce un posicionamiento aberrante de las neuronas, formando nódulos periventriculares heterotópicos. La migración neuronal también está alterada, con la sobremigración de neuronas causando ectopias en la subpia.
4. *FlnA* está altamente expresado en las capas germinales y en la placa cortical de la corteza de ratón y hurón durante el desarrollo, con una expresión altamente regionalizada en el hurón.
5. La reducción de expresión y la sobreexpresión de *FlnA* en la corteza en desarrollo del ratón causa un retraso en la migración neuronal. En el primer caso, esto resulta en la formación de ectopias subcorticales de neuronas de capas altas, mientras que en el segundo produce un leve defecto de laminación neuronal.
6. La reducción de expresión de *FlnA* en hurón altera la laminación neuronal en la corteza madura. El incremento en los niveles de expresión de *FlnA* disrumpe el cordón de adhesión apical ventricular, alterando Par3 y β-catenina, pero esto no es suficiente para causar nódulos periventriculares.
7. *Eml1* está altamente expresado en la placa cortical durante la corticogenesis del hurón. La expresión en las capas germinales es mucho menor, pero ésta aumenta más tarde en el desarrollo.
8. La sobreexpresión de *Eml1* causa un defecto leve en el posicionamiento de las neuronas corticales a estadios tempranos, lo que es corregido posteriormente en el desarrollo.
9. Las malformaciones de desarrollo cortical emergen con diferente severidad en distintas áreas corticales, indicando que éstas tienen una diversa robustez a agravios durante estadios tempranos del desarrollo.

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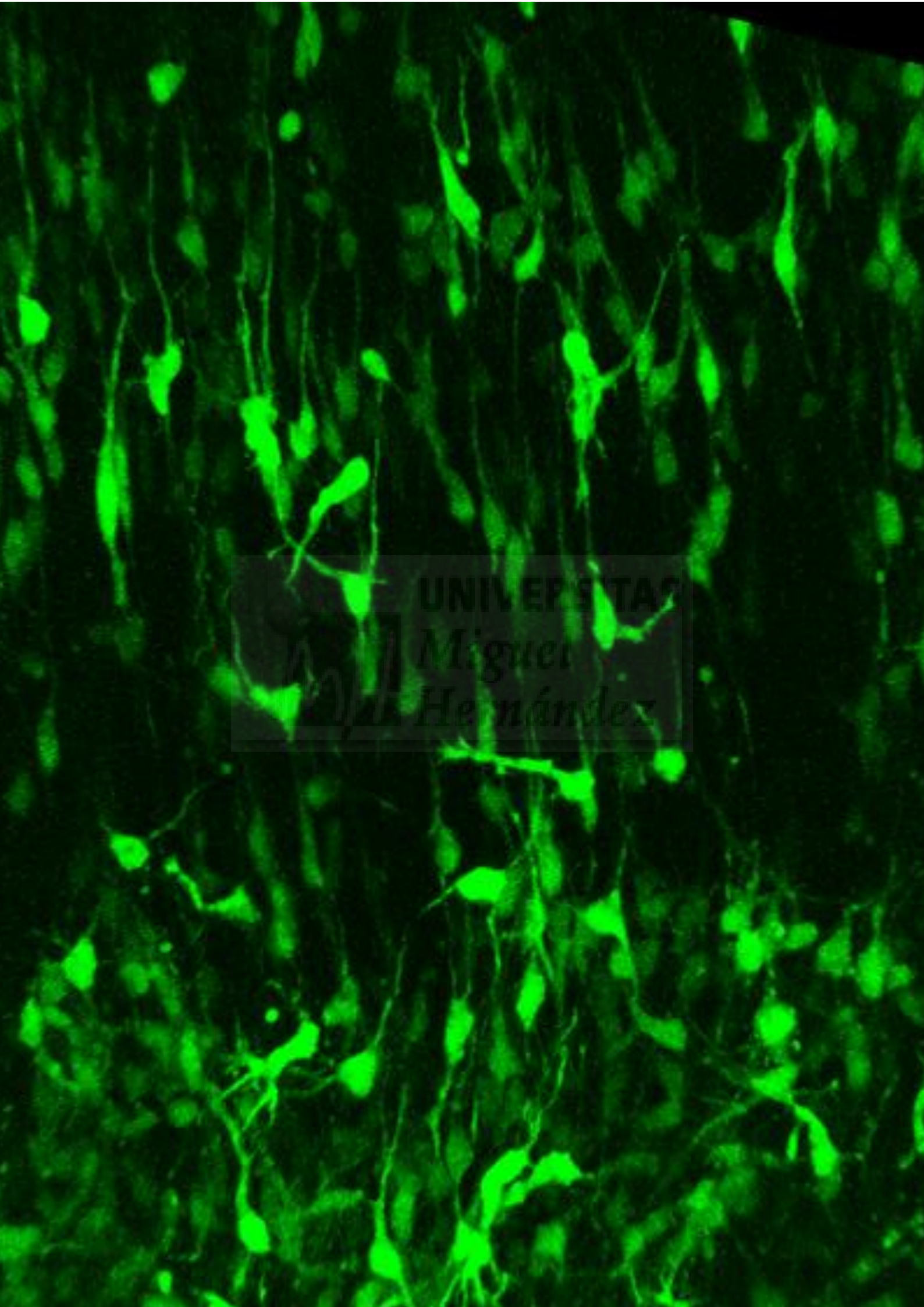

ANNEX 1. AUTHOR'S PUBLISHED SCIENTIFIC CONTRIBUTIONS

"Creo que Dios se enfada si pasas ante el color púrpura en el campo, sin fijarte"

El color púrpura, 1985

- Fernández, V., C. Llinares-Benadero and V. Borrell (2016). "Cerebral cortex expansion and folding: what have we learned?" The EMBO Journal 35(10): 1021-1044. **doi: 10.15252/embj.201593701**
- C. Llinares-Benadero and V. Borrell (2019). "Deconstructing cortical folding: genetic, cellular and mechanical determinants" Nat Rev Neurosci **doi: 10.1038/s41583-018-0112-2**

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Review

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Cerebral cortex expansion and folding: what have we learned?

Virginia Fernández†, Cristina Llinares-Benadero† & Víctor Borrell*

Abstract

One of the most prominent features of the human brain is the fabulous size of the cerebral cortex and its intricate folding. Cortical folding takes place during embryonic development and is important to optimize the functional organization and wiring of the brain, as well as to allow fitting a large cortex in a limited cranial volume. Pathological alterations in size or folding of the human cortex lead to severe intellectual disability and intractable epilepsy. Hence, cortical expansion and folding are viewed as key processes in mammalian brain development and evolution, ultimately leading to increased intellectual performance and, eventually, to the emergence of human cognition. Here, we provide an overview and discuss some of the most significant advances in our understanding of cortical expansion and folding over the last decades. These include discoveries in multiple and diverse disciplines, from cellular and molecular mechanisms regulating cortical development and neurogenesis, genetic mechanisms defining the patterns of cortical folds, the biomechanics of cortical growth and buckling, lessons from human disease, and how genetic evolution steered cortical size and folding during mammalian evolution.

Keywords evolution; ferret; gyrencephaly; humans; neocortex
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Introduction

In 1990, Wally Welker published a landmark book chapter updating on all that was known about the physiological, experimental, and pathological causes of cortical folding and misfolding, including comparative and phylogenetic considerations (Welker, 1990). Twenty-five years later, we have been invited to present an update of current knowledge on this matter. Welker's review was 134 pages long and cited 665 references, and much progress has been made since then. Clearly, if our review on cortical folding has to fit in this issue of *The EMBO Journal*, something has to be done. Our solution is to limit the size of this review by focusing and briefly touching on what we feel have been the most significant advances in this period leading to our current understanding of the biology of this problem.

One of the most prominent features of the human brain is the fabulous size of the cerebral cortex and its folding, visible as bulges and grooves on its external surface. Most animals with a large brain have a folded cortex, whereas most animals with a small brain have a smooth cortex, without folds. The cerebral cortex is a laminar tissue where neurons lie on the upper part, and the lower or inner part contains most of the wire connecting neurons between brain areas. In big brains, this sheet of neural tissue covering the outside of the brain is disproportionately larger than the deep brain structures it covers, and instead of adopting a balloon-like conformation it folds onto itself, minimizing total brain and cranial volume. In addition to minimizing brain volume, cortical folding is of key importance for the optimization of brain wiring and functional organization (Klyachko & Stevens, 2003), and alterations in cortical size or folding lead to severe intellectual disability and intractable epilepsy in humans (Walsh, 1999; Barkovich *et al*, 2012).

The process of cortical folding takes place during brain development, and thus, it is essentially a developmental problem. In this review, we will start describing exciting discoveries made over the last fifteen years on the central role of progenitor cell proliferation and survival in cortical size, the discovery of novel germinal zones and progenitor cell types, which are greatly overrepresented in gyrated brains, and their key function in cortical folding. This will be followed by our understanding of the genetic mechanisms regulating the patterns of cortical folding and the biomechanics of this process, what we have learned from human disease, and finally will close with a view of how genetic evolution may have steered cortical size and folding during mammalian evolution.

Cellular mechanisms leading to cerebral cortex expansion and folding

In order to understand the developmental mechanisms involved in cortical expansion and folding, we will frame these within the basic principles of cerebral cortex development as found in the most common animal model, the mouse.

Telencephalic neuroepithelium

In the early embryo (in mouse *c.* 9 days postconception, E9), the expansion of the rostral-most domain of the neural tube gives rise to the two telencephalic vesicles. The dorsal half of these vesicles is then

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molecularly specified as the primordium of the cerebral cortex. At this stage, the cortical primordium is solely composed of a monolayer of neural stem neuroepithelial cells (NECs; Bayer & Altman, 1991). NECs are highly polarized and attached to each other by adherens and tight junctions at the level of the apical domain (inner surface of the telencephalic vesicle) and which move their cell nucleus between the apical and the basal sides of the neuroepithelium in coordination with the cell cycle: basal-directed movement during G1, basal position during S-phase, apical-directed movement during G2, and mitosis at the apical surface. This cyclic movement is known as interkinetic nuclear migration and is completely asynchronous between NECs, conferring the neuroepithelium a pseudostratified appearance (Sauer, 1935; Bayer & Altman, 1991; Taverna & Huttner, 2010). NECs only undergo symmetric self-amplificative divisions, whereby each division generates two daughter NECs, hence exponentially increasing their number (Miyata *et al*, 2010; Fig 1).

Because NECs are the founder progenitor cells of the cerebral cortex, their pool size determines the numbers of their derived neurogenic progenitor cells and the final number of cortical neurons, and hence, it has a fundamental impact on the size of the mature cerebral cortex. Accordingly, already at this early stage, the size of the telencephalic vesicles is much larger in the human than in mouse embryo, reflecting significant differences in size of the neuroepithelium as a consequence of different NEC abundance (Sidman & Rakic, 1973; Rakic, 1995). NEC abundance may be increased by extending the time period of their self-amplification and delaying the onset of neurogenesis, as observed in primates compared to rodents (Rakic, 1995, 2009; Kornack & Rakic, 1998). The importance of NEC amplification on cortical size has been experimentally demonstrated in mouse, where NEC abundance in the embryonic cortex may be increased by either promoting their re-entry into cell cycle or preventing programmed cell death (Chenn & Walsh, 2002; Kingsbury *et al*, 2003). In both cases, increased NEC abundance leads to expansion in surface area and folding of the neuroepithelium.

Proliferation and neurogenesis

Immediately prior to the onset of neurogenesis, NECs start losing tight junctions and begin acquiring features typical of glial cells,

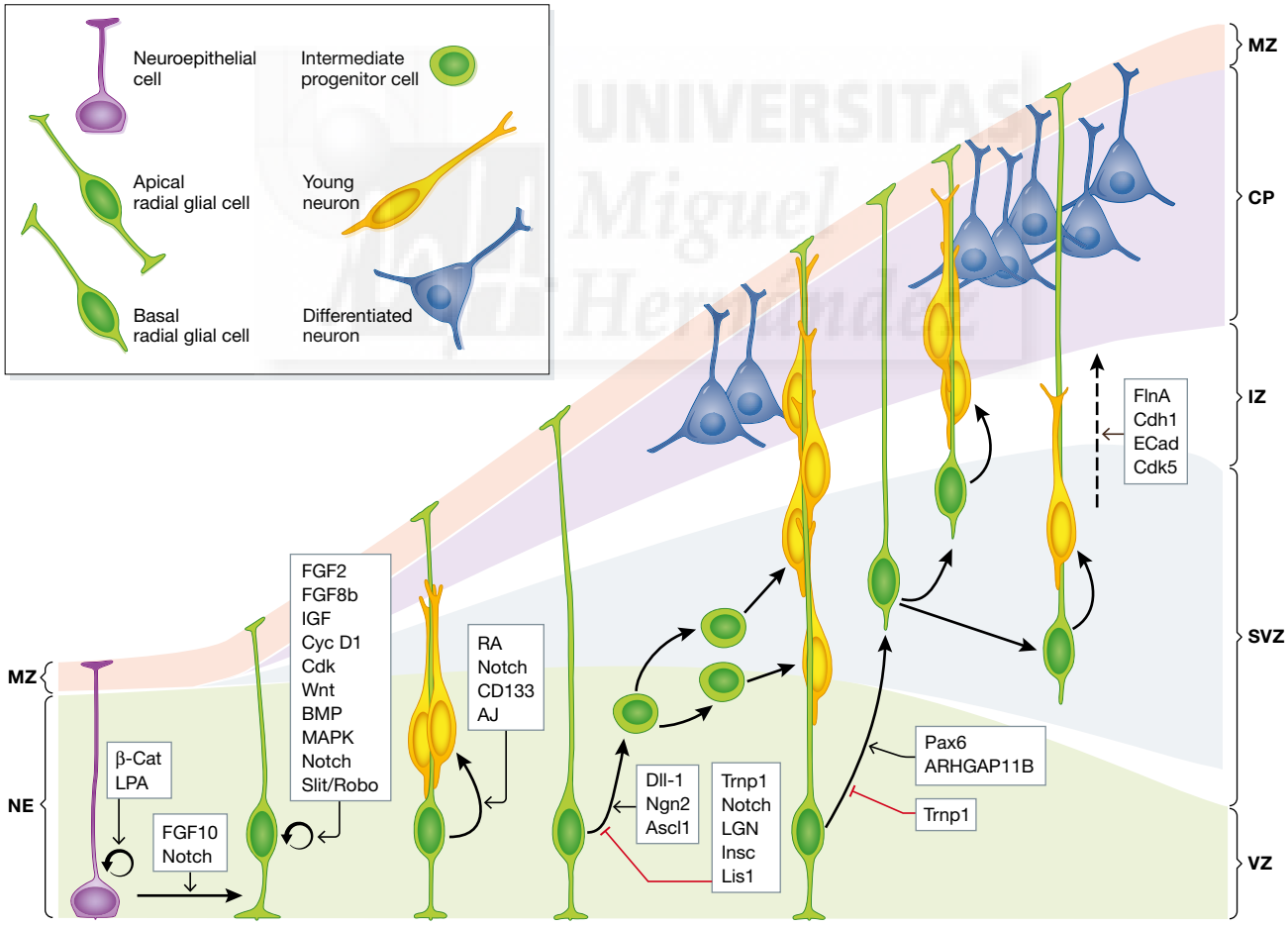


Figure 1. Stem cells in the developing cerebral cortex of gyrencephalic brains and their molecular regulation.
Schema depicting the main types of progenitor cells and their lineage relationships in the developing cerebral cortex. Arrows indicate lineage relationships demonstrated by time-lapse imaging and/or by retroviral lineage tracing. During the expansion phase, most neuroepithelial cells divide symmetrically to self-amplify to generate apical radial glial cells. During the neurogenic phase, most aRGCs divide asymmetrically to generate neurons, either directly or indirectly through intermediate progenitor cells or basal radial glial cells. Molecules or pathways regulating some of these steps are indicated. MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

including the expression of brain lipid-binding protein (BLBP), vimentin, and Pax6, thus becoming apical radial glial cells (aRGCs; see Taverna *et al* (2014) for a detailed review on the cell biology of this process). Like NECs, aRGCs undergo interkinetic nuclear migration, divide at the apical surface of the developing cortex and, at this early stage (*c*. E10 in mouse), also undergo self-amplifying divisions. However, aRGCs gradually start dividing asymmetrically to generate one aRGC plus a different cell. These new cells accumulate at the basal side of the cortical primordium, while the cell bodies of aRGCs remain in the apical side, forming the ventricular zone (VZ; Fig 1). With the accumulation of cells above the VZ, the basal process of aRGCs elongates while remaining attached to the basal lamina and is now termed radial glial fiber. Asymmetric aRGC divisions generate one aRGC plus either one neuron or one intermediate progenitor cell (IPC; Malatesta *et al*, 2000; Noctor *et al*, 2001, 2004; Haubensak *et al*, 2004; Miyata *et al*, 2004; Fig 1). IPCs are secondary progenitor cells without apical–basal polarity, do not undergo interkinetic nuclear migration, reside and divide in a location immediately basal to the VZ, the subventricular zone (SVZ), and contrary to aRGCs, they all express the transcription factor Tbr2 (Englund *et al*, 2005). In mouse, the vast majority of IPCs divide once to produce 2 neurons (neurogenic, self-consuming divisions), and hence, they are viewed as a strategy to amplify the production of cortical neurons. However, because each IPC self-consumes at mitosis, their relative abundance compared to aRGCs is quite low (Kowalczyk *et al*, 2009). IPCs in the cerebral cortex generate most cortical excitatory neurons (Attardo *et al*, 2008; Kowalczyk *et al*, 2009), whereas inhibitory interneurons are generated extra-cortically (Anderson *et al*, 1997). As neurogenesis progresses, there are a lower requirement for aRGC expansion/renewal and a greater need for neuron production, so there is a gradual predominance of asymmetric aRGC divisions producing IPCs (Noctor *et al*, 2004; Kowalczyk *et al*, 2009).

In addition to aRGCs and IPCs, the embryonic mouse cortex includes other much less abundant types of progenitor cells. Populating the VZ, we find apical intermediate progenitors (aIPs), which divide at the apical surface to produce neurons (Stancik *et al*, 2010; Tyler *et al*, 2015), and subapical progenitors (SAPs), which divide within the VZ, but away from the apical surface to generate IPCs (Pilz *et al*, 2013). Populating the SVZ, we find basal radial glial cells (bRGCs), which share many similarities with aRGCs including a basal process extended radially and contacting the basal lamina of the telencephalon, and expression of the transcription factor Pax6, but whose cell body is located and divides at basal positions in the SVZ (Shitamukai *et al*, 2011; Wang *et al*, 2011; Fig 1). As opposed to aRGCs, bRGCs in mouse do not self-amplify nor produce IPCs, but are highly neurogenic (Wang *et al*, 2011).

In gyrencephalic species like humans, monkey, or ferret, the abundance of aRGCs is much greater than in species with a smooth cortex like mouse, producing a more extended VZ. This is a direct consequence of the higher abundance of founder NECs at earlier stages (see above), further promoted by an increased self-amplification of aRGCs in these species. In addition to having an extended VZ, the most remarkable distinction of gyrencephalic species is having a thickened SVZ populated by an outstanding abundance of basal progenitors, especially at later stages of neurogenesis when these greatly outnumber apical progenitors (Smart *et al*, 2002; Lukaszewicz *et al*, 2005; Reillo *et al*, 2011; Reillo & Borrell, 2012). This abundance of basal progenitors is accompanied by the splitting of the SVZ in inner (ISVZ) and outer (OSVZ) subdivisions, not found in mouse (Smart *et al*, 2002; Fietz *et al*, 2010; Hansen *et al*, 2010; Reillo *et al*, 2011; Reillo & Borrell, 2012).

The OSVZ contains a wide diversity of progenitor cell types with high amplificative potential, and the combination of these two factors is considered key for cortical expansion and folding (Lui *et al*, 2011; Betizeau *et al*, 2013; Borrell & Gotz, 2014). Contrary to the lissencephalic mouse, in gyrencephalic species few basal progenitors are IPCs, but most are bRGCs (Hansen *et al*, 2010; Reillo *et al*, 2011; Reillo & Borrell, 2012; Betizeau *et al*, 2013). A seminal videomicroscopy study demonstrated that in macaque (a gyrated primate), bRGCs come in different modalities, which frequently transition between them and with IPCs, and all of these types of progenitors may self-amplify prior to generating neurons (Betizeau *et al*, 2013). Importantly, in gyrencephalic cortices like macaque and ferret, neurogenesis takes place during a period of time much longer than in rodents (up to tenfold; Takahashi *et al*, 1993; Kornack & Rakic, 1998; Lukaszewicz *et al*, 2005; Reillo & Borrell, 2012), allowing more rounds of cell division and increasing neuronal output (Dehay & Kennedy, 2007; Florio & Huttner, 2014). On these grounds, we and others have proposed that the OSVZ, with its wealth of neurogenic basal progenitors, plays central roles in the dramatically increased neurogenesis and folding in the cerebral cortex of higher mammals (Fig 2A; Fietz & Huttner, 2011; Lui *et al*, 2011; Borrell & Reillo, 2012; Borrell & Calegari, 2014; Borrell & Gotz, 2014; Florio & Huttner, 2014). This idea is well supported by experimental work in the gyrencephalic ferret, which demonstrates that forced overproliferation of OSVZ progenitors increases cortical surface area and folding, whereas blockade of their proliferation has the opposite effect (Reillo *et al*, 2011; Nonaka-Kinoshita *et al*, 2013). The central relevance of the OSVZ in cortical expansion and folding resides not only on its prominent contribution to increase neuron production, but also specifically on its high content of bRGCs, as explained in the next section.

