Function and Regulation of Bone Morphogenetic Protein 7 (BMP7) in Cerebral Cortex Development

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Dra. Soledad Alcántara Horrillo

Juan Alberto Ortega Cano

En los momentos de crisis,
sólo la imaginación es más importante que el conocimiento.

Albert Einstein

“Hay hombres que luchan un día y son buenos.
Hay otros que luchan un año y son mejores.
Hay quienes luchan muchos años y son muy buenos.
Pero hay los que luchan toda la vida:
Esos son los imprescindibles”

Bertolt Brecht

Es de ser bien nacido ser agradecido…

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ABBREVIATIONS

APCs: Astrocyte Precursor Cells

BDNF: Brain-Derived Neurotrophic Factor

BLBP: Brain Lipid-Binding Protein

BMPs: Bone Morphogenetic Proteins

BrdU: BromodeoxyUridine

CGE: Caudal Ganglionar Eminence

CC: Corpus Callosum

CNS: Central Nervous System

CNTF: Ciliary NeuroTrophic Factor

CP: Cortical Plate

CXCR4: CXC chemokine receptor 4

Dcx: Doublecortin

E: Embryonic day (i.e. E11: embryonic day 11)

EGF: Epidermal Growth Factor

FGFs: Fibroblast Growth Factors

GABA: Gamma AminoButiric Acid

GE: Ganglionar Eminence

GFAP: Glial Fibrillary Acidic Protein

GLAST: Astrocyte-specific glutamate transporter

GRP: Glial Restricted Precursor

GW: Glial Wedge

HAT: Histone Acetyltransferase

HDAC: Histone Deacetylase

H-I: Hypoxic-Ischemic

HIF1α: Hypoxia Inducible Factor 1α

ICC: Immunocitochemistry

IG: Indusium Griseum

IHC: Immunohistochemistry

IPC: Intermediate Progenitor Cells

IZ: Intermediate Zone

KO: Knock Out

LDL: Low Density Lipoprotein

LGE: Lateral Ganglionar Eminence

LIF: Leukemia Inducing Factor

MGE: Medial Ganglionar Eminence

MZG: Midline Zipper Glia

Nf1a: Nuclear factor 1 A

Nf1b: Nuclear factor 1 B

NGF: Nerve Growth Factor

Npn: Neuropilin

NRG: Neuregulin

NSC: Neural Stem Cells

NT: Neurotrophin (NT3, NT4 and NT5)

O-2A: Oligodendrocyte and type 2 Astrocyte precursor cell

OPCs: Oligodendrocyte Progenitors Cells

P: Postnatal day (i.e. P3: Postnatal day 3)

p75NTR: p75 Neurotrophin Receptor

PI-3kinase: Phosphatidylinositol-3-kinase

PACAP: Pituitary adenylate cyclase-activating polypeptide

PCR: Polymerase Chain Reaction

PDGF: Platelet-Derived Growth Factor

PDGFRα: Platelet-Derived Growth Factor Receptor alpha

PLC: Phospholipase C

PSA-NCAM: PolySialic Acid Neural Cell Adhesion Molecule

PP: Preplate

PVL: Periventricular Leukomalacia

RGC: Radial Glial Cells

RT-PCR: Real Time - Polymerase Chain Reaction

SC1: SPARC-like 1

SCS: Subcallosal Sling

SDF-1: Stromal cell-Derived Factor-1 (also CXCL12)

Sema: Semaphorin

Shh: Sonic hedgehog

SP: Subplate

SVZ: Subventricular zone

Tbr2: T-box-brain-2

TGF β: Transforming Growth Factor β

Trk: Tropomyosin-related kinase

TrkB: Tropomyosin-related kinase receptor B (also TrkA and TrkC)

VZ: Ventricular Zone

RESUMEN

INTRODUCCIÓN

EL DESARROLLO DE LA CORTEZA CEREBRAL

El sistema nervioso central (CNS) es el centro organizador de nuestras actividades motoras, sensitivas y comprensivas. Durante la evolución, los núcleos motores y sensitivos se han ido localizando de zonas más profundas a zonas más superficiales en el cerebro. La corteza cerebral es el último núcleo en emerger, variando su complejidad dependiendo de las necesidades de cada especie animal. La corteza cerebral humana es la más compleja dentro de los mamíferos, permitiendo el desarrollo de actividades motoras de alta complejidad y exclusivas del humano como son el lenguaje, la escritura, la pintura, el deporte, etc. La corteza cerebral es un manto de sustancia gris que cubre ambos hemisferios cerebrales. Está formada por diferentes capas horizontales de neuronas que al mismo tiempo están organizadas funcionalmente como columnas, las cuales responden de una manera conjunta a señales específicas (Rakic, 2007). Las neuronas de una columna están altamente interconectadas y comparten funciones comunes, actuando como una unidad básica que ejecuta un paquete de funciones corticales (Rakic, 2008). Las experiencias sensoriales al inicio de la vida post-natal son necesarias para la correcta maduración y organización columnar. Dicha organización es distinta en las diferentes áreas de la corteza cerebral, las cuales también se pueden distinguir por una cito-arquitectura, quimio-arquitectura, conectividad y expresión génica específicas.

Las *neuronas piramidales* son el principal tipo de neuronas en la corteza cerebral, representando el 80% de las neuronas del neocórtex. Se caracterizan por tener muchas dendritas apicales y laterales con un gran número de espinas dendríticas. De la base del soma en forma piramidal de estas neuronas, surge el axón que envía ramificaciones colaterales antes de llegar a la sustancia blanca subcortical. Las neuronas piramidales son también denominadas neuronas glutamatérgicas, debido a que sus axones liberan glutamato como neurotransmisor en la sinapsis, ejerciendo una acción excitatoria. El segundo tipo principal de células en la corteza cerebral está

formado por las *interneuronas*, que tienen una acción generalmente inhibitoria a través de la liberación del ácido gamma aminobutírico (GABA) al espacio sináptico. Dentro de este grupo de neuronas se pueden observar distintos tipos celulares con arquitecturas dendríticas diferentes.

De manera general se puede decir que la corteza cerebral de mamíferos consiste en seis capas neuronales diferentes, aunque el número, grosor y composición de las capas puede variar dependiendo de la región cortical. En las seis capas de la corteza de roedores se puede identificar: *Capa I*, la capa más superficial compuesta por un bajo número de células y un gran número de proyecciones terminales de dendritas de las neuronas piramidales y de axones provenientes de otras regiones corticales de uno u otro hemisferio así como del tálamo. Durante el desarrollo embrionario y hasta dos semanas después del nacimiento, en esta capa también encontramos las células de Cajal-Retzius. La c*apa II/III*, está compuesta por interneuronas pequeñas y neuronas piramidales de tamaño medio. Las neuronas piramidales de esta capa envían dendritas a la capa I y sus axones son proyectados a capas más profundas y otras áreas corticales, especialmente del hemisferio opuesto, generando la principal comisura cerebral, el cuerpo calloso. La *capa IV* es la capa que recibe la mayoría de las proyecciones extracorticales. En la *capa V* se encuentran las neuronas que proyectan sus axones hacia áreas subcorticales como el estriado, el tronco del encéfalo y la médula espinal, además de proyectar hacía el hemisferio contralateral. Finalmente, la capa VI, también recibe aferencias extracorticales aunque en menor medida que la capa IV (Valverde, 2002; Sansom and Livesey, 2009).

En este trabajo hemos usado el ratón como modelo animal por las facilidades que ofrece de manipulación, obteniéndose en poco tiempo un alto número de animales por parto, así como por la posibilidad de poder generar modelos transgénicos. Además hay una gran similitud entre las estructuras corticales y los mecanismos celulares implicados en el desarrollo de la corteza cerebral entre el ratón y el humano (Molnar et al., 2006). A parte de las lógicas diferencias de tamaño, existen dos diferencias principales entre la corteza de ratón y la de humanos: Primero, en la corteza de ratón las capas II y III no se distinguen como compartimentos distintos,

mientras que en primates ambas capas se pueden distinguir claramente; Segundo, las células de la zona subventricular aparecen antes durante el desarrollo de la corteza humana, produciendo una segunda región germinal mucho más amplia que en ratón. Además la zona subventricular en humanos está subestratificada en dos capas (la capa subventricular externa y la interna), compartimentación que no se observa en ratón (Smart et al., 2002). Esta mayor complejidad de la capa subventricular está asociada con una mayor diversidad y complejidad neuronal de la corteza cerebral en humanos (Tarabykin et al., 2001; Reillo et al., 2010).

Durante fases iniciales del desarrollo, el CNS de vertebrados surge de la región más anterior de la placa neural, que al plegarse da lugar al tubo neural. La parte interior de este tubo constituye los futuros ventrículos, y las paredes el neuroepitelio del telencéfalo. Las paredes telencefálicas forman una capa germinal de células adyacentes al ventrículo denominada zona ventricular (VZ), cuyas células comienzan a dividirse generando las primeras neuronas en el del proceso conocido como neurogénesis. Progenitores neuronales darán lugar a las neuronas de las diferentes capas corticales desde el día embrionario 11.5 (E11.5) hasta el día E17.5 en el ratón, siguiendo un proceso altamente controlado desde el punto de vista espacio-temporal. La primera oleada de neuronas corticales se acumula sobre la VZ dando lugar a la preplaca (PP) que contiene células de Cajal-Retzius, neuronas GABAérgicas y neuronas pioneras (Meyer et al., 2000). Las siguientes neuronas generadas invaden la PP formando la placa cortical (CP) que divide la PP en dos regiones diferentes separadas por la CP: la zona marginal justo debajo de la meninge, y una zona más profunda compuesta por las neuronas de la subplaca (Marin-Padilla, 1978). Ambas regiones son de vital importancia para el correcto desarrollo de la corteza cerebral. Por un lado la neuronas de la subplaca actúan como señal de guía para los axones talámicos aferentes que inervan la corteza (Wang et al., 2010). Por otro lado, la zona marginal está poblada por células de Cajal-Retzius que secretan relina, una glicoproteina de matriz extracelular necesaria para la migración de las neuronas piramidales (D'Arcangelo et al., 1995, 1997; Alcántara et al., 1998). Las siguientes oleadas de neuronas generadas en la VZ generarán las seis capas de la corteza siguiendo un programa estrictamente controlado. Estudios autoradiográficos realizados por

Angevine and Sidman en 1961 demostraron que dependiendo del día de en el que se generan las neuronas, estas se colocan en capas corticales diferentes. Las neuronas corticales siguen un modelo llamado "inside-out", donde las primeras neuronas en generarse en la VZ migran y forman las capas más profundas, mientras que las posteriores cohortes de neuronas pasaran entre las neuronas formadas anteriormente, asentándose en capas más superficiales.

NEUROGENESIS Y MIGRACIÓN NEURONAL

La neurogénesis es un proceso por el cual las células madre neurales (NSC) dan lugar a todas las neuronas que forman el CNS maduro. Según avanza el proceso de neurogénesis el neuroepitelio inicial se va transformando en un tejido multiestratificado donde los somas de las células progenitoras están adheridos a la parte más apical de la VZ. Durante el desarrollo cortical, los progenitores neuroepiteliales de la VZ dan lugar a células de glía radial (RGC) que se expanden desde la VZ hasta la meninge, manteniendo su polarización basal-apical a lo largo de la pared cerebral y convirtiéndose en la principal población de progenitores neurales. La aparición de las RGC en el telencéfalo dorsal se da al inicio de la neurogénesis (E12-13 en ratón) produciendo de manera directa o indirecta, a través de la generación de progenitores intermedios, la gran mayoría de las neuronas de proyección (Parnavelas et al., 2000). Mientras que en el telencéfalo ventral, los progenitores ventriculares promueven la formación de interneuronas GABAérgicas corticales (Anderson et al., 2001).

La producción de neuronas provenientes del epitelio ventricular pseudoestratificado está controlado por tres tipos diferentes de divisiones mitóticas: (1) División celular simétrica no-terminal, que produce dos células hijas que continúan proliferando sin salir de la superficie subventricular y por tanto, contribuyendo a la expansión de la población de precursores neuronales. (2) División asimétrica, donde una célula hija continúa proliferando en la VZ y la otra sale del ciclo celular y migra fuera de la VZ. (3) División celular simétrica terminal, la cual produce dos células hijas post-mitóticas que

migran fuera de la VZ, agotando la población de células proliferativas (Takahashi et al., 1996; Cai et al., 2002; Guillemot, 2005; Götz and Huttner, 2005). Durante la neurogénesis se dan los tres tipos de división dentro del neuroepitelio ventricular.

Las RGC generan neuronas corticales y progenitores intermedios. Las neuronas creadas migran hasta alcanzar su posición en la corteza, mientras que los progenitores intermedios se establecen por encima de la VZ generando una segunda capa proliferativa, la zona subventricular (SVZ), en fases tardías de la neurogénesis embrionaria (E15). Esta región proliferativa ayudará a aumentar la población de neuronas en el córtex mientras que las RGC irán desenganchándose de la VZ y se irán transformando en astrocitos. Durante el periodo post-natal, los progenitores subventriculares continúan proliferando y dando lugar a neuronas corticales y células de glía. Esta capacidad se mantiene durante el periodo adulto (Lois and Alvarez-Buylla, 1993), lo que hace que la SVZ sea una de los principales temas de estudio en el campo de la neurociencia por su potencial neuroregenerativo.

Durante el desarrollo cerebral, las poblaciones de neuronas se generan en una región diferente de donde finalmente se diferencian. Las neuronas corticales que se forman alcanzan su posición final siguiendo básicamente dos tipos de migración, la migración radial y la migración tangencial (Molineaux et al., 2007). En la *migración radial*, las neuronas piramidales que se forman en la VZ de la corteza cerebral utilizan la glía radial para alcanzar su posición final. En cambio la mayoría de las interneuronas GABAérgicas que se producen en la VZ/SVZ de la eminencia ganglionar lateral y medial (LGE y MGE) migran dorsalmente hasta alcanzar la corteza cerebral siguiendo diferentes señales químicas y sin utilizar la glía radial, en un proceso conocido como *migración tangencial* (Parnavelas, 2000; Marin and Rubenstein, 2001; Anderson et al., 2001; Rakic, 2001; Shu et al., 2004). Una vez que alcanzan la corteza, las interneuronas GABAérgicas también utilizan la glía radial para moverse ventralmente, hacia la VZ, o dorsalmente, hacia la meninge, y así llegar a su localización definitiva. Por lo tanto, podemos decir que las RGC juegan un papel vital en la histogénesis de la corteza cerebral, ya que a la vez que son las principales productoras de neuronas, son utilizadas por estas como raíl durante el proceso de migración.

Los mecanismos de reconocimiento entre neuronas y glía son esenciales durante la migración radial, pero también lo son el reconocimiento entre neuronas, y entre neuronas y matriz extracelular para los procesos de migración radial y tangencial. Neuronas, células de glía y matriz extracelular poseen una gran cantidad de elementos que parecen dirigir los diferentes pasos del complejo proceso de migración neuronal. Defectos en alguno de estos mecanismos pueden afectar gravemente el correcto desarrollo de la corteza cerebral y provocar déficits cognitivos y conductuales severos en el individuo.

EL CUERPO CALLOSO

Muchas de las neuronas de las capas II, III y V del neocórtex proyectan sus axones hacía el hemisferio contra-lateral dando lugar a una estructura esencial de la línea media, el cuerpo calloso (CC). Se estima que alrededor de 200 millones de proyecciones axonales forman la que es la estructura de sustancia blanca más grande del cerebro. El CC conecta neuronas de ambos hemisferios cerebrales y coordina el traspaso de información entre ellos. Individuos con malformaciones callosas inicialmente no parecen mostrar déficits intelectuales aunque en algunos niños se ha observado desordenes neuropsiquiátricos incluyendo dificultades en el aprendizaje, del sueño y de comunicación social entre otros.

En el CC se pueden distinguir la parte posterior llamada *esplenio*, la parte anterior llamada *rodilla* y entre ambas, el *cuerpo*. La parte anterior proyecta posterior e inferiormente desde la rodilla formando el *rostro*. Durante las primeras fases del desarrollo cerebral, el cerebro anterior está formado por una vesícula llamada prosencéfalo, la cual por procesos de proliferación y expansión celular dará lugar a dos vesículas prosencefálicas inicialmente independientes que constituirán los hemisferios cerebrales. La fusión de ambas vesículas es crítica para el correcto desarrollo del CC, y se realiza por regiones ventrales de la línea media antes de que las fibras callosas empiecen a cruzar hacia las respectivas regiones contra-laterales. Las primeras

regiones en formarse son el rostro y la parte anterior del cuerpo, formándose las estructuras más caudales un día más tarde (Volpe et al., 2009).

En ratón la formación del CC se inicia a E14-15 y está dirigida por diferentes poblaciones gliales localizadas en la línea media como la *midline zipper glia* (MZG), el *glial wedge* (GW) y el *indusium griseum* (IG) (Shu et al., 2003). Estas y otras poblaciones celulares particulares como el *subcallosal sling* (SCS) guían a los axones callosos para cruzar la línea media y alcanzar así su región diana definitiva. La MZG, localizada en regiones ventrales de la línea media, está directamente relacionada con la fusión de los hemisferios cerebrales (Lindwal et al., 2007). La glía que forma el GW deriva de la glía radial y está localizada en regiones ventriculares mediales. Mediante la secreción de Slit 2, una molécula de guía que ejerce una acción repelente sobre los axones callosos, evita la entrada de dichos axones hacía el septum, redirigiéndolos para que crucen la línea media (Shu and Richards, 2001; Shu et al., 2003). En la zona dorsal de la línea media encontramos el IG, un área formada por glía que también expresa Slit2 y que ayuda al proceso de guía de las fibras callosas. El SCS, es una población neuronal que forma una estructura en forma de "U" que delimita ventralmente el CC. Esta población celular migra desde la SVZ y se mantiene proliferativa hasta el inicio de la fase post-natal cuando desaparece. Su papel en el desarrollo del CC es todavía muy incierto, aunque algunos trabajos muestran las similitudes des SCS con las neuronas de la subplaca, las cuales están asociadas a procesos de guía axonal, regionalización y reconocimiento de patrones en el neocórtex (McConell et al., 1989, 1994). Los primeros axones en cruzar la línea media e inervar el hemisferio contralateral son los *axones pioneros*, que se originan en la región más medial de la corteza conocida como corteza cingulada (Koester and O`Leary, 1994). Estos axones son de gran importancia como señal de guía para los posteriores axones que cruzarán la línea media (Piper et al., 2009).

Durante la formación del CC hay un gran número de eventos clave, como es la formación y fusión de los hemisferios telencefálicos, el nacimiento y especificación de las neuronas comisurales, la correcta formación de estructuras gliales de la línea media y de la población de axones pioneros, así como la expresión de moléculas que

participan en el reconocimiento axonal de patrones. En todos estos pasos hay implicados una larga lista de genes cuya modificación puede provocar anomalías severas en el desarrollo del CC. Muchos trabajos basados en el modelo de ratón han ayudado a avanzar en el entendimiento de cómo participan cada uno de estos mecanismos en la formación de la comisura callosa. Además, análisis anatómicos, moleculares y de imagen en cerebros humanos fetales muestran las similitudes estructurales y genéticas que hay en los mecanismos de guía axonal en la línea media de ratón y humano (Ren et al., 2006). Por lo tanto, el ratón es un excelente modelo para estudiar el origen de las distintas anomalías callosas durante el desarrollo.

CITOQUINAS Y EL DESARROLLO DE LA CORTEZA CEREBRAL

En esta tesis se analiza el papel de factores solubles secretados por neuronas y células de glía que de una manera coordinada dirigen a las NSC para dar lugar a los distintos linajes neurales presentes en el CNS. Durante el desarrollo, la neuronas secretan BDNF (Brain Derived Neurotrophic Factor) que promueve diferenciación neuronal (Cheng et al., 2007), mientras que las BMPs (Bone Morphogenetic Proteins) secretados por neuronas y glía pueden promover la diferenciación de las NSC tanto hacia neuronas (Mabie et al., 1999) como hacia astrocitos (Gross et al., 1996), dependiendo del periodo del desarrollo y de los niveles de BMPs. Así durante la neurogénesis, la baja secreción de BMPs por parte de las neuronas induce a las células madre corticales a diferenciarse hacia neuronas, mientras que los astrocitos en fases post-natales secretan altos niveles de BMPs causando la diferenciación celular de las NSC hacia astrocito (Chang et al., 2002). Análisis iniciales en nuestro grupo sugieren que cambios en la expresión de BDNF modifica los niveles de expresión de BMP7. Esto nos hace pensar en la necesidad del cerebro de tener un patrón de expresión de citoquinas bien organizado para generar correctamente las diferentes poblaciones neurales en su debido lugar y a su debido tiempo.

A parte de las múltiples funciones de BDNF y BMPs durante el desarrollo del CNS, nos hemos interesado en estas dos citoquinas porque parece ser que juegan un papel

neuroprotector y neuroregenerativo importante en respuesta a daño nervioso (Chou et al., 2005; Meng et al., 2005; Tsai et al., 2007; Guo et al., 2008; Sabo et al., 2009; Tsai et al., 2010). La señalización celular activada por estas citoquinas en condiciones patológicas promueve supervivencia y procesos de recuperación celular como proliferación, diferenciación y crecimiento dendrítico y axonal (Esquenazi et al., 2002; Husson et al., 2005; Deumens et al., 2006; Tsai et al., 2007; Sánchez-Camacho and Bovolenta, 2009; Cate et al., 2010). Sin embargo, ambas citoquinas ejercen acciones pleiotrópicas en la corteza en desarrollo y el efecto real producido por la activación de sus vías de señalización es todavía bastante desconocido.

Son muchos los tipos de lesión durante la gestación que causan daño y disfunción cerebral en el feto. Nuestro grupo está especialmente interesado en patologías perinatales asociadas a cambios en los niveles de BDNF. La hipoxia es una condición patológica que encaja en este perfil. El daño hipóxico puede tener múltiples orígenes como pueden ser la oclusión del cordón umbilical, una función placentaria anormal debido a hipertensión materna, el desgarre parcial placentario o la oclusión de la arteria uterina (Ress et al., 2011). En bebés prematuros el problema hipóxico suele ser debido a la falta de madurez de sus pulmones. Aunque la tasa de supervivencia de los individuos afectados ha mejorado, estos pacientes todavía presentan en un alto porcentaje retraso mental, desordenes de la conducta o parálisis cerebral. Estas lesiones son producidas por niveles bajos de oxígeno durante el periodo perinatal que alteran la diferenciación neuronal, la sinaptogénesis y debido a una apoptosis excesiva, producen la perdida de neuronas, glía y de sus progenitores (Curristin et al., 2002; Madri, 2009). Otro contratiempo observado en estos individuos es la falta de factores neuroprotectores como son las neurotrofinas, las cuales son aportadas por la madre durante gran parte del periodo gestacional. Los niños prematuros por ejemplo, muestran una muy baja disponibilidad de muchos de estos factores (Malamitsi-Puchner et al., 2004). Son ampliamente conocidos los efectos positivos de BDNF en el CNS frente al daño hipóxico materno (Cheng et al., 1997; Galvin et al., 2003; Golan et al., 2004; Sun et al., 2008). Se ha observado que lesiones hipóxico-isquémicas (H-I) inducen aumentos inmediatos de los niveles de BDNF que posteriormente sufren una caída durante la posterior fase de normoxia (Lindval et al., 1992; Korhonen et al., 1998;

Hubold et al., 2009). Es por eso que el pre-condicionamiento, tratamiento basado en exponer al individuo a hipoxias moderadas que producen una subida sostenida de los niveles de BDNF, antes de un episodio H-I severo, produce una mejor recuperación (Ran et al., 2005; Madri, 2009). El mantenimiento de niveles altos de BDNF preserva las propiedades neuroprotectoras de esta citoquina reduciendo así el daño hipóxico (Hubold et al., 2009).

De todos modos, niveles altos de BDNF no siempre están relacionados con efectos positivos. Se han observado niveles altos de BDNF en suero y líquido cefalorraquídeo de niños que padecen desórdenes penetrantes del desarrollo (PDD), entre los cuáles encontramos el autismo (Tsai et al., 2005; Connolly et al., 2006). Estos trastornos se caracterizan por déficits en el comportamiento social interpersonal y problemas con el desarrollo del lenguaje, entre otros problemas cognitivos. Así, un aumento de la expresión de BDNF observada bajo condiciones patológicas puede contribuir a mecanismos protectores asociados a la acción trófica de BDNF o puede participar en el funcionamiento cerebral anómalo asociado al daño nervioso producido (Kuczewski et al., 2009). Hay una gran cantidad de lesiones relacionadas con la desregulación de BDNF y de los mecanismos celulares que controla esta citoquina. El conocimiento del conjunto de genes producto de la señalización activada por BDNF es especialmente interesante para desarrollar nuevas dianas terapéuticas contra un gran número de enfermedades neurológicas originadas durante el desarrollo perinatal.

En esta tesis muestro como BDNF modula la expresión de BMP7, y como ambas citoquinas parecen estar fisiológicamente muy correlacionadas. Como BDNF, BMP7 tiene un papel neuroprotector y neuroregenerativo en respuesta a daño nervioso (Tsai et al., 2007; Sabo et al., 2009; Tsai et al., 2010). BMP7 promueve recuperación funcional a través de inducir neurogénesis, mejora de circulación sanguínea y de la utilización de glucosa en el cerebro en respuesta a daño H-I (Liu et al., 2001; Chang et al., 2003; Harvey et al., 2005; Chou et al., 2006). Frente a este tipo de lesión, tanto BDNF como BMP7 son importantes para restablecer nuevas sinapsis promoviendo crecimiento y remodelación dendrítica (Esquenazi et al., 2002; Deumens et al., 2006). Sin embargo la acción protectora de estos factores depende de la dosis y de las

condiciones espacio-temporales. Husson y colaboradores describieron que administrando diferentes dosis a diferentes días de desarrollo post-natal, BDNF tiene efectos totalmente distintos sobre la sustancia blanca en respuesta a daño cerebral (Husson et al., 2005). De igual manera, las propiedades neuroprotectoras y neuroregenerativas de las BMPs dependen del tipo de tratamiento aplicado. Mientras que la administración de BMPs es ampliamente aceptada como una terapia neuroprotectora frente a daño nervioso, en ciertas situaciones se ha observado que la inhibición de las vías de señalización de BMPs favorecen la recuperación funcional a través de una mejor regeneración de la sustancia blanca (Hampton et al., 2007; Jablonska et al., 2010; Dizon et al., 2011).