Radial migration

Newborn cortical excitatory neurons must travel (migrate) from their layer of birth to the vicinity of the cortical surface, where they will coalesce into nascent neuronal layers. This process is named radial migration (Rakic, 1972). The scaffold of radial glial fibers, which span perpendicular between the ventricular and the pial surface of the cortex, provides the necessary substrate and guide for these neurons during their radial migration, much like the rail tracks for a train. This cell–cell interaction is under tight molecular regulation, and its disruption leads to severe defects of neuronal positioning and layer formation in the cortex (Sidman & Rakic, 1973; Rakic *et al*, 1974; Rakic, 1978; Anton *et al*, 1997, 1999; Elias *et al*, 2007). Due to this dependence of radially migrating neurons on radial glial fibers, the trajectory of these fibers largely defines the migratory route and final location along the cortical surface of new neurons (Rakic, 1995). Consequently, sibling neurons born from one progenitor normally occupy neighboring positions in the mature cerebral cortex (Fig 2B; Soriano *et al*, 1995; Gupta *et al*, 2003; O'Leary & Borngasser, 2006; Gao *et al*, 2014).

In contrast to the mouse cerebral cortex, a characteristic feature of folded cortices during development is their much greater surface area on the pial than on the ventricular side (Sidman & Rakic, 1973; Kriegstein *et al*, 2006; Rakic, 2009). This expansion of the pial

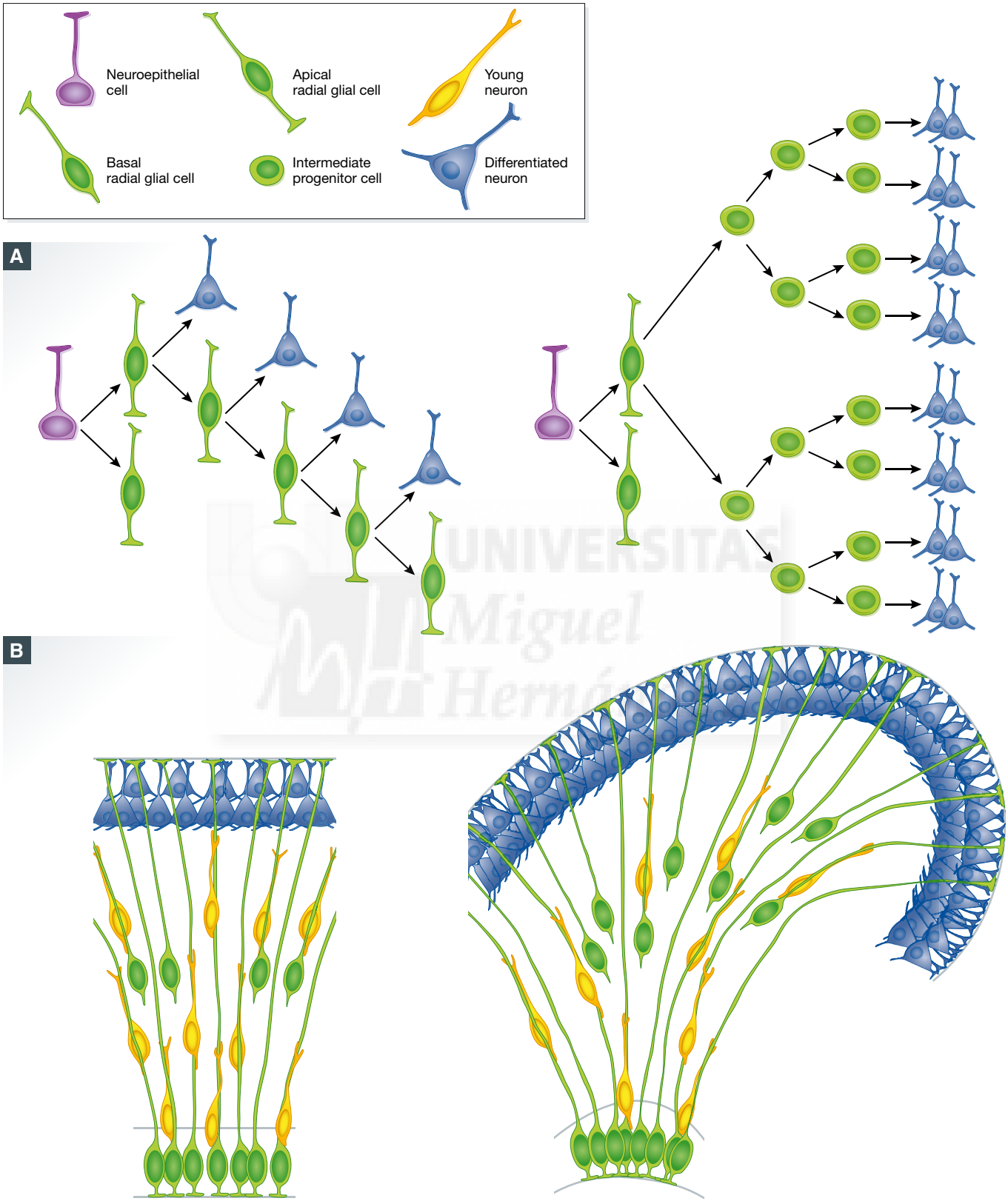


Figure 2.

Figure 2. Patterns of cell division in the embryonic cerebral cortex and mechanisms for tangential versus radial expansion.
(A) Two patterns of cortical progenitor cell division with opposite neurogenic outcome: The total number of neurons produced is proportional to the generation of intermediate progenitor cells, very small in species with a smooth cortex (left) and large in species with a folded cortex (right). (B) Difference in the general arrangement of the radial fiber scaffold in the cerebral cortex undergoing tangential (left) versus radial expansion (right). In species with a smooth cortex like mouse (left), radial glial fibers are parallel (green) and there is no net lateral dispersion of radially migrating neurons (yellow) with respect to the positions of their progenitor aRGCs (green). In gyrencephalic species, the radial fiber scaffold becomes divergent due to the intercalation of radial fibers from bRGCs. As a result, radially migrating neurons follow divergent trajectories which cause their lateral dispersion; this increases cortical surface area and ultimately promotes folding (modified from Borrell & Reillo, 2012).

surface relates to the increased numbers of cortical neurons, largely based on the expansion of basal progenitors. Several hypotheses have been proposed to explain how basal progenitors may promote this difference between pial and ventricular surface during development (Kriegstein *et al*, 2006; Fietz & Huttner, 2011). However, a critical issue overseen in early hypotheses is that whereas the addition of basal progenitors explains increased neurogenesis without expanding the ventricular surface area, the amount of radial fibers from aRGCs also does not expand, which is problematic. If the radial fiber scaffold does not increase as neurogenesis increases, then radial fibers (the rail tracks for migrating neurons) become a limiting factor to be shared by the augmented population of radially migrating neurons. As a result, neurons pile up in thickened cortical layers without a significant lateral separation, and thus, surface area does not increase and folds do not form. Intriguingly, this is reminiscent of human lissencephaly, where cortical layers are several-fold thicker than normal and folds fail to form (see section below). However, this is not the case in gyrencephalic brains, where the outer surface area of the cerebral cortex increases several orders of magnitude more than thickness (i.e., the human cortex has 1,000 times more surface area than in mouse, but is only 10 times thicker) and without a similar increase in VZ surface area.

Thus, the radial fiber scaffold must be modified so that the increased numbers of radially migrating neurons do not pile in thick layers, but are distributed along the cortex. An elegant solution to this problem is to create a divergence in the array of radial fibers with additional, intercalated fibers. Under this circumstance, neurons migrating radially and in intimate association with radial fibers find additional paths for migration, which has two advantages: (i) overcrowding of the migratory paths is released so radial migration is not delayed and (ii) radially migrating neurons are delivered to distant positions along the cortical surface (Fig 2B). The source of additional radial fibers to create this divergence is bRGCs (Reillo *et al*, 2011). Basal RGCs have a radial fiber extended to the pial surface, their cell soma is located in ISVZ or OSVZ, basal from aRGCs, and they are not anchored to the apical VZ surface. Hence, bRGCs are in the optimal position to provide additional radial fibers to create the divergence and fanning out of the radial fiber scaffold, without increasing VZ surface area, as reported in ferret (Fig 2B; Reillo *et al*, 2011). Importantly, in spite of the divergence of radial fibers, thanks to bRGCs their density remains high through the cortical thickness, and many are even intercrossed (Rakic *et al*, 1974; Reillo *et al*, 2011), so neurons can disperse laterally while maintaining radial glia-dependent migration, as recently demonstrated in ferret (Gertz & Kriegstein, 2015). In the developing ferret cortex, the fanning of the radial fiber scaffold is significant in prospective gyrus, but not in prospective sulcus regions, which further supports that this is a driving force in cortical surface area expansion and folding (Lui *et al*, 2011; Reillo *et al*, 2011; Borrell & Reillo, 2012). Importantly, this "radial divergence model" has been validated by several laboratories, where bRGC abundance and proliferation have been experimentally manipulated in ferret and mouse. Partial blockade of OSVZ progenitor proliferation in the developing ferret cortex without a significant neuronal loss leads to a reduction in size of cortical folds (Reillo *et al*, 2011) and even lissencephaly (Poluch & Juliano, 2015). Conversely, forced overproliferation of OSVZ progenitor cells in ferret significantly increases cortical surface area and folding (Nonaka-Kinoshita *et al*, 2013). Finally, even in the naturally smooth mouse cortex, genetic manipulations forcing an increased abundance of bRGCs during embryonic development lead to the formation of cortical folds (Stahl *et al*, 2013; Florio *et al*, 2015).

Differentiation

Once neurons finish radial migration and detach from the radial fiber, they begin terminal differentiation. This process has a fundamental impact in the final size of the cerebral cortex, essentially by increasing the size of cell somas and the volume of the neuropile: growth and branching of apical and basal dendrites; extension, navigation, and branching of the axon; formation of spines and boutons for synaptic connectivity. Packing density of cortical neurons, cell body size, and extent of their dendritic and axonal arbors are remarkably different between mammals, correlating with brain size (Purves, 1988) and also contributing to cortical expansion (Reillo *et al*, 2011).

Molecular regulation of cellular mechanisms

Over the last two decades, hundreds of studies have deciphered some of the key molecular mechanisms regulating cerebral cortex development in mouse. Although this invaluable knowledge is frequently extensible to humans and other species at the level of basic cellular mechanisms, much less is really known about the molecular regulation of events key for cortical expansion and folding. In this section, we provide an overview of some of this knowledge, and how it may be extensive to cortical folding.

NEC amplification versus transition to RGC

At early stages of cortical development, several signaling cascades regulate the maintenance of NEC self-amplification. One of the most potent promoters of NEC self-amplification is the β-catenin pathway, such that its constitutive activation in mouse embryos leads to the overexpansion and folding of the cortical neuroepithelium (Chenn & Walsh, 2002). Expansion and significant folding of the mouse cortical neuroepithelium is also achieved by limiting developmental apoptosis, by means of lysophosphatidic acid signaling (Kingsbury *et al*, 2003). Conversely, the fibroblast growth factor (FGF) pathway, by means of FGF10, drives NECs into expressing radial glial cell markers and thus promotes their transition toward aRGC fate, terminating the phase of NEC amplification (Sahara & O'Leary, 2009).

Intriguingly, FGF signaling promotes retention of the aRGC fate, as it also inhibits the subsequent transition of aRGCs toward basal progenitors (Kang *et al*, 2009). The transition from NECs to aRGCs is also strongly promoted by the activation of the Notch signaling pathway (Gaiano *et al*, 2000; Hatakeyama *et al*, 2004; Fig 1).

Progenitor amplification

In addition to their role in blocking the maturation of cortical progenitors, FGF ligands promote their proliferation and inhibit neurogenesis by regulating the duration of the cell cycle. This role of FGF signaling is critical for cortical growth, as its loss at early stages accelerates neuron production and loss of RGCs, resulting in reduced cortical surface area (Rash *et al*, 2011). Conversely, overactivation of FGF signaling by infusion of FGF2 or FGF8b causes the overproliferation of cortical progenitors and cortical expansion in surface area (Rash *et al*, 2013; Fig 1). FGFs influence cortical progenitors via regulation of cell cycle proteins. FGF2 and insulin-like growth factor (IGF) 1 upregulate the expression of cyclin D1 and downregulate the expression of p27(kip1), a cyclin-dependent kinase (Cdk) inhibitor, thereby shortening the G1 phase of the cell cycle and promoting self-amplificative divisions (Raballo *et al*, 2000; Lukaszewicz *et al*, 2002; Mairet-Coello *et al*, 2009). Activation of this FGF signaling cascade drives cortical expansion, which is accompanied by the incipient folding of the otherwise smooth mouse cortex (Rash *et al*, 2013), and in the already gyrencephalic ferret cortex, it causes extra folding (Masuda *et al*, 2015). Not surprisingly, cortical progenitor populations may be expanded directly by overexpressing Cdk4 and cyclin D1 (Lange *et al*, 2009). However, in the mouse cortex, this promotes cortical growth and megalencephaly, but not folding (Nonaka-Kinoshita *et al*, 2013), which highlights the molecular and cellular complexity of the process of cortical folding (Borrell & Calegari, 2014).

Other important signaling pathways regulating cortical progenitor proliferation and self-renewal include the Wnt, BMP, MAPK, and Notch pathways. Unfortunately, none of these pathways have been found able to induce *bona fide* cortical folding in mouse, and there are no experimental data in naturally gyrencephalic species. One of the best-known pathways regulating the balance between progenitor proliferation and neurogenesis is Notch. In addition to promoting the transition from NECs to aRGCs at early developmental stages (Gaiano *et al*, 2000; Hatakeyama *et al*, 2004; Martynoga *et al*, 2012), activation of the Notch pathway at later stages inhibits the generation of IPCs from aRGCs (Mizutani *et al*, 2007; Ohata *et al*, 2011; Martynoga *et al*, 2012). Conversely, the onset of *Dll1* expression (the main ligand of Notch1) coincides with the expression of the proneural proteins Ngn2 and Ascl1, which are major transcriptional regulators of neurogenesis and also directly regulate *Dll1* expression (Castro *et al*, 2006; Martynoga *et al*, 2012). The Notch pathway can be additionally activated by Slit/Robo signaling, with similar consequences: impairment of neurogenesis at early stages by favoring the self-renewal of aRGCs (Borrell *et al*, 2012). Importantly, Notch signaling seems to be required for the self-renewal of OSVZ progenitors in the human cerebral cortex (Hansen *et al*, 2010).

Regarding Wnt, activation of this pathway promotes proliferation and self-renewal of aRGCs at early developmental stages (Machon *et al*, 2003; Woodhead *et al*, 2006; Zhou *et al*, 2006), while at later stages it promotes the maturation of aRGCs into IPCs (Viti *et al*, 2003; Hirabayashi *et al*, 2004) and it may even promote neurogenesis (Munji *et al*, 2011). Thus, the effects of Wnt signaling in cortical development are complex and time-regulated during development. The BMP pathway has similarly elusive and complex inputs into the regulation of cortical neurogenesis. At early developmental stages, BMP signals induce neurogenesis (Li *et al*, 1998; Mabie *et al*, 1999), while at later stages, they block neurogenesis to promote astrocyte differentiation (Gross *et al*, 1996). The Ras-MAPK-ERK pathway controls the mitogen-stimulated proliferation of cortical progenitors and its negative regulators, thus ensuring progenitor self-renewal and preventing premature differentiation (Phoenix & Temple, 2010). Finally, IGF-2 secreted into the cerebrospinal fluid by the choroid plexus is another potent mitogen promoting proliferation of VZ cortical progenitors (Lehtinen & Walsh, 2011; Lehtinen *et al*, 2011).

Recently, retinoic acid (RA) signaling has also been identified as important in regulating the balance between cortical progenitor self-renewal and neurogenesis, where RA secreted by the meningeal membranes promotes neurogenesis while limiting aRGC amplification (Siegenthaler *et al*, 2009). Contrary to the classical signaling pathways mentioned above, the RA pathway holds promise as an important regulator of cortical expansion and folding, as its genetic blockade induces remarkable folding of the mouse cortex (Siegenthaler *et al*, 2009). The downstream transducers of RA signaling in this context are not known, but the orphan nuclear hormone receptor CoupTF1 and the pro-neural transcription factors Ngn1 and 2 may be involved (Ribes *et al*, 2008; Harrison-Uy *et al*, 2013).

Progenitor cell lineage

Whereas cortical folding is positively correlated with increased brain size and greater numbers of neurons, mounting evidence from comparative neuroanatomy and human pathology strongly supports that increased neuron numbers are not sufficient, but cortical folding requires additional mechanisms of developmental regulation (Welker, 1990; Borrell & Reillo, 2012). As explained in the previous section, the tangential dispersion of radially migrating neurons seems key in the expansion of cortical surface area that leads to folding, and this depends on the relative abundance of bRGCs (Reillo *et al*, 2011; Pilz *et al*, 2013). Hence, lineage regulation of the different types of cortical progenitors, particularly the production and maintenance of bRGCs, is critical in this process (Borrell & Gotz, 2014).

In the cerebral cortex of mouse, ferret, and humans, bRGCs are generated from aRGCs in the VZ (Reillo *et al*, 2011; Shitamukai *et al*, 2011; Wang *et al*, 2011). This process is seemingly associated with, and controlled by, the mitotic spindle orientation of aRGCs (Shitamukai *et al*, 2011; LaMonica *et al*, 2013), which is known to have a strong influence on the acquisition of asymmetric cell fates by cortical progenitors (Postiglione *et al*, 2011; Xie *et al*, 2013). Progenitors in the early neuroepithelium divide mostly in perpendicular orientations while oblique cleavage planes augment as neurogenesis becomes predominant, and disruption of these orientations at early stages leads to depletion of the progenitor pool (Mitchison & Kirschner, 1984; Chenn & McConnell, 1995; Yingling *et al*, 2008). Orientation of the mitotic spindle in the mammalian cerebral cortex is regulated by a number of factors, including LGN, Insc, and Lis1 (Yingling *et al*, 2008; Postiglione *et al*, 2011; Shitamukai *et al*, 2011; Fig 1).

In addition to the cleavage plane orientation, there are other cellular mechanisms regulating cortical progenitor cell fate,

particularly symmetric versus asymmetric fates, which include the maintenance of the apical–basal polarity by Notch signaling, Par3, Par6, prominin-1 (CD133), and other proteins related to the apical adherens junctions (Hatakeyama *et al*, 2004; Gotz & Huttner, 2005; Costa *et al*, 2008; Bultje *et al*, 2009; Kriegstein & Alvarez-Buylla, 2009; Imayoshi *et al*, 2010). A decrease in apical junction proteins like Par3 or Par6 switch the mode of aRGC division from self-renewing to neurogenic, while their overexpression promotes aRGC self-renewal (Costa *et al*, 2008).

A landmark finding on the molecular regulation of cortical folding via control of progenitor cell lineage was the identification of *Trnp1*, a key player in this process (Stahl *et al*, 2013). Trnp1 is a DNA-associated protein initially identified as being strongly expressed in self-amplifying aRGCs (Pinto *et al*, 2008). Starting at high levels in the early embryo, Trnp1 expression decreases as aRGCs gradually stop self-amplifying to produce IPCs and neurons. Overexpression of Trnp1 promotes aRGC self-renewal at the expense of IPCs and neurogenesis. Conversely, knockdown of Trnp1 function increases IPCs and, in addition, it dramatically increases the abundance of bRGCs, otherwise very scarce in the mouse embryo. Most importantly, these changes are associated with a significant expansion of the cortical surface area and the formation of structures resembling *bona fide* cortical folds, including modification of the radial fiber scaffold and the divergent distribution of radially migrating neurons (Stahl *et al*, 2013). Analyses of *Trnp1* expression in the developing cortex of ferret and human embryos are consistent with these functional results, as *Trnp1* levels are specifically low in cortical regions prospectively undergoing tangential expansion and folding (Stahl *et al*, 2013; de Juan Romero *et al*, 2015). Thus, *Trnp1* was the first gene able to induce *bona fide* folding of the mouse cerebral cortex.

A second gene causing folding of the mouse cortex has been recently found: *ARHGAP11B* (Florio *et al*, 2015). This is a truncated paralog of *ARHGAP11A* that arose on the human evolutionary lineage after the divergence from the chimpanzee (Antonacci *et al*, 2014), and hence does not exist in mouse. Similar to the loss of function of *Trnp1*, experimental expression of *ARHGAP11B* in mouse embryos drives cortical aRGCs into massively producing basal progenitors, many of which are bRGCs, and this ultimately translates into folding of the mouse cerebral cortex. The seminal relevance of this study is the discovery of a gene with a likely central role in human brain evolution and uniqueness, contributing to further promote cortical expansion and folding in the hominid lineage. High levels of the transcription factor Pax6 have also been reported to promote the generation of bRGCs in mouse, but intriguingly this does not cause cortical folding (Wong *et al*, 2015).

Progenitor cell heterogeneity: single-cell transcriptomics

One of the classical limitations of studies searching for genes important in cortical development, expansion and folding was to analyze progenitor cell pools instead of individual cells. The advent of single-cell transcriptomics has revolutionized this field by allowing uncovering an extraordinary molecular heterogeneity of progenitor cell types in the developing cerebral cortex (Pollen *et al*, 2014). Importantly, this technology has revealed that aRGCs and bRGCs are molecularly more heterogeneous in the developing folded cortex of humans and ferret (3 classes of aRGC, 2 classes of bRGC) than in the smooth cortex of mouse (2 classes of aRGC, 1 class of bRGC; Camp *et al*, 2015; Johnson *et al*, 2015; Pollen *et al*, 2015). Although we are just beginning to profit from the power of this technology, single-cell transcriptomics is already highlighting the potential relevance of specific signaling cascades and molecular programs in the formation and maintenance of bRGCs in general and the OSVZ in particular. For example, in agreement with previous population-wide transcriptomic analyses and functional manipulations, the extracellular matrix and its components are being highlighted as central in stimulating cortical progenitor proliferation and self-renewal (Fietz *et al*, 2010, 2012; Stenzel *et al*, 2014; Pollen *et al*, 2015).

Genetic patterning of cortical folds

What defines the location and shape of cortical folds and fissures as they form in the developing cortex? The traditional view has been that cortical folds form randomly, essentially based on the idea that cortical growth exceeds cranial volume, and thus, cortical folding occurs purely as a mechanical consequence of cranial constraint (Welker, 1990). However, as discussed in a later section, evidence indicates that both notions are wrong. If any portion of the cerebrum fails to develop or grow, due to either pathological or experimental causes, the skull tends to conform to the size and shape of the remaining neural tissue, which in fact still folds, thus demonstrating that limited cranial volume does not force cortical folding (Welker, 1990). Further evidence demonstrating that cortical folds do not form randomly includes that (i) the pattern of folds and fissures is highly stereotyped and well conserved between individuals of a species, particularly in those with small gyrated brains (i.e., ferret, cat); (ii) folding patterns of phylogenetically related species follow remarkably similar trends (Welker, 1990; Borrell & Reillo, 2012); (iii) even in species with large cortices and very complex folding patterns, like humans, the deepest and earliest fissures to develop do so at strikingly conserved positions, particularly in monozygotic twins where the overall folding pattern is significantly well conserved (Lohmann *et al*, 1999, 2008). Taken together, cortical folding patterns appear subject to strong genetic regulation.

In species with a simple pattern of folds, like ferret and cat, the stereotyped location of cortical folds and fissures is preceded and mirrored by regional variations in progenitor cell proliferation, in all three germinal layers, but most prominently in the OSVZ (Reillo *et al*, 2011). Local manipulations of OSVZ proliferation in ferret have a significant impact on the size and shape of cortical folds, without altering cortical area identity nor lamination (Reillo *et al*, 2011; Ghosh & Jessberger, 2013; Nonaka-Kinoshita *et al*, 2013). To identify genes whose expression covaries with the stereotypic patterning of progenitor proliferation and cortical folds, microarray technology was recently used to compare the transcriptome of progenitors in prospective folds versus fissures of the developing ferret cortex (de Juan Romero *et al*, 2015). This analysis demonstrates the existence of thousands of genes differentially expressed (DEGs) between these regions, mostly in OSVZ and VZ. Identified DEGs include genes key in cortical development and folding like *Trnp1* or *Ccnd1*, as well as genes mutated in human malformations of cortical development. Many DEGs are expressed in modules along the OSVZ and other germinal layers of the gyrencephalic ferret and also the human embryo cortex, but not in the lissencephalic mouse cortex (Sansom & Livesey, 2009; Elsen *et al*, 2013; de Juan

Romero *et al*, 2015). Most remarkably, expression modules along the OSVZ map faithfully the eventual location of cortical folds and fissures (de Juan Romero *et al*, 2015). This strongly supports a role for the OSVZ and some of those DEGs on cortical patterning, particularly in its stereotyped folding (Kriegstein *et al*, 2006; Lui *et al*, 2011; Reillo *et al*, 2011; Albert & Huttner, 2015; Fig 3).

Multiple genetic maps seem to overlap across germinal layers, as modular expression patterns also exist for DEGs in VZ and ISVZ of ferret and humans (de Juan Romero *et al*, 2015). Given that these germinal zones are major sites of neurogenesis and neural fate determination, these gene expression patterns may contribute significantly to further define cortical folds and/or functional areas of the cerebral cortex (Lui *et al*, 2011; Reillo *et al*, 2011; Taverna *et al*, 2014; Dehay *et al*, 2015). Indeed, many DEGs between the prospective gyrus and sulcus are known to regulate progenitor proliferation, neurogenesis, or fate specification, including key signaling pathways such as Notch, Shh, MAPK, and Wnt, which directly regulate cortical growth (see above). In the case of cortical folds, modular patterns of expression for a combination of genes, possibly different depending on the specific gyrus or sulcus, may impose differential tissue growth between modules, eventually leading to the evagination of the cortex and formation of folds (Smart & McSherry, 1986; Fig 3).

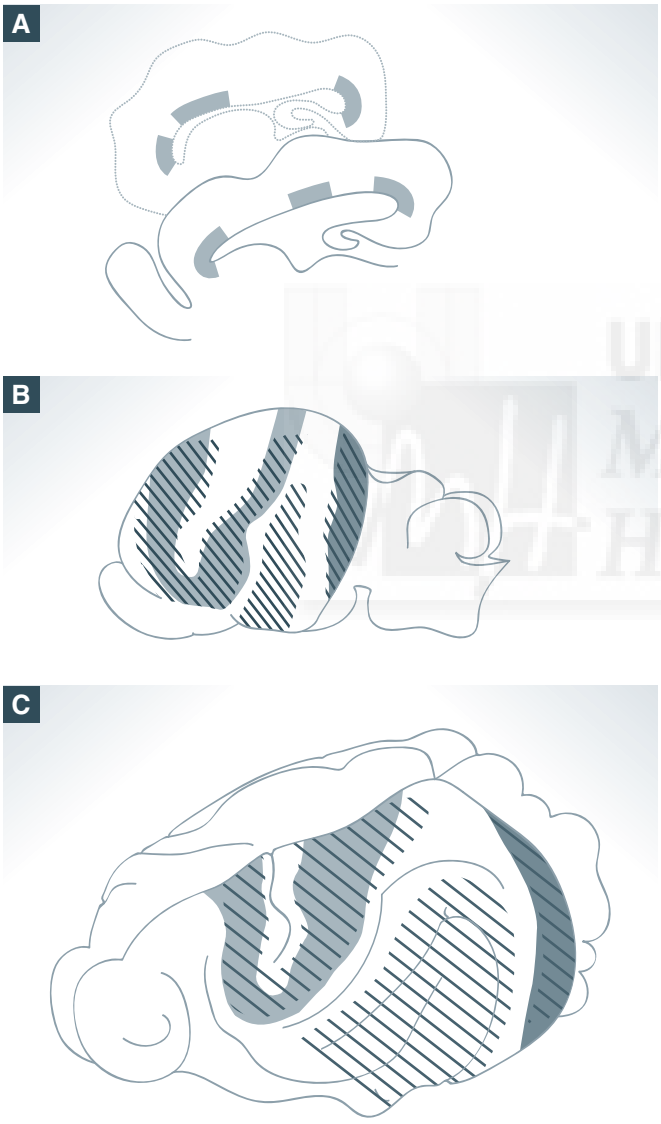


Figure 3. Patterns of gene expression map the prospective location of cortical folds in the developing ferret brain.
(A) Schema of sagittal sections of ferret brains at postnatal day P6 showing the modular pattern of mRNA expression for *Eomes* at the outer subventricular zone (shaded areas). (B, C) Representation of the ferret brain surface at postnatal day P2 (B) and adult (C) overlapped with the map of *Eomes* expression modules (shaded) and prospective gyri (striped pattern), showing the spatial correlation between *Eomes* expression and gyri (adapted from de Juan Romero *et al*, 2015).

Small enhancer elements have been recently identified to drive reporter gene expression in discrete modules, or protodomains, also in the embryonic mouse cerebral cortex (Visel *et al*, 2013; Pattabiraman *et al*, 2014). These enhancers have been proposed to integrate broad transcriptional information, including expression of several transcription factors regulating cortical patterning, to define gene expression in those protodomains (Nord *et al*, 2013; Pattabiraman *et al*, 2014). Variations in such small enhancer elements may be indeed at the core of cortical patterning (and folding) during development and evolution (Borrell & Gotz, 2014; see below). However, the definition of discrete cortical subdivisions requires the regional control over the expression of protein-coding genes or their interfering RNAs (i.e., cell cycle regulators, cell fate determinants, neuron terminal selectors) in protodomains along the embryonic germinal layers, as demonstrated in ferret and humans, but not in mouse (Dehay & Kennedy, 2007; Molyneaux *et al*, 2007; Hobert, 2011; de Juan Romero *et al*, 2015). Walsh and colleagues recently identified a key regulatory element for the expression of human *GPR56* which varies significantly across mammals and, when introduced in transgenic mouse embryos, drives different patterns of expression (Bae *et al*, 2014). Most importantly, *GPR56* expression levels regulate cortical progenitor proliferation, and mutation of this regulatory element disrupts human cortex folding around the Sylvian fissure, demonstrating the importance of the expression pattern of this gene in defining the pattern of folds. Similarly, mutations in the regulatory region of human *EOMES* lead to significant alterations of cortical size and folding (microcephaly with polymicrogyria; Baala *et al*, 2007). In agreement with the notion of a protomap of cortical folding, both *GPR56* and *EOMES* are expressed in modular patterns in the developing ferret and human cortex (de Juan Romero *et al*, 2015).

Biomechanics of cortical folding

Cranial pressure

One of the first hypotheses on the biomechanics of cortical folding proposed that cranial volume limits cortical size, so that as the cortical tissue grows in surface area the skull offers resistance to its outward expansion, forcing the neural tissue to fold onto itself (Le Gros Clark, 1945). The concept that brain morphology adapts to fit in a limited volume has been recently supported by experiments in chick embryos, where an experimentally expanded optic tectum folds to maintain cranial size (McGowan *et al*, 2012). However, studies focused specifically on the mammalian cerebral cortex have shown that cortical folding takes place even in the absence of

compressive constrain from the skull (Welker, 1990). For example, the removal of non-cortical brain tissue in sheep embryos alleviated any possible cranial pressure onto the growing cortex, and yet this did not suppress or simplify the formation of cortical folds (Barron, 1950; Muckli *et al*, 2009). On the contrary, development of the skull appears to be strongly influenced by brain growth, as cranial volume is significantly enlarged in pathologies where brain and cortical size are abnormally large, such as hydrocephalus or megalencephaly (Barkovich *et al*, 2012).

Axonal tension

The first attempt to mathematically model the biomechanical basis of cortical folding dates back four decades (Richman *et al*, 1975). This model proposed that cortical folding occurs as a result of differential growth between upper and lower cortical layers, which generates stress that is sufficient to induce cortical surface buckling. Thirty years later, Kriegstein and colleagues (Kriegstein *et al*, 2006) proposed that basal progenitors increase significantly neurogenesis at later stages, precisely when upper layers form, and that this enables the differential growth between layers and ultimately drives cortical folding. Whereas the Richman model showed that this principle is sufficient to convert a flat surface into wavy, it relies on such a difference in stiffness between neuronal layers that it seems unrealistic (Bayly *et al*, 2014).

Two decades passed before an alternative model was proposed to explain the biomechanics of cortical folding: the tension-based theory (Van Essen, 1997). This theory by Van Essen was conceptually based on D'Arcy Thompson's analysis on how tension and pressure can interact with structural asymmetries to determine the shape of biological structures (Thompson, 1917). Van Essen argued that cortico-cortical axons are under strong tension, exerting significant pulling forces capable of deforming the cortical mantle, and that these cortico-cortical connections are not symmetric or homogeneously distributed, but some areas are more strongly connected together than with others. Under this scenario, he proposed that those areas connected with a larger amount of axons withstand a greater pulling force, and thus come close together to form a fold, whereas areas poorly interconnected are relayed to the opposite banks of a sulcus. This hypothesis attracted the enthusiasm of many, not only for its simplicity but also for its coherence. Indeed, two simple but fundamental observations made this a very attractive model: (i) The cortical sheet is physically tethered in only one axis, initially by radial processes and followed by connections between cortex and subcortical nuclei (De Carlos & O'Leary, 1992). Tension along these processes may provide a cohesive force against intraventricular hydrostatic pressure, ensuring that the cortical mantle remains tightly wrapped around the subcortical interior. (ii) Specific and topographically organized cortico-cortical projections are established early in development while convolutions are forming (Coogan & Van Essen, 1996; Hilgetag & Barbas, 2006). Van Essen proposed that if developing CNS axons *in vivo* generate even a modest fraction of the specific tension measured *in vitro* (Dennerll *et al*, 1988), then populations of axons pulling together should have ample strength to cause folding of the highly pliable embryonic cortical sheet (Van Essen, 1997). Importantly, this hypothesis also provided a basis for individual variability in brain morphology.

Remarkably, the tension-based theory was widely accepted for more than a decade without rigorous experimental testing. But 14 years later, Taber, Bayly, and colleagues performed a series of very simple and elegant experiments that frontally challenged the foundations of this theory (Xu *et al*, 2010). Their idea was that if axonal tension between opposite sides of a gyrus pulls them together, then these should come apart if these axons are cut and tension is released. This was tested in living brain slices from developing ferrets of various ages throughout the period of gyrus formation. These experiments showed that axons are indeed under considerable tension in the developing brain, but most of this tension is found along axon bundles in deep white matter tracts, not within the core of individual gyri and thus too far to play a major role in initiating, sustaining, or maintaining cortical folding (Armstrong *et al*, 1991; Xu *et al*, 2010). In addition, other computational models show that cortical folding may occur in the complete absence of cortico-cortical fibers, only as an effect of buckling instability (see below; Toro & Burnod, 2005).

Tissue buckling

Folding of the cerebral cortex, like any other tissue, is limited by its physical–mechanical properties of rigidity and pliability, which define the relationship between cortical thickness and surface area. As a general principle, given a cortical surface area, the periodicity of folding is inversely correlated with gray matter thickness (Hofman, 1985; Toro & Burnod, 2005; Pillay & Manger, 2007). This applies to interspecies differences (i.e., ungulates have a thinner and more folded cortex than primates, whereas in the manatee, the cortex is thicker and rather smooth; Welker, 1990) and also to human pathology, where lissencephalic patients with reduced folding display an abnormally thick cortex, and polymicrogyric cortices are abnormally thin (Olson & Walsh, 2002; Barkovich *et al*, 2012).

A very recent study proposes a general law for this inverse correlation between thickness and folding, based on measurements from 62 different species and describing a mathematical relationship between total cortical surface area, thickness, and exposed area (Mota & Herculano-Houzel, 2015). The basic idea is that cortical folding is similar to crumpling a ball of paper, where the periodicity of individual folds will be high in a ball of thin smoking paper (small folds) and very low if using thick cardboard paper (large folds). According to this study, the combination of total cortical area and thickness grows with exposed cortical area to the power of 1.25, in a similar fashion as the area of a circle increases with its radius raised to the power of 2. This relationship is also proposed to represent the topological configuration involving the minimal energy, and therefore, cortical folding may settle into the configuration of least energy (Mota & Herculano-Houzel, 2015). But there are several fundamental problems with the paper ball analogy: (i) It describes the folding of a structure that no longer grows, whereas the cortex folds while it continues to develop and grow; (ii) it is based on physical models only valid for single-molecule thin materials; (iii) the theory considers that the whole brain folds, while folding only involves the cortical gray matter, not the rest of the brain (Mota & Herculano-Houzel, 2015). Finally, folding of the cerebral cortex does not occur randomly, as crumpling of a paper ball, but in a rather highly stereotyped process, defining patterns that are characteristic and distinct for each species. Prior to their appearance, folding patterns are delineated by regional variations of progenitor cell proliferation (Reillo *et al*, 2011), and by modules of differential gene expression along germinal layers, as recently demonstrated via

transcriptomic expression analyses (de Juan Romero *et al*, 2015). These fundamental heterogeneities of the developing cortex immediately prior to folding are completely ignored by the Mota and Herculano-Houzel model. Additional problems with this model, at the mathematical level, have been recently highlighted (De Lussanet, 2016).

Using a finite element computational model, Taber and colleagues suggested that a critical factor leading to the formation of outward folds is differential cortical growth (Xu *et al*, 2010), which precisely matches our experimental data and histogenic model of regional cortical expansion and folding in ferret (Reillo *et al*, 2011). This hypothesis where the local difference in tissue growth, particularly lateral/tangential expansion, is a key factor driving cortical folding (Reillo *et al*, 2011; Borrell & Reillo, 2012; Borrell & Gotz, 2014) is progressively gaining acceptance in the field (Ronan *et al*, 2014; Ronan & Fletcher, 2015; Striedter *et al*, 2015). Although these new findings contradict Van Essen's tension-based hypothesis of cortical folding, and axonal tension may not directly be the driving force of cortical folding, alternative computational models do propose that axonal tension may play an important role in tissue buckling (Chada *et al*, 1997). In this case, however, models propose that upon incipient folding, the mechanical stress created induces cell proliferation and differential growth, which further accentuates the folded appearance of the cortex and defines the shape of cortical folds (Toro & Burnod, 2005).

A major breakthrough in our understanding of the biomechanics underlying cortical folding came with a recent study by Tallinen and colleagues (Tallinen *et al*, 2014). Many of the ingredients of this model were already present in the mentioned models of Richman, Toro, and Taber (Richman *et al*, 1975; Toro & Burnod, 2005; Xu *et al*, 2010), but this new study is the first to propose a coherent framework with physical simulations, computational simulations, and a very compelling mathematical analysis of the problem. The key point of this model is that two different materials are sticking together while growing homogeneously, if one grows faster than the other, the system becomes unstable and will change shape by folding, which is an emergent property of any such mechanical system (Tallinen *et al*, 2014). Most interestingly, solely based on the slightly different physical properties of the two materials (i.e., upper versus lower cortical layers), homogeneous continuous growth is sufficient to lead the system to develop a dramatic change in shape, forming a heterogeneous pattern of stress that in turn can influence the biology of the tissue (i.e., cell proliferation, apoptosis, cell fate, and even axon guidance). Therefore, tissue buckling and cortical folding may ultimately result from the mutual influence between physical properties, biomechanics, and differential tissue growth.

Defects of cortical folding: human disease

The size and folding of the cerebral cortex have a fundamental impact on brain function. Significant changes (excess or defect) in these parameters are the most common cause of severe intellectual disability and intractable epilepsy (Guerrini *et al*, 2008; Andrade, 2009). Defects in human cortical development have been recognized as being originated by the disruption of some of the cellular and molecular mechanisms described above in this review (Barkovich *et al*, 2012), hence critically impacting on key developmental events. In this section, we briefly present these human malformations grouped according to the main affection: brain size, cortical folding, or the formation of ectopias (groups of cells in an abnormal location). It is important to note that cortical malformations usually appear as compound phenotypes, only very rarely as a single defect (Fig 4).

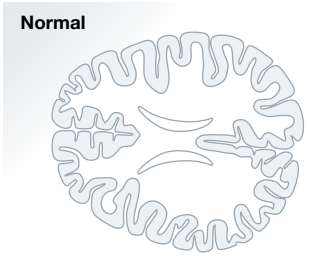
Brain size

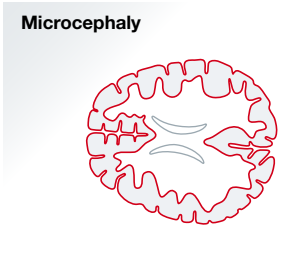
As explained in the first section, the pool size of founder and neurogenic cortical progenitors plays a central role in defining cortical size. Consequently, alterations in proliferation and/or survival of neural progenitors lead to abnormal brain size, either excessive (megalencephaly), defective (microcephaly), or imbalanced (dysplasia; Barkovich *et al*, 2012; Fig 4).