Todo esto nos lleva a pensar que un mejor conocimiento del papel que juegan estas dos citoquinas en el desarrollo cerebral y en respuesta a daño nervioso es vital para determinar cómo su regulación puede promover reparación, y que tipo de estrategias se podrían seguir en un futuro para hacer frente a lesiones del CNS en desarrollo.

OBJETIVOS:

1. Caracterizar las vías de señalización implicadas en la expresión de BMP7 dependiente de BDNF durante el desarrollo de la corteza cerebral.
2. Analizar la función de BMP7 sobre la diferenciación de progenitores neuronales y gliales de la corteza cerebral.
3. Determinar el efecto de BMP7 sobre la migración neuronal durante el desarrollo de la corteza cerebral.
4. Estudiar el papel de BMP7 en las poblaciones de la línea media involucradas en la formación del cuerpo calloso.
5. Analizar los cambios en el destino y diferenciación de los progenitores neurales inducidos por modificaciones de los niveles de BMP7 en respuesta a hipoxia sub-letal.

RESULTADOS

LA EXPRESIÓN DE BMP7 INDUCIDA POR BDNF/MAPK/ERK EN LA CORTEZA CEREBRAL INDUCE LA DIFERENCIACIÓN PREMATURA DE LA GLÍA RADIAL Y ALTERA LA MIGRACIÓN NEURONAL

En este estudio partimos de la idea que la regulación de los niveles de BDNF podría actuar como sensor de distintos estímulos fisiológicos, activando específicamente la transcripción de grupos de genes. En el presente estudio mostramos la inducción de la expresión de BMP7 por BDNF tanto *in vivo* como *in vitro*. La inyección de BDNF en el ventrículo lateral de embriones de ratón a E14 produce un aumento de la expresión de BMP7 en la corteza cerebral embrionaria. Para reproducir este efecto *in vitro* utilizamos cultivos neuronales primarios obtenidos a partir de cortezas cerebrales a E16 y cultivos gliales puros de ratones recién nacidos. El análisis mediante real time PCR demostró aumentos significativos en el mRNA de BMP7 a las 6h del tratamiento con BDNF en los cultivos neuronales pero no en los gliales. Para identificar el mecanismo de señalización tratamos los cultivos neuronales con diferentes inhibidores de vías de señalización de BDNF/ TrKB. El tratamiento con wortmanina, un inhibidor de la vía de PI3K/AKT no afectó al incremento de BMP7 inducido por BDNF. Sin embargo, el tratamiento con UO126, un inhibidor específico de la vía de MAPK/MEK, bloqueó completamente la inducción de BMP7 por BDNF.

Dado que recientemente se ha identificado a p53 y su familia de factores de transcripción como inhibidores transcripcionales de BMP7, utilizamos pifithrin-α como inhibidor de p53 y Nutlin 3 como activador de la vía de p53. Nuestros resultados indican que los factores de transcripción de la familia de p53 actuarían como co-represores de la expresión de BMP7 inducida por BDNF, y que en parte BDNF induciría la expresión de BMP7 bloqueando esta represión.

Para analizar la función de BMP7 durante el desarrollo de la corteza cerebral inyectamos la proteína recombinante en el ventrículo lateral de embriones de ratón a

E14. La inyección de BMP7 altera la distribución laminar de las neuronas piramidales corticales a E18, que se acumulan en la zona intermedia bajo la placa cortical, sin afectar a la distribución de las neuronas GABAérgicas. Seguidamente analizamos el estado de la glía radial, ya que es el principal substrato de migración de las neuronas piramidales. Utilizando marcadores de glía radial y de distintos estados de maduración astrocitaria mostramos que BMP7 induce la diferenciación prematura de la glía radial. La disminución de la glía radial unida al aumento de la expresión de la proteína anti-adhesiva *Sparc like 1* en los animales inyectados con BMP7, podría ser la causa del defecto observado en la migración radial de las neuronas piramidales de capas altas.

Finalmente proponemos un modelo fisiológico donde la expresión de BDNF inducida por el inicio de la actividad eléctrica produciría a su vez un aumento de BMP7, proporcionando así un mecanismo para la maduración sincronizada de neuronas y glía al final del periodo de neurogénesis.

NIVELES APROPIADOS DE BMP7 SON NECESARIOS PARA LA DIFERENCIACION DE CÉLULAS SEÑAL DE LA LÍNEA MEDIA INVOLUCRADAS EN LA FORMACIÓN DEL CUERPO CALLOSO

La formación del cuerpo calloso (CC) está dirigida por diferentes poblaciones celulares como el glial wedge (GW), el indusium griseum (IG), la midline zipper glia (MZG), y el subcallosal sling (SCS) de la línea media que dirigen a los axones callosos para que crucen correctamente la zona intermedia y alcancen su región diana (Shu et al., 2003). La agénesis del CC es la formación defectuosa de la comisura callosa, debido a alteraciones en alguno de los múltiples pasos implicados en el desarrollo del CC, entre ellas alteraciones en las poblaciones gliales de la línea media (Richards et al 2004; Paul et al., 2007; Donahoo and Richards, 2009). Los mecanismos de guía de estas poblaciones son todavía bastante desconocidos. Es por eso que estamos muy interesados en investigar BMP7, una citoquina que promueve diferenciación glial en el CNS (Gross et al., 1996; Li et al., 1998; Mabie et al., 1999) y cuya modificación en los niveles de expresión dan lugar a malformaciones en el CC. El uso de un modelo animal

transgénico con un alelo mutante de BMP7 ($Bmp7^{lacZ/Neo}$) permitió observar que esta citoquina se expresa en células de distintas poblaciones de la línea media como son el GW, el IG y el SCS.

Los animales knock out (KO) para BMP7 muestran agénesis del cuerpo calloso. Los axones callosos son incapaces de cruzar la línea media y quedan retenidos en el hemisferio ipsilateral formando un ovillo o Probst bundle. Se analizó la posibilidad de que BMP7 pudiese modificar el destino de las neuronas corticales que proyectan sus axones hacia el CC, sin embargo no observamos diferencias relevantes en la laminación cortical entre los animales KO y los animales control. También se estudió una posible actividad quimiotrópica de BMP7 sobre las neuronas de proyección, pero tampoco observamos cambios en el crecimiento axonal en presencia o ausencia de BMP7. Finalmente observamos que la ausencia de BMP7 afectaba a poblaciones gliales de la línea media (GW e IG) así como al SCS. El GW e IG estaban altamente desorganizados y anormalmente distribuidos a lo largo de la línea media, mientras que el número de células que forman el SCS estaba severamente reducido en la región cortico-septal entre los ventrículos laterales y la región medial.

Sorprendentemente, el modelo de ganancia de función de BMP7, donde embriones E14 eran inyectados con la proteína recombinante de BMP7 a nivel intraventricular, también mostraba agénesis del CC y un desarrollo anormal del GW e IG, marcado por una alta y prematura expresión del marcador astroglial GFAP. Al igual que en el modelo KO estaba muy reducido el número de células del SCS. Estos resultados demuestran la necesidad de unos niveles apropiados de BMP7 para la correcta formación de las poblaciones guía de la línea media, cuya alteración desemboca en un desarrollo anómalo del CC.

LOS NIVELES DE OXÍGENO MODULAN EL DESTINO DEL LINAJE GLIAL A TRAVÉS DE MODIFICACIONES EN LOS NIVELES DE EXPRESIÓN DE BMP7

La hipoxia perinatal es una de las mayores causas de discapacidad crónica, incluyendo parálisis cerebral, retraso mental o epilepsia en niños. Se ha observado que los niveles de expresión de BMP7 están alterados en respuesta a lesión H-I y que el tratamiento con esta citoquina mejora la recuperación funcional después de este tipo de daño cerebral. En esta tesis se han llevado a cabo modelos hipóxicos *in vitro* e *in vivo* para analizar posibles cambios de expresión de BMP7 inducidos por hipoxia sub-letal, y si estos cambios están asociados con modificaciones en los progenitores corticales.

Las BMPs son factores de regulación importantes para mantener el equilibrio entre las poblaciones astrogliales y oligodendrogliales. Durante el desarrollo perinatal, las BMPs promueven diferenciación astroglial en detrimento de diferenciación oligodendroglial a partir de progenitores neurales. Cultivos corticales sometidos a hipoxia mostraron una reducción en los niveles de expresión de BMP7, seguido por un aumento en el número de progenitores oligodendrogliales marcados con NG2. De igual manera, ratones sometidos a hipoxia hipobárica pre-natal (E16) mostraban una bajada inicial de los niveles de expresión de BMP7 y una immunoreactividad más alta para marcadores oligodendrogliales como el NG2 y Olig2 respecto a los animales control. Para corroborar los resultados obtenidos utilizamos sendos modelos de ganancia y pérdida de función de BMP7, mediante tratamientos con la proteína recombinante de BMP7 y uno de sus inhibidores, la folistatina (FST). Como esperábamos, el tratamiento con FST imita el efecto conseguido en condiciones hipóxicas, donde los niveles de BMP7 disminuyen. El tratamiento de FST aumenta la immunoreactividad por marcadores oligodendrogliales *in vivo* e *in vitro*, mientras que el tratamiento con BMP7 produce un efecto completamente opuesto en el desarrollo de las poblaciones gliales, aumentando la expresión del marcador astroglial GFAP y reduciendo los niveles de NG2 y Olig2.

Estos resultados demuestran que los niveles de BMP7 deben estar altamente regulados para la correcta diferenciación de los progenitores corticales durante el

desarrollo perinatal. Un desequilibrio entre diferentes poblaciones corticales en la corteza cerebral causado por niveles de expresión anormales de BMP7, puede llevar a una disfunción en los circuitos cerebrales y desencadenar desordenes cognitivos y conductuales como los descritos en algunos casos de hipoxia hipobárica pre-natal (Simonová et al., 2003; Maiti et al., 2006). El mejor conocimiento del efecto de BMP7 sobre la corteza cerebral inmadura puede ayudar a identificar tratamientos potenciales que prevengan desordenes neurológicos producidos durante el desarrollo perinatal.

DISCUSIÓN

BDNF INDUCE LA EXPRESIÓN DE BMP7 A TRAVÉS DEL RECEPTOR TrkB Y LA VÍA DE SEÑALIZACIÓN DE MAPK/ERK

Este trabajo demuestra que BDNF es uno de los factores que modulan la expresión de BMP7 durante la corticogénesis. BDNF induce la expresión de BMP7 básicamente en neuronas pero no en células de glía. Esta diferencia se puede explicar porque las neuronas expresan el receptor completo de TrkB mientras que la glía expresa una isoforma truncada que carece del dominio intracelular tirosina quinasa (Cheng et al., 2007; Islam et al., 2009). La unión de BDNF a la forma completa de TrkB produce la dimerización y posterior activación quinasa-dependiente que inicia un proceso de autofosforilación del receptor. Esto da lugar a la activación de las vías intracelulares como las vías proteína quinasa (1) Ras-Raf-ERK (extracellular signal-regulated kinase), (2) fosfatidilinositol-3-quinasa (PI-3kinase)/Akt, y (3) fosfolipasa C (PLC)-γ1 (Kaplan and Miller 2000). El análisis realizado mediante inhibidores de TrkB y de las diferentes vías de señalización indican que la expresión de BMP7 dependiente de BDNF se produce a través de la activación de TrkB y de la vía MAPK/ERK.

Además se observó que la familia de proteínas de p53 reprime la expresión de BMP7. La inhibición de la actividad transcripcional de p53/73 promueve la regulación al alza

de BMP7, mientras que la inducción de la actividad de estas proteínas genera una reducción proporcional al aumento observado con el tratamiento inhibidor. Estos datos indican que miembros de la familia de p53 tienen una actividad co-represora sobre la expresión de BMP7 y que BDNF induciría la expresión de BMP7 en parte liberando esta actividad represora. Por otro lado, el hecho de no bloquear completamente la expresión de BMP7 con ninguno de los inhibidores utilizados sugiere que hay otros mecanismos que participan en la modulación de la expresión de esta citoquina.

FUNCIONES DE BMP7 EN EL DESARROLLO DE LA CORTEZA CEREBRAL

Los datos obtenidos demuestran que el aumento de los niveles de BMP7 induce cambios en la maduración glial *in vivo* e *in vitro*. Aumentos de los niveles de BMP7 durante la corticogénesis embrionaria, a través de inyecciones intraventriculares a E14, produce alteraciones en la migración de las neuronas piramidales destinadas a capas altas. Ensayos inmunohistoquímicos contra marcadores específicos de capas altas descartaron que BMP7 estuviera alterando la distribución de estas neuronas en la corteza cerebral a través de cambios en su determinación laminar. Las neuronas corticales de capas altas se generaban con normalidad pero no eran capaces de alcanzar su correcta localización laminar. Observamos que los animales inyectados con BMP7 mostraban una clara perdida de radialidad del andamio de glía radial, estructura vital para el proceso de migración radial llevado a cabo por las neuronas corticales. Esta anomalía se produce por una prematura transformación de la glía radial hacia astrocito. Además, los animales inyectados con BMP7 mostraban otros síntomas de maduración cortical prematura, como la temprana expansión de la SVZ, la cual empieza a expandirse en fases más tardías en el desarrollo normal. Durante la neurogénesis, los progenitores que se encuentran en la SVZ dan lugar a progenitores neurogénicos amplificadores que aumentan la población neuronal en la corteza (Reillo et al., 2010), aunque durante el desarrollo post-natal temprano esta región es también una fuente de células astrogliales y oligodendrogliales (Levison and Goldman, 1993). Los animales tratados con BMP7 muestran un mayor número de células progenitoras

desplazadas de la VZ a la SVZ que los animales control, así como una reducción de progenitores subventriculares destinados a producir neuronas. Estos datos sugieren que BMP7 induce una temprana transformación de la glía radial hacia progenitores gliales de la SVZ. Además, los animales inyectados con BMP7 mostraban un aumento de la expresión de SC1, una proteína de matriz extracelular producida por astrocitos, confirmando el aumento de maduración astroglial observada en los animales tratados. SC1 es una proteína con características anti-adhesivas distribuida en zonas altas de la placa cortical que parece estar relacionada con el desenganche de la glía radial por parte de las neuronas en migración (Gongidi et al., 2004). Por tanto, la prematura maduración de la glía radial hacia astrocito inducida por BMP7, acompañada por el aumento de expresión de SC1, explicaría la acumulación ectópica de neuronas corticales en la zona intermedia en vez de en capas altas.

El efecto de BMP7 sobre la maduración glial es también crucial para el correcto desarrollo de las poblaciones gliales de la línea media que participan en la formación del CC. Animales con niveles anormales de BMP7 muestran agénesis del CC, que se caracteriza por la incapacidad de las fibras callosas de cruzar la zona intermedia y la formación de un ovillo fibroso conocido como Probst bundle. La ausencia de BMP7 produce una marcada disminución en la expresión de marcadores astrogliales en las poblaciones de la línea intermedia como el GW y el IG, mientras que los animales inyectados con BMP7 muestran una fuerte diferenciación astroglial en estas estructuras mediales. También se observa una importante disminución del número de células del SCS producida tanto por niveles anormalmente altos como bajos de BMP7 durante el periodo de formación del CC. Por tanto es necesaria una expresión temporal y espacialmente controlada de BMP7 para la correcta generación y diferenciación de las poblaciones de la línea media.

Es de suponer que cambios en estas poblaciones celulares vayan acompañados de modificaciones en la liberación de moléculas señal que modulan el crecimiento y expansión de los axones callosos. Inicialmente nos centramos en Slit2, un ligando quimio-repelente expresado por las células del GW e IG, que guían a los axones callosos a cruzar la línea media evitando que estos entren en la región del septum (Shu

and Richards, 2001; Shu et al., 2003b). Aunque tanto las estructuras del GW como la del IG estén severamente afectadas por niveles anormales de BMP7, los niveles de expresión de Slit2 parecían bastante normales. Sería entonces interesante analizar otras moléculas secretables como Wnts, Netrins, Ephrins, Draxin y Semaphorins, conocidas por jugar un papel esencial en mecanismos de guía axonal así como también componentes de la matriz extracelular, como heparan sulfato proteoglicanos o condroitin sulfato proteoglicanos, capaces de captar las moléculas de guía secretadas con las cuáles pueden establecer gradientes para los procesos de guía axonal.

Todos estos datos demuestran la necesidad de un estricto control tanto de BDNF como de BMP7 para el desarrollo de diferentes estructuras cerebrales. En esta tesis se sugiere un sistema de maduración neuronal y glial sincronizado por la acción conjunta y coordinada de BDNF y BMP7 durante el desarrollo cerebral. Durante el periodo perinatal, las señales externas que recibe el individuo y que se traducen en una alta actividad eléctrica, llevarían a un aumento en la expresión de BDNF. Está ampliamente descrito que BDNF promueve supervivencia neuronal, diferenciación neuronal, crecimiento neurítico y plasticidad sináptica (Revisado por Huang and Reichard, 2001; Numakawa et al., 2010). A su vez BDNF induce la expresión de BMP7 en la corteza, la cual promovería la maduración astroglial durante las primeras semanas de desarrollo post-natal. De esta manera ambas citoquinas sincronizarían la supervivencia y diferenciación neuronal y astroglial a la llegada de nuevos axones producida durante las fases de actividad cortical inicial. La alteración del patrón de expresión espacio-temporal de estos factores podría afectar de una manera importante la citoarquitectura cortical y por consiguiente el correcto funcionamiento cerebral. De hecho diferentes condiciones patológicas asociadas al desarrollo embrionario y perinatal están relacionadas con el desequilibrio de los niveles de BDNF y BMP7, y de sus respectivos receptores (Madri et al., 2009; Lindval et al., 1992; Korhonen et al., 1998; Hubold et al., 2009; Lewen et al., 1997; Charytoniuk et al., 2000; Setoguchi et al., 2001; Hampton et al., 2007). Ambas citoquinas juegan un papel neuroprotector y neuroregenerativo en respuesta a distintos daños en el CNS (Cheng et al., 1997; Galvin et al., 2003; Sun et al., 2008; Chou et al., 2005; Meng et al., 2005; Tsai et al., 2007; Guo et al., 2008; Sabo et al., 2009; Tsai et al., 2010). Sin embargo, cambios de expresión en

estas citoquinas puede tener efectos secundarios en el altamente regulado programa de desarrollo cerebral.

LA REDUCCIÓN DE LOS NIVELES DE BMP7 EN RESPUESTA A HYPOXIA PERINATAL PROMUEVE LA DIFERENCIACIÓN DE LOS PROGENITORES CORTICALES HACIA UN LINAJE OLIGODENDROGLIAL

La hipoxia es un condición patológica capaz de inducir cambios en los niveles de expresión y señalización de BDNF y BMPs (Martinez et al., 2001; Schmidt-Kastner et al., 2002; Pistollato et al 2007, 2009; Panchision, 2009). Individuos que han sufrido traumas hipóxicos fetales muestran unos niveles de BDNF reducidos, lo cual está asociado a distintos desordenes mentales como es el caso de la esquizofrenia (Cannon et al., 2008). En nuestros modelos hipóxicos hemos observado también una reducción en la expresión de esta neurotrofina. BDNF tiene una gran influencia en los procesos cognitivos y conductuales a través de su función como potente inductor de la supervivencia celular, la morfogénesis, y la plasticidad sináptica (Numakawa et al., 2010). Es por ello que es plausible asociar los efectos negativos generados por la hipoxia con la reducción de los niveles fetales de BDNF.

Los efectos deletéreos de una hipoxia perinatal se piensa que son consecuencia de alteraciones en la diferenciación neuronal, sinaptogénesis así como en una excesiva muerte celular de neuronas, células de glía y de sus respectivos progenitores (Curristin et al., 2002). Efectos que pueden ser atenuados a través de la rápida aplicación de BMP7 después del evento traumático (Chang et al., 2003; Chou et al., 2005; Chou et al., 2006). Los resultados que aquí se presentan muestran como una hipoxia sub-letal sostenida durante 12 horas produce una inicial regulación a la baja de los niveles de expresión de BMP7 *in vitro* e *in vivo.* En el modelo *in vivo* se observa como la hipoxia hipobárica prenatal puede inducir cambios en la señalización de BMPs en la VZ/SVZ. Pistollato y colaboradores (2007, 2009) observaron un efecto similar con otros BMPs y postularon que esta represión es debida a la sobreactividad de HIF1α, el mediador en respuesta a oxígeno mejor descrito. Niveles bajos de oxígeno promueven la

estabilización de HIF1α el cual ejerce un efecto represivo sobre la vía de señalización de BMPs bloqueando la activación de Smad1/5/8 (Pistollato et al., 2007, 2009; Panchision, 2009).

La inhibición de la señalización de BMP7 producida por hipoxia se ha asociado al mantenimiento del estado auto-renovador de los progenitores neurales. En cambio el aumento de expresión de BMP induce una rápida regulación a la baja de HIF1α como ocurre en condiciones hiperóxicas (en cerebro se considera hiperoxia por encima de valores de 5-7 % de oxígeno), lo que lleva a la depleción de los precursores corticales y a una diferenciación astroglial (Pistollato et al., 2007, 2009; Panchision 2009). De acuerdo con estos resultados, los datos aquí presentados muestran como BMP7 y los niveles de oxígeno dentro de las regiones germinales de la corteza cerebral pueden modular el balance entre auto-renovación/diferenciación de los progenitores neurales durante el desarrollo cortical. BMP7 es capaz de modular la decisión de destino glial de la glía radial y de los progenitores subventriculares que dan lugar a astrocitos y oligodendrocitos durante las primeras semanas de desarrollo post-natal. Los oligodendrocitos derivan de los precursores O-2A que se encuentran en la corteza cerebral y que pueden dar lugar también a astrocitos tipo-2. El comportamiento plástico de los precursores O-2A se puede modular por diferentes factores entre ellos las citoquinas, que pueden controlar el periodo de extensión y producción de oligodendrocitos modulando el equilibrio entre la auto-renovación y la diferenciación de los precursores O-2A en división (Smith et al., 2000). En nuestro modelo de hipoxia, observamos que la reducción de los niveles de expresión y señalización de BMP7 van acompañados de una mayor diferenciación de los progenitores corticales hacia oligodendroglia en detrimento de la formación de astrocitos.

Los resultados de esta tesis indican que cambios en la expresión de BMP7 durante el periodo crítico de desarrollo perinatal, pueden modificar de manera importante el destino celular de los progenitores corticales en el rigurosamente controlado programa de desarrollo cerebral. Estas modificaciones pueden comprometer seriamente el correcto funcionamiento de los circuitos cerebrales en el adulto.

CONCLUSIONES

1. BDNF induce la expresión de BMP7 en neuronas a través del receptor TrkB y la vía de señalización MAPK/ERK. Un mecanismo de inducción que está mediado en parte por la liberación de la actividad represora ejercida por la familia de proteínas de p53.

2. BMP7 promueve maduración astroglial *in vivo* e *in vitro*.

3. La función fisiológica de la inducción de BMP7 dependiente de BDNF sería la de establecer un mecanismo de maduración neuronal y glial sincrónico en respuesta a actividad al inicio de la sinaptogénesis.

4. Niveles anormalmente altos de BMP7 durante el desarrollo cortical induce la maduración prematura de la glía radial hacia astroglía y altera la migración de las neuronas piramidales de capas altas, las cuales se acumulan de una manera anómala en capas corticales más bajas.

5. El correcto patrón de expresión de BMP7 es necesario para una correcta maduración de las estructuras intermedias como son el glial wedge, el induseum griseum y el subcallosal sling, las cuales aportan las señales de guía necesarias para la formación del cuerpo calloso.

6. La hipoxia sub-letal reduce los niveles de expresión y señalización de BMP7 en la corteza cerebral. Esta reducción induce la diferenciación de los progenitores corticales hacia el linaje oligodendroglial en detrimento del astroglial.

INTRODUCTION

1. THE CEREBRAL CORTEX

1.1. CYTOARCHITECTURE OF THE CEREBRAL CORTEX

The central nervous system (CNS) is the organizer center of our motor, sensitive and comprehensive activities. During evolution, motor and sensitive nuclei appear, being located from deeper to more superficial positions in the brain. Cerebral cortex is the nucleus that emerges the last, and its complexity varies depending on the animal necessities. The human cerebral cortex is the most complex within the mammals, allowing extremely complex motor activities such as language, writing, painting, sport, etc., that are exclusively in human. The cerebral cortex, also named pallium (from the latin term pallidum that means mantle) is a grey matter mantle that covers both cerebral hemispheres. An anatomical classification divides cerebral cortex in lobuli, giri, surci and fissures, while a phylogenetic classification classify the different cortical areas depending on its age in the zoological scale, distinguishing between *archicortex, paleocortex* and *neocortex*. The function and the number of layers where the bodies of cortical neurons and axons are distributed are dramatically different between different cortical areas. The archicortex in human is formed by hippocampus and dentate gyrus. It is related with emotional instincts and is composed of only three cortical layers. The paleocortex is the pallium associated with the sense of smell and can be composed of three to five cortical layers. Finally, the neocortex is the most evolved and complex cortex that occupies the most part of the cerebral cortex. From functional point of view, neocortex is associated with the most complex integrative functions, such as language and intellectual activity (Valverde 2002).

The neocortex is formed by a sheet of neurons in six different organized horizontal layers intersected by vertical or radial columns that respond to specific signals (Rakic 2007). The neurons within a given column are highly interconnected and share a common function, acting as basic units that exert a set of common static and dynamic cortical operations (Rakic 2008). Sensorial experiences at the beginning of postnatal life are necessary for a correct maturation of columnar organization. Cortical columns

are organized into distinct functional areas, such as motor control, vision and hearing (Fig.1). The distinct cerebral cortex areas can be also distinguished by their particular cytoarchitecture, chemoarchitecture, connectivity and gene expression.