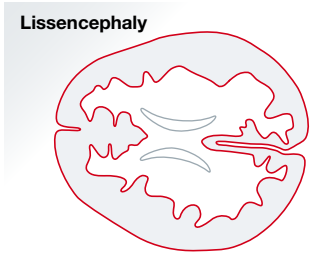
Microcephaly Microcephaly is a rare developmental disorder in which affected individuals display a significantly reduced brain size compared to controls (Bond *et al*, 2002; Gilmore & Walsh, 2013). This condition may be mild (only brain size is affected) or severe (small brain size and altered cortical folding; Bilguvar *et al*, 2010; Yu *et al*, 2010; Adachi *et al*, 2011). Microcephaly has been associated with mutations in genes important for a wide variety of cellular processes: DNA repair efficiency; cell cycle length; mitotic spindle positioning; and centrosome maturation, duplication, and position (Table 1). For example, the most common causes of primary microcephaly are mutations in microcephalin (*MCPH1*), which lengthens the cell cycle and alters chromosome alignment during mitosis (Jackson *et al*, 2002; Woods *et al*, 2005; Gruber *et al*, 2011), and *ASPM*, which is important to maintain the orientation of the mitotic cleavage plane (Kumar *et al*, 2004; Shen *et al*, 2005; Fish *et al*, 2006; Gul *et al*, 2006; Table 1). For an extensive review on the role of different genes identified in microcephalic patients on the emergence of this condition, please refer to Bizzotto and Francis (2015).

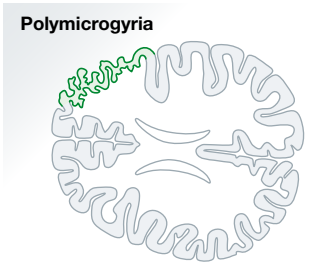
Megalencephaly Megalencephaly is characterized by an abnormal enlargement of the brain, which has been related to an excessive production of progenitor cells and cortical neurons due to a decreased apoptosis, or a shortening of cell cycle and increased cell cycle re-entry (Dehay & Kennedy, 2007; Hansen *et al*, 2010; Wang *et al*, 2011; Barkovich *et al*, 2012). As in other malformations, severe forms of megalencephaly may occur together with altered patterns of cortical folding. Usually, the increased abundance of progenitor cells and neurons results in polymicrogyria or excessive cortical folding (Barkovich *et al*, 2005). In fact, megalencephaly normally occurs in syndromes, in combination with other alterations of development, such as MPPH (macrocephaly, polymicrogyria, polydactyly, hydrocephalus), M-CMTC (macrocephaly cutis marmorata telangiectasia congenita), and MCAP (macrocephaly capillary malformation; Mirzaa *et al*, 2004; Conway *et al*, 2007; Tore *et al*, 2009). The genetic causes of megalencephaly are only partially understood, but recent progress highlights the importance of phosphatidylinositol 3-kinase (PI3K)-Akt signaling, which seems to play a central role in controlling brain size (DiLiberti, 1998; Lee *et al*, 2012; Riviere *et al*, 2012; Mirzaa *et al*, 2013; Table 1).

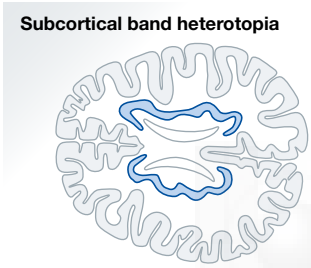
Dysplasia A very common group of malformations of cortical development related to epilepsy is focal cortical dysplasia (FCD), which classically has included patients showing a variety of histologic

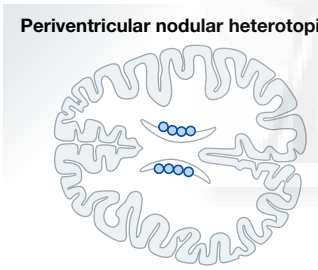












MALFORMATION	SIZE	FOLDING	ECTOPIA
Microcephaly	Reduced	Mild – Normal	No
		Severe – Altered	Yes
Megalencephaly	Increased	Mild – Normal	No
		Severe – Altered	No
Dysplasia	Altered	Increased	Abnormal layers
Lissencephaly type I	Normal	Reduced	Abnormal layers
	Reduced		
Lissencephaly type II Cobblestone	Normal	Increased	Cortical surface
Polymicrogyria	Increased	Increased	Abnormal layers
	Reduced		
Subcortical band heterotopia Double cortex	Reduced	Normal	White matter
Periventricular heterotopia	Normal	Normal	Periventricular
	Reduced	Normal	

Figure 4. Human cortical malformations and their phenotypic manifestations.
Schematic of horizontal sections through the cerebral cortex of a normal human brain compared to those of patients with cortical malformation: microcephaly, lissencephaly type I, polymicrogyria, subcortical band heterotopia (double cortex), and periventricular nodular heterotopia. The table summarizes the phenotypic manifestations associated with each malformation regarding brain size, cortical folding, and the formation of ectopias. The most representative effects are highlighted and color-coded: Features negatively affected by the pathology are in red, features augmented in green, and particularities are in blue. Uncolored cells indicate additional alterations that may be associated with the primary defect.

alterations such as cortical disorganization and architecture (abnormal layering, polymicrogyria) and cells with abnormal location or morphology (neuronal heterotopia, balloon cells, neuronal cytomegaly; Palmini *et al*, 2004; Barkovich *et al*, 2005). These alterations may appear in any part of the cortex and affect regions of different size, even multiple cortical lobes (Tassi *et al*, 2002), which determine the semiology of seizures. Genes in the mTOR pathway are emerging as important players on the origin of developmental cortical dysplasias (Crino *et al*, 2006; Barkovich *et al*, 2012). FCDs are distinguished in three different varieties (Blumcke *et al*, 2011; Barkovich *et al*, 2012): (i) type I (isolated), with disrupted cortical lamination that may be radial (Ia), tangential (Ib), or both (Ic); (ii) type II (isolated), presenting dysmorphic neurons with balloon cells (IIb) or without them (IIa); (iii) type III, associated with another main lesion, such as hippocampal sclerosis (IIIa), glial or glio-neuronal tumor (IIIb), vascular malformation (IIIc), and others (trauma, ischemic injury, encephalitis) (IIId) (Blumcke *et al*, 2011).

Cortical folding

Alterations of cortical folding in the human brain have been classically attributed to defects of neuronal migration (Ross & Walsh, 2001). However, disruption of neuron migration and positioning also leads to other cortical defects with a mild alteration of cortical folding, such as lissencephaly type II and subcortical band heterotopia. The latter will be discussed in the next section focused on ectopias.

Lissencephaly (smooth brain) This includes several disorders collectively characterized by the simplification of the folding pattern: agyria (complete absence of folds), pachygyria (simplified pattern of folds), and subcortical band heterotopia (gyral pattern is either

Table 1. Types of human cortical malformation, molecular mechanisms altered, and genes associated.

	Malformation	Molecular mechanism	Genes	References
Size	Microcephaly	DNA repair efficiency	MCPH1, PNKP, PNCT	Woods *et al* (2005); Griffith *et al* (2008); Sheen *et al* (2010); Gruber *et al* (2011)
		Cell cycle length	ASPM, STIL, AKT3	Boland *et al* (2007); Desir *et al* (2008); Kumar *et al* (2009); Passemard *et al* (2009)
		Mitotic spindle positioning	ASPM, STIL, WDR62, NDE1, TCOF1, DYNC1H1, TUBG1, KIF5C, KIF2A	Feng & Walsh (2004); Bilguvar *et al* (2010); Nicholas *et al* (2010); Yu *et al* (2010); Sakai *et al* (2012); Poirier *et al* (2013)
		Centrosome maturation, duplication, and position	NDE1, CDK5RAP2, CENPJ, ASPM, CMPH1, WDR62, STIL, CEP152, CEP63	Abrieu *et al* (2000); Alkuraya *et al* (2011); Bhat *et al* (2011); Bond *et al* (2005); Graser *et al* (2007); Bakircioglu *et al* (2011); Marthiens *et al* (2013); Mirzaa *et al* (2014); Nicholas *et al* (2010); Sir *et al* (2011); Thornton & Woods (2009); Yao *et al* (2000)
	Megalencephaly	Cell growth	PI3K-AKT signaling AKT3, PIK3R2, PIK3CA	DiLiberti (1998); Lee *et al* (2012) #7409; Mirzaa *et al* (2013); Poduri *et al* (2013); Riviere *et al* (2012)
	Dysplasia	Cell cycle and growth, ribosome biogenesis, mRNA translation	mTOR pathway activation (tuberous sclerosis complex 1–tuberous sclerosis complex 2)	Crino *et al* (2006); Barkovich *et al* (2012)
Folding	Lissencephaly type I	Radial migration	LIS1, DCX, TUBB3, TUBA1A, RELN	D'Arcangelo *et al* (1995); Sapir *et al* (1997); Pilz *et al* (1998); Caspi *et al* (2000); Dulabon *et al* (2000); Hong *et al* (2000); Rice & Curran (2001); Fallet-Bianco *et al* (2008); Morris-Rosendahl *et al* (2008); Kumar *et al* (2010)
		Cortical lamination	RELN	D'Arcangelo *et al* (1995); Dulabon *et al* (2000); Hong *et al* (2000); Rice & Curran (2001)
	Polymicrogyria	Cell adhesion, regulation of phosphorylation, cell motility, synaptogenesis, angiogenesis	SPRX2	Roll *et al* (2006)
		Gene regulator	GPR56	Piao *et al* (2002, 2004, 2005); Bae *et al* (2014)
		Cytoskeleton regulation	TUBB2B, TUBB3, TUBA1A, TUBA8, KBP	Abdollahi *et al* (2009); Jaglin & Chelly (2009); Jansen *et al* (2011); Tischfield *et al* (2011); Poirier *et al* (2013); Valence *et al* (2013); Squier & Jansen (2014)
		Neurite outgrowth	KBP	Valence *et al* (2013)
		DNA repair efficiency	NHEJ1	Cantagrel *et al* (2007)
			Microdeletions in 22q11	Robin *et al* (2006)
		Suggested: centrosomal role	WDR62	Yu *et al* (2010)
Ectopia	SBH/double cortex	Cytoskeleton regulation/ neuronal migration defects	DCX, LIS1, TUBA1A, TUBG1, EML1	Gleeson *et al* (1998); Francis *et al* (1999); Sicca *et al* (2003); Keays (2007); Mineyko *et al* (2010); Kielar *et al* (2014)
	Lissencephaly type II (cobblestone)	Pial surface stability	POMT1; POMT2; FKTN, FKRP, LARGE, POMGNT1, LAMB1	Brockington *et al* (2001); Yoshida *et al* (2001); Beltran-Valero de Bernabe *et al* (2002); Longman *et al* (2003); van Reeuwijk *et al* (2005b); Roscioli *et al* (2012); Willer *et al* (2012); Kariminejad *et al* (2013)
	Periventricular heterotopia	Actin cytoskeleton	FLNA	Fox *et al* (1998); Sheen *et al* (2001); Parrini *et al* (2006); Ferland *et al* (2009)
		Vesicle trafficking	ARFGEF2	Sheen *et al* (2004); Ferland *et al* (2009)
		Neuronal migration	C6orf70	Conti *et al* (2013)
		Molecular adhesion	FAT4	Cappello *et al* (2013)
		Molecular adhesion	DCHS1	Cappello *et al* (2013)
		(unknown)	Microdeletions in 22q11	Kiehl *et al* (2009)

normal or simplified with broad convolutions and a thickened cortex; Guerrini & Marini, 2006; Fig 4). Lissencephalies are classified into two main types: type I or classic, caused by mutations in genes related to the cytoskeleton and affecting cell migration; newborn neurons fail to migrate properly and, instead of forming the characteristic six layers, they accumulate below the preplate in only four distinguishable layers, resulting in a largely disorganized and thickened cortex (Golden & Harding, 2004). Type II, or

cobblestone, is caused by alterations in the interaction between radial glia and the pial surface, which result in the disruption of the cortical surface and the overflow of neurons above the meninges (Bizzotto & Francis, 2015).

Most cases of type I lissencephaly are due to mutations in *LIS1* or *DCX* (Pilz *et al*, 1998). These are proteins that interact with the tubulin cytoskeleton allowing its polymerization and stability (Sapir *et al*, 1997; Caspi *et al*, 2000). This is also the case for *TUBB3* and *TUBA1A*, mutated in 1–4% of type I lissencephalies (Morris-Rosendahl *et al*, 2008; Kumar *et al*, 2010) and 30% of lissencephalies with cerebellar hypoplasia (impaired growth). Interestingly, the loss and simplification of folds displayed by these patients are very similar to those associated with mutations in *LIS1*, suggesting a shared molecular pathway (Barkovich *et al*, 2012). A small number of patients with autosomal recessive type I lissencephaly with cerebellar hypoplasia have mutations in *RELN*, also a gene essential for radial migration and normal cortical lamination in mouse and humans (D'Arcangelo *et al*, 1995; Dulabon *et al*, 2000; Hong *et al*, 2000; Rice & Curran, 2001; Table 1).

Polymicrogyria (many small folds) This includes a group of cortical malformations characterized by the formation of abnormally abundant and small cortical folds. It usually also involves the interdigitation of white matter resulting in abnormal lamination (Barkovich *et al*, 1999; Walsh, 2001). The defects in cortical lamination may be either simplification, with four layers similar to type I lissencephaly, or complete disruption and disorganization. Most frequently polymicrogyria (PMG) phenotypes are very complex and combined with other alterations such as microcephaly (Bilguvar *et al*, 2010; Yu *et al*, 2010). Due to this phenotypic complexity, the causative genes for human PMG have been very elusive. Genetic mutations linked to PMG include alterations in *SPRX2* (Roll *et al*, 2006), microsomal deletions in 22q11 (Bassett *et al*, 2005; Robin *et al*, 2006), and mutations in a number of cytoskeleton-associated genes (Table 1). Nongenetic causes of PMG have also been identified including insults during embryogenesis such as hypoxia, hypoperfusion, and congenital infections (Jacobs *et al*, 1999; Squier & Jansen, 2014).

Ectopia

The proper position of cortical neurons depends on a complex cellular and molecular regulation of two variables: where and when. Neurons must migrate through the entire cortical thickness and stop precisely near the cortical surface, a process determined by the time and place of their generation. Altering these events leads to misplaced neurons, a malformation generically called ectopia (out of place; Fig 4).

Subcortical band heterotopia/double cortex This type of ectopia is characterized by the accumulation of neurons in the cortical white matter (Barkovich *et al*, 2001; Ross & Walsh, 2001). Typically, the cluster of ectopic neurons forms a thick band of cells below an otherwise normal cortical gray matter (Gleeson *et al*, 1998; Francis *et al*, 1999). Importantly, double cortex is accompanied by a reduction in the size of the cerebral cortex due to the loss of neurons from the normocortex. This frequently affects cortical surface area and thickness, and in some cases, it even results in microgyria (Barkovich *et al*, 2012). Genetic mutations causative of double cortex affect a variety of cytoskeleton-interacting proteins (Table 1).

Cobblestone Whereas most types of heterotopia are due to deficient neuronal migration, cobblestone (type II lissencephaly) is caused by their excessive migration. In this case, the anchoring and attachment of the radial fiber of RGCs to the pial surface is disrupted, thus altering the basement membrane (Yamamoto *et al*, 2004; Luo *et al*, 2011). Given that the cortical basement membrane and the attachment of RGCs to it are the finish line for radially migrating neurons, this disruption leads to their overmigration, which continue moving up to the meningeal space, thus resembling cobblestones on the cortical surface (van Reeuwijk *et al*, 2005a). Several complex syndromes cause cobblestone lissencephaly: Fukuyama congenital muscular dystrophy (FCMD), muscle–eye–brain disease (MEB), and Walker–Warburg syndrome (WWS). In spite of this wide spectrum of phenotypes, mutations linked to cobblestone are found in genes involved in the attachment of the radial glial fiber to the pial surface (Li *et al*, 2008; Luo *et al*, 2011), or associated with reduced glycosylation of alpha dystroglycan, which is fundamental to anchor the dystrophin complex to the extracellular matrix (van Reeuwijk *et al*, 2005a; Roscioli *et al*, 2012; Buysse *et al*, 2013). Six major genes have been identified encoding putative or confirmed glycosyltransferases (Table 1).

Periventricular heterotopia Contrary to cobblestone, in periventricular heterotopia (PH) cortical neurons are unable to undergo radial migration. Due to defective remodeling of the actin cytoskeleton, newborn neurons cannot perform the changes in cell shape and locomotion required for their migration and completely fail to leave the germinal zones, remaining in the vicinity of the ventricular surface clustered into nodules, which eventually act as epileptic foci (Sheen *et al*, 2001, 2005; Sheen & Walsh, 2005; Sarkisian *et al*, 2006, 2008; Andrade, 2009). These periventricular nodules may appear in a variety of locations and conformations: bilateral, unilateral, laminar, sub-ependymal, and subcortical white matter (Andrade, 2009; Ferland *et al*, 2009). Remarkably, most of the cortex appears completely normal and patients show no gross defects in intellectual development or performance. PH may appear alone or as part of complex syndromes, associated with other cortical malformations such as microcephaly (Parrini *et al*, 2006). The most frequent genetic alterations linked to periventricular nodular heterotopia affect *FLNA* and *ARFGEF2* (Table 1; Fox *et al*, 1998; Sheen *et al*, 2001, 2004; Parrini *et al*, 2006; Ferland *et al*, 2009). Although these two proteins have very different cellular functions (FLNA acts on the actin cytoskeleton; ARFGEF2 has a role in intracellular membrane and vesicle trafficking), they may act in a common pathway and even interact directly (Sheen *et al*, 2004; Ferland *et al*, 2009). It has been proposed that the disruption of vesicle trafficking due to alterations of the cytoskeleton may impair cell adhesion and the integrity of the apical adherens junctions, thus leading to the formation of the periventricular nodules (Ferland *et al*, 2009).

Evolution of cortical folding

Brain size varies in several orders of magnitude between mammalian species, which is mostly the result of a disproportionate difference in size of the cerebral cortex (Finlay & Darlington, 1995). Increased cortical size is largely due to increased surface area (Rakic, 1995), and this is accompanied by cortical folding and fissuring, which in part allow its effective packing within a minimal

cranial volume (Welker, 1990; Albert & Huttner, 2015). Based on our previous sections, evolution of cerebral cortex size and topology may be attributable to modifications in the abundance and behavior of cortical progenitor cells (Borrell & Reillo, 2012). Changing the duration of the cell cycle, generating IPCs, and increasing their abundance would augment exponentially the production of neurons and therefore brain size, whereas generating bRGCs would augment cortical surface area and folding (Borrell & Calegari, 2014; Lewitus *et al*, 2014).

Early gyrification and secondary loss

Classically, the remarkable expansion and folding of the mammalian cerebral cortex along evolution has been viewed as a unidirectional process, where the small and smooth cortex of a primitive ancestor (presumed similar to mouse) gradually evolved to be larger, more complex, and folded, and from there on, it further evolved to have an increasing number of folds, like the human cortex (Kriegstein *et al*, 2006; Rakic, 2009). However, this view was challenged recently with the hypothesis that gyrencephaly might be an evolutionarily ancient trait, expressed in a common ancestor to all mammals and retained during speciation (Borrell & Reillo, 2012). This hypothesis is based on two facts: a) gyrencephaly develops in species from across mammalian phylogeny, ranging from monotremes and marsupials to ungulates, carnivores, primates, and even rodents, and b) during embryonic development of the cerebral cortex, gyrencephaly differs from lissencephaly in two critical features: subdivision of SVZ into ISVZ and OSVZ and high abundance of basal progenitors (bRGCs and IPCs in ISVZ+OSVZ) that greatly outnumber apical progenitors (aRGCs in VZ; Reillo *et al*, 2011; Borrell & Reillo, 2012; Kelava *et al*, 2012; Reillo & Borrell, 2012). Based on these facts, it seems most parsimonious to propose that gyrencephaly emerged in the stem mammal ancestor upon the innovative generation of bRGCs and OSVZ, and these traits were retained along mammalian speciation. This hypothesis of early gyrification was subsequently supported by studies using a phenomic character matrix of living placental orders and fossil species, which conclude that the ancestor of placental mammals was a small gyrencephalic animal (O'Leary *et al*, 2013). In this scenario, the smooth cortex of lissencephalic mammals (namely rodents and lagomorphs) would have emerged by the simplification, or phenotypic reversal, of gyrencephaly. This reversal may have occurred by reducing the abundance and self-amplificative capacity of basal neurogenic progenitors and bRGCs, as supported by recent studies (Kelava *et al*, 2012, 2013; Martinez-Cerdeno *et al*, 2012; Borrell & Gotz, 2014; De Juan Romero & Borrell, 2015). There are various examples of phenotypic and genomic reversals documented (Teotonio & Rose, 2001), one of its attributes being the ability to acquire new evolutionary trajectories (Borowsky & Wilkens, 2002). The seeming ability of the mammalian brain to undergo significant phenotypic reversals and change in various directions during evolution may explain the remarkable adaptability of mammals along this process (Kelava *et al*, 2013).

Molecular evolution

As discussed previously, the biology of cortical progenitor cells is regulated by the coordinated action of multiple proteins, and the experimental manipulation of these proteins has a profound influence on cortical size and folding. However, it remains to be defined whether the development of folded versus smooth cortices is due to the differential regulation of these genes between species during their normal development, and if so how they became differently regulated during evolution. Only recently, we have begun to identify molecular changes occurred during evolution that provide answers to these questions.

Human accelerated regions (HARs) Our understanding of the genetic basis of cerebral cortex evolution was jump-started by the generalized interest in identifying the genetic determinants of human uniqueness (Dorus *et al*, 2004). Strategies to identify the genetic and molecular mechanisms underlying the distinction of humans focused on searching for variations between the genome of human and immediate relatives in phylogeny. In a seminal study, Lahn and colleagues compared the genomic sequence of humans, chimpanzee, rat, and mouse and found that the genome is moderately well conserved across these species, but the human genome showed sites of uniquely high divergence rate (Dorus *et al*, 2004). Subsequently, they also found evidence demonstrating that genetic evolution is still ongoing in humans, with hotspots in genes related to brain development and also relevant in pathology of human cortical development, such as *ASPM* and *MCPH1* associated with microcephaly (Evans *et al*, 2004, 2005; Mekel-Bobrov *et al*, 2005).

Whereas sequence changes in protein-coding genes might seem the most straightforward mechanism to drive brain evolution (Hill & Walsh, 2005), further analyses have revealed an even more likely role for regions regulating gene expression (Prabhakar *et al*, 2008; Bae *et al*, 2014). Improved genomic comparisons between humans and chimpanzees identified hundreds of small DNA segments, the sequence of which diverged rapidly in humans (Pollard *et al*, 2006a,b). These segments were called "human accelerated regions" (HARs), in reference to their uniquely high rate of nucleotide substitution in the immediate human lineage. This accelerated evolution was proposed to have contributed to acquiring the unmatched size and complexity of the human brain in a relatively brief period (Pollard *et al*, 2006a). Importantly, instead of being part of protein-coding genes, HARs are mainly located in introns and intergenic regions, strongly suggesting their role in gene regulation (Bejerano *et al*, 2006; Pollard *et al*, 2006b). Significantly, relevant genes nearby HARs code for transcription factors and other DNA binding proteins involved in development and morphogenesis. In fact, HAR1, the HAR with the highest level of difference, contains an RNA gene (HAR1F) expressed in Cajal–Retzius cells during development, a peculiar type of cell essential for neuronal migration and lamination of the cerebral cortex (Pollard *et al*, 2006b).

Novel regulatory sequences Recent analyses of HARs focusing on their sequence, histone modification, chromatin state, and transcription factor binding sites conclude that at least 30% of HARs are developmental enhancers (Capra *et al*, 2013). Indeed, the activity of 29 noncoding HARs was tested by generating transgenic mice, which demonstrated that the majority of them are active enhancers in humans and chimpanzee. This confirms that HARs are good candidates as human-specific regulatory regions and also that human-specific brain evolution might be particularly associated with changes in spatial–temporal gene expression, instead of changes in protein sequence (King & Wilson, 1975; Mouse Genome

Sequencing Consortium *et al*, 2002; Lunter *et al*, 2006; Pollard *et al*, 2006b; Ponting & Lunter, 2006; Prabhakar *et al*, 2008).

In order to investigate how the activity of enhancers influences the developing telencephalon, Visel and colleagues performed an *in vivo* digital atlas of transgenic mouse embryos using 145 selected enhancers to drive reporter gene expression (Visel *et al*, 2013). Using a similar strategy, Pattabirman and colleagues generated stable transgenic mouse lines to characterize the gene-promoting activity of putative enhancers and demonstrated that these exhibit sharp boundaries of reporter gene expression in the E11.5 mouse pallium (Pattabiraman *et al*, 2014). These mouse lines were also used to determine the regional fate map of the mouse telencephalon, which demonstrated the existence of distinct progenitor protodomains defined by the activity of those enhancers at various developmental stages.

More recently, the evolution of active genomic enhancers was demonstrated with the analysis of the human *GPR56* locus, mutated in malformations of cortical development including polymicrogyria (Bae *et al*, 2014). In this study, a collection of transgenic mice was generated driving GFP expression from a portion of the same *GPR56* enhancer from various species (humans, mouse, marmoset, dolphin, and cat). The enhancer from these gyrencephalic species drove a similar GFP expression pattern, but this was different from the endogenous pattern in the lissencephalic mouse. Given that mouse and primates share a phylogenic ancestor more recent than with carnivores and cetaceans (Bininda-Emonds *et al*, 2007), the similar regulation of *GPR56* expression among these gyrencephalic species suggests convergent evolution.

Novel non-protein-coding genes In addition to variations in enhancer sequences, an extra level of control over the spatial–temporal patterns of gene expression is provided by non-protein-coding RNAs. Mounting evidence demonstrates the relevance of noncoding RNAs (miRNAs, lncRNAs, etc.) on cerebral cortex development (Aprea & Calegari, 2015; Liu & Sun, 2015), and their significance on evolution is supported by their increased number at evolutionary divergence points (Heimberg *et al*, 2008). As for the regulation of cerebral cortex expansion and folding, hundreds of miRNAs have been found expressed in apical and basal progenitor cells of the developing cortex of macaque embryos, but not in mouse (Arcila *et al*, 2014). These primate miRNAs prominently target proteins regulating the cell cycle and neurogenesis, hence contributing to the differential growth, amplification, and complexification of germinal layers and cortical areas, including the OSVZ (Lukaszewicz *et al*, 2005; Dehay & Kennedy, 2007). Altogether, the coevolution of emergent miRNAs and their target genes suggests that novel miRNAs became integrated into ancient gene circuitry to exert additional control over cortical progenitors, ultimately leading to the acquisition of primate-specific cortical features and, potentially, higher brain performance (Dehay *et al*, 2015).

Novel protein-coding genes As shown by Lahn and colleagues (Dorus *et al*, 2004), biological evolution also comes from variations in the sequence of protein-coding genes (True & Carroll, 2002). Looking for gene innovations that might have been involved in cortical expansion and folding during evolution, the focus has recently turned specifically onto cortical progenitor cells. A transcriptomic search for genes differentially expressed in radial glial cells between species identified 56 genes expressed in human, but not mouse, RGCs (Florio *et al*, 2015). Among these, *ARHGAP11B* was uniquely intriguing because it had the highest degree of radial glia-specific expression, and there is no mouse ortholog. In fact, *ARHGAP11B* arose from partial duplication of the *ARHGAP11A* gene on the human lineage after separation from the chimpanzees, and thus, it is a hominid-specific gene (Antonacci *et al*, 2014). Most impressively, experimental expression of *ARHGAP11B* in aRGCs of the embryonic mouse cortex promoted the generation of self-renewing basal progenitors (particularly bRGCs) and, in turn, increased cortical surface area and induced the formation of cortical folds in mouse (Florio *et al*, 2015). Therefore, the emergence of new protein-coding genes along mammalian phylogeny, such as *ARHGAP11B* in the hominid lineage, may have contributed significantly to the evolutionary expansion and folding of the mammalian cerebral cortex.

Concluding remarks and open questions

The expansion and folding of the mammalian cerebral cortex during embryonic development is a rather complex process regulated by multiple factors, where the abundance, type, and lineage of cortical progenitor cells play central roles. These cellular mechanisms are subject to molecular regulation by multiple proteins and signaling pathways, the expression of which is tightly controlled by a variety of enhancer elements and non-protein-coding genes. The specific spatial–temporal expression patterns of some of these proteins on the embryonic cortex faithfully map the prospective pattern of folds and fissures, and their mutation frequently leads to malformations of cortical size and folding in human patients. Yet, we are still far from identifying the specific role of these genes and their spatial–temporal regulation on the normal development of the human cerebral cortex.

Studying cortical development and folding across species helps us to understand the evolution of this complex process, and in return, we hope that this helps us to understand the aspects of cortical development critical to its expansion and folding during embryogenesis. Again, more and more refined molecular and genomic analyses are shedding some light on this problem, and emerging experimental animal models in this field like the ferret are of great help, but the truth is that we remain far from having a significant level of understanding.

Given the complexity of the developmental mechanisms involved in the expansion and folding of the cerebral cortex, and thus its tremendous cost in terms of genetic, cellular, and histogenic evolution, the ecological advantages must be more than remarkable. But what are the advantages of cortical folding? Clearly, a bigger cortex contains more neurons and more neuropile, and thus in principle, it provides greater computational power. Folding brings together highly interconnected cortical areas, thus minimizing cortical wiring and favoring high speed associational communication, ultimately optimizing brain circuitry (Klyachko & Stevens, 2003). However, the human cortex is neither the largest nor the most folded among mammals, as compared with elephants or dolphins, for example, and yet it is assumed that humans have a higher intelligence over any other earthly species. Are we really more intelligent than these other species? (Roth & Dicke, 2005) Or it is only that we have the

advantage of speech and manual ability to express our intellectual abilities? Is there a trade-off between cortical size, folding, and intellectual performance? Modern humans and Neandertals co-existed until 30,000 years ago, but we persisted and the latter perished. Was there some critical (albeit small) disadvantage in the organization or folding of the Neandertal brain? Was their extinction related to a less competitive intelligence? Were their cortical circuits or synapses less efficient? With the genomes of Neandertal and modern humans at hand, the specific differences in their DNA sequence identified (Green *et al*, 2010; Prufer *et al*, 2014), in combination with novel *in vitro* models of human brain development like cerebral organoids (Lancaster *et al*, 2013), these fascinating questions may soon be approachable. Hopefully, our understanding of the mechanisms and consequences of cortical expansion and folding will be much deeper 25 years from now.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Deconstructing cortical folding: genetic, cellular and mechanical determinants

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Abstract | Folding of the cerebral cortex is a fundamental milestone of mammalian brain evolution and is associated with dramatic increases in size and complexity. New animal models, genetic tools and bioengineering materials have moved the study of cortical folding from simple phenomenological observation to sophisticated experimental testing. Here, we provide an overview of how genetics, cell biology and biomechanics shape this complex and multifaceted process and affect each other. We discuss the evolution of cortical folding and the genomic changes in the primate lineage that seem to be responsible for the advent of larger brains and cortical folding. Emerging technologies now provide unprecedented tools to analyse and manipulate cortical folding, with the promise of elucidating the mechanisms underlying the stereotyped folding of the cerebral cortex in its full complexity.

Gyri
Also known as 'folds' or 'convolutions'. Rounded elevations of cortical tissue between two sulci that contain all six neuronal layers bending outwards, such that the deep layers on either side of a gyrus come close together.

Sulci
Also known as 'fissures'. Depressions or grooves of cortical tissue that contain all six neuronal layers bending inwards, such that the superficial cortical layers come close together on either side of a sulcus.

Gyrencephalic
The characteristic of a brain presenting cortical folds, giving a convoluted or wrinkled appearance.

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One of the most prominent and characteristic features of the human brain is the folding of the cerebral cortex into gyri and sulci. However, we still have a very limited understanding of how this emerges during development. Unfortunately, the tangibility of this phenomenon has in the past prompted many seemingly intuitive misconceptions regarding its origins. Intense research over the past decade has exponentially increased our understanding of cortical folding, identifying some of the key genetic, cellular and mechanical mechanisms involved. Until now, cortical folding has been viewed and approached as a phenomenon that is either purely mechanical (that is, resulting from physical forces) or purely biological (resulting from cellular or molecular factors) (see REFS[1–5] for recent reviews). However, it is becoming evident that, although important for cortical folding, neither of these two aspects is in itself sufficient. Relevant cellular and molecular mechanisms occur days to weeks before actual tissue folding[6], and the effect of mechanical factors depends on specific initial conditions that must be set in advance by early developmental processes[7]. Hence, the initial simplistic view is rapidly changing towards an integrative one in which cortical folding depends on genetics and cell biology to set the stage for tissue mechanics, as supported by recent experimental evidence[8].

In this rapidly evolving scenario, we present here an updated, comprehensive and integrative review of determinants of cortical folding. Following a description of the anatomical features of cortical folding, we review models and evidence concerning the mechanics of this process and the cell biological processes underlying stereotyped cortical shaping. We then focus on the genetics and transcriptomics that define and regulate these cellular and mechanical factors and review the current understanding of the evolution of cortical folding. Two main themes of our Review are the importance of mutual interactions between mechanical and cellular or genetic factors, and the importance of the regulation of gene expression in the development, evolution and disease of cortical folding.

Anatomical features

The folding of the cerebral cortex of gyrencephalic species is characterized by the alternation of folds (outward bending) and fissures (inward bending) of the cortical mantle. In the mature brain, fissures display remarkable periodicity across the cortex, with highly regular spacing between them, measured as folding wavelength. Cortical folding wavelength across species correlates with thickness of the grey matter: brains with thicker neuronal layers have wider folds than those with thinner grey matter[9–11]. This correlation emerges from the necessity of folds to contain the full complement of all six neuronal layers plus their descending axons, which varies considerably between species. For example, cetaceans have remarkably thinner grey matter and a much smaller folding wavelength than other clades, translating to a very high gyrification index (GI) (for example, 5.55 in the Pacific pilot whale compared with 2.56 in humans)[9].

In highly folded brains, including those of humans, cortical fissures are hierarchically organized into primary fissures, which are the deepest and first to form during development; then secondary fissures, which form by subdivision of the former; then tertiary fissures; and so on. The sulcal pits of primary fissures

occur at highly stereotyped locations between individuals[12]. In animals in which folding involves only the formation of primary fissures, their pattern is similarly well conserved[10]. Different mechanisms seem to underlie the formation of each type of fissure, as presented in the following sections.

The thickness of the neuronal layers varies considerably along folds, being thickest at the gyral crown and thinnest at the sulcal fundus, whereas the thickness along the lateral walls remains relatively constant[10,13] (FIG. 1a). The combination of these variations in shape and thickness of the neuronal layers along cortical folds corresponds with variations in the morphology and the arrangement of their constituent elements. In gyral crowns, the layers and columns of neurons are more sharply defined, cell bodies are less densely packed and myelinated fibres are denser and more vertically oriented than in fissures, where the latter mostly run horizontally[13,14]. Pyramidal neurons in crowns have longer and more elaborately branched apical and basal dendrites than do pyramidal neurons in sulcal fundi. Moreover, in the gyral crowns, these neurons are more vertically oriented and show an elongated cell soma and basal dendrites in vertical or oblique orientations[15]. By contrast, the morphology of pyramidal neurons at sulcal fundi largely follows the tangential axis, with a flattened cell soma, short apical dendrites with long collateral branches, and tangentially extended basal dendrites[15] (FIG. 1a). Primary descending axons follow similar overall patterns of orientation, with predominantly radial orientation in folds and tangential orientation in fissures. In lateral walls, most of these features are intermediate between those of the gyri and sulci. As an exception, the most superficial and cell-sparse layer 1 is thicker in fissures than in folds[13], and this difference is proposed to be the consequence of the high density of apical dendritic tufts of pyramidal neurons in fissures[10]. The meningeal membranes, which are associated with a protein-rich extracellular matrix (ECM) on the surface of the brain, faithfully follow the basal border of layer 1 at the cortical surface, fully overlaying all cortical folds and fissures.

In contrast to the remarkable folding of the six layers of cortical grey matter, the outer surface area of which dramatically expands during development, the cortex inner surface, which, in the adult brain, is composed of white matter and ependymal cells, is completely smooth[1]. The smoothness of this inner cortical surface derives from the equally smooth embryonic ventricular zone (VZ), the primary germinal layer.

These two distinct features of cortical folding under physiological conditions — outer folding and inner smoothness — are reproduced in some experimental models but not all (reviewed elsewhere[1]). Bona fide cortical folding occurs, by definition, in naturally gyrated model species such as ferrets, cats, sheep, macaques and obviously humans[13,16–18]. Several experimental models also faithfully recapitulate these features: in vivo in transgenic mice[19–21] and through transient manipulation of the developing cortex in ferrets and mice[22–29], and recently in vitro, through manipulation of early human brain slices[8]. By contrast, other models fail to reproduce these features of true folding, including transgenic mice in vivo[30–33] and in vitro cultures of mouse cortex or human cerebral organoids[34–36].

Several pathological conditions alter normal folding, and most of these largely affect the number and periodicity of folds[37]. Such malformations usually result from abnormal cortical development and include severe alterations of the above-mentioned architectonic features, such as the layering, density and orientation of cortical neurons, as well as the formation of cellular ectopias (BOX 1).

Mechanical factors

Cortical folding is a long process that takes place in the very last period of forebrain development, coincident with the cytoarchitectural differentiation of cortical areas and synapse formation[2,6,10,38,39]. Several hypotheses have been proposed over the past decades to explain the process of cerebral cortical folding. Early ideas proposed that cortical folding could be the passive result of external mechanical forces acting on the expanding and developing brain, including hydraulic pressure from the cerebrospinal fluid, the impression from major blood vessels or growth constraints from the limited volume of the cranium[10]. These ideas were soon abandoned for important reasons. For example, cranial sutures do not ossify until the brain has finished growing, and experimental evidence in cats demonstrates that alleviation of cranial pressure does not reduce cortical folding[10]; in fact, abnormally large brains can force the expansion of the surrounding cranium[30]. In 1997, Van Essen published one of the most attractive and influential hypotheses on the biomechanics of cortical folding, in which he proposed that internal hydraulic pressure and patterned axonal tension between specific cortical areas were the driving forces to initiate and shape cortical folding[40] (FIG. 1b). This notion was consistent with the fact that axons in the living brain exist and grow in a state of tension[41–43] and was consistent with axonal tracing evidence in the macaque[44]. However, subsequent direct experimental measurements very elegantly and definitively refuted the idea by showing that, contrary to the basis of the Van Essen hypothesis, the tensile forces from cortical axons do not act along the orientation that would be required to bring the walls of developing gyri together[42].

An alternative hypothesis proposed that cortical folding is the result of forces originating from the differential growth in the developing cerebral cortex, in which an outer shell of tissue undergoes faster tangential expansion than an inner core[45] (FIG. 1c). Subsequent theoretical modelling with more sophisticated mathematical methods, and recent experimental testing, have provided further support to this idea[7,42,46–49]. For example, circular slices of gel, in which an outermost layer expands by swelling, have been used as a physical model of differential growth, showing that this outer layer folds with wavelength depending on its thickness and elastic modulus[46]. In a series of remarkable experiments, these experimental studies of differential growth were extended to three dimensions by combining two polymer gel preparations: one forming an inner core and

Gyral crown
Also known as the 'gyral crest'. The top or outermost part of a gyrus.

Sulcal fundus
Also known as the 'sulcal pit'. The bottom or deepest part of a sulcus.

Lateral walls
Portions of cortex between gyral crowns and sulcal fundi.

Cellular ectopias
Also known as 'cellular heterotopias'. Cells positioned in an abnormal location.

Hydraulic pressure
Force exerted by a fluid onto the surrounding tissue that contains it under pressure.

Cranial sutures
Fibrous joints between the cranial bones.

Modulus
Measure of a mechanical property of a material. The elastic modulus is the measure of the resistance of an object to being deformed elastically after stress is applied.