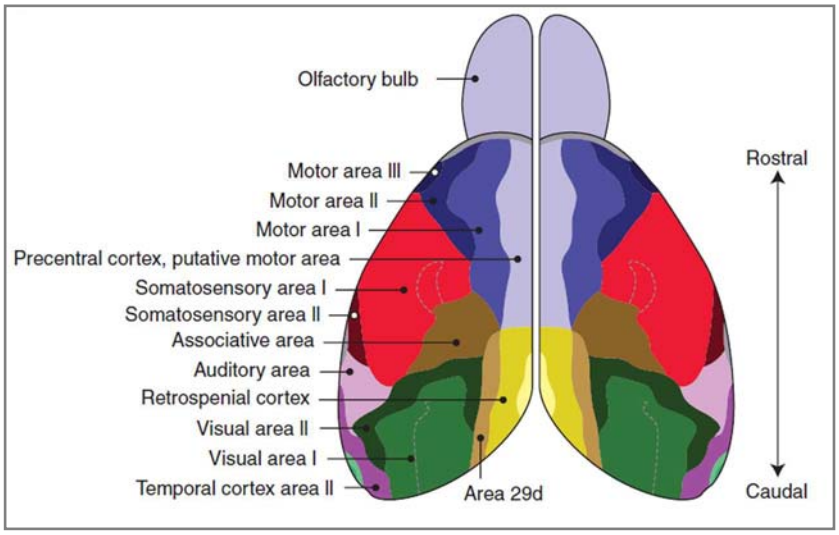

Fig.1. Cerebral cortex is organized into different functional areas that can be distinguished by their histological characteristics. Adapted from *Sansom and Livesey, 2009.*

The number of neurons in the whole cerebral cortex varies between $2{,}6x10^{9}$ and $1{,}6x10^{10}$ depending on the counting technique used. The main cell type that represents 80% of neurons in the neocortex is known as *pyramidal neurons*, characterized by a long axon and pyramidal shaped soma with a 10 to 50 µm size. There are particular exceptions within this group of neurons, such as *Betz cells* (giant pyramidal cells) with a soma size of 100 µm, or *fusiform cells* with abnormal elliptic cell bodies. Another characteristic of pyramidal neurons is that they have many apical and lateral dendrites with branches full of dendritic spines. From the base of the pyramid or from one of the biggest dendrites emerges the axon that sends many collateral ramifications before arrive to subcortical white matter. Pyramidal neurons are also named glutamatergic neurons, due to the fact that their axons release glutamate as a neurotransmitter in the synapsis, exerting an excitatory action. Pyramidal neurons can be also classified according to the type of connection they establish: 1. *Projecting neurons* connect with subcortical regions such as striatum, brainstem, spinal cord or thalamus. 2. *Associating neurons* establish connections with other cortical neurons of

the same hemisphere. 3. *Comissural neurons* send axons to the opposite hemisphere (Valverde 2002).

The second main cell group in the cerebral cortex is composed by cortical *interneurons*. Within this group of neurons it can be observed different types of cells that show particular dendritic architectures (Fig.2). In the cortical layer IV there are the *stellate cells* that own dendritic spines and exert an excitatory action probably by secreting glutamate, unlike the rest of interneurons which act as inhibitors secreting gamma aminobutiric acid (GABA) in their synapsis. *Basket cells* are another type of interneurons that laterally branch their axons around pyramidal neurons. *Martinotti cells* are distributed in deeper layers with axons projecting to the pial, while *Cajal-Retzius cells* are a transitory population observed in the most superficial cortical layer during embryonic an early postnatal development (Greenstein and Greenstein, 2000). Cajal Retzius cells utilize glutamate as a neurotransmitter although their networking action is still rather unknown (del Rio, Martinez et al. 1995; Hevner, Neogi et al. 2003; Soriano and Del Rio 2005).

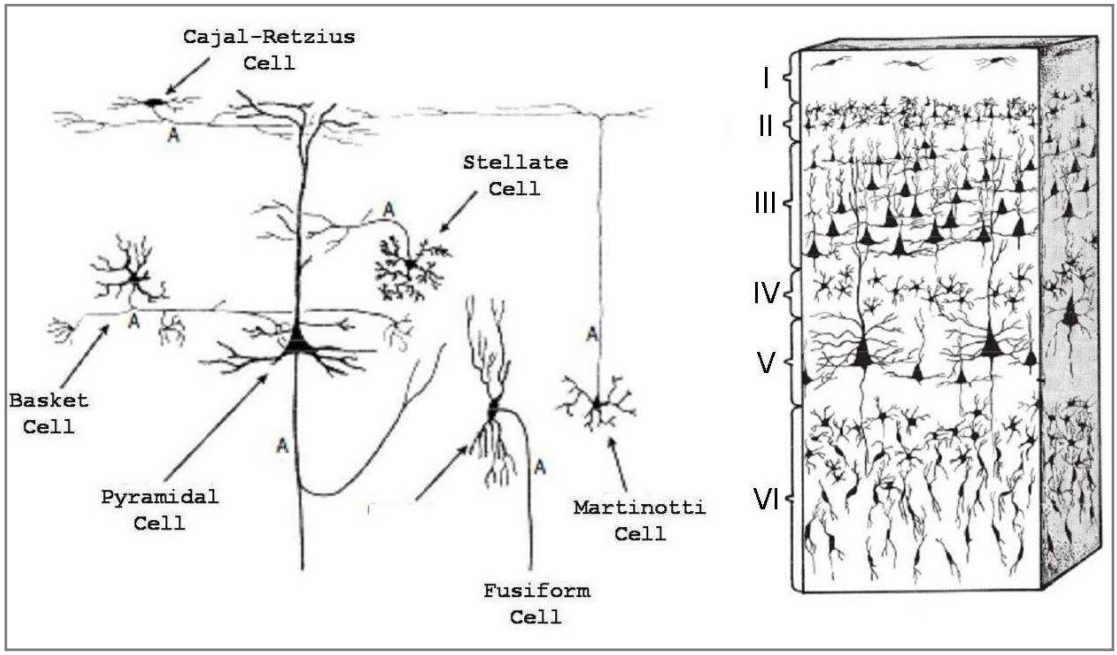

Fig.2. Cellular morphology and relative cortical distribution of the different neuron types observed in the mammalian cerebral cortex. (A) indicates the axonic projection. Modified from *John A. Kiernan, Barr's The Human Nervous System: An Anatomical Viewpoint, Ninth Edition*.

The mammalian neocortex consists of six different neuronal layers which have different thickness and neuronal composition depending on the cortical region. In the six main layers of isocortex we can identify: *Layer I,* is the most superficial layer composed by a low number of cells and large number of terminal projections of dendrites from pyramidal neurons and axons mainly projected from other cortical regions of the one and the opposite hemisphere, and also from the thalamus. Deep inhibitory Martinotti cells also send axons to this layer. Among dendritic and axonal terminations there are few number of Cajal-Retzius cells during cortical embryonic development up to two weeks after birth. *Layer II/III* are composed by little interneurons as well as little and medium pyramidal neurons. Layer II/III pyramidal neurons send dendrites to the layer I and their axons are projected to deeper layers and to other cortical areas, especially to the opposite hemisphere generating the main cerebral comissure, the corpus callosum (Fig.3). In *layer IV* there are basically stellate cells and few pyramidal cells. Most extracortical projections innervate layer IV. In layer V there are interneurons and bigger pyramidal neurons than those from layer II/III. *Layer V* neurons project their axons to subcortical areas such as striatum, brainstem and spinal cord. Finally, *layer VI* is composed by fusiform cells and Martinotti interneurons. Output axons from this layer connect with the thalamus, and as some axons from layer V, also project to contralateral hemisphere. Extracortical afferents arrive to layer VI although in a lesser degree than in layer IV (Valverde 2002; Sansom and Livesey 2009).

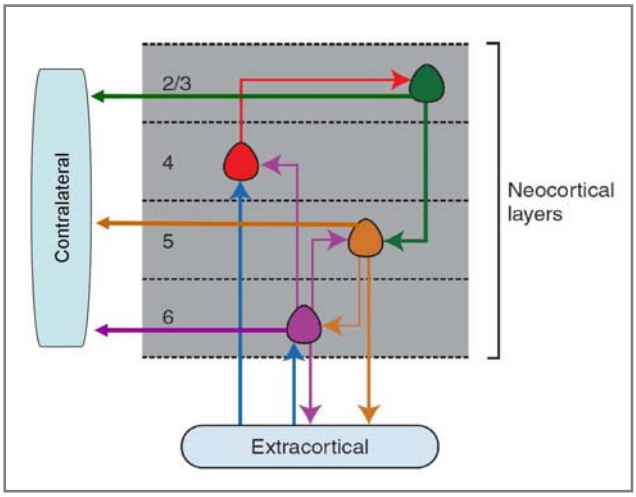

Fig.3. The basic cortical axonal tracts observed in cerebral cortex. Modified from *Sansom and Livesey, 2009.*

Functionally, within the cortical columns it can be distinguished association layers from projection layers. Association layers receive inputs from neighbouring regions, while projecting layers send information to distant regions such as basal nuclei, brainstem, thalamus and spinal cord. Thus, it can be said that layers I, II and III act as an intracortical associating region, receiving projections from the same cortex, whereas layer IV receives specific axons from the thalamus. On the other hand, layer V and VI are efferent layers that project axons to cortical areas of the same and the opposite hemisphere, and to subcortical nuclei (Fig.3) (Murayama, Perez-Garci et al. 2009; Sansom and Livesey 2009; Tau and Peterson 2009). The correct organization of cerebral cortex is vital for a correct functioning. On the contrary, and as we will review in section 4, a disruption of the cerebral cortex histogenesis can provoke important neurological disorders as epilepsy, mental retardation and distinct behavioural disorders.

In this work I have used the mouse as an animal model because of the facilities in manipulation and progeny obtaining as well as the possibility of generate transgenic models. The most interesting point is the similarity in cortical structures and cellular mechanisms implicated in the cerebral cortex development between mouse and human. Although rodent and human cerebral cortex are different in dimension, configuration and developmental timing of the germinal zones, both the pallial origin and radial migration of glutamatergic neurons as well as the subpallial origin and tangential migration of GABAergic neurons are surprisingly conserved across both mammalian species (Molnar, Metin et al. 2006). A part from the logical difference in size, two main differences are observed between mouse and human cerebral cortex (Fig.4). First, in the mouse cerebral cortex, the neocortical layers II and III are not typically distinguished as distinct compartments, while they are expanded and obviously distinct in primates (Fame, MacDonald et al. 2011). Second, human subventricular zone (SVZ) cells are observed earlier in corticogenesis and show a much greater expansion than in mouse during development. Human SVZ is a specialized cortical structure formed by two different regions, the outer and the inner SVZ, while in mouse this subventricular stratification is not observed (Smart, Dehay et al. 2002). Histologically, the outer SVZ is very different to SVZ in rodents. The specialized

compartimentation of SVZ in humans may be associated to a higher neuronal diversity of neocortical superficial layers (Tarabykin, Stoykova et al. 2001). The contribution of the neocortical SVZ to neuronal production seems to increase during evolution with the increasing complexity of the cerebral cortex among species (Reillo, de Juan Romero et al. 2010).

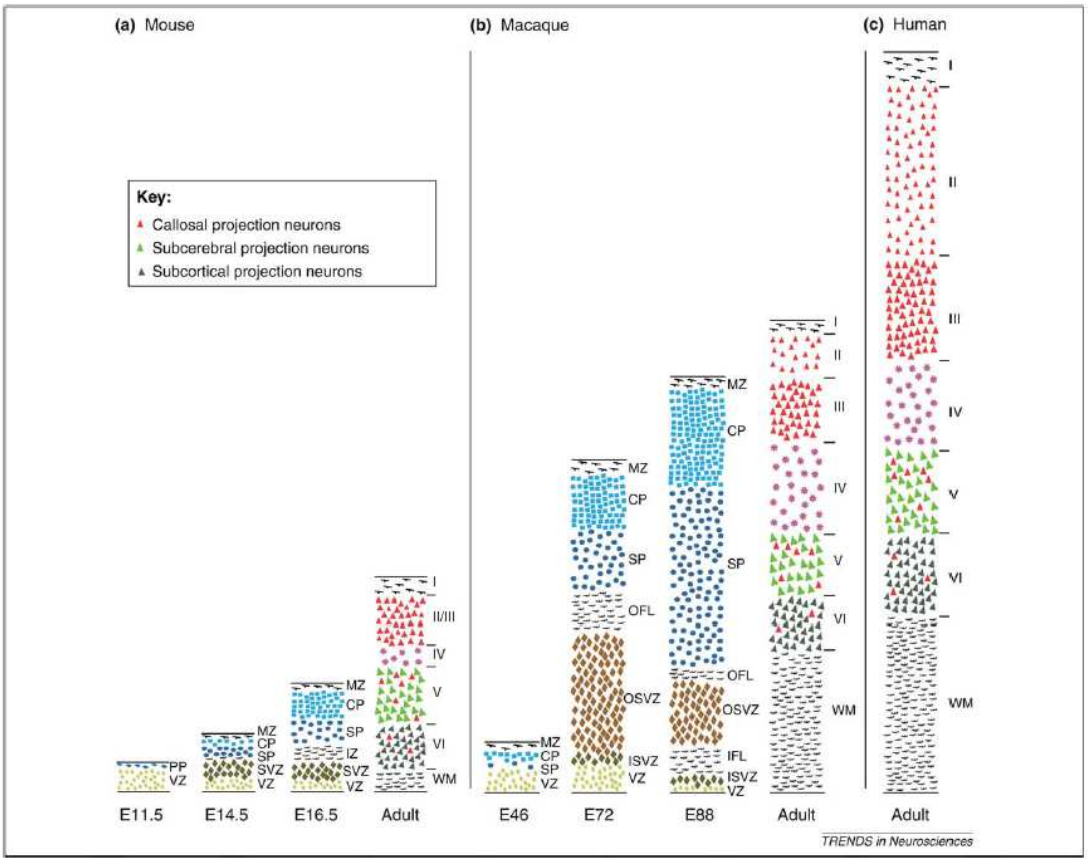

Fig.4. Comparison of developing and adult cerebral cortex of mouse, macaque and human. Main differences are observed in layer II/III and SVZ between mouse and human cerebral cortex. Adapted from *(Fame, MacDonald et al. 2011).*

1.2. CEREBRAL CORTEX HISTOGENESIS

In the early embryonic development, vertebrate CNS arises from a specialized part of embryonic ectoderm located in the most anterior part of the neural plate, which folds giving rise the neural tube. The inner part of this tube develops in the future ventricles, and the walls are composed by neuroepithelial cells of the telencephalon. In the

telencephalic wall a single proliferating cell layer close to ventricles constitutes the ventricular zone (VZ). This germinal region starts to divide asymmetrically generating first neurons that migrate away. As neurogenesis proceeds, an additional proliferative region known as the subventricular zone (SVZ), is formed above the VZ. Progenitors from both regions generate the neurons of the different neocortical layers in a tightly controlled temporal order from embryonic day (E) 11.5 to E17.5 in the mouse. Newborn neurons will reach their final positions following defined modes of radial and tangential migration (Molyneaux, Arlotta et al. 2007).

The first wave of cortical neurons accumulates on the top of the VZ giving rise to the preplate (PP) which contains early appearing Cajal Retzius cells, GABAergic neurons and pioneer neurons (Meyer, Schaaps et al. 2000). Subsequent generated neurons invade the PP forming the cortical plate (CP) and dividing the PP in two different regions separated by the CP: the marginal zone (MZ) just beneath pial surface, and a deeper zone composed by subplate (SP) neurons and incoming axons of the intermediate zone (IZ) (Marin-Padilla 1978). Both layers are essential for a correct cerebral cortex development. On the one hand subplate neurons act as guidepost for afferent thalamic axons which innervates the cerebral cortex (Wang, Hoerder-Suabedissen et al. 2010). On the other hand, the marginal zone is populated during development by Cajal-Retzius cells which secrete reelin, an extracellular matrix glycoprotein required for radial migration of newborn glutamatergic neurons (D'Arcangelo, Miao et al. 1995; D'Arcangelo, Nakajima et al. 1997; Alcantara, Ruiz et al. 1998). Next newly born neurons from VZ will generate the 6-layered cortex according to a very organized program (Fig.5). Autoradiographic studies performed by Angevine and Sidman in 1961 demonstrated that depending on the birth date of the newborn neurons, they will be finally located in different cortical layers. The accepted model about cortical layer formation is based in an *inside-out* model, where the earliest born neurons generated in the VZ migrate radially constituting the deeper layers, while later neuronal cohorts pass the previously form neurons and settle within most-upper layers.

This is a valid model of genesis and migration for excitatory projection neurons, but other cortical neurons do not follow it. Many studies have revealed that most local GABAergic inhibitory neurons emerge from the VZ/SVZ of the lateral and medial ganglionic eminences (LGE and MGE) (Parnavelas, Anderson et al. 2000; Anderson, Marin et al. 2001; Marin and Rubenstein 2001; Xu, Cobos et al. 2004). From these ventral forebrain regions they migrate dorsally to reach their final cortical position without using the radial glia, in a process known as tangential migration (Rakic 2001).

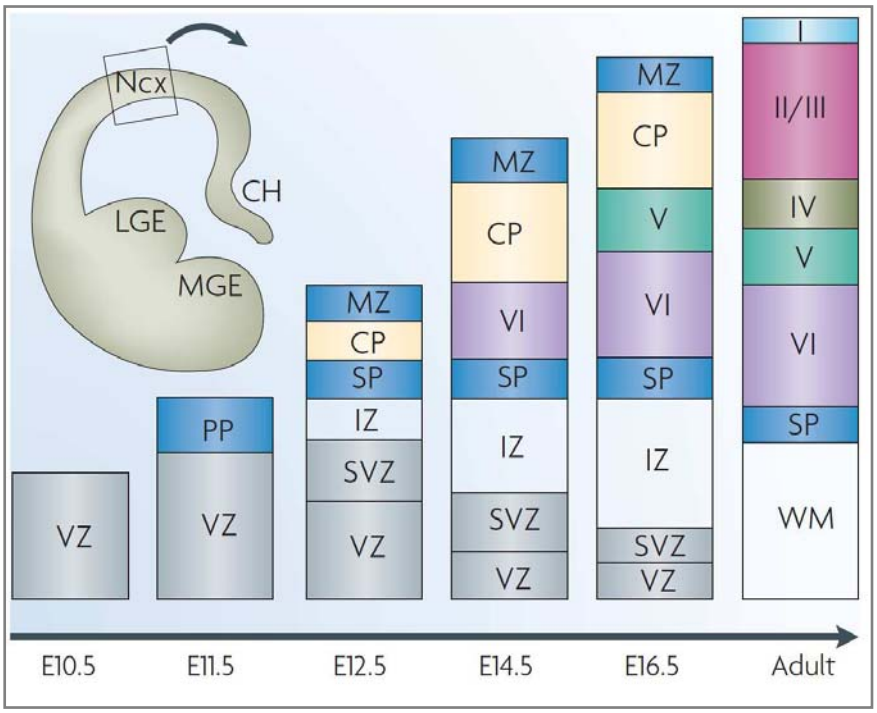

Fig.5. Diagram representing cerebral cortex histogenesis. The earliest born neurons form the preplate (PP), split into the more superficial marginal zone (MZ) and the deeper subplate (SP). The cortical plate (CP) develops in between these two layers, and later born neurons arriving at the cortical plate migrate past earlier born neurons forming the multilayered cerebral cortex. CH, cortical hem; Ncx, neocortex; IZ, intermediate zone; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; SVZ, subventricular zone; VZ, ventricular zone; WM, white matter. Adapted from *Molineaux et al., 2007*.

1.3. NEUROGENESIS

Neurogenesis is a process by which neural stem cells (NSC) give rise to all the neurons that will form the mature CNS. During embryonic development there are two main neurogenic regions: the ventricular zone (VZ) and the subventricular zone (SVZ). In rodents, embryonic neurogenesis is initiated at embryonic day 12 (E12) and VZ is the major proliferative zone until late corticogenesis (E15), when starts to decline its activity and a secondary proliferative zone, the SVZ, assumes an increasingly important role on neuron production.

1.3.1. THE VENTRICULAR ZONE (VZ)

As neurogenesis proceeds, the neuroepithilium changes into a multi-cell-layered tissue, where progenitor cell bodies are attached to the most apical cell layer forming the VZ. This pseudostratified neuroepithilium was detailed described already by His and Ramon y Cajal at the beginning of the twentieth century, defining this region as a structure with several cell diameters in thickness and mitotic figures adjacent to the ventricular surface. Both scientists agreed on postulate that the mitotic figures were germinal cells which, after continual division, give rise to two daughter cells; one would remain attached to ventricular surface and the second would develop into a new neuron. The cells attached to the ventricular epithelium would form a syncytium, origin of glial cells, and the separated ventricular cell population would give rise to neuroblast.

More than one century later, and after hundreds of studies, is known that neuroepithilial progenitors give rise to radial glial cells (RGC). Both types of cells are highly related although are notably different at the same time, basically by the expression of astrocyte markers by RGC (Kriegstein and Götz 2003; Gotz and Barde 2005). A variety of proteins like vimentin, and nestin, and transcription factors like Pax6, can be used as markers to identify RGC. Furthermore, taking advantage of the proliferative nature of RGC, S-phase markers such as Bromodeoxyuridine (BrdU, a

Timidine analogous), and different proteins present in particular phases of the cell cycle as Ki67 and phosphorilated histone H3 (M-phase marker), also allow identify these progenitor cells.

RGC span from the ventricular zone to the pia, maintaining the basal-apical polarization along the cerebral wall, and being the major neuronal progenitor population. In the dorsal telencephalon, RGC appear at the beginning of neurogenesis (at embryonic day 12-13 in mice) and they give rise directly or indirectly, through the generation of basal progenitors, to most of cortical projection neurons (Parnavelas 2000). While in the basal telencephalon, ventricular progenitors promote the formation of cortical interneurons (Anderson, Marin et al. 2001).

In mouse, during six-day neocortex formation, ventricular progenitors carry out 11 cell cycles, becoming their length longer after each cycle. During the cell cycle, RGC execute interkinetic movements throughout the epithelium of the VZ (Fig.6 and Fig.7). The nucleus of a progenitor cell moves from the ventricular surface at the beginning of G1 to the border of the VZ when S-phase starts. The germinal cell goes back to the ventricular surface during G2, entering there on M-phase and giving rise to two daughter cells. One of these daughter cells quit the cell cycle, and it becomes a post-proliferative cell, a postmitotic neuron that will migrate away from the VZ to populate the future mature brain (Fig.6) (Noctor, Martinez-Cerdeno et al. 2004; Guillemot 2005). The output of neurons and other cell types that will form the mature brain from the ventricular zone, is basically controlled by the number of proliferating cells, the length of their cell cycle, the period of time that the proliferative population exists, and the rate between the number of daughter cells that quit the cell cycle and the daughter cells that remain proliferative (Cai, Hayes et al. 2002; Gotz and Huttner 2005).

It has been also proposed, that the output of neurons from the pseudostratified ventricular epithilium is controlled by three different possible types of mitotic divisions: First, symmetric nonterminal cell division, which produces two daughter cells that continue proliferating without going out of the subventricular surface and so

expanding the pool of neuronal precursor cells; Second, asymmetric cell division, where one daughter cell continues proliferating in the VZ, and the other quits the cell cycle and migrates out of VZ; and third, symmetric terminal cell division, which produces two post-mitotic daughter cells that migrate out of the VZ, thus depleting the pool of proliferative cells (Cai, Hayes et al. 2002; Gotz and Huttner 2005; Guillemot 2005). It is generally accepted that all three types of division occurs within the ventricular neuroepithilium (Fig6).

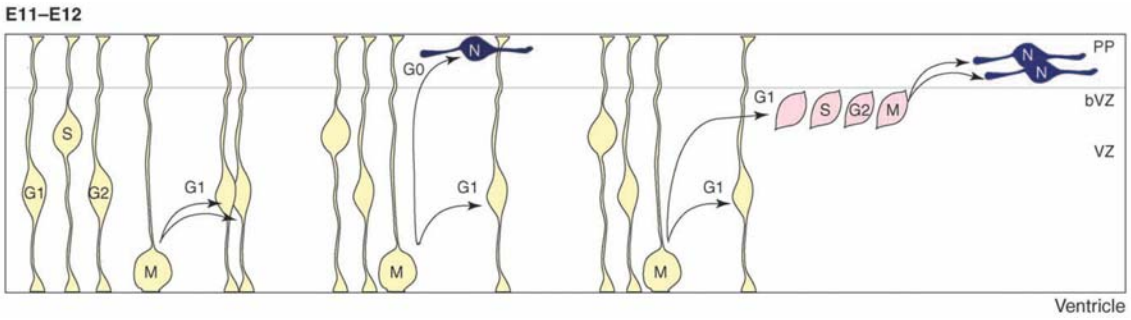

Fig.6. During neurogenesis ventricular progenitors undergo interkinetic nuclear migration and divide symmetrically to generate two new neuroepithelial cells or asymmetrically to produce both a neuroepithelial progenitor and a new neuron. Adapted from *Guillemot, 2005*.

RGC generate cortical neurons and intermediate or basal progenitors. Post-mitotic newborn neurons originate from RGC migrate to reach their corresponding position in the developing cortex, while basal progenitors appear at the onset of neurogenesis and also migrate away from the ventricular surface where they establish in a second proliferative layer at the basal side of the VZ generating the SVZ (Fig.7). During postnatal period, this secondary proliferative region will aid to increase the neuronal pool in the cortex whereas RGC lose their ventricular attachment and they transform into astrocytes.