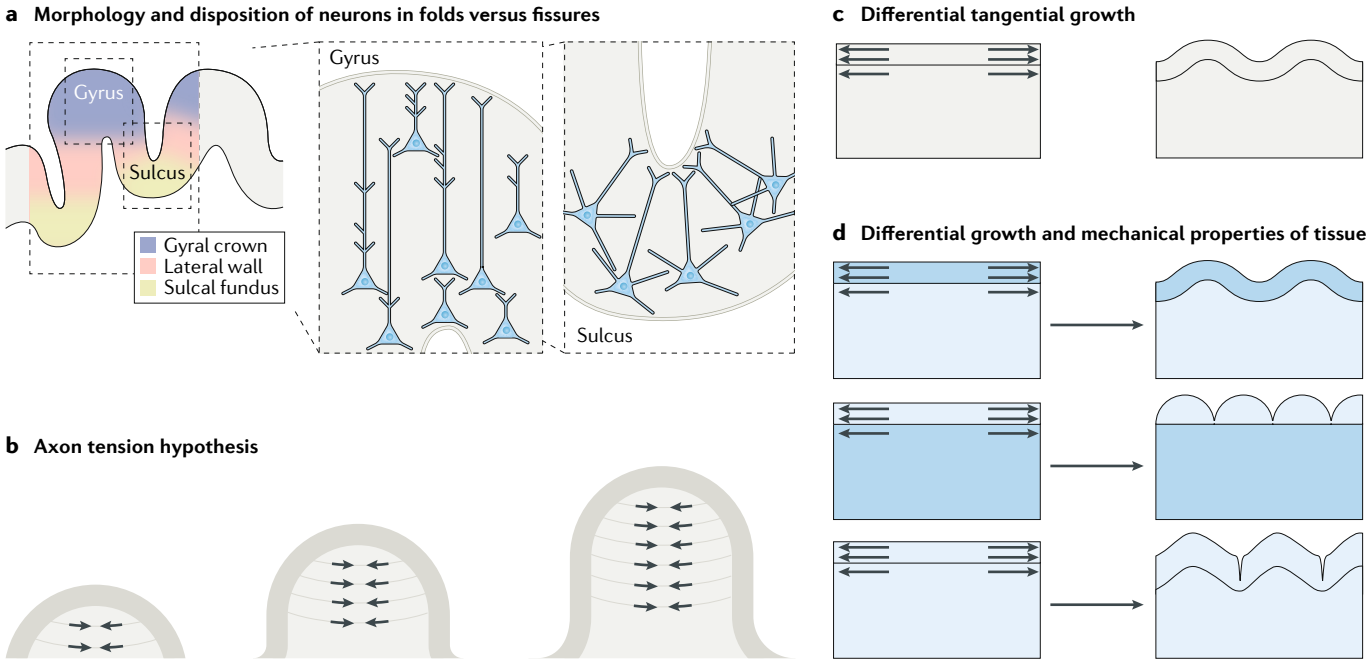


Fig. 1 | **Anatomy and mechanics of cortical folding: push, not pull. a** | Key cortical folding terms are illustrated, along with a schematic of the general morphology and orientation of cortical neurons within gyri and sulci. Cells have a different orientation and a more extensive horizontal branching of apical and basal dendrites in sulci compared with gyri. **b–d** | Biomechanical hypotheses of cortical folding. According to the axon tension hypothesis (part **b**), axons connecting cortical areas are under tension, and the more connected these regions are (grey lines), the greater the tension (arrows) and the closer the regions become, resulting in increased folding. According to the differential tangential growth hypothesis (part **c**), the cerebral cortex is divided into two regions, with the outer layer growing faster than the inner core. This differential growth is predicted to result in the folding of the outer layer. Another hypothesis of cortical folding is based on the notion that, on top of differential growth, differences in the mechanical properties of the tissue (such as stiffness and elasticity) define its capacity to fold more or less (part **d**). Darker blue indicates the stiffer material; lighter blue indicates the softer material. The cerebral cortex is intrinsically connected with subcortical structures that have different mechanical properties. Variations in the difference of stiffness between superficial and deep regions influence the degree and mode of folding. Wrinkling occurs in a smooth, sinusoidal way. Evagination of the grey matter leads to the formation of cusped folds. If the grey and white matter have similar properties, both will fold. Part **a** is adapted from REF.[10], Springer Nature Limited. Part **d** is adapted with permission from REF.[49], Proceedings of the National Academy of Sciences (PNAS).

another forming an outer layer, bound to the former. Upon exposure to a solvent, the outer shell polymer expanded faster than the inner core. When the stiffness of the outer material was similar to that of the inner material, it wrinkled similarly to a folded cortex[49] (FIG. 1d). When the physical size, shape and material properties (that is, stiffness) of this compound gel structure were all made similar to those of the smooth fetal human brain before folding, the fast-expanding outer shell formed folds with remarkable similarity to those in the human cortex, in both wavelength and pattern[7]. Indeed, it is generally held that the stiffnesses of grey and white matter in the human brain are not as different as initially postulated[45]. Importantly, gel models and in silico 3D simulations demonstrate that certain initial conditions are crucial for determining the eventual pattern and wavelength of cortical folds, whereby even subtle differences in these parameters lead to dramatically different outcomes. These parameters include the initial size and precise geometry of the expanding brain, the thickness of the outer zone and factors that affect the rate of tangential expansion of the outer zone relative to the inner zone (for example, differences in solvent absorption)[7,48–50].

What are the actual biological substrates involved in the differential expansion models of the mechanical forces driving cortical folding? Current hypotheses propose that the neuron-dense cortical plate (CP), or grey matter, may act as the rapidly expanding outer shell, whereas the underlying, transient embryonic layers, including the fibrous intermediate zone and the germinal zones, which eventually become the white matter, may act as the slowly expanding inner zone[2]. Differences in stiffness and expansion rate between these two general compartments seem compatible with these factors being driving forces for cortical folding, but developmental changes in these and other variables that may also have a critical impact on the process remain to be evaluated[2]. Folding has also been observed in the neuroepithelium of various cerebral organoid preparations[35,36], and biomechanical measurements in these systems support the differential expansion model[36]. Notably, as mentioned above, the degree of cortical folding varies considerably between mammalian orders, and this variability may result from variations in the thickness of grey matter, which will change the mechanical resistance of the cortex to folding[51]. Likewise, substantial variations in white-matter

Stiffness
Property of a material that defines its resistance to being deformed after force is applied to it.

Cortical plate
(CP). Transient layer of the developing cortex, located beneath the marginal zone and containing the neurons that most recently finished radial migration.

Viscoelastic instability
Property of nearly inertia-less, non-Newtonian, flowing, complex fluids, such as polymer melts and solutions.

Anisotropy
The characteristic of materials of having different physical or mechanical properties when measured along different axes.

volume or thickness (for example, as observed between elephants and manatees[52]) may also exert effects similar to variations in grey-matter thickness on the mechanics and degree of cortical folding.

The differential expansion hypothesis depends on the rapid tangential growth of the CP. This fast tangential growth of the CP was recently proposed to emerge from the differentiation of its constituent neurons, the growth of their cell bodies and surrounding neuropil, the elaboration of dendritic and axonal arborizations, and the formation of synapses[53]. This notion is well supported by the temporal correlation between the developmental trajectories of cortical maturation and folding in ferrets, macaques and humans[2,53–55]. Accordingly, variations in the initial packing of neurons in the CP may have a fundamental impact on the cortical folding phenotype.

Remarkable as they are, these differential expansion models fail to replicate two important features of cortical folding: the stereotyped location of primary folds and fissures across individuals, and the growth of the subcortical white matter. At a microscopic level, axons elongate in response to, and maintain a small level of, tension[43,56–58]; similarly, at the macroscopic level, white matter probably responds to stress by growing in the direction of axonal fibres — consistent with experimental observations[41,42] — and thus may affect macroscopic folding. Taking this growth into consideration, the (inner) subcortical tissue can be modelled as a viscoelastic material that displays a distinct viscoelastic instability (buckling on a viscoelastic foundation) in response to growth of the CP. That is, mechanical stress in the (inner) subcortical tissue induced by relatively rapid CP growth will relax with a characteristic time constant, depending on the ratio of viscous resistance to elastic stiffness. This instability, and the wavelength of the resulting surface, depend on the rate of cortical growth relative to the rate of mechanical relaxation of the subcortical tissue[4]. Computer models that incorporate tensile-stress-induced growth reproduce several qualitative features of cortical folding, including its dependency on cortical thickness, growth rate and stiffness of the outer layer[47]. More elaborate mathematical models that represent subcortical white matter as a 3D continuous viscoelastic foundation can predict the effects of different growth rates on the wavelength of cortical folds, such that the larger the ratio of cortical to subcortical growth rates, the shorter the folding wavelength[48,50,59]. This model of tensile-stress-dependent growth was also further extended to include subcortical anisotropy, such that axons elongated more in the prevailing direction of their orientation than in other directions[60]. This model predicted that tissue anisotropy is also an important parameter in cortical folding, as the changes in the orientation of subcortical axons could alter the location of gyri and sulci.

Cellular mechanisms

A key corollary from the section above is that cortical folding varies considerably depending on the conditions immediately before the onset of folding, particularly the shape of the cortex and regional variations in neuron density. What previous developmental events, then, influence these onset conditions? Cortical shape and the density of neurons in the CP depend on two fundamental processes: neurogenesis and neuron migration.

Neurogenesis

Studies in ferret, macaque and human brain show that, in species with a folded cortex, the rate of neurogenesis is heterogeneous along the developing cortical mantle. Regions that exhibit high neurogenesis go on to undergo great surface area expansion and folding, and alternate

Box 1 | Human malformations of cortical folding

Cortical development involves a plethora of mechanisms that are under fine genetic control. Mutations in genes controlling these processes cause malformations in cortical development that may affect cortical folding in several ways, either increasing or decreasing the number or size of folds.

Polymicrogyria is a heterogeneous malformation characterized by the generation of many small folds, usually confined to a particular area. Depending on the extent of the malformation, it may be unilateral or bilateral, symmetric or asymmetric, and may occur in combination with other pathologies or as a part of a complex syndrome. Lissencephaly ('smooth brain') includes various disorders of varying severity, in which the folding pattern is either simplified (leading to pachygyria) or absent (leading to agyria). Both mild and severe forms may appear together in the same affected individual, with gradients in cortical fold simplification usually appearing along a rostro-caudal or caudo-rostral axis. Cases of polymicrogyria are usually associated with thinning of the cortex, whereas lissencephaly generally coincides with increased cortical thickness.

Genes mutated in patients with these pathologies are listed here and are grouped by the molecular mechanism in which they are implicated. Variants of the same gene — for example, *LAMC3* or one of the tubulin-encoding genes (*TUBB2B*, *TUBB3*, *TUBA1A* and *TUBA8*) — can have differential effects on cortical folding, depending on the specific mutations or context.

Molecular mechanism	Mutated genes or genomic mutation	Refs
Polymicrogyria		
Synaptic function	*GRIN1* and *SRPX2*	94,179
Regulation of cortical patterning	*GPR56* and *RTTN*	169,180–184
Specification and proliferation of IPCs and their progeny	*TBR2*	107
DNA repair	*NHEJ1*	185
Neuronal migration and cortical lamination	*TUBB2B*, *TUBB3*, *TUBA1A*, *TUBA8* and *LAMC3*	186–189
Vesicle transport	*KBP* and *KATNB1*	188,190
Tight junction maintenance	*OCLN*	191
Unknown	*WDR62*, microdeletions in 22q11, monosomy 1p36 and duplication of Xq26.1–26.2	192–197
mTOR pathway	*CCND2*, *PIK3CA* and *PIK3R2*	91,198
Pachygyria–agyria		
Neuronal migration and cortical lamination	*RELN*, *VLDLR*, *ACTB*, *LIS1*, *ACT1*, *DCX*, *TUBA1A*, *TUBB2B*, *TUBB*, *TUBA8*, *TUBB3* and *LAMC3*	91,120,122, 199–207
Vesicle and organelle motility and mitosis spindle assembly	*DYNC1H1*, *KIF2A*, *KATNB*, *KIF5C* and *CDK5*	187,190,208–210
Maintenance of specific neuronal types	*ARX*	211,212
Neuronal apoptosis	*CRADD*	206
Centrosome duplication and the formation and function of mitotic spindle	*YWHAE*, *NDE1*, *LIS1* and *TUBG1*	187,206,209,213

IPC, intermediate progenitor cell; mTOR, mechanistic target of rapamycin.

with regions of low neurogenesis that go on to show limited expansion and folding[17,61–63]. Regional differences in neurogenesis rate are particularly striking in the basal germinal zones (away from the ventricular surface), the so-called inner and outer subventricular zones (ISVZ and OSVZ, respectively). Regional differences in embryonic neurogenesis translate, in part, into differences in the density of immature neurons in the CP before cortical folding[1,17]. However, neuronal density and layer thicknesses are quite homogeneous across the mature cortex (except area 17 in macaque, which shows twofold neuron density compared with other areas[62,64]), seemingly as a result of greater surface area expansion and folding of regions with higher neurogenesis[17,63]. This notion is consistent with the current biomechanical model in which the cortex folds owing to a greater tangential expansion of the CP as a consequence of neuronal differentiation and neuropil growth[2].

Tangential dispersion

In addition to differences in neuron density in the CP, patterned expansion and folding of the cortical surface area also result from the tangential dispersion of neurons during their radial migration. The importance of radial neuron migration for cortical folding was first made evident by genetic studies of cortical malformations in humans[65] (BOX 1) and was further highlighted recently by studies showing that alterations to the dynamics of radial migration can induce folding of the mouse cortex[19] or the loss of folds in the ferret cortex[27].

Newborn cortical neurons migrate radially from the germinal layers to the CP along a scaffold of radial glial fibres (RGFs), the basal processes of radial glial cells (RGCs). In species with a smooth, non-folded cortex, such as the mouse, RGFs follow strictly parallel trajectories, such that neurogenic progenitors and their daughter neurons remain radially aligned[66,67]. In species with a folded cortex, the trajectory of RGFs varies across regions, being dramatically divergent in regions where the CP will later undergo the greatest expansion and folding[17]. As a result, radially migrating neurons exhibit considerable tangential dispersion in these regions, thus greatly contributing to expansion of the local cortical surface area[17,68].

The divergence of the RGF scaffold emerges from the generation of basal RGCs (bRGCs)[17], also known as outer RGCs[69]. These are neural stem cells that are very similar to the classical apical RGCs (aRGCs) in the VZ — in that they show similar marker expression and both have a radial process extending to the pial surface of the cortex — but instead have the cell soma located in the ISVZ or OSVZ, where they undergo mitosis[17,69–71]. bRGCs are generated by delamination from aRGCs in the VZ[17,72–74] or by self-amplification in the ISVZ and OSVZ; in both cases, newly generated bRGCs extend a new radial fibre that intercalates among the pre-existing RGF scaffold[17,75,76]. The generation of large numbers of bRGCs, each with their new radial fibre, leads to the divergence of this scaffold in the layers that are superficial to the OSVZ[17,77]. As a consequence, cortical areas with greater proliferation in the OSVZ and ISVZ before cortical folding exhibit the greatest divergence of radial fibres, the greatest tangential dispersion of radially migrating neurons and the highest degree of surface area expansion and folding[78] (FIG. 2), again with the exception of A17 in macaque[62]. Moreover, the degree of cortical folding in a given species increases exponentially with the relative abundance of proliferation in the OSVZ[17,79].

The above model has been tested experimentally in mice and ferrets. In ferrets, which have a folded cortex and abundant bRGCs, genetically enhancing OSVZ progenitor proliferation in a particular cortical region leads to a considerable increase in the surface area and folding of that region[24], whereas reducing the proliferation of bRGCs leads to smaller folds[17]. In mice, which naturally have a smooth cerebral cortex and very rare bRGCs[73], genetically increasing bRGC abundance consistently leads to the formation of cortical folds[20,22,23].

Neurogenesis is not enough

In gyrencephalic species such as ferrets and macaques, much neurogenesis takes place in the OSVZ[62,80,81], particularly from bRGCs[75,76]. An alternative possibility to the model of RGF divergence[77] is that cortical folding depends solely on the number of cortical neurons, which would suggest that the main role of the OSVZ and bRGCs is to increase neuron production rather than to modify and build the RGF scaffold. This might be the case in the human cortex at late stages of development, where it has been recently reported that the RGF scaffold splits between the VZ and ISVZ[82]. This recent finding remains controversial, as it is in conflict with evidence in ferrets, macaques and humans from multiple laboratories[17,38,62,83,84]. Neurons born from aRGCs need RGFs to migrate from the VZ to the CP. If, in human embryos, the RGF scaffold is truncated at late stages and aRGCs cease to contribute to it, late aRGC-born neurons cannot migrate all the way to the CP and thus cannot become part of the cortical grey matter. In that case, only late neurons born in the OSVZ have an RGF scaffold uninterrupted to the CP, and therefore bRGCs in the OSVZ may be the only, and thus key, contributors to late neurogenesis in our species[52,82]. Contrary to the notion that cortical folding depends solely on the number of cortical neurons, however, human lissencephaly (that is, a pathological absence of cortical folds) can occur without a substantial reduction in brain volume and cortical size, as in lissencephalic mutant ferrets[85]. Similarly, the marmoset is a New World monkey with thick cortical layers, rich in neurons, but a relatively low proportion of bRGCs located in the OSVZ and a near absence of folding[79,86,87]. Conversely, the considerable reduction in neuron number and brain size observed in human microcephaly (pathological small brain size) may occur with seemingly normal cortical folding (BOX 1). Likewise, in ferrets, microcephaly can be induced through early genetic depletion of VZ progenitor cells, without leading to a loss of folding or a loss of bRGCs in the OSVZ[85,88]. Thus, cortical folding is driven by differences in neurogenesis and the tangential dispersion of radially migrating neurons along the developing cortex and by the accumulation of neurons at different densities at the CP.

Delamination
Detachment from the apical adherens junction belt, followed by basal movement, away from the ventricular zone.

Lissencephaly
The characteristic of a brain without cortical folds, smooth or unfissured.

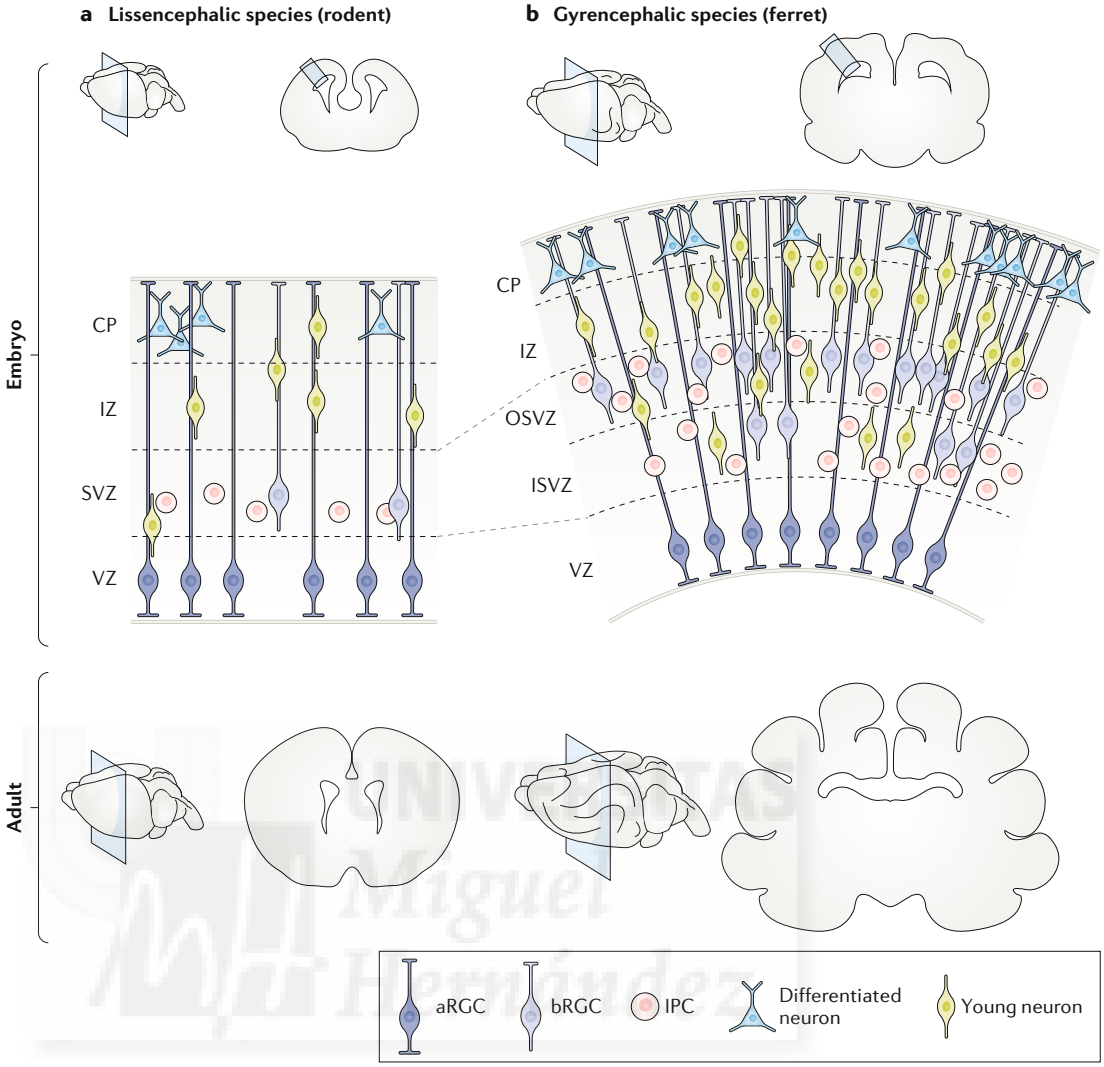


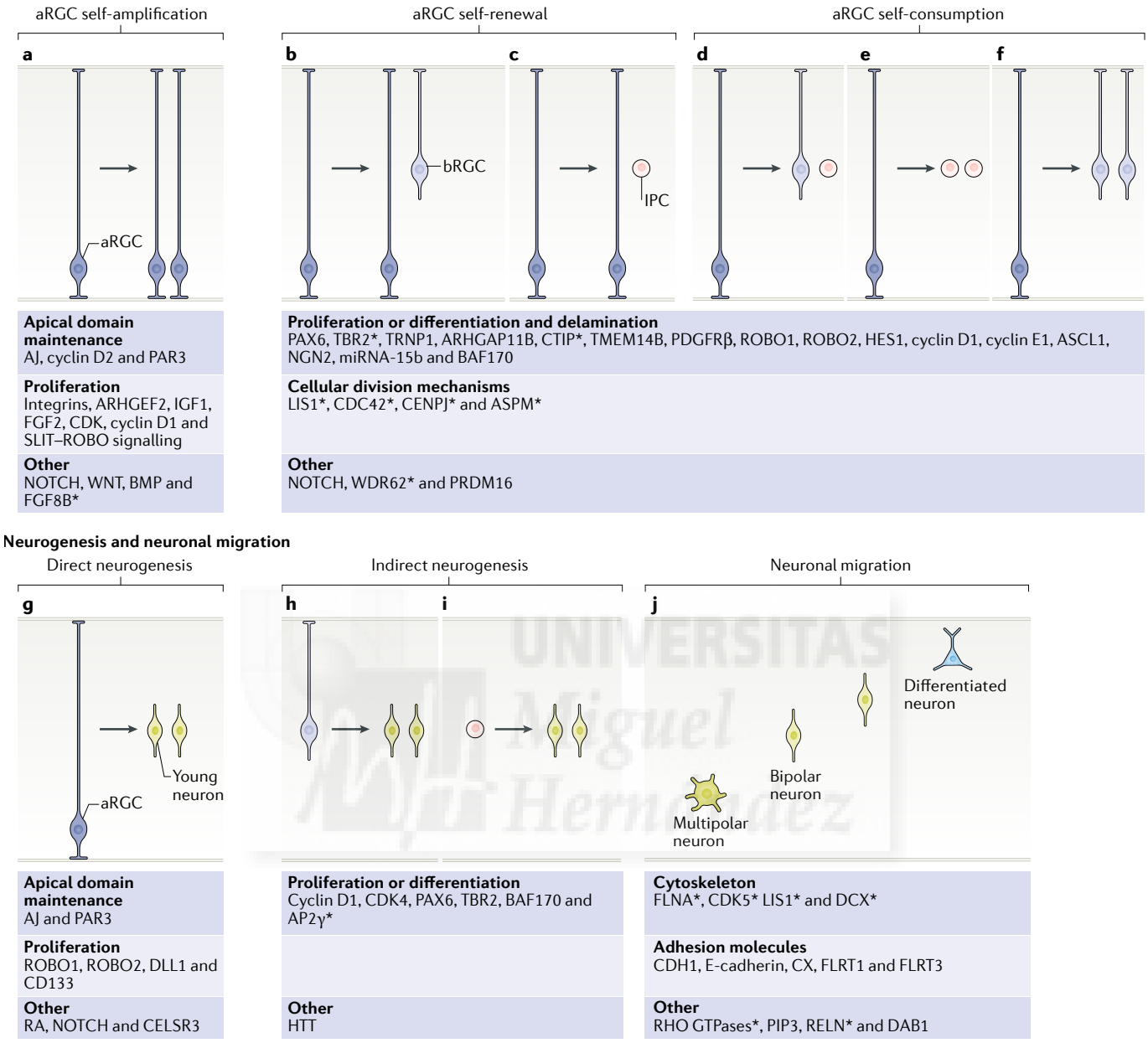
Fig. 2 | **Cellular mechanisms of cortical growth and folding: progenitor cells.** Different scenarios in lissencephalic (part **a**) and gyrencephalic species (part **b**) at embryonic and adult stages. Row 1 shows a representation of a rodent (part **a**) and ferret (part **b**) brain at embryonic stages in external view and coronal section. Row 2 shows schematics of the cellular composition and organization of the developing cerebral cortex. In lissencephalic species, most cortical progenitors are apical radial glial cells (aRGCs) and intermediate progenitor cells (IPCs), whereas basal radial glial cells (bRGCs) are scarce. After neurons are generated, they migrate intimately associated with radial glial fibres, which follow strictly parallel trajectories. In gyrencephalic species, the subventricular zone (SVZ) is greatly expanded and specialized in two germinal layers: the inner subventricular zone (ISVZ) and the outer subventricular zone (OSVZ). The ISVZ and OSVZ are both rich in bRGCs and IPCs. Species with a folded cortex present a much greater abundance of bRGCs than do lissencephalic species, particularly in the OSVZ. Each bRGC extends its own basal fibre, which creates a dramatic divergence of the radial fibre scaffold. This divergence leads to the tangential dispersion of radially migrating neurons (light green) and hence to the tangential expansion and folding of the cortical surface. Row 3 shows a representation of the adult brains of both types of species in external view and in coronal section: a smooth cortex in lissencephalic species in contrast to an expanded and folded cortex in gyrencephalic species. CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone.

Genetic factors

Over the past two decades, human genetic studies have identified an ever-increasing list of mutations that severely perturb the development of the human cerebral cortex[37]. These mutations result in macroscopic malformations of cortical development and are usually associated with severe defects of cortical folding that underlie intellectual disability and epilepsy (BOX 1). Thus, the cellular mechanisms involved in cortical folding during brain development are under tight genetic regulation[89]. Moreover, the expression of genes involved in cortical folding is itself precisely regulated in both time and space[3].

Genetic regulation of cellular mechanisms

Progenitor cell proliferation versus neurogenesis. Regulation of the balance between cortical progenitor cell proliferation and neurogenesis (in all germinal layers) is under the control of multiple signalling pathways, but few thus far have been tested for an effect on cortical folding (FIG. 3).



* Variants associated with cortical malformations in humans.

Fig. 3 | **Genetic regulation of cortical folding: progenitor cells and neuronal migration.** The division of cortical progenitor cells may result in different lineage outcomes. Regarding progenitor production, the main type of cortical progenitor cell, apical radial glial cells (aRGCs), may self-amplify (part **a**); self-renew (parts **b**,**c**), generating one aRGC plus either a basal radial glial cell (bRGC) or an intermediate progenitor cell (IPC); or undergo self-consumption by producing two basal progenitor cells (in each combination; parts **d**–**f**). In addition, aRGCs may produce neurons via direct neurogenesis (part **g**). Basal progenitors may also generate neurons (so-called indirect neurogenesis) (parts **h**,**i**). Newborn neurons change their morphology from multipolar to bipolar before starting their migration through the cortical thickness to reach their final position in the cortical plate, where they differentiate (part **j**). Listed are some of the gene products that are critical for these lineage decisions and mechanisms. Cortical malformations in humans are associated with mutations in the genes encoding the products that are marked with an asterisk. AJ, adherent junction complex; AP2γ, transcription factor AP2γ; ARHGAP11B, RHO GTPase-activating protein 11B; ARHGEF2, RHO guanine nucleotide exchange factor 2; ASCL1, achaete-scute homologue 1; ASPM, abnormal spindle-like microcephaly-associated protein; BAF170, BRG1-associated factor 170 (also known as SMARCC2); BMP, bone morphogenetic protein; CD133, prominin 1; CDH1, cadherin 1; CDK, cyclin-dependent kinase; CELSR3, cadherin EGF LAG seven-pass G-type receptor 3; CENPJ, centromere protein J; CTIP, CTBP-interacting protein (also known as RBBP3); CX, connexin; DAB1, disabled homologue 1; DCX, neuronal migration protein doublecortin; DLL1, Delta-like protein 1; FGF2, fibroblast growth factor 2; FLNA, filamin A; FLRT, leucine-rich repeat transmembrane protein; HES1, transcription factor HES1; IGF1, insulin-like growth factor 1; HTT, huntingin; LIS1, lissencephaly 1 protein; NGN2, neurogenin 2; PAR3, partitioning defective 3 homologue (also known as PARD3); PAX6, paired box protein 6; PDGFRβ, platelet-derived growth factor receptor-β; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PRDM16, PR domain zinc finger protein 16; RA, retinoic acid; RELN, reelin; ROBO, Roundabout homologue; TBR2, T-box brain protein 2; TMEM14B, transmembrane protein 14B; TRNP1, TMF-regulated nuclear protein 1; WDR62, WD repeat-containing protein 62.

Overactivation of fibroblast growth factor (FGF) signalling by infusion of FGF2 or FGF8 in the telencephalic ventricle increases progenitor proliferation and induces cortical folding in mice, or polymicrogyria (extra folding) in ferrets, respectively[25,28]. In agreement with a role for FGF signalling in regulating cell cycle proteins, direct overexpression of *Cdk4* and *Ccnd1* also causes extra folding in ferrets. However, this manipulation was not sufficient to induce folding in mice[24], highlighting the molecular and cellular complexity of this process[90]. Retinoic acid signalling also regulates the proliferation of cortical progenitors and induces folding in the otherwise smooth mouse cortex[31].

Other genes involved in signalling pathways that regulate progenitor proliferation are mutated in human malformations of cortical folding; for example, mutations in *SRPX2*, or *PIK3CA* and *PIK3R2* (which encode components of the mechanistic target of rapamycin (mTOR) pathway) induce polymicrogyria in children[91–94] (BOX 1). Although mice with mutations in these genes do not develop cortical folds, the emergence of transgenesis in more suitable animal models, such as ferrets and marmoset monkeys, should aid understanding of the contributions of these and other genes in progenitor proliferation and cortical folding[85,88,95–97].

Formation and amplification of bRGCs. New bRGCs may form de novo from aRGCs, or from basal progenitors, usually by self-amplification[72,75,76] (FIG. 3b). The formation of bRGCs from aRGCs involves two main processes: asymmetric division of aRGCs and cell delamination from the VZ. Changes in the orientation of the mitotic cleavage plane from vertical to horizontal usually lead to asymmetric cell division, although this kind of cell division may also happen from vertical cleavage planes, as does symmetric cell division. The orientation of the cleavage plane is regulated by factors such as the mosaic protein LGN, inscuteable protein homologue (INSC), nuclear distribution protein-like 1 (NDEL), lissencephaly 1 protein (LIS1) and the WNT–planar cell polarity (PCP) pathway, and strongly influences the acquisition of asymmetric cell fates by cortical progenitors[74,98–101]. However, these factors influence cell fate differently. Changes in mitotic cleavage plane orientation by LGN and cadherin 1 (CDH1) regulate the formation of bRGCs from aRGCs[74,76], whereas INSC favours the formation of intermediate progenitor cells (IPCs)[100]. The asymmetric division of aRGCs is also regulated by FGF and ROBO signalling, but this signalling promotes the formation of IPCs or neurons rather than bRGCs[102–104], a difference in cell fate decision that is not well understood.

Once bRGCs are born from aRGCs, they must delaminate from the VZ and translocate to the ISVZ and OSVZ. Most genes that have been manipulated or knocked in to induce cortical folding in mice, or that are necessary for cortical folding in ferrets, are involved in the delamination of basal progenitors, including bRGCs. These genes include those encoding the cell-adhesion molecules CDH1 and contactin 2 (CNTN2; also known as TAG1)[76,105], FGF receptors[26], insulinoma-associated protein 1 (INSM1)[106], sonic hedgehog (SHH) signalling factors[20] and TMF-regulated nuclear protein 1 (TRNP1)[22,76]. The transcription factors scratch, paired box protein PAX6 and T-box brain protein 2 (TBR2; also known as EOMES) also regulate cell delamination and the formation of bRGCs, and misregulation of TBR2 expression in humans produces severe folding abnormalities[107]. By contrast, manipulating these factors in mice has mild or no effects on cortical folding[108–110]. Importantly, some of the few newly emerged genes in the recent primate and hominid lineages, such as *ARHGAP11B*, *TBC1D3* and *TMEM14B*[21,23,111,112], induce the abundant formation of bRGCs and folding of the mouse cortex, whereas others, such as *NOTCH2NL*, amplify IPCs but not bRGCs[113–115].

The amplification of bRGCs seems to be regulated by various molecular pathways, some of which have been identified, including Notch signalling and integrin signalling[69,71]. Many other signalling mechanisms have been identified in recent targeted transcriptomic analyses — including those comparing developing cortical layers in mice and humans[116], different progenitor cell populations in mice and humans[23], and single-progenitor cells in humans and ferrets[113,117–119]. Although the signalling mechanisms identified in these analyses have not yet been shown to affect cortical folding, they may also be relevant in the bRGC amplification that leads to it.

Neuronal migration. Several genes essential for neuronal migration have a substantial impact on cortical folding[65] (FIG. 3j). For example, in humans, mutations affecting the reelin signalling pathway or in the gene encoding the neuronal migration protein doublecortin (DCX) — two key regulators of radial migration and cortical lamination — severely impair cortical folding[120–124], and *Dcx*-knockout ferrets completely lack cortical folds[85]. Overexpression of a dominant negative mutant allele of *Cdk5*, which is important in mouse neuron migration, impairs the radial migration of upper-layer neurons and the formation of cortical folds in ferrets[27]. Leucine-rich repeat transmembrane protein 1 (FLRT1) and FLRT3 are cell-adhesion molecules important in radial migration of cortical neurons[125], and mice lacking both FLRT1 and FLRT3 develop cortical folds[19].

Genetic regulation of mechanical tissue properties

The stiffness and other viscoelastic properties of biological tissues are determined by their molecular composition[126]. This molecular composition is related to protein density and composition of the ECM, as well as to cell adhesion, either between cells or with the ECM. Thus, the mechanical properties of the developing brain, which strongly contribute to cortical folding, depend on the expression levels and half-lives of various ECM, cell-adhesion and cytoskeletal proteins. Whereas much remains to be investigated on this front, transcriptomic analyses have identified several integrins, collagens and laminins that are differentially expressed between layers of the developing human cortex[116] and between prospective gyral and sulcal regions in the developing ferret brain[127]. Importantly, FLRT1 and FLRT3 are differentially expressed between gyri and sulci in ferrets, whereas in mice they are

Intermediate progenitor cells
(IPCs). Germinal cells born from apical radial glial cells that populate the subventricular zone (basal from the ventricular zone) and produce neurons.

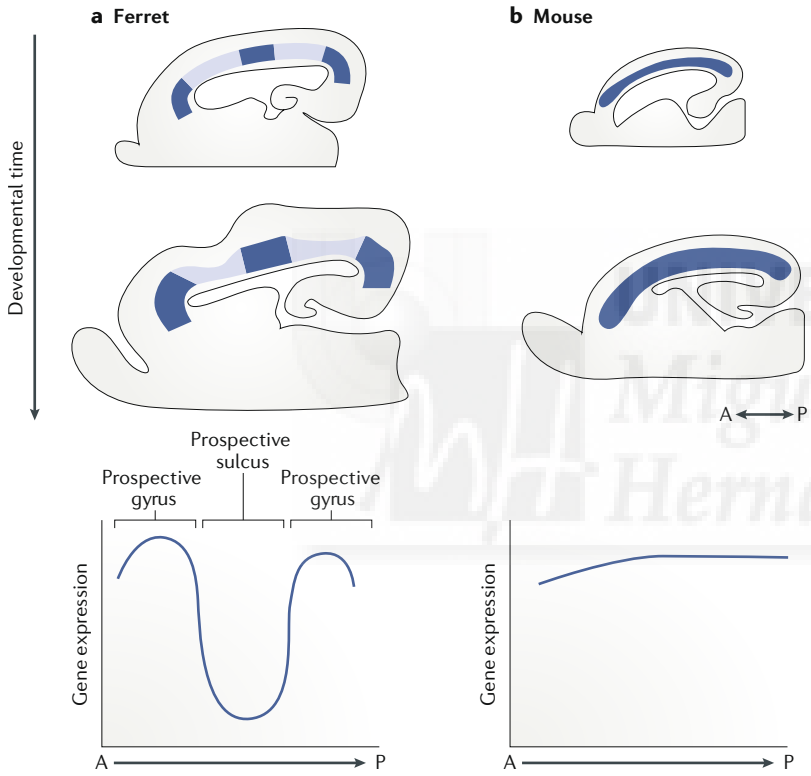
expressed by only a subset of cells distributed in a 'salt-and-pepper' pattern. In mice lacking FLRT1 and FLRT3, the neurons that were deficient for these molecules (which were genetically labelled) instead clustered together to form columns, and cortical folds developed[19], strongly suggesting that the modular arrangement of the neurons expressing these genes is important to drive cortical folding.

Patterned gene expression

Cortical folds form in stereotyped patterns among individuals of a given species, with a predetermined location, size and shape. This stereotypy is very clear in species with few folds, such as ferrets and cats[10]. In species with a highly folded cortex, such as humans, these patterns are more complex and variable, but primary fissures, which develop the earliest and end located at the deepest positions (sulcal pits), are still highly conserved[12,128]. Folding patterns also follow remarkably conserved trends among phylogenetically related species (for example, among carnivores)[10,77]. Altogether, these similarities indicate that the patterns of cortical folding are under strong genetic regulation, during development and across evolution[129].

In ferrets and cats, the locations along the developing cortex where primary folds will form display increased neurogenesis, radial fibre divergence and amplification of bRGCs — particularly in the OSVZ — compared with the locations of future primary fissures, suggesting a possible causative link[17]. In support of this idea, local genetic manipulations of OSVZ proliferation in ferrets substantially alter the size and shape of the overlying folds[17,24]. Insights into the genetic regulation of cortical folding versus fissuring first came from transcriptomic analyses in developing ferrets. A comparison of the transcriptional signatures of germinal layers in prospective folds and fissures in ferrets identified thousands of differentially expressed genes (DEGs), mostly in the OSVZ and VZ[127]. DEGs include genes that are important for cortical progenitor proliferation, neurogenesis and folding, such as *Trnp1, Ccnd1, Tbr2, Flrt1, Flrt3, Fgfr2* and *Fgfr3*, as well as genes encoding components of the Notch, SHH, MAPK and WNT signalling pathways. DEGs from this analysis also included 80% of the genes that are homologous to those mutated in humans with cortical malformations, such as *RELN, DAB1, CDK5, PAX6, PAFAH1B1, TUBA8, TUBA1A, TUBB2B* and *GPR56* (REF.[127]) (BOX 1). Strikingly, in the germinal layers of ferret and human cortex, but not in lissencephalic mouse cortex, many of these DEGs are expressed in modular patterns, with alternating blocks of high and low expression[127,130,131] (FIG. 4). These patterns faithfully map the prospective location of folds and fissures, strongly supporting a role for the OSVZ and DEGs in defining the stereotyped patterning of cortical folds[17,52,127,129,132].

The OSVZ is important in cortical expansion and folding, as explained above. The abundance of progenitor cells in the OSVZ correlates positively both with cortical size and the degree of folding, both within and between species[17,79]. Studies in ferrets demonstrate that the OSVZ forms through the seeding of bRGCs generated from aRGCs in the VZ[76]. Unlike seeding of the ISVZ, this seeding of the OSVZ from the VZ is transient, occurring during a very brief time window of embryonic development, after which the founder bRGCs self-amplify until the OSVZ reaches its full size[76]. This critical time period for OSVZ formation is defined genetically, with the expression of genes that are important for the formation and delamination of bRGCs from the VZ — namely, *Cdh1* and *Trnp1* — precisely regulated[76]. Thus, in ferrets and primates[75,76], the developmental stage at which the OSVZ is seeded, the duration of the seeding period and the duration of the subsequent period when the OSVZ self-amplifies are all proposed to dramatically affect the size of the OSVZ and hence the degree and complexity of cortical expansion and folding.



c DEGs

High in SG				High in LS
Human and ferret		Ferret		Ferret
• *TBR2*	• *FGFR3*	• *Lhx2*	• *St3gal1*	• *Cdh8*
• *GPR56*	• *FLNA*	• *Lmo7*	• *Slitrk6*	• *Nrxn3*
• *FGFR2*	• *TRNP1*	• *Cdk6*		

Fig. 4 | **Genetic patterning of cortical folds.** In gyrencephalic species such as the ferret, certain genes are expressed in modular patterns, with alternating high and low expression levels (relative grey tone) along germinal layers of the developing cortex (part **a**). By contrast, in lissencephalic species such as the mouse, gene expression levels are quite homogeneous (part **b**). Modules of expression levels concur with the prospective location of folds and fissures. Variations in expression are associated with differences in proliferation and neuron migration and, eventually, cortical expansion and folding. Differentially expressed genes (DEGs) reported in ferrets by in situ hybridization are listed (part **c**), distinguishing between those expressed at high levels in the splenial gyrus (SG) with respect to the lateral sulcus (LS), or vice versa. Some of these genes also display modular expression in the fetal human cortex. A, anterior; P, posterior. Parts **a** and **b** are adapted with permission from REF.[3], Elsevier.

A mechanical and molecular tango

Several lines of evidence suggest that mechanical forces affect various important events during neural development, including progenitor cell proliferation, neuronal migration, cell differentiation and the formation of synapses. In chick embryos, neuroepithelial cells proliferate in response to mechanical impact[133,134]. The differentiation of stem cells into neurons in vitro is strongly affected by physical tension and the mechanical properties of surrounding growth substrates[135,136]. Neurite outgrowth in chicks, and synapse formation in *Drosophila melanogaster*, are strongly promoted by increased axonal tension[58,137]. In the developing rat cerebral cortex, mechanical pressure onto the basal process of aRGCs triggers calcium signalling and promotes their proliferation[138].