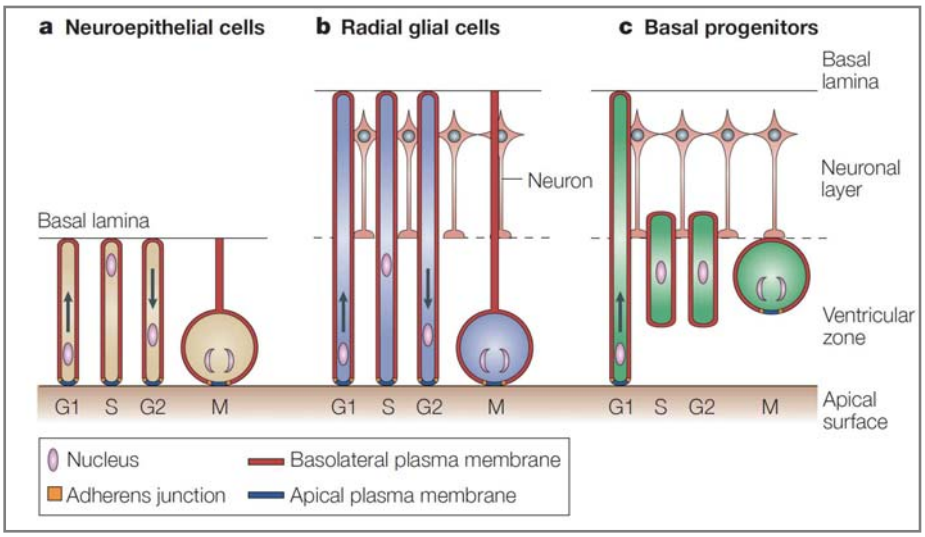

Fig.7. Summary of the three neuronal progenitors observed in the ventricular surface during cerebral cortex development. The image also shows the interkinetic nuclear movements and the loose of apical contact by basal progenitors. Adapted from *Götz and Huttner, 2005*.

1.3.2. THE SUBVENTRICULAR ZONE (SVZ)

As development proceeds, VZ diminish and its role it is carried out by a second proliferative zone overlapped with basal VZ and extended into the intermediate zone, the subventricular zone (SVZ). SVZ will continue proliferating and giving rise to cortical neurons and glial cells. In fact, SVZ maintain its neurogenic capacity during adulthood (Lois and Alvarez-Buylla 1993), being one of the main studied issues for its neural regenerative potential.

SVZ cells were firstly described by Schaper and Cohen in 1905 as mitotic figures clearly distant from VZ. It was already seen, that they differ from VZ progenitors basically because they are not attached to each other as a pseudostratified epithilium, and they do not exert interkinetic nuclear migration during their cell cycle (Altman and Bayer 1990). Moreover SVZ cells have shorter processes instead long radially oriented processes as RGC have (Rakic 1974). Recent studies demonstrate that both types of cell progenitors, ventricular and subventricular progenitors, are responsible of generating the majority neurons in the cerebral cortex during the embryonic period

(Noctor et al., 2007). The populations of ventricular and subventricular progenitors share common markers like phosphorilated histone H3 (PH3), an M-phase marker, hence demonstrating their proliferative nature.

Initial studies identified this secondary proliferative population as the origin of glial progenitors (Takahashi, Nowakowski et al. 1995). During early postnatal development, subventricular progenitors are the main source of astroglial and oligodendroglial cells (more detailed information in chapter 2). Nevertheless, recent video time-lapse studies identified neurogenic subventricular progenitors, named intermediate progenitor cells (IPC), which divide in basal positions in the VZ, SVZ and intermediate zone, producing two neurons in one or two mitotic cycles (Noctor, Martinez-Cerdeno et al. 2004; Englund, Fink et al. 2005; Pontious, Kowalczyk et al. 2008). From midstages of cortical neurogenesis onwards, while VZ starts to diminish in size SVZ expands as a result of symmetric proliferative IPC division (Fig.8) (Noctor, Martinez-Cerdeno et al. 2008; Pontious, Kowalczyk et al. 2008). In fact, IPC has been proposed as a neurogenic transit amplifying progenitors which would exponentially increase the neuronal pool in the cortex (Reillo, de Juan Romero et al.). During mammalian evolution SVZ enlarges in line with cortical expansion (Molnar, Metin et al. 2006). Some studies have pointed out that the early loss of IPC produces a dramatic decrease in cortical surface expansion and thickness along the cortical wall demonstrating the importance of this progenitor population (Reillo, de Juan Romero et al. 2010; Sessa, Mao et al. 2008).

Apart from the differences in morphology and cortical distribution between cortical IPCs and RGCs, both progenitor populations can be distinguished by the expression of characteristic transcription factors. Englund and collaborators clearly described the sequential expression pattern of Pax6→Tbr2→Tbr1 during the differentiation of RGC (Pax6+) to IPC (Tbr2+) and the resulting newborn neuron (Tbr1+) (Englund, Fink et al. 2005). Other transcription factors allow distinguish RGC from IPC too: Emx2 and Sox2 are expressed exclusively in RGC, while Cux1-2, Lmo4 and Svet are exclusive to IPC (Tarabykin, Stoykova et al. 2001; Nieto, Monuki et al. 2004; Gotz and Huttner 2005).

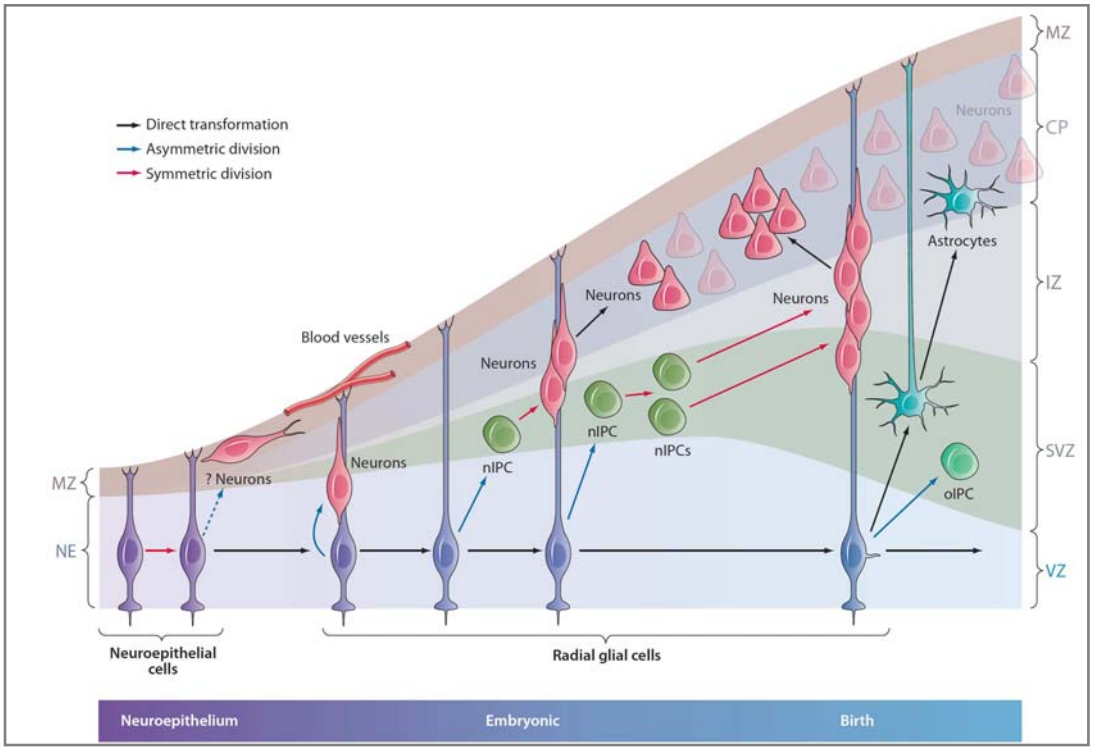

Fig.8. Ventricular radial glial cells are able to generate neurons and intermediate progenitors (nIPC), which in turn amplify the neuronal pool from SVZ. In addition, during postnatal development different pool of subventricular glial progenitors derived from RGC also generate astrocytes and oligodendrocytes. Adapted from *Kriegstein and Alvarez-Buylla, 2009.*

T-box-brain-2 (Tbr2, NCBI: Eomes) has been widely used as a specific IPC marker. Little is known about the function of this IPC marker during cerebral cortex development because Tbr2 mutant embryos are not able to differentiate trophoblast cell linage and stop their development (Russ, Wattler et al. 2000; Strumpf, Mao et al. 2005). Tbr2 gene encodes a member of the T-box family of transcription factors that are characterized by the common feature of the evolutionary highly conserved T-box DNA-binding domain. During corticogenesis Tbr2+ subventricular progenitor cells are observed in the SVZ from E10.5 to birth, giving rise to upper layer neurons exclusively as defend Tarabykin (Tarabykin, Stoykova et al. 2001) and Cubelos (Cubelos, Sebastian-Serrano et al. 2008) among others (Pinto, Drechsel et al. 2009). Nonetheless there is another model that postulates that these IPC contribute indirectly to all cortical layers (Englund, Fink et al. 2005; Kowalczyk, Pontious et al. 2009). This theory is strongly supported by studies that put forward that conditional ablation of Tbr2 leads to IPC loss and a dramatic neuronal reduction observed in all cortical layers (Sessa, Mao et al.

2008). Further studies also carried out by Sessa and collaborators even demonstrate that Tbr2-positive intermediate (basal) neuronal progenitors would exert an additional function that consist on attracting subpallial GABAergic interneuron to dorsal regions of cerebral cortex (Sessa, Mao et al. 2010).

Finally, SVZ in the walls of the lateral ventricles is also the most prominent neurogenic region in the adult mammals. Its capacity to give rise to cortical neurons and glial cells during adulthood makes it one of the main studied issues in the neuroscience field to understand brain development and adult neurogenesis and to develop new strategies for brain repair (Lois and Alvarez-Buylla 1993; Kriegstein and Alvarez-Buylla 2009).

1.4. NEURONAL MIGRATION DURING CEREBRAL CORTEX DEVELOPMENT

During brain development, neuronal populations are initially generated in a region different than where they finally differentiate. The two main neuronal populations in the cerebral cortex, pyramidal neurons and GABAergic interneurons, follow different patterns of migration in order to reach their final position. Pyramidal neurons generated in the cortical VZ use radial migration to reach their proper position, while GABAergic interneurons, generated in the ganglionar eminence, initially migrate in a tangential mode following different chemical cues until they reach the cerebral cortex. The main difference between both migratory processes is that in radial migration migrating neurons closely interact with RGC using them as a scaffold for move through cerebral cortex, while in tangential migration migrating neurons do not require this physical support (Noctor, Martinez-Cerdeno et al. 2004).

Once in the cerebral cortex, GABAergic neurons also use radial glia scaffold to move ventrally, toward VZ, or dorsally, toward pial surface, to reach their final position. So, RGC play a key role in cerebral cortex histogenesis because they produce cortical neurons and they serve as a rail for the migration of these newborn neurons. Recognition mechanism between neurons and glia are essential in radial migration, but it is also vital the recognition between neurons, and neurons with the extracellular

matrix, in radial and tangential migration. Neurons, glial cells and extracellular matrix own many elements that seems to direct many processes within the neuronal migration (Marin and Rubenstein 2003).

1.4.1. RADIAL MIGRATION

Cortical neurons migrate radially into the cortical plate by two possible modes: translocation and glial-guide locomotion. Soma translocating neurons are directly attached to pial surface with a long process, and once they loose their ventricular attachment translocating cell moves its nucleus to reach their appropriate location within the cortical plate (Nadarajah and Parnavelas 2002). Translocation is observed during early stages of corticogenesis. By contrast, locomoting neurons have a relatively short leading process (100-200 µm) and their migration is guided by RGC. The morphology and distribution of RGC along cerebral cortex are essential for being the main support of locomoting neurons. RGC are minutely arranged forming a scaffold with a particular environment that altogether allow the correct locomotion of migrating neurons. Migratory locomoting neurons establish specialized membrane contacts with RGC such as interstitial junction, punctae adherentia or junctional domains that are essential for direct a correct migration on radial glia substrate (Gregory, Edmondson et al. 1988; Cameron and Rakic 1994; Anton, Marchionni et al. 1997). In addition, surface and extracellular matrix signals play a critical role during neuronal migratory process. All these signals direct the stages of radial migration communicating where and when a neuron may stop, detach or differentiate.

From their origin site to the specific laminar location, neuronal locomotion passes through three sequential phases: initiation, maintenance of migration and termination at the appropriate cortical layer. Initiation of migration entails the detachment of the newborn neuron from the ventricular surface and its displacement towards the cortical plate. Actin cytoskeleton seems to be essential in this process since mutations in actin-binding protein filamin 1, which participates in actin filaments network dynamics and is

highly expressed in VZ cells, were observed to produce accumulation of neuroblasts in the VZ (Ferland, Batiz et al. 2009).

Once newborn neurons leave the ventricular surface many processes such as adhesion mechanism, cell-cell recognition, transmembrane signalling and cell motility, control the maintenance of radial migration. Migrating neurons initially attach to RGC by specialized junctional domains in their leading process and cell soma. In this phase microtubule cytoskeleton is vital for the stability of neuron-radial glia joint (Gregory, Edmondson et al. 1988; Cameron and Rakic 1994). Microtubules generate the necessary force for cell movement and also participate in the elaboration and maintenance of junctional domains. Several mutations in proteins related with microtubules cytoeskeleton dynamics are related with abnormal neuronal migration and the subsequent aberrant cerebral cortex lamination (Fig.9). One of these mutations is related with Lis1, a protein that binds microtubules with dynein, a microtubule based motor protein, and other microtubule interactors such as dynactin, NUDEL and mNudE. In humans, Lis 1 mutations lead to lyssencephaly, a brain formation disorder with lack of development of grooves and folds caused by defective neuronal migration. In mice, mutations in Lis1 lead to slower neuronal migration and cortical plate disorganization (Cahana, Escamez et al. 2001). It was observed that neurons with an aberrant Lis 1 fail to aggregate dynein and organize microtubules network around the centrosome properly (Smith, Niethammer et al. 2000). Nonetheless, it is still poorly understood the direct connection between microtubules network defects and nuclear translocation fails in migrating neurons caused by mutations in Lis 1 gene. Doublecortin (Dcx) is another mitrotubule-associated protein expressed by neuronal precursor cells and immature neurons in embryonic and adult cortical structures that can cause lissencephaly and double cortex syndrome in humans. Dcx facilitates the polimerization and stabilization of the microtubules and its mutation cause aberrant neuronal migration during corticogenesis, characterized by a second underlying layer of neurons in the cerebral cortex (Friocourt, Marcorelles et al.; Gleeson 2000).

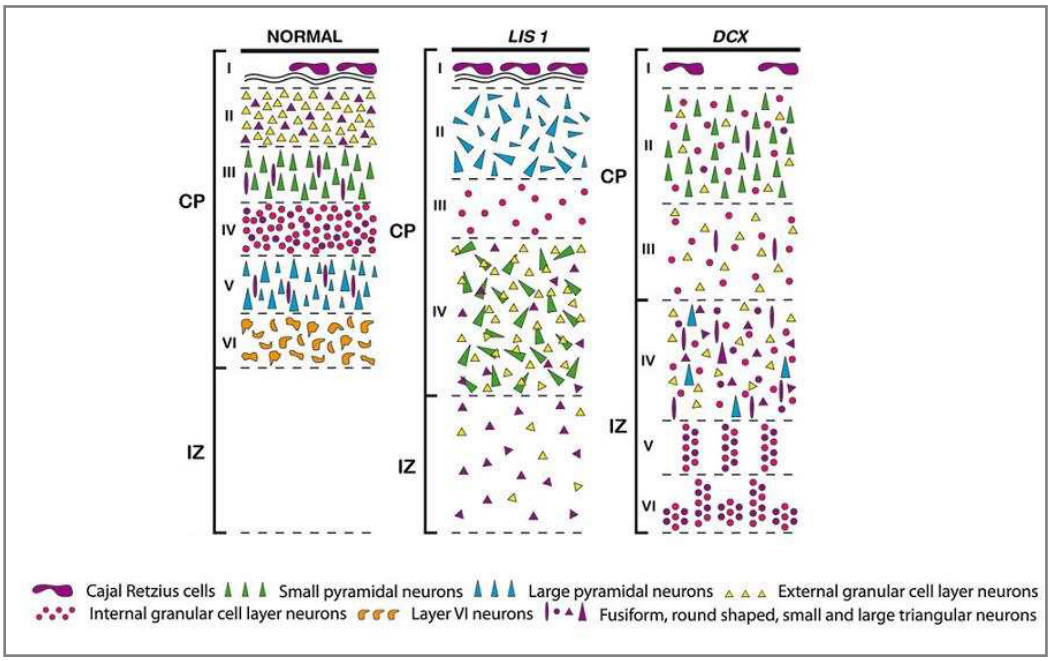

Fig.9. Scheme of cortical lamination defects produced by mutations in genes associated with microtubules cytoskeleton regulation. Adapted from *Friocourt et al., 2011*.

In radial migration maintenance, it is also very important the extracellular environment surrounding migrating neurons. External signals such as glutamate, GABA, neuregulins and neurotrophins are detected by neurons during their migration and can modulate radial-guided neuronal migration (Anton, Marchionni et al. 1997; Komuro and Rakic 1998; Marin and Rubenstein 2003). Extracellular cues can trigger activation of internal pathways involved in cytoskeletal changes that modulate the mechanics of migration. One of the most known neuronal receptors related with direct link between extracellular cues and internal modifications in the cytoeskeleton are integrins. Integrins receptors are composed by heterodimers of α subunits that preferently dimerize with β_1 subunit, which is ubiquitously expressed in the developing cerebral cortex. Different integrin heterodimeric receptors are related with different aspects of neuronal migration during corticogenesis. Mutational studies have allowed finding out the roles of distinct integrins subunits in neuronal migration. For instance, homozygous mice for a mutation in the α_3 integrin show a complete lose of neuronal organization along the cerebral cortex. The function of α_3 integrin is associated with neuron-glia recognition by the migrating neurons. Their absence produced a switch in neuronal adhesive preferences that might lead to the abnormal neuronal distribution observed

in the mutant animals (Anton, Kreidberg et al. 1999). Other mutations in α subunits more related with transient cell-matrix interactions and cell-anchoring mechanisms have demonstrated their importance in the neuronal migration process during cortical development. The β_1 integrin can dimerize with at least 10 different α subunits, and its deficiency is lethal early in development. Conditional transgenic animal experiments, that allow get knock out for β_1 integrin from E10, also demonstrate the association of this integrin subunit with neuronal radial migration. The absence of β_1 integrin leads to an error in radial glial end feet formation triggering and abnormal distribution of neurons in the cerebral cortex (Graus-Porta, Blaess et al. 2001).

The final stage of neuronal migration is when neurons reach the top of the cortical plate and they stop abruptly, detach and differentiate into their respective cortical layer. It has been proposed that the termination signal can be provided by the afferent axons which are found by migrating neurons during their route (Pearlman and Sheppard 1996; Super and Uylings 2001). Although the environment in the interface between the cortical plate and marginal zone, or the change in cell surface properties of the radial glia at the top of the cortical plate seem to play a very important role during the termination of neuronal migration too (D'Arcangelo, Miao et al. 1995; Anton, Marchionni et al. 1997; Gongidi, Ring et al. 2004; Tachikawa, Sasaki et al. 2008; Chai, Forster et al. 2009).

Studies about termination of migration have used reeler mouse, so named because its characteristic gait. This is caused by deep hypoplasia of the cerebellum, in which the normal cerebellar folia disappears. This animal is also characterized by an inverted cortex with late-born neurons located in deeper layers and newborn neurons in the more superficial cortical layers (Caviness and Sidman 1973). Subplate neurons fail to stop and invade the uppermost layer, creating the so-called superplate in which they mix with Cajal-Retzius cells and some cells normally destined for the second layer. Reeler phenotype is due to a mutation in the locus that encodes Reelin, an extracellular matrix protein secreted by the marginal zone Cajal-Retzius cells (D'Arcangelo, Miao et al. 1995). Reelin binds to VLDLR and ApoER2 receptors, members of the low density lipoprotein (LDL) receptor superfamily which are present

on both neurons and glial cells. On the one hand, VLDLR and ApoER2 receptors have a NPXY motif, which is implicated in clathrin-mediated endocytosis. On the other hand, VLDLR and ApoER2 receptors bind Dab1 intracellulary, and the binding of reelin to these receptors leads to Dab1 phosphorylation and the subsequent activation of a complex regulation mechanism of neuronal migration (Fig.10). When Dab1 becomes phosphorylated stimulates actin cytoskeleton to change its configuration, affecting the proportion of integrin receptors on the cell surface, which promotes a change in cell adhesion. Phosphorylation of DAB1 also leads to its ubiquitination and subsequent degradation, and this explains the heightened levels of DAB1 in the absence of reelin (Feng, Allen et al. 2007). Mutations in this three main molecules implicated in the reelin signalling (VLDLR, ApoER2 and Dab1) caused the same phenotypic defects than reeler gene mutation. Although it remains unclear how reelin acts on migration or adhesion of cortical neurons, different theories hypothesize its function on cortical layering. Firstly, reelin may control cerebral cortex organization directing the preplate splitting into marginal zone and subplate as reeler mice cerebral cortex show cortical neurons underneath subplate neurons (Sheppard and Pearlman 1997). Secondly, a gradient of reelin from the marginal zone act as attractive or at least permissive cue in a multistep functional process of radial neuron migration, allowing late-born neurons to arrive to the most superficial layer of the cortical plate crossing through the earlier born neurons (Soriano and Del Rio 2005). Thirdly, recent studies propose a new view of reelin function on neuronal migration, regulating at the same time somal translocation and detachment from radial glia in a very coordinated fashion (Cooper 2008; Huang 2009). Finally, some *in vitro* studies have shown that reelin may induce the stop of neuronal radial migration at the marginal zone acting together with α_3 integrin signalling (Dulabon, Olson et al. 2000; Huang 2009). In glial-guided migrating neurons, α_3 integrin may interact with glial cell surface molecules such as fibronectin or laminin-2. However, once neurons reach the top of the cortical plate, the preferences of α_3 integrin may change from extracellular matrix glial molecules to reelin, promoting detachment of migrating neurons from radial glia rail (Hack, Bancila et al. 2002). Thus, Reelin may orchestrate the entire process of neuronal migration from the beginning to the end, controlling the timing of activation and termination signalling.

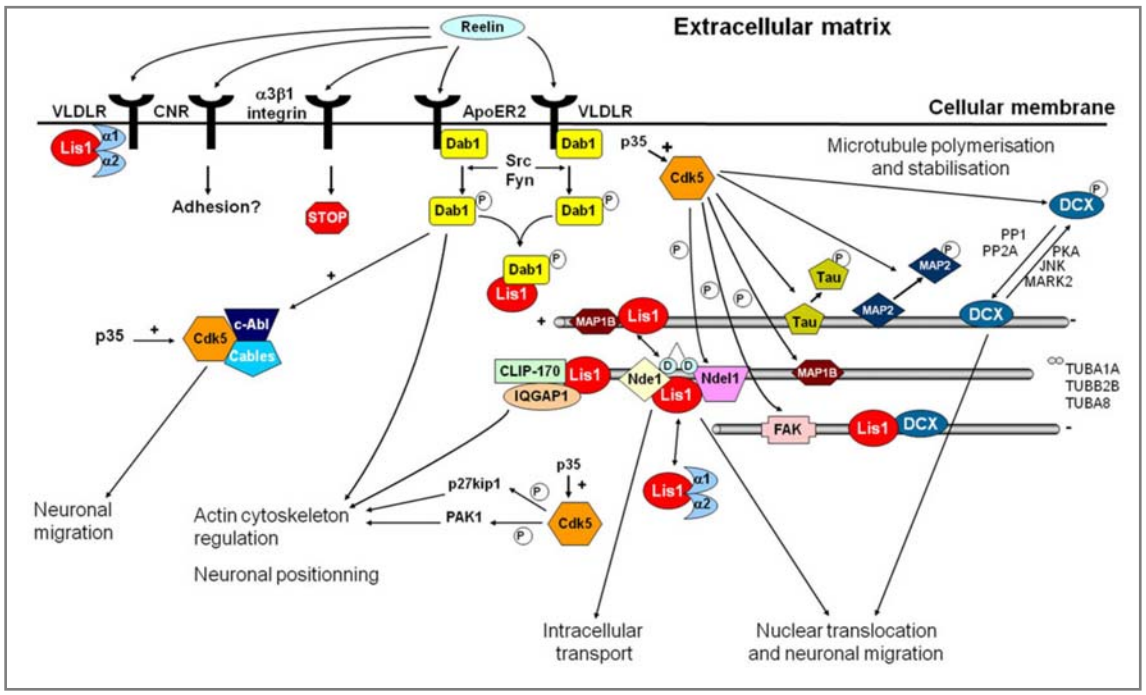

Fig.10. Scheme showing the complex regulation of cytoskeletal dynamics exerted by reelin linked with neuronal migration during cerebral cortex development. Adapted from *Friocourt et al., 2011*.

There are other less studied extracellular molecules expressed in the region between the top of the cortical plate and the marginal zone that exert a vital function in termination of neuronal migration. SPARC-like 1 (SC1), is an extracellular matrix protein identified in the radial glia surface, in the boundary of upper cortical plate. SC1 is particularly interesting by its temporal and spatial distribution during corticogenesis but also by its anti-adhesive properties. Experiments carried out by Gongidi and collaborators (Gongidi, Ring et al. 2004) clearly show SC1 as one of the most important cues for cessation of migration, enabling the detachment of migrating neurons from their radial glial guide at their proper cortical layer.

1.4.2. TANGENTIAL MIGRATION

Tangential migration is referred to as a nonradial mode of neuronal translocation without requiring direct contact with RGC. Initially, it was thought that the majority of the neurons reached their final position through radial migration (Rakic 1978). Nevertheless, posterior studies demonstrated that depending on neural linage, neurons followed different migratory route. Thus, most glutamatergic pyramidal neurons produced in the cortical ventricular zone migrate radially, whereas most GABAergic neurons produced in subpallial ventricular zone reach the cerebral cortex by tangential migration.