Cellular mechanotransduction is mediated by a number of elements, including through focal adhesions and/or by the ECM, primary cilia and cytoskeletal proteins[135,139,140]. During embryonic development, variations in the cytoskeletal system that result from mechanotransduction ultimately alter the intracellular localization and function of proteins, including transcription factors. For example, in developing *D. melanogaster*, mechanical pressure induces ectopic expression of the transcription factor Twist throughout the entire embryo[141]. Similarly, tension forces drive the activity of tension-induced proteins (TIPs) that, owing to their histone acetyltransferase activity, modify chromatin structure and thus substantially alter gene expression[142]. These changes in gene expression alter the genetic profile of neural cells during development and, consequently, their identity and biology.

The ECM is a key structural component of tissues that, depending on its specific molecular composition, helps to define their specific mechanical properties and functions[126]. For example, the assembly of complex fibronectin-based fibrillary structures in the ECM increases its stiffness, which is essential to support the movement and change in shape of cells during gastrulation[143]. Transcriptomic analyses of the developing cerebral cortex have identified differences in gene expression patterns between the smooth mouse cortex and the folded human cerebral cortex[23,116,118,119,144,145]. These analyses have shown that the expression of genes encoding ECM proteins is upregulated in human cortical germinal zones and progenitor cells, especially in the OSVZ[23,116]. In vivo and in vitro studies have demonstrated that ECM receptors can regulate the proliferation of cortical progenitor cells in mice and ferrets[71,146]. Most importantly, one recent in vitro study showed that application of a combination of three ECM components that are highly expressed in the human embryonic cerebral cortex (namely, hyaluronan and proteoglycan link protein 1 (HAPLN1), lumican and collagen I) onto human fetal brain slice cultures could induce cortical folding[8]. This treatment increases hyaluronic acid (HA) in the CP, and loss of HA in vitro reduces the nascent physiological folding of human fetal cortex. Moreover, treatment with these ECM components induced changes in stiffness of the cortical tissue, coherent with the localization of folds and fissures[8].

In summary, regulation of gene expression during cortical development may help to establish the necessary conditions to initiate folding, including patterned neurogenesis, neuron migration and differentiation as well as tissue stiffness and mechanical stress (tension or pressure). In turn, cellular strain and stress resulting from cortical folding may cause considerable changes in gene expression, as observed in several systems. Thus, the intimate and mutually influential relationship between genetics, cell biology and mechanical forces is likely to define their respective dynamic changes during development and to finally affect the cortical phenotype, both in health and disease[1,2].

The evolution of cortical folding

The advent of high-throughput genome sequencing and improved bioinformatics methodologies has enabled the phylogenetic relationships between extant mammals to be properly established[147]. By combining genomic information with measurements of cortical folding in different species, the evolution of cortical folding seems not to be a simple or linear story.

Starting from *Homo sapiens* and our closest relatives, analysis across the New and Old World primates shows that our common ancestor had a GI of 1.50–1.75 (REF.[87]). From this ancestor, Old World monkeys and primates evolved towards an exponential increase in cortical folding up to a GI of 2.56, as in humans, whereas New World monkeys followed the opposite trajectory and exhibit simplified cortical folding with a GI as low as 1.17, as in marmoset monkeys[87]. This divergence was confirmed independently by a phenomic character matrix (set of physical traits) of living placental orders and fossil species, which concluded that the ancestor of placental mammals had a folded cortex[148]. A specific analysis of cortical folding in 102 mammalian species including marsupials further confirmed that gyrencephaly is an ancestral mammalian trait (with the common mammalian ancestor estimated to have a GI of 1.36 ± 0.16) and that trajectories towards increased folding or decreased cortical folding, as in primates, have occurred multiple times across mammalian clades over the past ~100 million years of brain evolution[149]. Secondary loss of gyrencephaly may have occurred as a result of reductions in the number of basal neurogenic progenitors and bRGCs[77,87,150]. The ability of the mammalian brain to undergo dramatic phenotypic reversals and differential changes in cortical folding during evolution may reflect the remarkable adaptability of mammals along this process[151].

The genomic changes that occurred during evolution and that were directly responsible for the observed variations in cortical folding are unclear. The human genome shows sites of uniquely high sequence divergence from the genomes of close evolutionary relatives[152]. In fact, genetic evolution is still ongoing in humans; there are several hot spots in genes related to cortical development, including *ASPM* and *MCPH1*, and mutations in these hot spots are associated with microcephaly[153–155]. Recent transcriptomic analyses have identified 15 genes that exist only in primate genomes and that show enriched expression in cortical progenitor cells[113]. A subset of these genes emerged uniquely in the human

Gastrulation
Phase of early embryonic development during which the single-layered blastula is reorganized into a multilayered gastrula.

Hot spots
Regions in the genome that exhibit elevated rates of a specific event. In evolutionary hot spots, the local sequence of DNA has changed rapidly during evolution.

lineage during evolution through certain mechanisms — mostly through whole-gene duplication, as in the case of *NOTCH2NL*[114,115,156,157], or through partial gene duplication, as with *ARHGAP11B*[23,111,158,159]. In most other cases, new genes emerged through small insertions into or deletions from the ancestral genome that may have eliminated pre-existing stop codons, caused frameshifts or introduced new sites of RNA splicing[113]. Even within genes that are conserved between species, small differences in coding DNA sequences — including synonymous and non-synonymous base pair changes — have also occurred over evolution and may or may not have led to modified amino-acid sequences.

In addition to the appearance of new genes, changes in the regulation of gene expression have also contributed considerably to cortical evolution. A comparison of the human genome and the genome of the chimpanzee (which has a GI of 2.45) identified hundreds of human accelerated regions (HARs) — small DNA segments that exhibit highly divergent sequences in humans and rapid

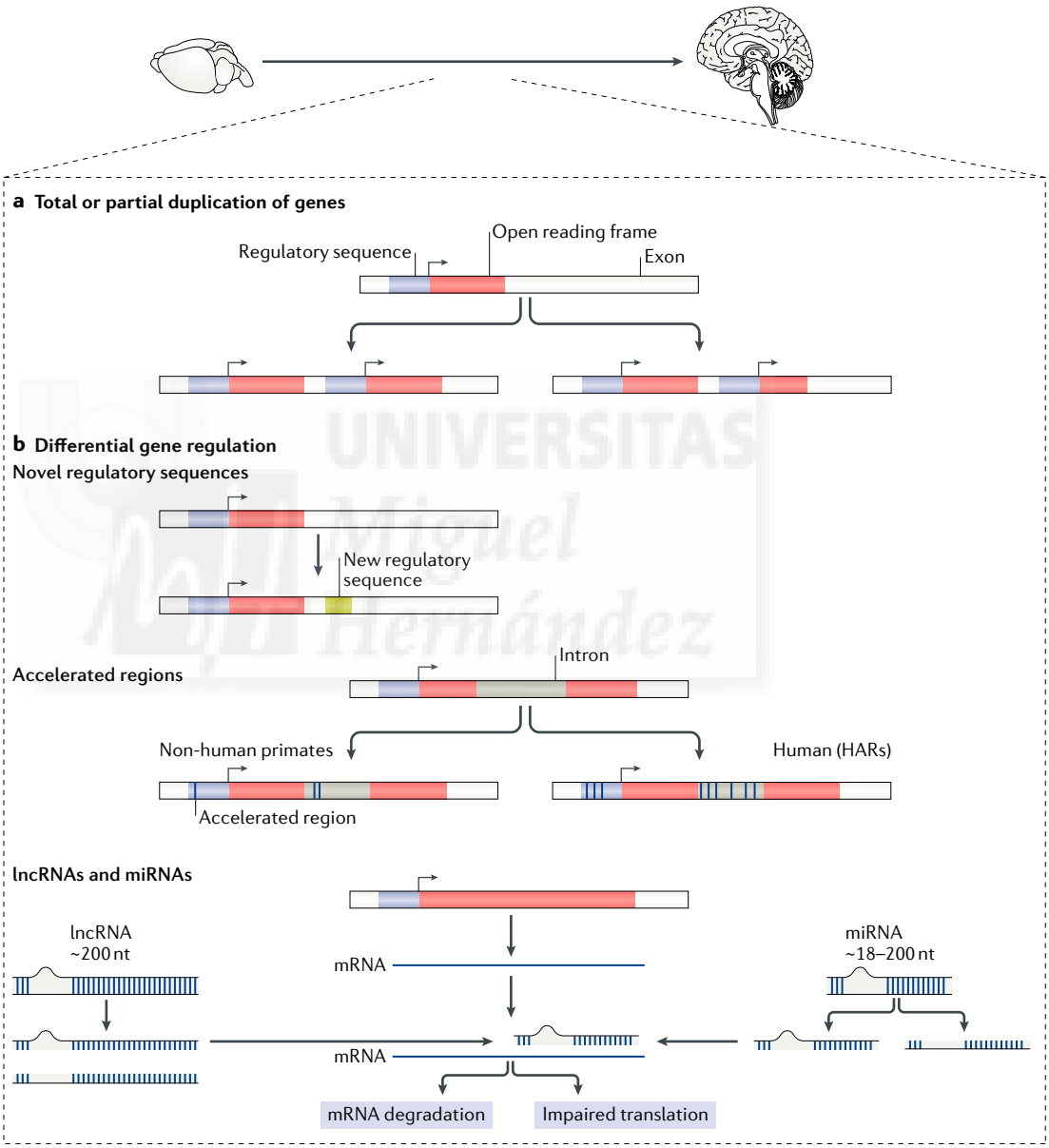


Fig. 5 | **Genetic evolution and cortical folding.** Different genetic variations that have occurred during evolution and affect cortical folding are summarized here in two main categories: duplication and gene regulation. **a** | Duplication (total or partial) of genes, both protein-coding and non-coding, allows the modification of the existing sequence without altering the original gene, preserving its functionality. One example of such duplication is *ARHGAP11*, which occurred specifically in the human lineage and resulted in the emergence of the new, human-specific gene, *ARHGAP11B*. **b** | The expression of different genes may be differentially regulated through the generation of novel regulatory sequences, including promoters or enhancers, accelerated regions and those encoding long non-coding RNAs (lncRNAs) and microRNAs (miRNAs). Interestingly, some of these regulatory elements are primate or human-specific, and many of these are related to cell cycle and neurogenesis. Blue, regulatory sequence; red, open reading frame; light grey, non-coding sequence; dark blue, accelerated regions. HAR, human accelerated region; nt, nucleotide.

nucleotide substitution in the human lineage — that are proposed to regulate gene expression and thus contribute to human-specific brain expansion and complexity[160–162]. At least 30% of HARs encode developmental enhancers that show activity in humans and chimpanzees[163] and therefore are good candidates as regulatory regions that may define human-specific spatiotemporal patterns of gene expression[160,164–168]. Although not a HAR, one study assessed the activity of the enhancer sequence at the *GPR56* locus, which is crucial for normal cortical folding, in different species[169]. In transgenic mice, reporter gene expression driven by the mouse *Gpr56* enhancer sequence displayed a pattern different from that driven by the enhancer sequences from species with folded cortices, such as dolphins, cats or humans. Gene expression is also regulated by non-coding RNAs, which have central roles in cortical development, and long non-coding RNAs have been proposed to be important in the evolution of gene regulation[170–172] (FIG. 5). Accordingly, in apical and basal progenitor cells of the developing macaque cortex, but not in the developing mouse cortex, hundreds of microRNAs are expressed that regulate the cell cycle and neurogenesis, particularly in the OSVZ, and thus may contribute to primate cortical complexification and possibly folding[62,81,173,174].

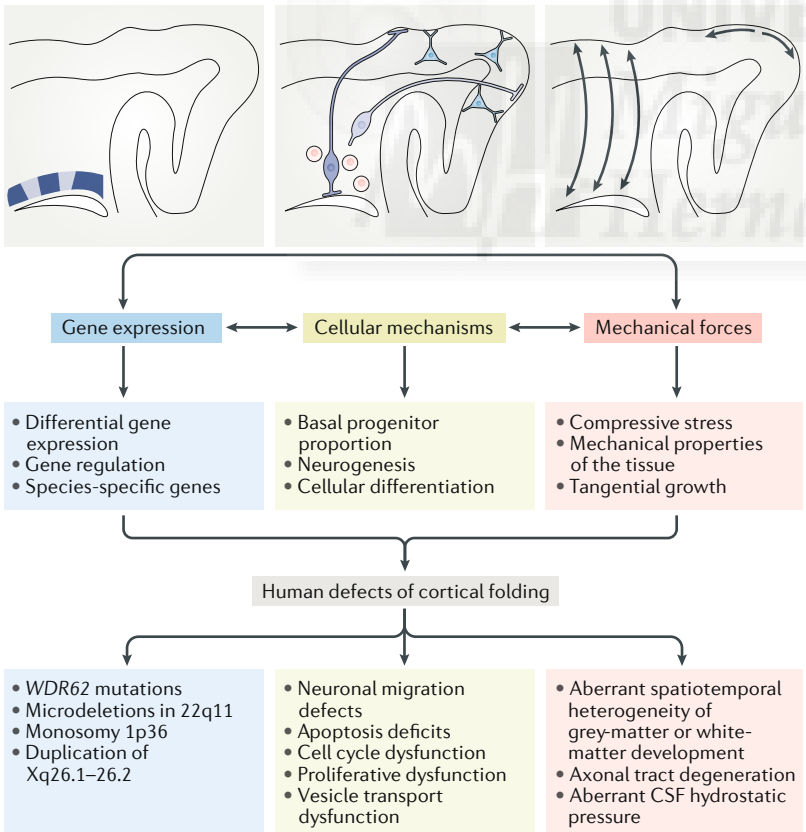


Fig. 6 | **Three key players in cortical folding: gene expression, cellular mechanisms and mechanical forces.** Each component and their specific role in cortical folding is represented schematically in the upper part of the figure. Arrows between gene expression, cellular mechanisms and mechanical forces represent their mutual influence. Some of the main mechanisms from each component are listed. The bottom panel lists examples in which alterations at any one of these three levels lead to defective folding of the human cortex (bottom). CSF, cerebrospinal fluid.

These changes in the regulation of gene expression translate into transcriptomic differences between species, as demonstrated in comparative analyses of single-progenitor cell transcriptomes that show greater similarity between humans and ferrets than between humans and mice[117]. On this basis, variations in enhancers and other regulatory elements may be at the core of the evolution of precise spatiotemporal patterns of gene expression that define cortical patterning and folding during development[3,76,127].

Future directions

The folding of the mammalian cerebral cortex remains one of the most fascinating phenomena of developmental neuroscience and evolution, but only with the combined efforts of several scientific disciplines are we beginning to clarify its most basic aspects. Not surprisingly, these efforts reveal that cortical folding results from the combined actions of factors at different levels and their interactions with one other — a notion that seems directly relevant for human disease (FIG. 6). Now that some of the key cellular and genetic elements involved in cortical folding have been identified, ongoing research is focusing on suitable experimental animal models, such as ferrets, marmosets and macaque monkeys, and in vitro systems such as cerebral organoids[35,36,85,96,175]. Taking full advantage of genome-editing techniques, gene-knockout and transgenic ferrets and marmosets are already being generated[85,88,176], and these will undoubtedly be one of the driving forces of this field in the future. In the quest for genetic mechanisms that regulate cortical development, and particularly progenitor cell diversity and lineage, considerable efforts are being made using high-throughput single-cell analyses (for example, Drop-Seq)[177,178]. Performing these analyses in ferrets, humans and non-human primates holds great promise for the identification of cell populations that are enriched in prospective folds or fissures and that may hence underlie differential cortical expansion rates and folding. Such cell populations may have distinctive features such as the potential to self-amplify, to produce bRGCs or neurons, or to undergo tangential dispersion. Single-cell analyses in various gyrencephalic species may reveal differences in expression of proteins that endow cells, and the tissue immediately surrounding them, with particular mechanical properties relevant to drive tissue buckling, including adhesion molecules, cytoskeletal proteins or ECM components[8].

We are beginning to capture the outstanding complexities of the developing cerebral cortex as a biological and mechanical system, with dynamic developmental trajectories and feedback mechanisms between cell biology, mechanics and genetics. Unfortunately, our knowledge in these fields is still too fragmentary to be combined in a unifying model. We have some detailed understanding of genetic and cellular events that occur early in development and that precede and underlie the eventual folding of the cortex. However, much less is known about the events that occur during folding, such as any changes in cell size and density, neuropil or ECM. By contrast, we have some notions about the mechanical features and constraints of tissue deformation that

are involved in actual cortical folding late in development, but not about how these emerge during early developmental stages and change later. Understanding cortical folding will require a full and quantitative characterization of all these relevant features throughout cortical development.

Once a unifying model is built that explains cortical folding, a great challenge will be to validate it by experimentally analysing the interactions between genetics, cell biology and mechanics and their highly dynamic changes during development. Which cells generate mechanical forces before and during folding, and how is this achieved? In turn, how do these cells respond to mechanical force during folding, and what are the effects of physical forces on the gene expression or function of progenitor cells and migrating neurons at different stages of cortical development and folding? Only by understanding these parameters and interactions in the developing cerebral cortex in vivo, at a quantitative level, will we finally elucidate the mechanisms that underlie the emergence of stereotyped folding of the mammalian cerebral cortex.

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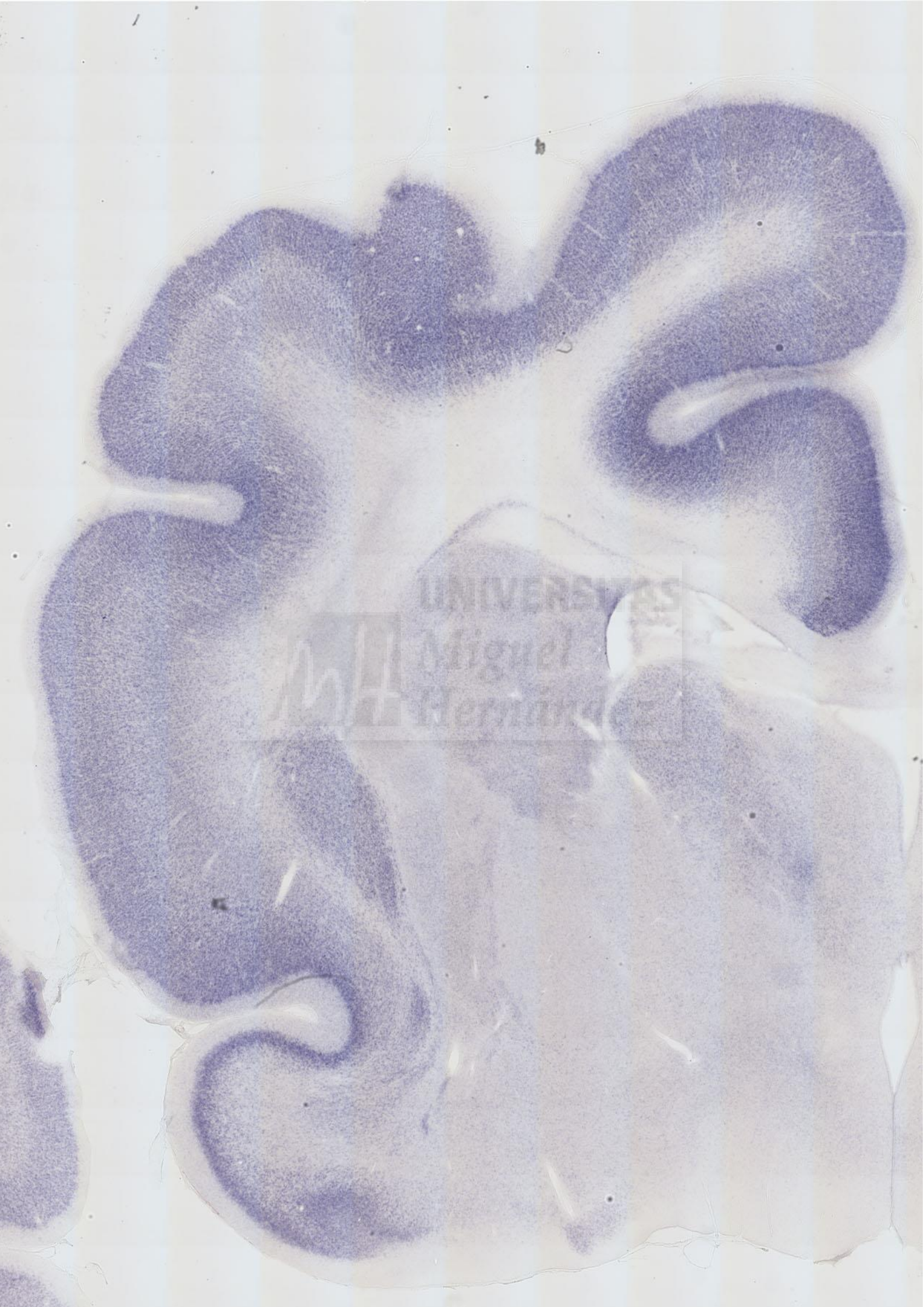

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