The origin of cortical GABAergic neurons was determined after mutational studies of Dlx1/2 double knockout mice, two transcription factors expressed in ventricular and subventricular progenitor cells of the subpallial ganglionar eminence (GE). The lack of homeodomain proteins Dlx1/2 produces the absence of cell migration from the subcortical telencephalon to the neocortex and an acute reduction of GABA-expressing cells in the neocortex (Anderson, Eisenstat et al. 1997). The GE is a structure located in the ventral telencephalon or subpallium that can be divided into three neuroanatomical substructures: the medial (MGE), the lateral (LGE) and the caudal (CGE) region. These three regions may represent three different sources of cortical interneurons that can also be distinguished by a distinct expression pattern of transcription factors (Nakajima 2007). Cajal at the beginning of twentieth century demonstrated that there is an important diversity within the population of interneurons with particular morphological, physiological and molecular properties. By recent tracing analysis it has been observed that GABAergic cells generated in the MGE migrate tangentially into the most dorsal region of the cerebral cortex during intermediate stages of corticogenesis giving rise the majority of interneurons of the cortex including GABA-, parvalbumin- or somatostain-expressing interneurons (Anderson, Marin et al. 2001; Wichterle, Turnbull et al. 2001; Nakajima 2007). Later than MGE cells, LGE cells migrate ventrally and anteriorly giving rise to different GABAergic neurons in different brain regions such as striatum, nucleus accumbens and olfactory bulb. Finally, CGE showed a distinct pattern of migration contributing to give

rise to interneurons in different nuclei from those populated by the MGE and LGE such as amygdala, the posterior striatum or globus pallidum (Nery, Fishell et al. 2002). CGE has been also proposed as a source of hippocampal interneurons and calretinin-containing cortical cells (Fig.11.)(Xu, Cobos et al. 2004).

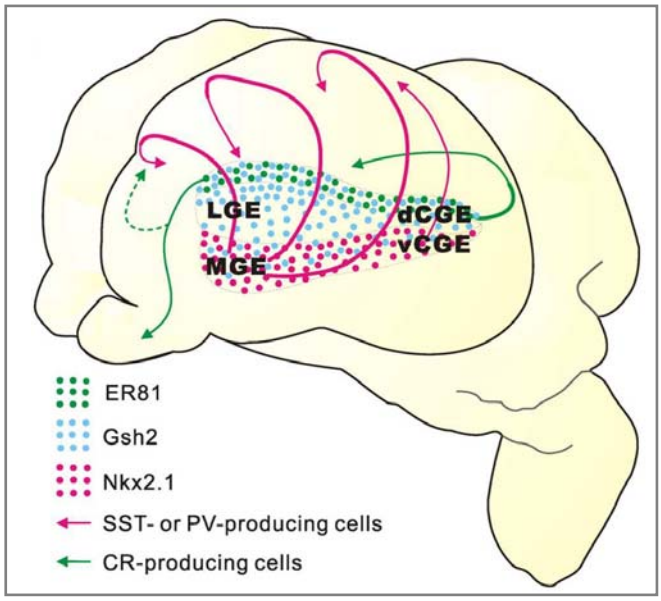

Fig.11. Main migratory pathways followed by interneurons produced in the ganglionar eminences (LGE: lateral; MGE:medial; CGE: caudal) to reach the cerebral cortex. Adapted from *Nakajima, 2007.*

Time-lapse imaging studies have revealed the existence of multiple tangential migration pathways (Metin, Baudoin et al. 2006). They demonstrated that GABAergic interneurons can reach cerebral cortex migrating tangentially mainly through the IZ/SVZ, subplate and marginal zone (Fig.12) (Denaxa, Chan et al. 2001; Ang, Haydar et al. 2003). At the early stages interneurons appear almost exclusively in the MZ while at later stages the routes are at the level of IZ/SVZ and SP (after the split of the MZ). In the MZ, interneurons show varied orientations and enter the developing CP by downward migration. Cells that enter the cortex through the lower streams migrate to the CP directly or after a short pause in the VZ. Subsequently, these interneurons migrate radially to reach their proper cortical layer (Fig.13).

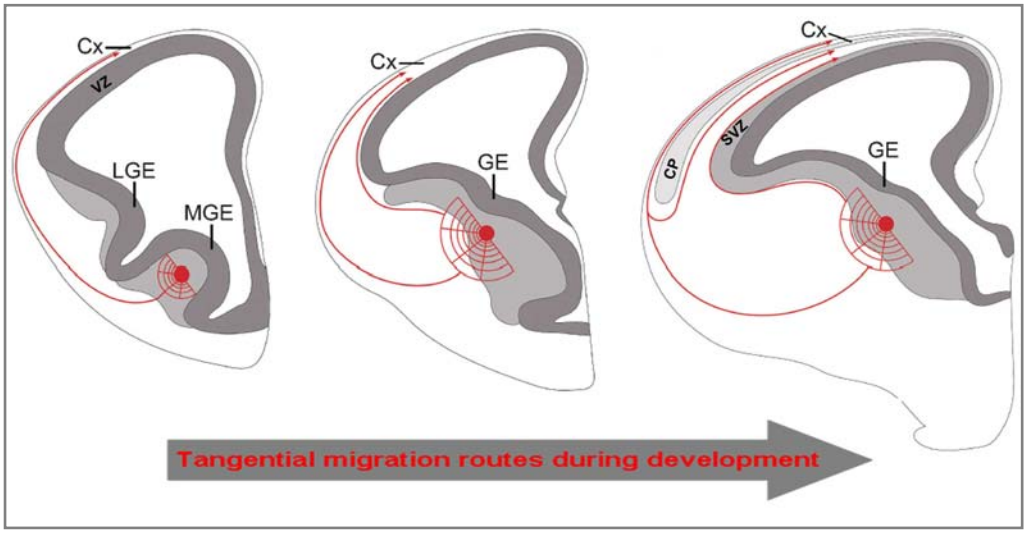

Fig.12. Interneurons reach the cerebral cortex by different tangential migration routes. At the early stages, interneurons almost exclusively enter by MZ while at later stages, when PP splits into MG and SP, they also follow routes at the level of IZ / SVZ and SP. Modified from *Métin et al., 2006.*

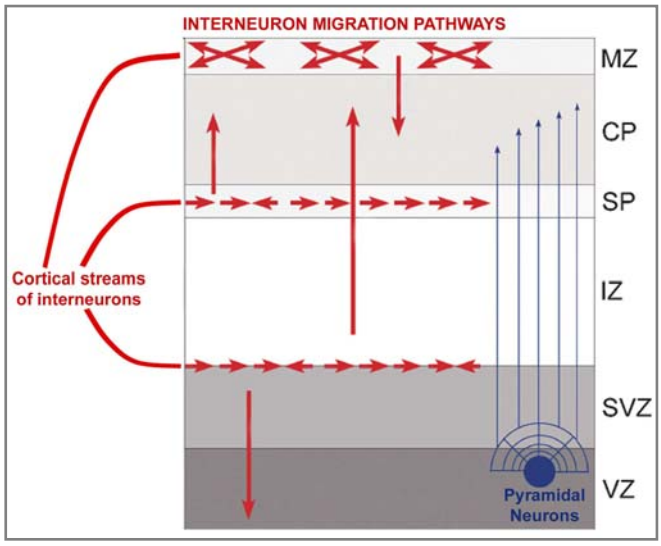

Fig.13. Migratory interneurons follow different orientations within the cerebral cortex depending on their entrance route in the cortex. Modified from *Métin et al., 2006.*

1.4.3. SUBSTRATES OF INTERNEURONS FOR TANGENTIAL MIGRATION

Interneurons do not use RGC processes as a scaffold during their migration to the cortex. However it has been described that interneurons may interact with corticofugal axons through TAG-1, a neural cell-adhesion molecule that plays a key role in this non-radial neural migratory mode (Denaxa, Chan et al. 2001). Otherwise, it has been described many extracellular signals that control in different ways interneuron tangential migration in the forebrain. Several factors along migrating pathway act as a regulators of the motility of migrating interneurons. Hepatocyte growth factor (Powell, Mars et al. 2001) and neurotrophins such as brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT4) (Polleux, Whitford et al. 2002) are considered potent motogenic factors and have been observed to stimulate the migration of cortical interneurons. There are also guidance cues composed by factors such as Sema 3A, Sema 3F, Neuropilin and Slits, that through chemotactic mechanisms direct interneuron migration from GE to the cortex and avoid their entrance into striatum. Studies performed by Marin and collaborators (Marin, Yaron et al. 2001) shown that class 3 semaphorins are distributed along the striatum and its receptor, neuropilin (Npn1 and 2) is expressed by future cortical interneurons. This work demonstrated that class 3 semaphorins may exert a chemorepulsive action, making the striatum as region of exclusion for interneurons destined to cerebral cortex.

On the contrary, there are also chemoattractive factors for cortical interneurons, such as neuregulin (NRG1) and stromal cell-derived factor-1 (SDF-1), which create a permissive environment for migrating GABAergic neurons in their way toward the cortex. During normal development, a diffusible and membrane bound isoforms of NRG1 are expressed along LGE, making an attractive corridor used by MGE-derived interneurons which express the NRG1 receptor, ErbB4 (Fig.14) (Flames, Long et al. 2004). Stumm and colleagues (Stumm, Zhou et al. 2003) observed that CXC chemokine receptor 4 (CXCR4) expressed by migrating interneurons is vital for the correct arrival of these cells to the cortex. Meninges secrete the ligand of CXCR4, SDF1 which acts as a potent chemoattractant for these CXCR4 expressing migrating interneurons.

Interneurons in mice deficient in SDF-1 and CXCR4 fail to reach superficial cortical layers and they ectopically populate deeper cortical regions.

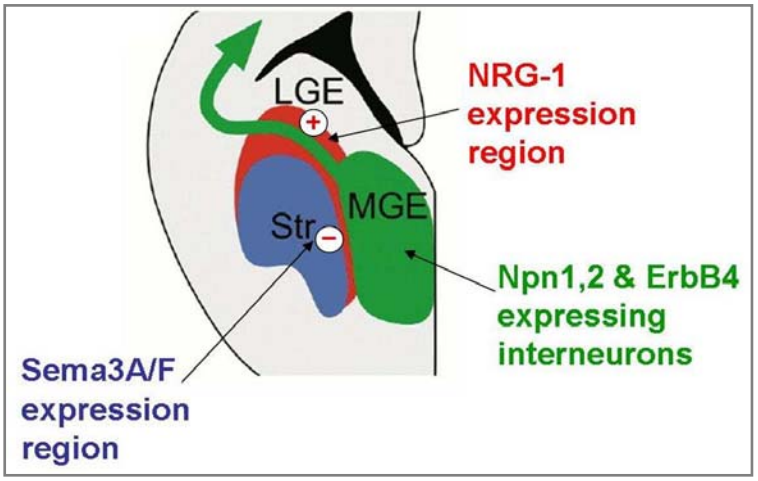

Fig.14. GABAergic neurons are guided by different positive and negative guidance cues thorough their migratory route. Modified from *Flames et al., 2004.*

Once in the cortex, radial migration of GABAergic neurons is controlled by different signals cues such as BDNF, a neurotrophin expressed by Cajal-Retzius cells. Studies carried out by Alcántara and collaborators (Alcantara, Pozas et al. 2006) demonstrated that the overexpression of BDNF during corticogenesis period produced an impairment of the final radial migration of interneurons within the cerebral cortex. Nowadays increasing evidences suggest that interactions between migrating interneurons and pyramidal projection neurons may also direct the cortical distribution of GABAergic neurons. Glutamate secreted by the growing corticofugal axons could activate calcium-permeable AMPA receptors expressed by tangentially migrating neurons (Metin, Denizot et al. 2000), receptors that are also activated by GABA released from migrating interneurons themselves (Poluch and Konig 2002). Behar and coworkers have described in distinct works how GABA and glutamate can control the motility of migrating neurons in the developing cortex (Behar, Li et al. 1996; Behar, Schaffner et al. 1998; Behar, Scott et al. 1999; Behar, Schaffner et al. 2000). Some *in vitro* studies have shown that the activation of AMPA receptor leads to neurite retractions that could be considered as a stop migration signal for migrating interneurons in their final cortical location (Poluch, Drian et al. 2001).

1.5. THE CORPUS CALLOSUM, THE MAIN BRAIN FIBER TRACT THAT CONNECT BOTH CORTICAL HEMISPHERES

Many neurons in the cerebral neocortex, especially from cortical layers II, III and V, project their axons to the contralateral hemisphere forming the midline structure known as corpus callosum (CC). It is estimated that around 200 million axonal projections form the CC which is considered the largest white matter structure in the brain. It connects neurons from both cerebral hemispheres and coordinates transfer of information between them. Individuals with CC malformations do not initially seem to show intellectual deficits albeit in some children has been observed behavioral and neuropsychiatric defects including learning difficulties, sleep and social communication disorders among others.

In the CC can be distinguished the posterior part called splenium, the anterior called the genu (or Knee) and between them, the truncus (or body) (Fig.15). The anterior part projects posteriorly and inferiorly from the anteriormost knee forming the rostrum. The axons of the knee connect with the prefrontal cortex while splenium axons interconnect areas of premotor and supplementary motor areas and motor cortex. Finally, posterior body sends somatosensory information between parietal lobe and visual center at the occipital lobe (Richards, Plachez et al. 2004).

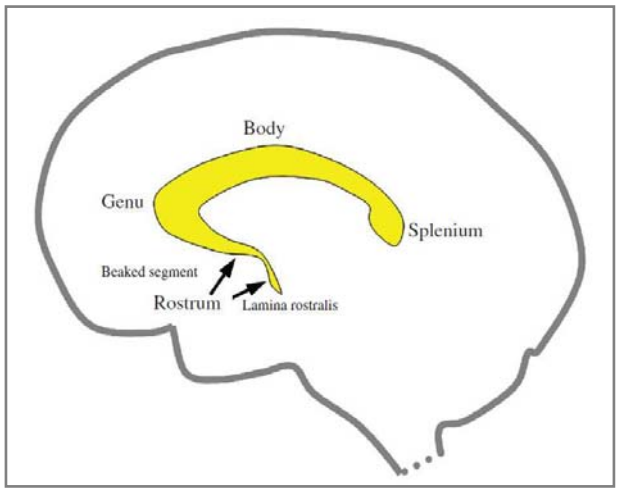

Fig.15. Rostro-Caudal view of human corpus callosum. Adapted from *Richards et al., 2004.*

At the first stages of brain development, forebrain is a single vesicle called prosencephalon. Two cerebral hemispheres arise from cellular proliferation and expansion from the single prosencephalic vesicle. Both telencephalic vesicles are fused in dorsal regions ventrally by midline area before callosal fibers cross forming the CC. Indeed, the fusion of both cerebral vesicles is critical for the correct axonal crossing and the resulting CC formation. The first regions to form are the lamina rostralis, the ventralmost part of rostrum, and the anterior region of the body that cross over the hippocampal commissure. More caudal regions are generated one day later in the developing CC (Volpe, Campobasso et al. 2009).

In mice, CC formation occurs at E14-E15 and is initially directed by different factors secreted by a glial population located in midline region named *midline zipper glia* (MZG) (Shu, Puche et al. 2003). Other midline populations identified in both mouse and human, such as glial wedge, the indusium griseum glia and the subcallosal sling, also participate in the genesis of the CC, guiding callosal axons to cross the midline into their final target region (Fig.16).

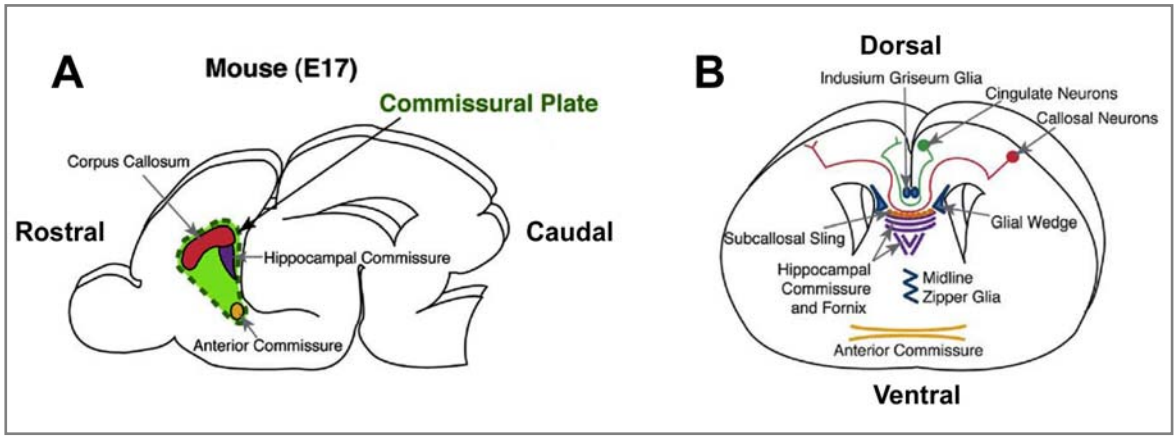

Fig.16. (A) Scheme of commissural plate in E17 mouse, an anatomical forebrain region traverse by interhemispheric commissures during development. (B) Coronal section showing the cellular populations that direct corpus callosum formation. Modified from *Donahoo and Richards, 2009.*

Glial wedge (GW) is wedge-shaped glia located in the medial ventricular region that is derived from radial glia scaffold and so they express typical radial glia marker as RC2, BLBP, Nestin and GLAST. In fact, GW cells are the first rostral cells expressing GFAP (protoypical glial marker) as early as E13, and as development proceeds they lose their

pial attachment (Shu, Puche et al. 2003). GW is essential for expressing Slit 2, a guidance molecule for crossing axons. Slit 2 exerts a repellent action on Robo (Slit2 receptor) expressing callosal axons, preventing the entrance of these axons into the septum instead of crossing the midline (Shu and Richards 2001; Shu, Sundaresan et al. 2003).

Dorsally to CC there is the *indusium griseum* (IG), an area formed by glia that also expresses the axonal guidance molecule Slit2. Pathfinding defects were observed in animals that showed aberrant IG glial cells translocation from ventricular zone to the prospective IG region (Smith, Ohkubo et al. 2006), demonstrating the importance of this glial population.

Subcallosal sling (SCS,) is a cell population described in both rodent and human brain. Although SCS was previously known as glial sling, it is a cellular lineage more related to neural than glial phenotype (Shu, Puche et al. 2003). SCS form a "U" shape structure that is ventrally delimiting the CC. This neural population migrates from the SVZ and remains proliferative until initial postnatal stages when it disappears. The developmental function of SCS remains rather unclear. Different hypothesis take into account the fact that sling neurons label with many of the same markers of SP neurons, which are known to be involved in axon guidance, regionalization and patterning of the neocortex (McConnell, Ghosh et al. 1989; McConnell, Ghosh et al. 1994). Sling structure is formed simultaneously with the pathfinding process of crossing callosal axons, and that is matter of discussion where ones postulate that SCS serves as a cue for crossing axons and others that callosal axons direct sling cells migration.

The first axons to cross through the midline called *pioneering axons* are originated in the medial-most part of the cortex, known as the cingulate cortex. Pioneering axons innervate the contralateral cingulate cortex at E14-15 in mice (Koester and O'Leary 1994) and have been described to be important as a guide post for later crossing axons. Piper et al., 2009 (Piper, Plachez et al. 2009) demonstrated the importance of pioneering axons from cingulate cortex in CC formation by studies with Npn1, a

membrane receptor located in pioneering axons membrane. This receptor is able to recognize Semaphorin 3 (Sema3) family protein which is expressed in the cortical midline during CC formation. The absence of Npn-Sema signalling provokes aberrant formation of CC. Sema3A and 3C would be required to direct the first axonal crossing tract through the midline by their repulsive and attractive respective effect on pioneering axons.

During callosal commissure formation, neocortical axons follow many environmental cues present in the midline region more or less associated with the previously described midline glial structures. Knockout studies have demonstrated that the absence of secreted guidance molecules such as Slit2 and Sema3A, and their receptors (Robo1/2 and Npn1 respectively) cause defects in CC development. Extracellular-matrix molecules as heparan sulfate proteogycans and chondroitin sulfate proteoglycans attach secreted pathfinding cues and play a key role in establish diffusible gradients for incoming callosal axons. The expression pattern of these proteoglycans is tightly regulated, and coincides spatially and temporally with commissural formation periods (Lindwall, Fothergill et al. 2007; Conway, Howe et al. 2010).

Changes in cytoeskeleton dynamics can disrupt the correct commissural extension, too. Growth-associated protein-43 (GAP-43) is a single copy gene that is a major neuronal substrate of protein kinase C (PKC) and modulates F-actin in growth cones (He, Dent et al. 1997; Oestreicher, De Graan et al. 1997). Although GAP-43 mutant normally form midline glial populations, axons fail to cross the midline forming the typical Probst bundles (Shen et al., 2002). The absence of GAP-43 severely affects actin cytoskeleton of commissural axons which have growth cones with smaller lamellas and reduced F-actin immunoreactivity.

Transcription factors have been also observed to be critical for commissural development by three different mechanisms: (1) the specification of neurons as callosal projection neurons, (2) promote or repress the expression of specific molecules and receptors implicated in axon growth in response to external cues, and

(3) control the development and expression of guidance factors of midline glial populations, which in turn regulate callosal axon pathfinding (Lindwall, Fothergill et al. 2007). Emx1, Emx2 and Satb2 are transcription factors expressed in dorsal telencephalic neurons, particularly in specific upper-layer callosal neurons. Knock out animals for these transcription factors show an aberrant cortico-callosal tract formed by neurons that normally express Emx1, Emx2 and Stab2, demonstrating the importance of a correct cellular fate determination (Fishell and Hanashima 2008; Piper, Plachez et al. 2009). There are other transcription factors expressed in cortical plate but not in callosal neurons, which action is focused in regulate midline glial population formation and the correct sling cells migration. Nuclear factor 1 A (Nf1a) and B (Nf1b) are transcription factors related with this function and their absence produces severe reduction of GW and IG structures, and abnormal SCS cells migration. As a result, callosal axons of Nf1a and Nf1b mutant animals fail to cross the midline region and abnormally project to the septum (das Neves, Duchala et al. 1999; Shu, Puche et al. 2003; Lindwall, Fothergill et al. 2007).

So, a number of guidance events are critical to CC formation including the correct formation of midline glial structures and pioneering axon population, the expression of molecules for a correct midline patterning, the formation of telencephalic hemispheres, and the birth and specification of commissural neurons. All the steps controlling the correct genesis of the CC require a large list of implicated genes, and their disruption leads to a callosal development failure. Many works based on rodent models have allowed advance in the better understanding of all these mechanisms implicated in commissural development. In addition, imaging, anatomical and molecular analysis carried out in developing human fetal brain have confirmed the similarities in the midline structures and the expression of genes that mediate midline axonal guidance between mice and humans (Ren, Anderson et al. 2006). These studies demonstrate that the mouse is an excellent model for studying CC agenesis.

2. GLIAL CELLS, THE SUPPORTIVE AND MODULATOR CELLS OF THE CENTRAL NERVOUS SYSTEM

2.1. ASTROCYTES, A KEY REGULATOR OF CORTICAL DEVELOPMENT

Astrocytes with oligodendrocytes are the two major macroglial cell types in the adult brain. Astrocytes were already described at the beginning of the 20th century as a star-shaped glia that acts maintaining the homeostasis in the brain by Santiago Ramón y Cajal (1909). They are uniformly distributed along the adult CNS parenchyma surface, where they exert distinct functions such as regulation of extracellular ions concentration, modification of synaptic efficacy, inactivation of neurotransmitters, induction and maintenance of the blood-brain barrier and glial limitans, and provide nutrients and trophic support for neurons and oligodendrocytes. Apart from contact with other astrocytes, astroglial cells are associated with oligodendrocytes, neurons, blood vessels and the pial membrane. Astrocytes are communicated through gap junctions formed by connexins, which allow ions and small molecules flow between connected cells. Thus, astroglial cells form a syncytium that can transport signals along large distance in the CNS. They also associate with blood vessels connected endothelial cells as well as neuronal bodies, dendrites and synapses (Fig.17).

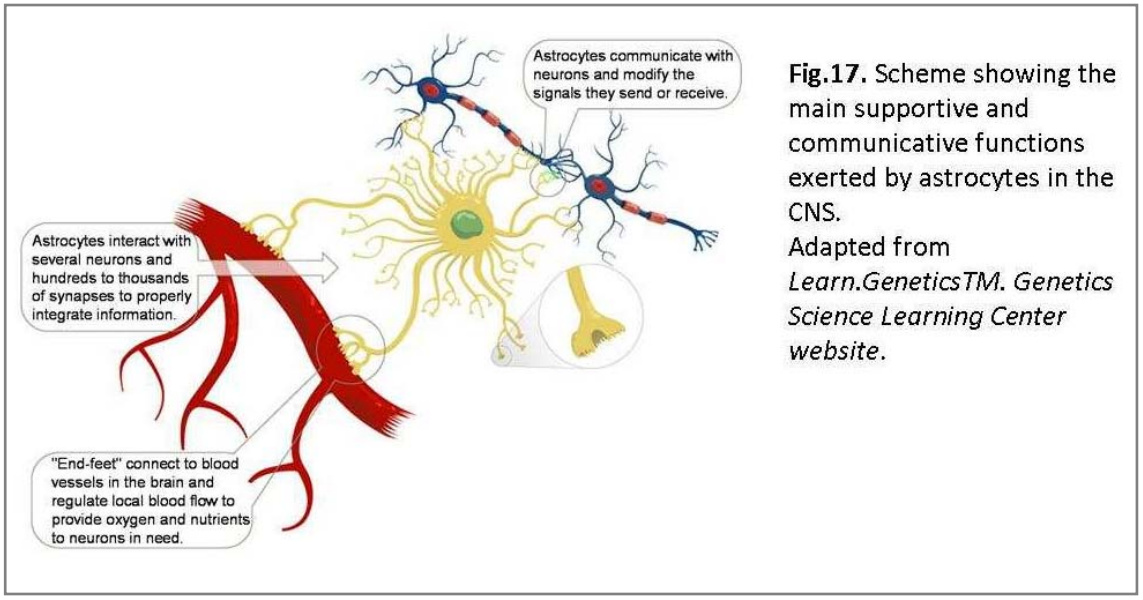

Fig.17. Scheme showing the main supportive and communicative functions exerted by astrocytes in the CNS.
Adapted from *Learn.GeneticsTM. Genetics Science Learning Center website*.

From a morphological point of view, in the adult, astrocytes are classified into fibrous and protoplasmic. *Fibrous astrocytes* are located in the white matter and have a star-like morphology, with many cylindrical processes radiating symmetrically from the soma. They form an end feet at the nodes of Ranvier and on the capillaries. They are also characterized by containing abundant intermediate filaments and relatively low density of organelles. Unlike fibrous astrocytes, *protoplasmic astrocytes* have fewer intermediate filaments and a high density of organelles. They have a more complex morphology with highly branched membranous sheets that involve neuronal prolongations and cell bodies. Moreover, as fibrous astrocytes, they also form end feet on capillaries and at the pial surface.

However, this classification is very simplistic because many intermediate glial cell forms have been described between oligodendrocytes and astrocytes. In addition, specific astrocytic forms can be found depending on the nervous system region, such as Bergmann glia in the cerebellum. Bergmann glia is located in the cerebellar Purkinje cell layer, and projects long processes through the molecular layer, ending at the pial surface and at large blood vessels. These glial cells have a bean-shaped nucleus and their cytoplasm contains intermediate filaments, random oriented microtubules, glycogen and scattered ribosomes. Nowadays the fibrous and protoplasmatic astrocytes dichotomy is still used to generally classify the astrocytes, although if there are functional differences between them, or if they come from the same progenitor cells remain unknown (Levison et al., Developmental Neurobiology 4th Ed. 2005).

Immunological molecular markers have been used in order to classify in a reliable way the different mature astrocytes putting aside morphological discrepancies (see below in point 2.1.3). Possibly the most known astroglial marker is the intermediate filament *GFAP, glial fibrillary acidic protein*, that with vimentin are the two major intermediate filaments of astrocytes. Although GFAP has been often used as a major criterion for classify astrocytes, some studies pointed that is not conclusive the use of antibodies against this intermediate filament for astroglial identification. These works postulate that GFAP (1) is barely expressed in early astrocyte development (Schnitzer, Franke et al. 1981), (2) is expressed at lower levels in gray matter than in white matter (Ong,

Garey et al. 1995) and (3) non neural cells, such as myoepithelial cells, osteocytes and chondrocytes also expressed this intermediate filament (Hainfellner, Voigtlander et al. 2001). Consequently others markers have been developed to identify astrocytes. Two examples are: *Enzime glutamine synthethase (GS)*, enriched in fibrous and protoplasmic astrocytes and the *calcium-binding protein S-100β*. The real situation is that in the CNS the macroglial populations composed by astrocytes, oligodendrocytes and their precursors have several typologies and complexities. All of them are characterized by distinct patterns of glial markers expression, being so complicated a fully reliable characterization.

2.1.1. VENTRICULAR AND SUBVENTRICULAR ZONE, THE ORIGIN REGIONS OF ASTROCYTES

Astrocytes are originated from precursors located in two main germinal regions, the primitive neuroepithelium of the VZ and SVZ. From the VZ arise RGC that are able to generate both neuronal and non-neuronal cells, including astrocytes and oligodendrocytes (reviewed en previous chapters). During early forebrain development radial glia processes form a scaffold that serves for migrating neurons to move and reach their final position (Rakic 1971; Rakic 1972). Nonetheless, during the perinatal period RGC undergo a transformation into astrocytes (Fig.18).

Indeed, RGC have cellular and molecular characteristics of astroglia, sharing molecular markers and also ultrastructural similarities with astocytes that are absent in neuroepithelial cells (Mori, Buffo et al. 2005). RGC express a number of molecules that are observed later in immature astrocytes. Among them, there are intermediate filament proteins, such as nestin, vimentin and GFAP (Bramanti, Tomassoni et al. 2010). They also express brain lipid-binding protein (BLBP) (Hartfuss, Galli et al. 2001), astrocyte-specific glutamate transporter GLAST (Shibata, Yamada et al. 1997), glutamine synthase (GS) and the post-translation modifications of intermediate filament nestin, RC1 and RC2 (Mori, Buffo et al. 2005).

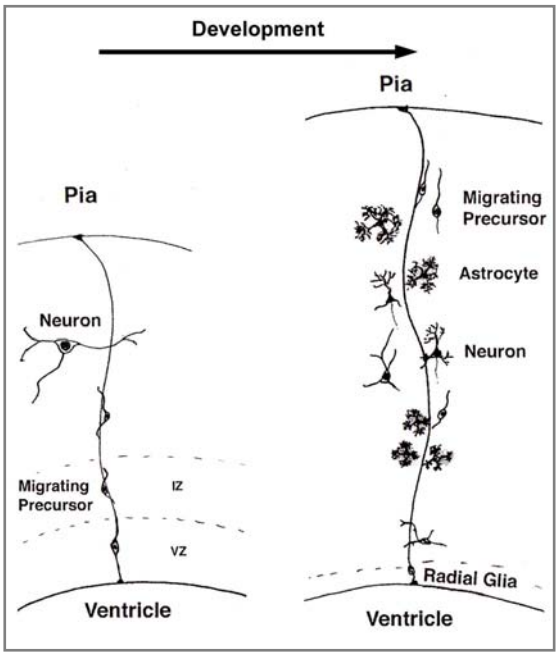

Fig.18. In the embryonic cerebral cortex, radial glial cells are able to give rise neurons and support their migration, whereas during postnatal development the transform into astrocytes. Adapted from *Gray and Sanes (1992).*

It is thought that RGC identity is maintained by signals sent out by neurons during their radial migration. Different molecular pathways described the crosstalk established between RGC and migrating neurons. One comprises the transmembrane receptor Notch, present on VZ radial cells, and one of their ligands, Delta-1 which is highly expressed by immature neurons. Gaiano and collaborators carried out experiments where the activated Notch intracellular region was enough to induce the maintenance of RGC phenotype (Gaiano, Nye et al. 2000). A second pathway refers to another transmembrane receptor highly expressed during development by RGC, erbB receptor, and its ligand neuregulin (NRG), expressed by migrating neurons (Anton, Marchionni et al. 1997). The downregulation of erbB2 receptor leads to the transformation of RGC into astrocytes and *in vitro* assays demonstrated that the addition of NRG induce astroglial dedifferentiation into more radial glia phenotype (Schmid, McGrath et al. 2003). A model proposed by Patten and colleagues describes a sequential signalling through Notch and erbB pathways in RGC induced by neurons. Initial contact may provoke the release of Notch ligands by neurons activating astrocytic Notch receptors. Notch signalling then induces expression of BLBP and erbB2 in the glia. The increase in

erbB receptor expression makes the astrocyte more responsive to neuron-derived NRG, which subsequently induces the glia to adopt a radial morphology and to support neuronal migration (Patten, Peyrin et al. 2003).

At the end of neurogenesis, when the radial scaffold is no longer required for neuronal migration, RGC transform into astrocytes. They gradually become morphologically more complex and start to express typical astrocyte markers. The inner accumulation of GFAP in transforming cells was strong evidence that supported this developmental transition. Immunostaining assays for different antigens expressed by RGC and immature astrocytes, precisely describe how during neurogenesis the expression of the radial precursors markers change, increasing the expression of more astrocytic related proteins, such as BLBP or GLAST (Fig.19)(Hartfuss, Galli et al. 2001). Linage-tracing assays carried out by Gray and Sanes (Gray and Sanes 1992) based on retroviral injection using reported genes also corroborated that RGC differentiate into astrocytes. Initially infected RGC express radial markers such as vimentin and RC2, and once development proceeds, infected cells acquired astrocytic morphologies.

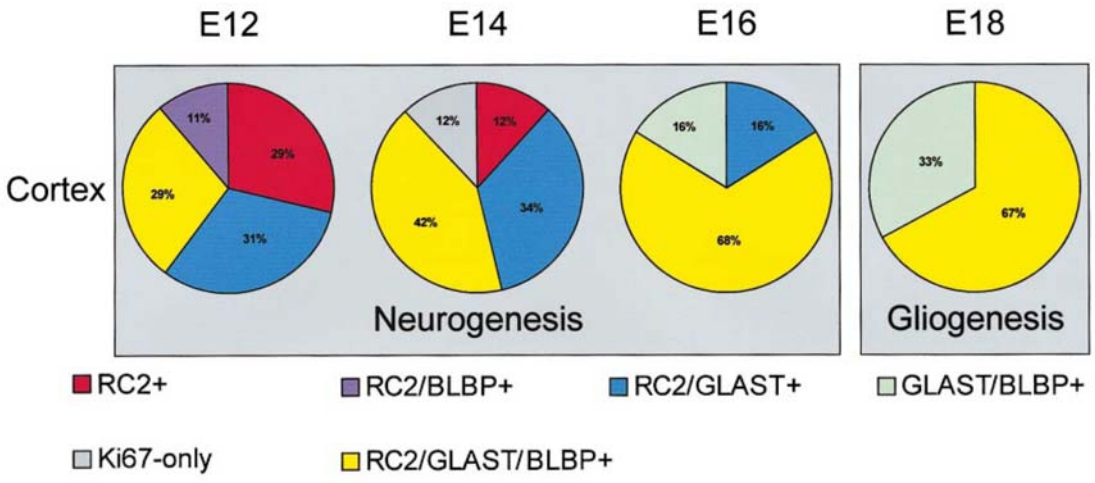

Fig.19. During embryonic development there are changes in the composition of cortical precursors. Adapted from *Hartfuss et al., 2001.*

During the initial postnatal period, astrocytes and oligodendrocytes are generated in the highly proliferative SVZ. The SVZ precursors can be rather easily distinguished from RGC, because most of the more characteristic radial markers, such as nestin, vimentin,

BLBP, GLAST, RC1 and RC2, are absent in subventricular progenitors. In contrast, two-thirds of SVZ cells that will generate astrocytes and oligodendrocytes express markers such as polysialic acid neural cell adhesion molecule (PSA-NCAM), which is not expressed by RGC.

Initial linage-tracing experiments, where subventricular progenitors were labelled by retroviruses infection at birth, showed labelled cells dispersed throughout the forebrain one month later. Morphological and immunostaining analysis identified protoplasmic astrocytes, myelinating and nonmyelinating oligodendrocytes as the descendents of initially labeled subventricular progenitors (Levison and Goldman 1993). Video time-lapse studies showed that astrocytes derived from SVZ progenitors colonize all cortical layers, up to the pial surface and striatum, while SVZ derived cells that migrate into the white matter differentiate into oligodendrocytes. Further studies propose that SVZ cells are not irrevocably committed to an astrocytic or oligodendrocytic fate, because they demonstrate that SVZ progenitors are able to give rise to both glia and neurons (reviewed by Kriegstein and Alvarez-Buylla 2009). Indeed, these progenitors are constantly receiving signals, even during their migration, and that is the reason why some of them do not make a final fate decision until they have stopped migrating. A clear example of how many local environmental factors determine the fate decision of a progenitor cell is the differentiation into astrocyte of cultured progenitors obtained from optic nerve when they contact with endothelial cells (Mi, Haeberle et al. 2001). These studies reproduce the interaction established between blood vessels and SVZ migrating cells, which will form the classic astrocyte end foot around the vessel. Thus, intrinsic but also extrinsic signals are playing an important role during neuronal and glial differentiation process.

During early postnatal period, subventricular progenitors use radial glia scaffold to migrate into their final destination. Progenitors addressed to generate astrocytes and oligodendrocytes migrate radially into the overlying white matter and cortex, while progenitors destined to produce neurons will migrate to the olfactory bulb where they will develop into interneurons (Suzuki and Goldman 2003). Emigrating glioblasts can migrate parallel to axons or radially into the cortex, where they can continue or turn to

migrate tangentially (Fig.20). Nevertheless, RGC transform into astrocytes at postnatal day 14, and despite subventricular progenitors still have the ability of migrate into the subcortical white matter or the striatum, they are unable to reach the neocortex. Thus, without RGC subventricular progenitors are restricted from colonizing dorsal forebrain structures (Levison, Chuang et al. 1993).

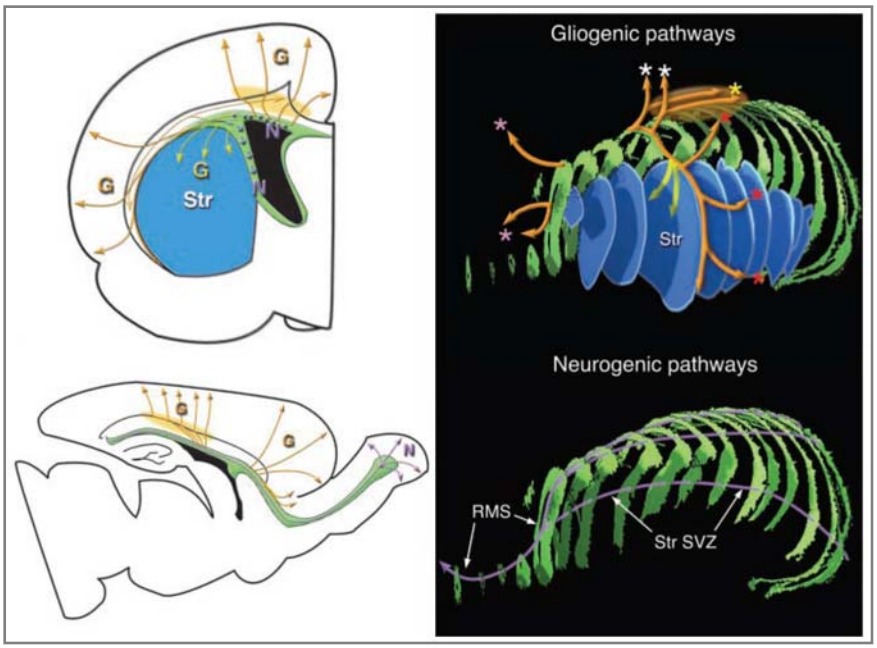

Fig.20. Migratory pathways followed by neurons and glial cells generated in the SVZ**.** Adapted from *Suzuki and Goldman, 2003.*

2.1.2. ASTROCYTE LINEAGES

Astrocytes constitute a heterogeneous group of cells which show different morphology and molecular pattern, including levels of intermediate filaments (mainly GFAP), growth factors, ions channels and neurotransmitter transporters. It is still unknown if these differences are determined by their origin (RGC or SVZ progenitors) or by local environmental factors (white matter or gray matter). Studies in optic nerve cultures performed by Martin Raff and colleagues (Raff, Miller et al. 1983) proposed another classification for astrocytes. They distinguished between two subtypes of astrocytes depending on their lineage and antigenic features:

Type 1 Astrocytes are GFAP positive cells with a flat polygonal morphology. It has been suggested that Type 1 astrocytes are generated from RGC because it is known that they develop early during gliogenesis and they initially express RC1 marker. In fact, there is clear evidence that RGC would generate a subset of astrocyte-restricted precursors with radial-shape that initially retain RC1 epitope and posteriorly express GFAP and acquire stellate-shape typical of astrocytes (Culican, Baumrind et al. 1990). In experiments carried out by Mi and collaborators identified by immunostainings other astrocyte-restricted precursor population purified from optic nerve (Mi, Haeberle et al. 2001). This progenitor population that is clearly different from immature astrocytes and from O-2A (oligodendrocyte and type 2 astrocyte precursor cell) progenitor cells and was called astrocyte precursor cells (APCs). When APCs were stimulated with ciliary neurotrophic factor (CNTF) they differentiated into type 1 polygonal astrocytes and not into type 2 astrocyte. Unlike O-2A, type 1 astrocytes express fibronectin and the rat neural antigen-2 (ran-2) in rodents, and proliferate in response to epidermal growth factor (EGF). Nonetheless many differences have been observed in the expression pattern of markers in *in vitro* studies as well as in different CNS regions and different developmental periods.

Type 2 Astrocytes appear during postnatal period and come from a bipotential O-2A progenitor (able to give rise to type 2 astrocytes and oligodendrocytes). CNTF and astrocyte-inducing molecule (AIM) present in the fetal bovine serum are very well-known factors able to differentiate O-2A progenitors into type 2 astrocytes. Ganglioside A2B5, proteoglycan NG2, growth associated protein GAP-43 among others, are specific markers for type 2 astrocyte and O-2A progenitors.

More recently new markers have been described for distinguish between additional astrocyte types identified in forebrain and spinal cord. Some studies conducted in spinal cord identified the earliest glial precursors that do not generate neurons and are able to give rise to type 1 astrocytes, oligodendrocytes and under appropriate conditions, type 2 astrocytes. These glial restricted precursors (GRPs) were observed in the developing spinal cord and can be identified as early as E12 by their A2B5 and nestin immunoreactivity (Rao and Mayer-Proschel 1997; Liu and Rao 2004). Thus, the

pluripotent neuroephitelial progenitors may generate GRPs that subsequently differentiate into even more restricted glial precursors, the O2A cells and APC cells. All these cells can be distinguished from each other on the basis of their antigenic properties (Fig.21).

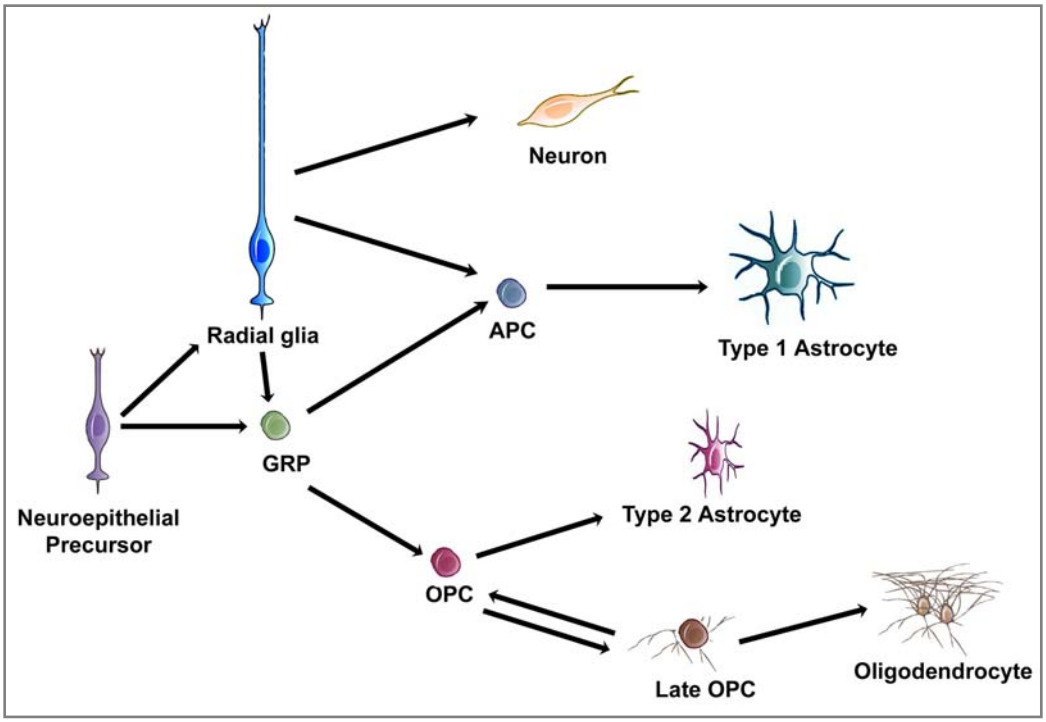

Fig.21. Hypothetic association observed between glial lineages and neural progenitors according to *in vitro* studies and *in vivo* observations.

Astrocytes continue to be generated in the adult CNS. In fact, some astrocyte population act as a primary progenitors generating new neurons and glial cells in restricted germinal regions in the adult mammalian brain (Alvarez-Buylla and Lim 2004). Recent studies demonstrate that a subset of astrocytes located in the VZ, called type 1 cells, are essential for the formation of new olfactory bulb interneurons and some oligodendrocytes in CC, fimbria and striatum (Alvarez-Buylla, Kohwi et al. 2008; Jackson and Alvarez-Buylla 2008). Many efforts are put in the study of this adult population of progenitors because of their potential as regenerative therapy.

2.1.3. ASTROCYTIC FATE IS REGULATE BY MULTIPLE SIGNALS

Several factors have been reported to be implicated in astrocyte differentiation: (1) alpha helical family of cytokines (CNTF and LIF), (2) Bone morphogenetic proteins (BMPs), (3) Delta and Jagged ligands and Notch receptors, (4) Fibroblast growth factors (FGFs), (5) Epidermal growth factor (EGF) family member ligands and the erbB family of receptors, and (6) Pituitary adenylate cyclase-activating polypeptide (PACAP) and the PAC1 receptor.

Alpha helical family of cytokines

CNTF and leukemia inducing factor (LIF), are two members of alpha helical family of cytokines that induce astrocyte differentiation through their receptor which is expressed by cells located in the VZ and SVZ of the cerebral cortex. LIF seems to be more essential than CNTF, because deficient mice for CNTF do not show defects in astrocyte production while the absence of LIF produces reduction of GFAP positive cells at E19 (Koblar, Turnley et al. 1998). The classical downstream pathway of alpha helical cytokines basically implicates the janus kinases (JAKs), which are associated with the receptors, and the transcription factors STAT1 and STAT3. The binding of the ligand to the receptor induces the sequential phosphorilation of JAK and STAT effectors, which once phosphorilated will form a complex with CBP/p300 that will move to the nucleus. Once there, this complex can activate or repress genes implicated in astrocyte differentiation (Fig.22) (Fukuda and Taga 2005).

Bone morphogenetic proteins (BMPs)

BMPs are members of the TGFβ superfamily that are implicated in a variety of roles in the developing and mature nervous system. BMPs promote differentiation of neurons early during development and astrocytes at later stages (Samanta and Kessler 2004). In later development stages, when there is an intense gliogenesis, BMP stimulation enhances astrogenesis and inhibits oligodendrogenesis from late embryonic SVZ progenitors or neuroepithelial cells (Gross, Mehler et al. 1996; Nakashima, Takizawa et al. 2001; Yanagisawa, Takizawa et al. 2001). BMPs are known to signal through heterotetrameric serine/threonin kinase receptors composed by type 1 and 2 subunits,

which are highly expressed in VZ and SVZ from as early as E12 (Gross, Mehler et al. 1996). The canonical pathway comprises transcription factors called receptor-restricted Smads (Smad1, Smad5 and Smad8) that are phosphorilated when the receptor is activated. The complex Smad1/5/8 binds to Smad 4, a mediator that allows the movement of the whole complex to the nucleus where it leads to transactivation of target genes. Through the control expression of transcription factors such as Id1 and Id3, BMPs repress the neurogenesis and concomitantly induce astrocytogenesis of neuroepithelial cells (Nakashima, Takizawa et al. 2001; Yanagisawa, Takizawa et al. 2001). BMPs also promote astrocyte differentiation through the competition with pro-neurogenic factors such as neurogenin, for the Smad co-factor CBP/p300, necessary for transactivation of pro-astrocytic genes (Fig.22) (Sun, Nadal-Vicens et al. 2001).

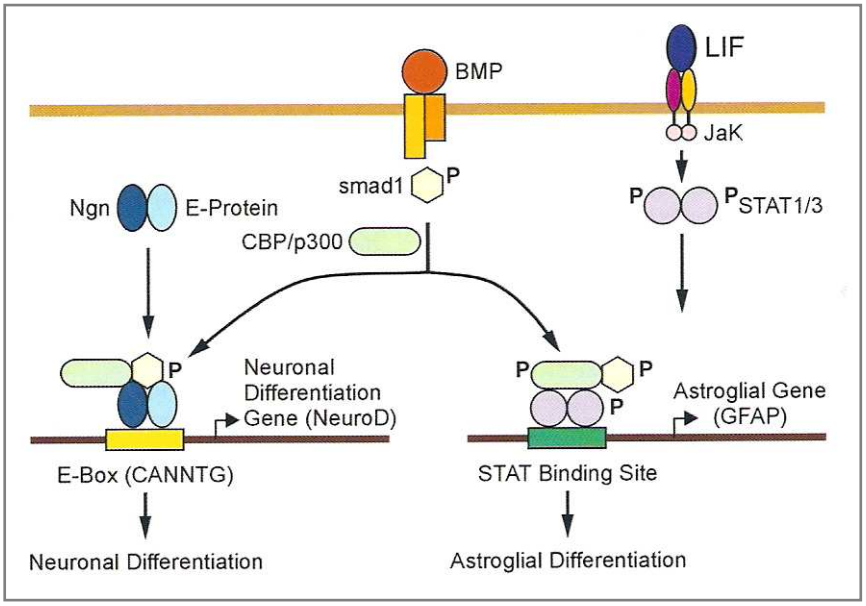

Fig.22. BMP and LIF pathways are importantly associated with the developmental switch from neurogenesis to gliogenesis. Adapted from *Levison et al., 2005, Developmental Neurobiology 4th ed*.

Fibroblast growth factors (FGFs)

There are at least 23 FGF members; ten have been identified in the brain where they exert multiple functions. For instance, FGF-2 is a potent mitogen for type 1 astrocytes, and mice deficient for FGF2 show a dramatic decrease of astrocytes in the hindbrain (Perraud, Labourdette et al. 1988; Irmady, Zechel et al. 2011). Activation of FGF

receptors triggers several intracellular signalling cascades that play important roles in neurogenesis, axon growth, and differentiation.

Epidermal growth factor (EGF) receptor

As FGF, EGF receptor signalling is known to activate the cell cycle machinery and induce neural stem cell proliferation through MAPK pathway (Vaccarino, Fagel et al. 2007). EGF receptor also promotes differentiation of glial progenitors into astrocytes (Ayuso-Sacido, Moliterno et al. 2010; Gampe, Brill et al. 2011). The induction of astrocyte differentiation triggered by EGF receptors starts when decrease the levels of NRG expressed by migrating neurons through radial glia. NRG decreased levels occur during neural maturation and allow RGC to become receptive to astrocyte differentiating signals, such as the signal leaded by EGF receptor and LIF. Raising levels of EGF receptor observed at the end of neurogenesis may confer competence to progenitor to respond to LIF and so, to generate astrocytes (Viti, Feathers et al. 2003).

Pituitary adenylate cyclase-activating polypeptide (PACAP)

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide that has been observed to promote proliferation in reactive astrocyte where its receptor PAC1 is highly expressed after brain injury (Nakamachi et al., 2010). PACAP and PAC1 are also highly expressed in the VZ during late gestation period. *In vitro* assays demonstrated that the overactivation of PACAP-PAC1 pathway, elevating cAMP levels and the concomitant phosphorilation of the transcription factor CREB, elevated the levels of GFAP as well as decreased nestin levels in cultured neural precursor cells (McManus, Chen et al. 1999).

Thus, the mechanism of astroglial differentiation seems to be regulated by the intrinsic competence of neural precursors to respond to extracellular cues, modulated by the balance between neurogenic and gliogenic transcription factors in the cell, and extrinsic signalling molecules surrounding the precursor cell in a particular spatio-temporal context.

2.2. OLIGODENDROCYTES

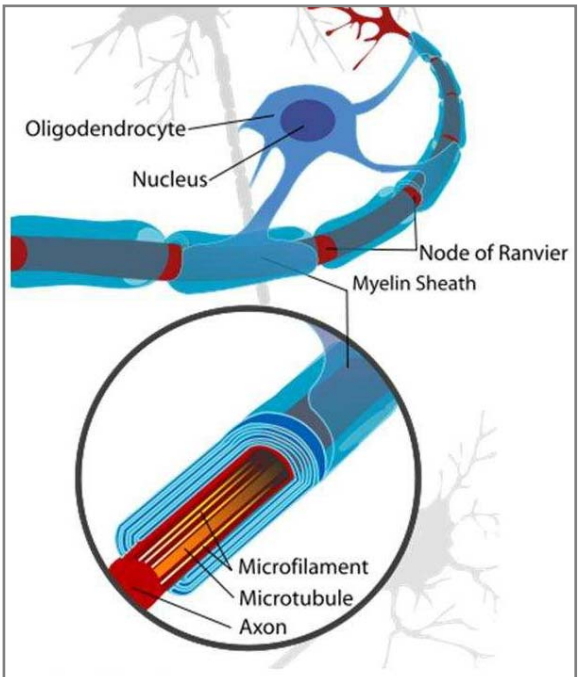

Fig.23. Oligodendrocytes produce the myelin sheath around the axon that allows the rapid and efficient conduction of electrical impluse. *Learn.GeneticsTM. Genetics Science Learning Center website.*

Oligodendrocytes are the myelinating cells of the CNS. Myelin is a fatty insulation composed of modified plasma membrane surrounding the axons that allow the rapid and efficient conduction of electrical impulse. Oligodendrocytes are cells that have a particular physiology/metabolism and a developmental program that includes complex and precisely timed program of proliferation, migration, differentiation and myelination to finally produce the sheath of axons. All these intrinsic particularities make them the most vulnerable cells of the CNS. Disruption in myelination caused by injury, pathological degeneration, or genetic reasons is associated with abnormal impulse transmission that leads to neurological functionality failure and loss of neurons themselves.

2.2.1. OLIGODENDROCYTES ORIGIN

In the adult, oligodendrocytes are distributed throughout the CNS, including both white and grey matter. During development, oligodendrocytes are believed to arise from a precursor that is able to generate oligodendrocyte progenitors (OPCs) and a particular subset of astrocytes (type 2 astrocytes). For this reason, these precursors were named oligodendrocyte type 2 astrocyte (O-2A) progenitor cells (Raff, Miller et al. 1983). Each developmental step in the oligodendrocyte maturation, from precursor stage to mature myelinating oligodendrocyte, can be identified by changes in their

morphology and expression pattern of particular markers (Fig.24) (de Castro and Bribian 2005).

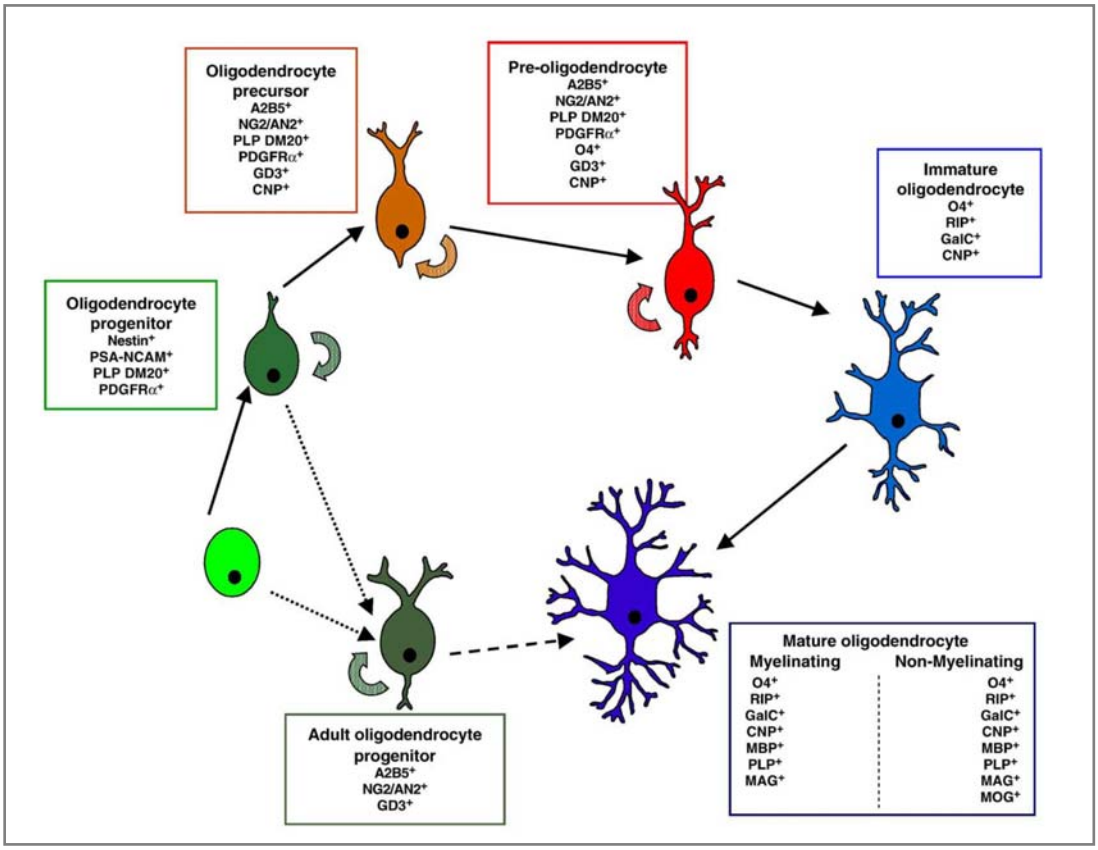

Fig.24. Each step of oligodendroglial maturation process is characterized by a particular battery of cellular markers and cellular morphologies. Adapted from *De Castro and Bibrián, 2005.*

Morphogenetic cues and transcription factors play a key role in the patterning and regional specification of the neural tube during the early oligodendrogliogenesis period (Richardson, Kessaris et al. 2006). Initial transplant studies performed in spinal cord determined that ventrally-derived precursors produce most or all oligodendrocytes in the spinal cord and forebrain. These studies also suggested that the initial appearance of oligodendrocyte precursors is regulated by two general mechanisms, the cell intrinsic potential to generate oligodendrocytes and the local signals that instruct neighboring cells to assume an oligodendrocyte fate (Trousse, Giess et al. 1995).

In fact, evidence suggests that signalling molecules secreted from notochord/floor plate, involving sonic hedgehog (Shh), are necessary and sufficient to induce the

development of ventrally derived oligodendroglia (Pringle, Yu et al. 1996). Shh morphogen may contribute to the initial progression towards the oligodendrocyte lineage, acting through induction of cell-type specific transcription factors such as the basic helix-loop-helix proteins Olig-1 and Olig-2 (Zhou, Wang et al. 2000). Conversely, elevated BMP expression derived from dorsal skin and neural tube inhibits the appearance of ventral oligodendrocyte precursors (Mekki-Dauriac, Agius et al. 2002). *In vitro* assays based on O-2A precursor cells cultures, demonstrated that specifically BMP2 and BMP4 appear to inhibit oligodendrocyte development, and promote the generation of astrocytes instead (Mabie, Mehler et al. 1997; Mehler, Mabie et al. 2000).

During cerebral cortex development, immature OPCs initially migrate from the VZ of the LGE as well as MGE and CGE (He, Ingraham et al. 2001; Marshall and Goldman 2002; Richardson, Kessaris et al. 2006). Those cells first appear in the neuroepithelium of the MGE and express oligodendrocyte lineage markers such as OLIG1, OLIG2, SOX 10 and platelet-derived growth factor receptor (PDGFRα). Later, these oligodendrocyte precursor cells seem to migrate laterally and dorsally from MGE and the nearby anterior entopeduncular area into all parts of the developing forebrain, including the cerebral cortex. The first precursors arrive to the cortex around E16, whereas the entire cortex invasion is observed at E18 when a second wave of oligodendrocyte precursors arrives from the LGE and/or CGE. The third wave of OPCs, finally arises from subventricular progenitors and migrates radially and tangentially toward the pial surface populating all regions of the cerebral cortex (Fig.25)(Levison, Young et al. 1999; Kessaris, Fogarty et al. 2006). Cortical populations of OPCs are functionally redundant and when one of them is destroyed, the remaining cells fill the gap and restore the normal OPCs distribution. Thus, a normal complement of oligodendrocytes and myelin can be produced, and the animal develops, survives and behaves normally (Kessaris, Fogarty et al. 2006).

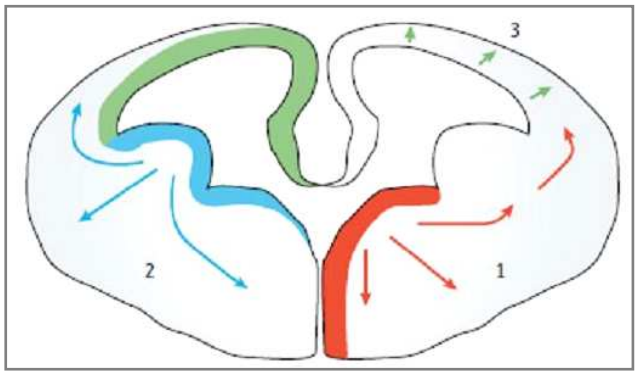

Fig.25. The three main origins of oligodendrocytes. During early cerebral cortex development (E12.5) oligodendrocytes precursors are located in the ventricular zone of the MGE (1) and few days later in the LGE (2). After birth OPCs derived from the cortical SVZ (3). Adapted from *Richardson et al., 2006.*

2.2.2. REGULATING MECHANISMS IMPLICATED IN FOREBRAIN OLIGODENDROCYTES MIGRATION AND DIFFERENTIATION

The generation of oligodendrocytes from ventral forebrain stem cells is modulated by the sequential actions of ventral and dorsal gradient of Shh and BMPs respectively. In the subpallium, oligodendrocytes are coupled to GABAergic neurons, and a local and spatio-temporal modulation of BMP signalling may regulate the sequential elaboration of cortical GABAergic neurons and oligodendrocytes after tangential migration of ventral forebrain progenitors (Yung, Gokhan et al. 2002). Shh promotes GABAergic neuronal/Oligodendrocyte lineage restriction while BMPs inhibit oligodendrocyte lineage elaboration and enhance the specification of late-born cortical GABAergic neurons from subpallial progenitors.

The motility of OPCs is promoted by Platelet-Derived Growth Factor (PDGF), the same growth factor that promotes their proliferation and survival, but due to its ubiquitous distribution in CNS is unlikely to guide OPCs migration (McKinnon, Smith et al. 1993; Wilson, Onischke et al. 2003). During development of the cerebral cortex, immature OPCs have been suggested to migrate from their origin regions along preexisting axon tracts, in which case, the final destinations of oligodendrocytes would be defined by axonal topography. This hypothesis would make sense, because OPCs follow precise

patterns of migration and the establishment of synapses also requires high rate of precision. The contact between OPCs and preexisting axons implicates different adhesion molecules, extracellular matrix proteins and receptors that would serve as a cue for migrating OPCs. In explant studies it was observed that the removal of PSA-NCAM inhibits the dispersal of oligodendrocyte precursors (Wang, Pralong et al. 1996). Other *in vitro* analysis showed that fibronectin and the laminin family member merosin promote migration, whereas tenascin-C selectively inhibits the migration of oligodendrocytes (Frost, Kiernan et al. 1996). Furthermore, studies in optic nerve suggested that OPCs migration can be guided by secreted molecules such as Netrins (Netrin-1) and semaphorins (Sema 3A). These well-known chemotropic factors may act as chemorepellent signal for OPCs migration (Sugimoto, Taniguchi et al. 2001), although this hypothesis awaits further confirmation. Another chemotactic signal for OPCs is CXCL1, a chemokine with a spatio-temporal expression pattern coincident with OPC migration. Tsai and colleagues observed that CXCL1 inhibits OPCs migration and the blockage of its signalling pathway disrupts oligodendrocyte development (Tsai, Frost et al. 2002). These data proposed CXCL1 as a stop signal candidate for migrating OPCs, and suggested that CXCL1 contributes with PDGF to oligodendroglial cell patterning in the developing CNS (Tsai, Frost et al. 2002).

Once located at their final destination, some OPCs persist in the adulthood while the vast majority differentiates to myelin-producing oligodendrocytes. Oligodendrocytes have only a brief period of time for myelination early during differentiation, because once they are mature, they are unable of enseath the axon. Differentation process involves Jagged 1 signalling located on the axonal surface, and oligodendrocyte Notch1 receptor. The ensheatment is a highly coordinated event, where oligodendrocytes select axons following different molecular cues. The electrical activity of neurons seems to provide a vital signal for a correct myelination. The action potential triggers ATP and adenosine release which can mediate neuron-glia communication. On the one hand, adenosine is known to inhibit proliferation and promotes OPCs differentiation and myelin formation (Stevens, Porta et al. 2002). On the other hand, ATP released from firing axon potentials triggers the release of LIF from astrocytes, which promotes myelination by mature oligodendrocytes (Ishibashi, Dakin et al. 2006).

The observation that OPCs act and response to inducers signals in a different way in distinct CNS regions suggests that there are more signalling pathways contributing to the generation of oligodendrocytes that still remain unknown. Microenvironment could be a very important factor controlling the balance between self-renewal and differentiation. Signalling molecules such as CNTF, Tyroid hormone, retinoic acid or neurotrophins, and the own redox state of the precursor cell, can control the timing and extent of oligodendrocyte production (Smith, Ladi et al. 2000). Some developmental studies suggest that the different classes of oligodendrocytes may be derived from distinct precursors (Mallon, Shick et al. 2002), adding a new level of complexity in oligodendrogenesis mechanisms.

3. CHEMOKINES IN CNS DEVELOPMENT

In this chapter will be analyzed the role of soluble factors secreted by neurons and glial cells that coordinately direct neural stem cells to differentiate into the different neural lineages present in the CNS. During CNS development, differentiated neurons secrete BDNF and NT3 that promote neuronal fate (Cheng, Coksaygan et al. 2007), while BMPs secreted by both neurons and glia, can force stem cells to differentiate into either neurons (Mabie, Mehler et al. 1999) or astrocytes (Gross, Mehler et al. 1996) depending on the developmental period. Neuronal BMPs secretion is low, inducing cortical stem cells to differentiate into neurons, whereas high BMPs levels secreted by astrocytes produce cell differentiation into astrocytes (Chang, Son et al. 2003). Previous analysis in our group suggests that changes in BDNF expression directly affect the expression levels of BMP7. Altogether show us the necessity of the brain of having a well organized cytokines expression pattern to establish the different cell populations in their correct spatio-temporal timing.

Apart from their multiple implications in several steps of CNS development, we are also interested in BDNF and BMPs, because it has been recently observed that they play neuroprotective and neuroregenerative role in response to distinct CNS injuries (Tsai, Pan et al.; Meng, Zhiling et al. 2005; Chou, Harvey et al. 2006; Tsai, Weng et al. 2007; Guo, Kim et al. 2008; Sabo, Kilpatrick et al. 2009). The cell signalling activated by these cytokines in pathological conditions leads to promote cell survival accompanied by recovery cellular changes such as cellular proliferation and differentiation, neurite outgrowth and axonal sprouting (Cate, Sabo et al.; Esquenazi, Monnerie et al. 2002; Husson, Rangon et al. 2005; Deumens, Koopmans et al. 2006; Tsai, Weng et al. 2007; Sanchez-Camacho and Bovolenta 2009). Nonetheless, these cytokines exert pleiotropic actions in the developing cerebral cortex and the real effect produced by the activation of their pathways is far to be elucidated.

3.1. BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF)

The main description of a neurotrophic factor initially postulates that neurotrophins ensures a balance between the size of a target organ and the number of innervating neurons in the targets of innervation (Purves, Snider et al. 1988). Generally, neurotrophins are considered regulators of neural survival, development, function, and plasticity (Huang and Reichardt 2001; Numakawa, Suzuki et al. 2010). The brain-derived neurotrophic factor (BDNF) is the second member of the neurotrophic factors family discovered after the nerve growth factor (NGF) discovery in the early 1950s. The broad expression on developing and adult brain as well as the huge number of functions exerted by BDNF makes it one of the most studied molecules in the neurodevelopment field.

3.1.1. FROM GENE TO MATURE BDNF PROTEIN

The BDNF gene is mapped in humans in chromosome 11 while in mice is in chromosome 2. Human and mouse sequence are composed by at least nine exons showing good alignments (mouse exons correspond to each human sense BDNF exon except exon VII). Although there is only one BDNF protein form, mouse BDNF gene can give rise to multiple different transcripts that are expressed in a developmentally regulated and tissue-specific manner (Fig.26) (Kuczewski, Porcher et al. 2009). These splicing variants could be detected in most brain regions, being I, II and III the most expressed in CNS (Liu, Walther et al. 2005). The transcription of BDNF gene can be initiated by multiple activity-dependent and tissue-specific promoters (Aid, Kazantseva et al. 2007), such as promoter IV-dependent BDNF transcript, which is the most expressed in cerebral cortex in response to activity (Timmusk, Belluardo et al. 1994; Tao, West et al. 2002). In addition, BDNF transcripts can also suffer regulated polyadenylation (Timmusk, Palm et al. 1993) allowing obtaining a larger number of different BDNF transcripts (Fig.26).

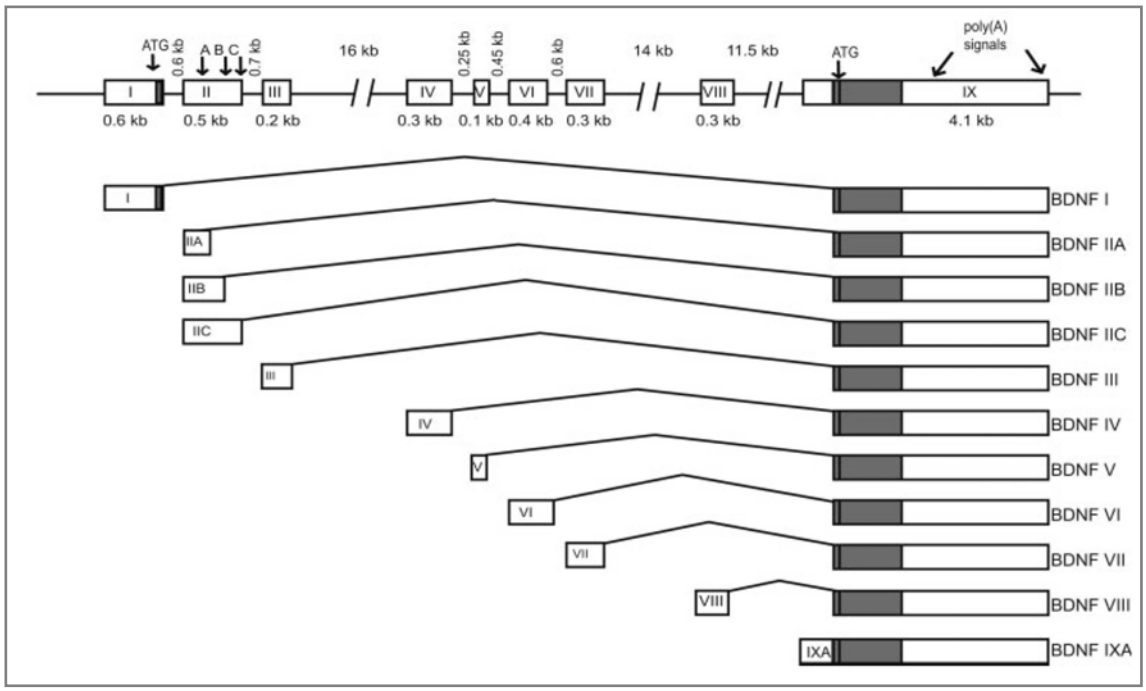

Fig.26. BDNF gene structure and splicing variants. Boxes represent exons, lines show the possible splicing, and arrows indicate within-exon splice and alternative polyadenylation sites. Adapted from *Aid et al., 2007.*

BDNF shares about 50% peptidic sequence with other neurotrophins such as NGF, NT-3 and NT4/5. Each neurotrophin consists of a non-covalently linked homodimer which contains a signal peptide following the initiation codon and a pro-region containing an N-glycosilation site. BDNF is initially synthesized in endoplasmic reticulum as a precursor (preproBDNF) which is cleaved, eliminating the signal peptide and producing a new precursor form of BDNF, proBDNF (M.W. 30kDa aprox.). This precursor is transported to the Golgi where trans-Golgi endoproteases such as furin, convert ProBDNF into the mature BDNF (M.W. 14kDa aprox.). This final process could be also done in the immature secretory granules by proprotein convertases (Mowla, Pareek et al. 1999) and even in the extracellular matrix, where tissue plasminogen activator secreted by the same cell could perform the conversion of pro- to mature form of BDNF (Nagappan, Zaitsev et al. 2009). It is generally accepted that the mostly secreted and biologically active form is the mature BDNF (Matsumoto, Rauskolb et al. 2008) although some reports suggest that proBDNF can also be released by neurons (Yang, Siao et al. 2009), and it might exert important functions in late-phase long-term potentiation (L-LTP) and long-term memory (Pang and Lu 2004). In fact some studies

about synaptic formation pointed out that both mature and proBDNF could even exert opposite functions (Woo, Teng et al. 2005).

In the trans-Golgi, BDNF also undergoes the corresponding sorting process to be released by either constitutive or regulated secretory vesicles. The sorting of BDNF into both pathways depends on two amino acidic motifs of the mature and the pro domain of the protein. The interaction between the motif of the mature domain and the receptor carboxipeptidase E (CPE) directs BDNF toward the regulated secretion pathway, while BDNF is directed to the constitutive pathway when this interaction does not exist (Fig.27)(Lou, Kim et al. 2005). The interaction of the pro-domain motif with the protein sortilin also directs BDNF toward the regulated pathway (Chen, Patel et al. 2004).

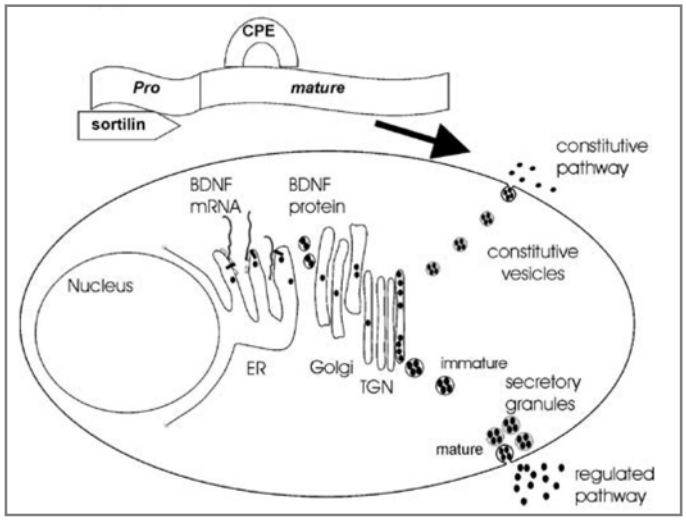

Fig.27. Scheme of constitutive and regulatory BDNF secretion pathway. Adapted from *Kuczewski et al., 2009.*

3.1.2. BDNF SIGNAL TRANSDUCTION

Neurotrophins generally activate two different receptors, the p75 neurotrophin receptor (p75NTR), a member of tumor necrosis factor (TNF), and tropomyosin-related kinase (Trk) receptors, members of the family of receptor tyrosine kinases (Fig.28). Each neurotrophin molecule can bind to one or more Trk receptors triggering distinct

intracellular signalling cascades regulating the multiple biological responses associated to neurotrophins (Patapoutian and Reichardt 2001; Huang and Reichardt 2003; Schramm, Schulte et al. 2005). Thus, NGF binds to TrkA, BDNF and neurotrophin 4 (NT4) to TrkB and neurotrophin 3 (NT3) to TrkC (Chao 2003). NT3 can also activate the others Trk receptors although with less efficiency in certain cellular contexts. In contrast, p75NTR binds each mature neurotrophins but with low affinity, although it is able to form complexes with Trk receptors generating higher affinity neurotrophin binding sites (Esposito, Patel et al. 2001) Furthermore, there are truncated isoforms of TrkB and TrkC, lacking their essential tyrosine kinase domain. It has been suggested that these receptors would help to regulate the effectiveness of neurotrophins to be presented to the full-length Trk receptors (Eide, Vining et al. 1996) and the surface expression of full-length TrkB (Haapasalo, Sipola et al. 2002).

p75NTR was the first neurotrophin receptor isolated, but its analysis were complicated because it can interact directly with Trk, and its action is modified by the coincident activation of Trk receptors. Thus, p75NTR can promote apoptosis following injury during development when Trk receptors are inactive or sub-optimally activated (Kaplan and Miller 2000). p75NTR is essential for rapid and appropriate developmental cell death and for maintaining the specificity of neuronal survival responses to different neurotrophins (Majdan and Miller 1999). Apoptotic pathways promoted by p75NTR are silenced by Trk signalling, although p75NTR in presence of Trk activation is able to modulate neuronal growth, regulating the specificity or density of axonal growth (Kaplan and Miller 2000; Park, Grosso et al. 2010).

Neurotrophin-Trk receptor (full-length) binding produces the receptor dimerization and the posterior kinase activation. Thereafter a receptor autophosphorylation occurs generating multiple tyrosine residues, which are docking sites for adapter proteins with phosphotyrosine binding (PTB) or src-homology-2 (SH2) domains (Barbacid 1994). These adapter proteins connect Trk receptor with different intracellular transducing signal pathways, including Ras-Raf-ERK (extracellular signal-regulated kinase) protein kinase pathway, the phosphatidylinositol-3-kinase (PI-3kinase)/Akt kinase pathway, and phospholipase c (PLC)-γ1 (Fig.28) (Kaplan and Miller 2000; Reichardt 2006).

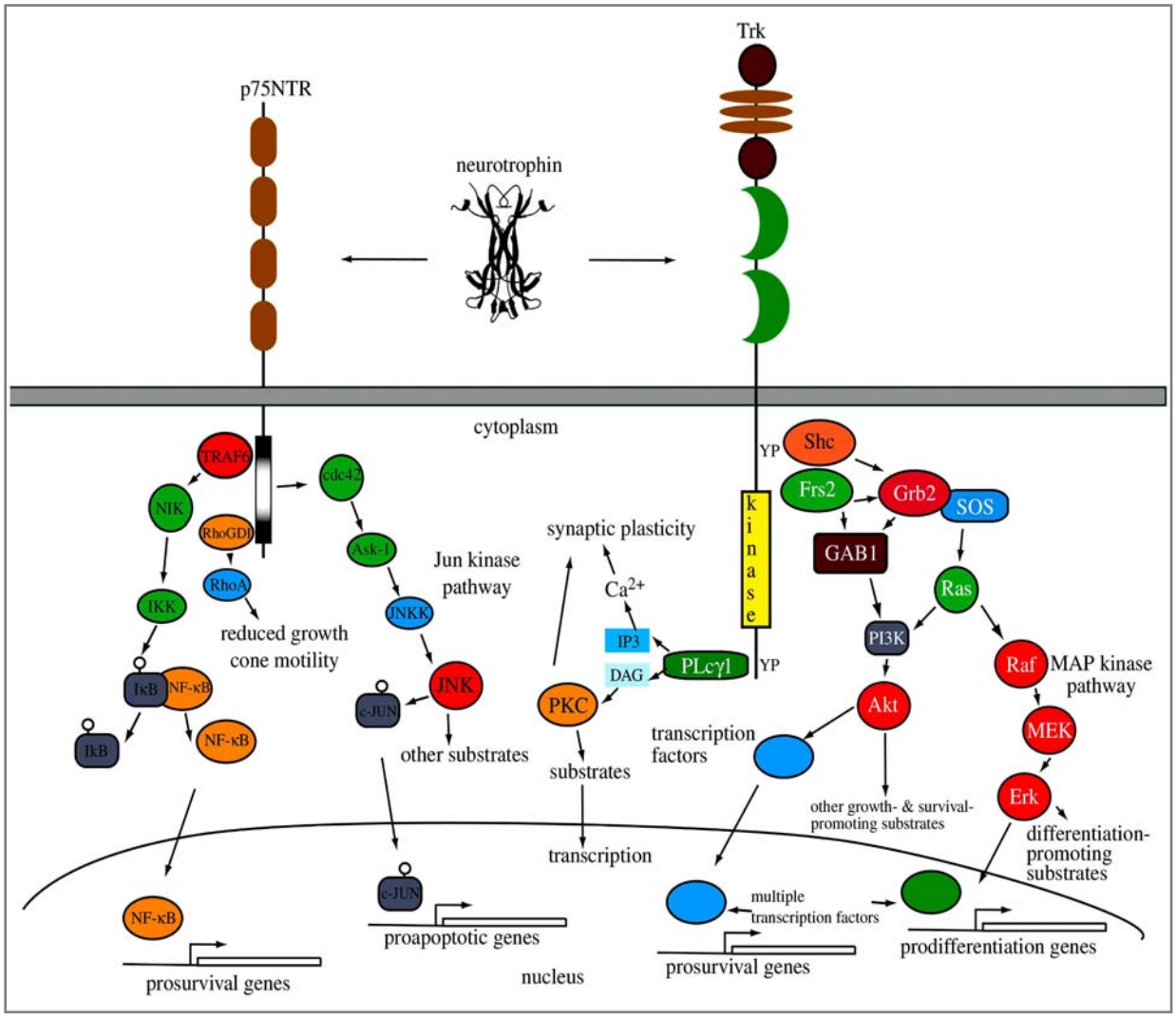

Fig.28. Neurotrophin signalling through p75NTR and Trk receptors. Adapted from *Reichardt, 2006.*

Ras-MAP kinase signalling commences with Ras activation by the adaptor protein Grb2 complexed with SOS which has been recruited by the phosphorilation of Y490 domain of Trk receptor. Ras activation stimulates the classic pathway formed by Class I PI3-kinases, Raf, and p38MAP kinase. Subsequently follows the sequential phosphorilation of MEK1 and/or MEK2 by Raf, and the posterior phosphorylation of Erk1 and Erk2 by MEK1 or MEK2 (English, Pearson et al. 1999). This pathway comprises many roles in neurons, including synaptic plasticity, long-term potentiation and cell survival (Grewal, York et al. 1999; Minichiello, Calella et al. 2002).

Phosphatidylinositol-3-kinase (PI-3kinase)/Akt kinase pathway starts with the recruitment of Gab1 by phosphorylated Grb2, and the subsequent binding and

activation of PI3-kinase. PI3-kinase can be also activated by Ras, and by insulin receptor which is phosphorylated by Trk signalling. Once PI3-kinase is activated it generates 3-phosphorylated phosphoinositides which can activate phosphatidylinositide-dependent protein kinase (PDK1). Together direct the activation of the protein kinase Akt (also known as PKB), which in turn controls, through phosphorylation, the activities of several important proteins for cell survival. Although PI-3K/AKT pathway is the major survival-promoting mechanism (Kaplan and Miller 2000; Huang and Reichardt 2003), it also controls vital neuronal mechanism such as the motility through the modulation of actin cytoskeleton (Tornieri, Welshhans et al. 2006). The 3-phosphoinositides phosphorylated by PI3 kinase are able to recruit to the membrane the Cdc42-Rac-Rho G proteins, involved in rapid cytoskeleton cell responses to growth factors.

Phospholipase C (PLC)-γ1 is activated when it binds to phosphorilated Y785 site on TrkA and analogous sites on TrkB and TrkC. PLC- *γ*1 activated is able to hydrolyse phosphatidylinositides and to generate inositol tris-phophate (IP3) and diacylglycerol (DAG). IP3 activates enzymes such as some protein kinase C isoforms and calmodulin-regulated protein kinases, enzymes that are modulated by cytoplasmic calcium released from cellular stores produced by IP3 action. DAG induces DAG-regulated protein kinase C isoforms, which is required for NGF-dependent activation of neurite outgrowth (Corbit, Foster et al. 1999; Huang and Reichardt 2003). Moreover signalling through this pathway controls expression and/or activity of many proteins, including ion channels and transcription factors (Toledo-Aral, Brehm et al. 1995; Minichiello, Calella et al. 2002). Electrophysiological experiments in transgenic animals with PLC- *γ*1 signalling disrupted showed the importance of this pathway for the initiation and maintenance of hippocampal Long Term Potentiation (LTP) (Corbit, Foster et al. 1999).

So, BDNF signalling through TrkB receptor is necessary for survival and morphogenesis of CNS neurons as well as neuronal plasticity. Some of these functions are directly exerted by intracellular BDNF-stimulated pathways and others by the BDNF-induced expression of a huge number of genes that coordinately with BDNF activate synaptic function, morphological change, survival or differentiation programs (Reichardt 2006;

Numakawa, Suzuki et al. 2010). Previous studies in our group suggested that alterations produced by abnormal higher levels of BDNF during cerebral cortex development might be due by dramatic changes in the expression of proteins associated to distinct developmental processes.

3.1.3. BDNF EXPRESSION AND FUNCTION IN THE CNS

Depending on the spatio-temporal context, neurotrophins exert assorted functions related with axon growth and guidance, dendrite structure, regulation of cell fate and survival, synapsis and synaptic plasticity. Neurotrophins expression sites and levels can be regulated by cellular interactions depending on the tissues (Patapoutian, Backus et al. 1999). Each of the neurotrophin genes is regulated through multiple promoters and enhancers (Aid, Kazantseva et al. 2007) as well as multiple extrinsic signals such as Wnt and TGF-b family members, thyroid hormone, steroids and inflammatory cytokines (Buchman, Sporn et al. 1994; Toran-Allerand 1996; Koibuchi, Fukuda et al. 1999). Moreover, BDNF gene is also regulated by neuronal activity (Aid, Kazantseva et al. 2007; Greer and Greenberg 2008). It is known that Calcium (Ca^{2+}) influx through via L-type Ca^{2+} channels activates BDNF transcription through protein kinase-cascades that result in the activation of several BDNF-activating transcription factors (Gorba, Klostermann et al. 1999; Chen, West et al. 2003; Greer and Greenberg 2008).

BDNF is broadly expressed in the developing and adult mammalian brain, being especially abundant in the hippocampus, cerebral cortex, cerebellum and amygdala. In rodents BDNF expression can be initially observed at embryonic day 13 (E13) in the cerebral cortex at low levels. During embryonic period BDNF is expressed in the marginal zone of the cerebral cortex by Cajal-Retzius cells. Both Cajal-Retzius cells and GABAergic neurons express TrkB (Fukumitsu, Furukawa et al. 1998; Gorba and Wahle 1999) that is why they are morphological, neurochemical and physiologically modulated by this neurotrophin (Numakawa, Suzuki et al.; Marty, Carroll et al. 1996; Fukumitsu, Furukawa et al. 1998; Aguado, Carmona et al. 2003). BDNF expression increases from postnatal day 2 (P2) and peaks at P14, remaining at high expression

levels during adulthood (Timmusk, Belluardo et al. 1994; Yan, Rosenfeld et al. 1997). BDNF expression is found predominantly in glutamatergic neurons, not in interneurons or astrocytes under physiological conditions, although its expression can be detected in cultured astrocytes (Ohta, Kuno et al.; Gorba and Wahle 1999).

Neurotrophins are involved in the activity-dependent development of cortical connectivity. Multiple experiments in cultured hippocampal neurons have shown that BDNF is implicated in the gene regulatory events required to synaptic plasticity in the hippocampal formation (Hu and Russek 2008). Apart from their function in the maintenance of LTP in hippocampal neurons (Lu, Christian et al. 2008), BDNF also promotes glutamatergic synapsis through PLC *γ* pathway (Numakawa, Yamagishi et al. 2002) whereas induces GABA release and the expression of synaptic proteins through MAP kinase pathway in cortical neurons (Matsumoto, Numakawa et al. 2006). Despite the fact that BDNF modulates both GABA and glutamate release, it differentially regulates pyramidal neuron and interneuron firing rates (Rutherford, Nelson et al. 1998).

GABAergic neurons tangential migration is strongly stimulated by BDNF during corticogenesis (Polleux, Whitford et al. 2002). Studies performed in our group demonstrated that BDNF also participates in the final interneuron migration period, in the radial migration and layer recognition process. Abnormal high levels of BDNF produce aberrant segregation of GABAergic neurons in the cortical marginal zone, forming ectopic cluster of GABAergic/Cajal-Retzius cells spaced by empty stretches, fact that leads to an aberrant cerebral cortex lamination (Fig.29) (Alcantara, Pozas et al. 2006). In addition, BDNF downregulates reelin, an essential glycoprotein for proper cortical lamination produced in the marginal zone by Cajal-Retzius cells and by GABAergic neurons in the cortical plate (Ringstedt, Linnarsson et al. 1998; Alcantara, Pozas et al. 2006). It has been proposed that BDNF and Reelin act in parallel during brain development and maintenance. Thus, BDNF through Cajal-Retzius cell survival and Reelin expression, directly or indirectly controls radial migration in the neocortex (reviewed by Ringstedt, 2008).

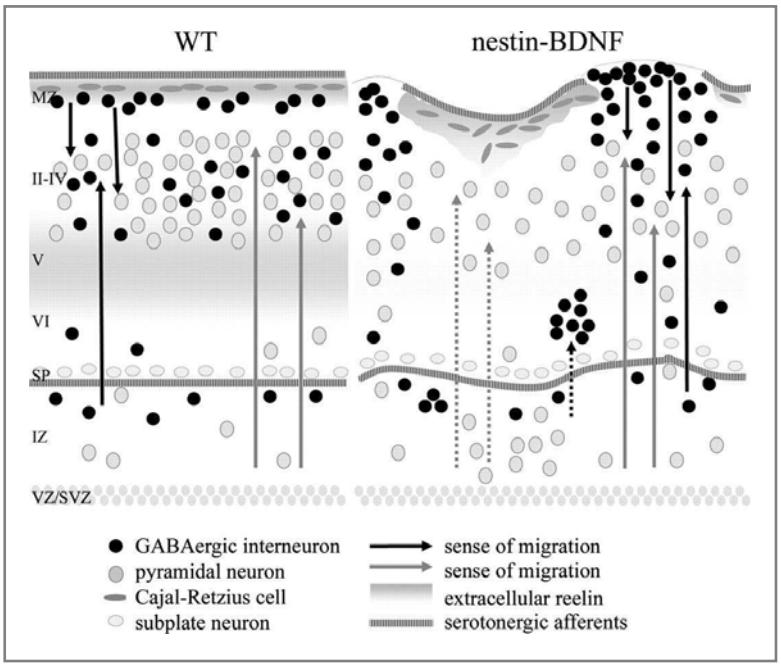

Fig.29. Overexpression of BDNF dramatically affects the distribution of cortical populations. Adapted from *Alcántara et al., 2006.*

Other approaches also support that BDNF plays an important role in the correct lamination of the cerebral cortex. Transgenic studies with TrkB knock-out animals demonstrated the importance of TrkB signalling for a correct timing of new generated neurons migration (Medina, Sciarretta et al. 2004). In these animals they observed an altered cortical lamination, with an increased number of upper layer neurons in deeper cortical layers, indicating a delay in neuronal migration.

During cortical development, neurotrophins regulates neural precursors in terms of cell proliferation and differentiation. Experiments carried out by Ohmiya and colleagues based on BDNF intrauterine injection and bromodeoxyuridine (BrdU) assays observed that the generation of the layers IV-V, but not II/III layers, was affected. They concluded that BDNF affects particular progenitors at limited stages (Ohmiya, Shudai et al. 2002). Further experiments using BDNF *in utero* microinjection suggest that BDNF might determine neural laminar fate of neurons by modifying the timing of cell cycle-exit and the expression of transcription factors important for newborn neuron commitment (Fukumitsu, Ohtsuka et al. 2006). *In vitro* experiments demonstrated that BDNF also promotes the differentiation of cortical stem cells predominantly into

neurons (Chang, Son et al. 2003). Indeed, BDNF is expressed in precursor cells of the cortical neuroepithelium (Barnabe-Heider and Miller 2003; Fukumitsu, Ohtsuka et al. 2006) which also express Trk receptors. Experiments carried out by Bartkowska and colleagues inhibiting Trk receptors pointed out that Trk signalling is essential for cortical precursor cell proliferation and neurogenesis in the cortical neuroepithelium (Bartkowska, Paquin et al. 2007). Further studies have demonstrated that not functional TrkB receptors promote differentiation of neural stem cells toward astrocytes, inhibiting neuronal fate (Cheng, Coksaygan et al. 2007). Differentiation of other neural lineages, such as oligodendrocytes, is also influenced by BDNF (Du, Fischer et al. 2003).

All these data suggest that BDNF is an important environmental cue that regulates survival, proliferation and differentiation of neuroepithelial precursors as well as their ability to regulate cell positioning and plasticity in the brain that makes it one of the most likely candidates in the aetiology of mental illness.

3.2. BONE MOPHOGENETIC PROTEIN 7 (BMP7)

The Bone Morphogenetic Protein 7 (BMP7), also known as Osteogenic Protein 1 (OP1), is a member of the Transforming Growth Factor β (TGF β) superfamily. Despite the fact that BMP7 was initially isolated from bovine bone and characterized as an osteogenic factor, its role in developing tissues is not limited to bone formation. Indeed, during development BMP7 is widely expressed and participates in skeletal muscle, nerves, blood vessels and perichondrium formation (Lyons, Hogan et al. 1995; Dudley and Robertson 1997; Godin, Takaesu et al. 1998). Different studies using knockout mice pointed out the lethality caused by the absence of BMP7 and the importance of this morphogen in the development of kidney, eye and hindlimb among other structures (Dudley, Lyons et al. 1995; Jena, Martin-Seisdedos et al. 1997; Adams, Karolak et al. 2007). Recently BMPs have emerged as a crucial regulator of nervous system development. This thesis aims to define the role of BMP7 in cerebral cortex development.

3.2.1. BMP7 CHARACTERIZATION

BMP7 gene is located on chromosome 2 in the mouse, and on chromosome 20 in humans (Hahn, Cohen et al. 1992). It is composed by 7 exons and northern analyses revealed four separate splice forms of BMP7 (Adams, Karolak et al. 2007). In general, BMPs expression is highly regulated by cis-regulatory elements (Dudley, Lyons et al. 1995; Dudley and Robertson 1997; Solloway, Dudley et al. 1998). Studies of BMP7 expression in kidney, eye and hindlimb carried out by Adams and colleagues identified an approximately 480 base pair evolutionarily conserved enhancer island within intron 1 of the BMP7 locus governing BMP7 tissue-specific expression. The activity of this enhancer is entirely dependent on the presence of a stretch of 10 base pairs that contains a predicted binding site for the transcription factor FOXD3 (Adams, Karolak et al. 2007). *In situ* hybridation studies revealed that although BMP7 is expressed in most organ systems, its expression pattern within each organ is intricately regulated during development and in the adult (Oxburgh 2009).

The expression of BMP7 has been described to be also modulated by epigenetic mechanisms (Wang, Lin et al. 2007; Yoshikawa, Hishikawa et al. 2007; Marumo, Hishikawa et al. 2008; Nott, Watson et al. 2008). The increase of histone acetylation, which is controlled by the balance between the activities of histone acetyltransferase (HAT) and histone deacetylase (HDAC), generally stimulates gene transcription by relaxing chromatin structure. In kidney, the activity inhibition of different HDACs promotes BMP7 expression, and is proposed as a regenerative strategy for ischemic insults (Marumo, Hishikawa et al. 2008).

BMP7 protein is a heterogeneous mixture of differentially processed proteins resulting from multiple proteolytic recognition sites. BMP7 is synthesized as a large precursor protein, with the mature protein present in the carboxy-terminal that undergoes proteolysis at RXXR sites giving rise to mature active homodimers linked by disulfide bonds. Each monomer of BMP7 contains seven cysteine residues conserved in the C-terminal region, six of these cysteins build a ringed structure termed cysteine knot, and the seventh is used for dimerization with a second monomer (Fig.30). The cysteine

knot constitutes the core of the monomer and consists of three difulfide bonds. In the three-dimensional structure of BMP7 are also observed four strands of antiparallel β-sheets that form two finger-like projections. An α-helix is located on the opposite end of the knot, with a perpendicular orientation respect the axis of the two fingers. The active BMP7 dimer is formed by the association of the α-helix of one monomer with the finger regions of the second monomer, and is stabilized by a single interchain disulfide bond (Griffith, Keck et al. 1996).

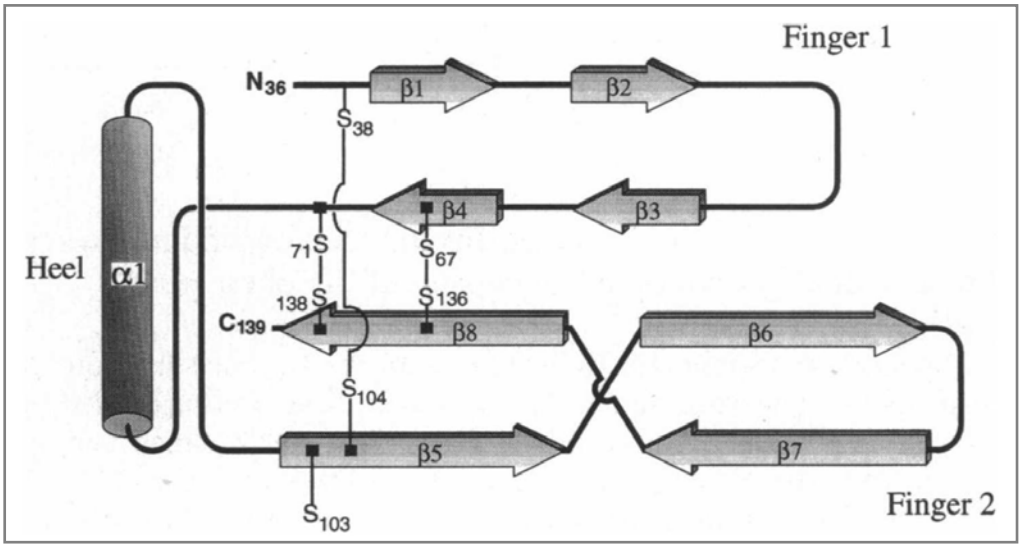

Fig.30. Schematic structure of BMP7 monomer. Adapted from *Griffith et al., 1996.*

Human BMP7 precursor is a 431 amino acid glycoprotein that has a native signal of targeting to the endoplasmic reticulum, and a prodomain region. In the endoplasmic reticulum the protein folds into a homodimeric complex, which is then proteolytically processed by Furin-like paired basic proprotein convertase (PACE). The primary cleavage event results in the prodomain removal which will remain non-covalently associated to the covalently-linked mature homodimer. Indeed, it is reported that the prodomain is essential for directing BMP7 to the extracellular matrix through the interaction of the prodomain with fibrillin microfibrils in the extracellular matrix (Gregory, Ono et al. 2005). This avidly binding of BMPs to extracellular matrix proteins limits their diffusion. Moreover, BMPs are not particularly soluble, hence their biological actions are likely to be quite local (Hall and Miller 2004).

3.2.2. BONE MORPHOGENETIC PROTEINS SIGNALLING

BMPs act through hetero-oligomeric complexes formed by serine/threonine kinase receptors type I and type II. Binding of the BMP to at least one Type-I and one Type-II receptor is necessary for the activation of the BMP signal. There are seven Type-I receptors identified (ALKs, activin receptor-like kinases), and five Type-II receptors (Fig.31). BMPs have low affinity for type I and II receptors but require both for high affinity binding and signalling. It is also known that BMPs bind with different affinity to specific Type-I and Type-II receptors. The preference of some BMPs for particular receptors may explain the hypothesis that the affinity of BMPs to their receptors is important for the activation of particular transduction signals. It is also known that different cells express different receptor combinations and downstream signalling molecules that contribute to the diversity of BMP signalling responses (Ebendal, Bengtsson et al. 1998; Nohe, Keating et al. 2004). Regarding BMP7, it seems to bind well to BMPR-II and forms a signalling complex in the presence of ActR-I or BMPR-IB. At high doses, BMP7 is also able to bind to ActR-II and IIB when ActR-I, BMPR-IA, or IB are present (Yamashita, ten Dijke et al. 1995). In the brain BMPR-II, ActR-I and ActR-II are highly expressed, suggesting the importance of BMP7 signalling for the formation and normal function of the CNS.

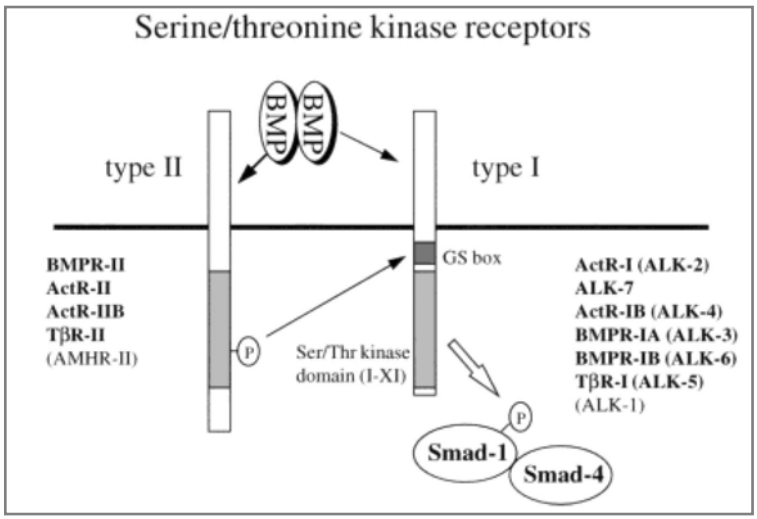

Fig.31. Seven Type-I receptors (ALKs, activin receptor-like kinases), and five Type-II BMP receptors have been identified. Adapted from *Ebendal et al., 1998.*

The canonical BMPs intracellular pathway starts once they bind to at least one Type-I and one Type-II receptors, then the Type-II receptor phosphorylates the Type-I receptor leading to the recruiting of intracellular Smad proteins. Once receptor activated-Smads (R-Smads: Smads1, 5 and 8) associate with Type I receptor, they undergo direct phosphorylation at their C-terminal SSXS motif. Then R-Smads are released from the receptor and recruit the common mediator Smad (Co-Smad: Smad4) forming a complex that migrates into the nucleus and activates the transcription of specific target genes. R-Smads are present predominantly as monomers in the steady state. Ligand stimulation promotes R-Smads to form homo- or hetero-oligomers that are composed of R-Smads alone or together with Co-Smads (Nohe, Keating et al. 2004). In the nucleus, Smad proteins exert transcriptional activity through direct binding to DNA as well as through association with other DNA-binding proteins. Although BMPs are known to induce most of their multiple functions through Smad pathway, alternative pathways activated by BMPs have been reported. An example is the BMP-dependent activation of LIM Kinase 1, a key downstream effector of Rho GTPases that can regulate important CNS cellular mechanisms such as neurite outgrowth and synapse formation (Lee-Hoeflich, Causing et al. 2004).

BMP dependent Smad-pathway signals are modulated by cellular intrinsic inhibitory molecules, the inhibitory Smads (I-Smads). In the absence of ligand, I-Smads are located predominantly in the nucleus, but after ligand stimulation, they are exported rapidly into the cytoplasm. I-Smads can interact directly with activated Type-I receptors preventing the access of R-Smads. It has also been demonstrated that the I-Smad6 can inhibit BMP signalling by competing with Smad4 for heteromeric complex formation with activated Smad1 (Fig.32).

In addition to the tissue-specific expression of BMPs and their receptors, BMP signalling is modulated by BMP antagonists. These extrinsic molecules bind directly to BMPs avoiding the association between BMPs and their receptors in the extracellular space (Fig.32). The list of proteins classified as BMP antagonists is large. These molecules have a secretory signal peptide and a cysteine arrangement that directs the formation of the cystine-knot structure, which depending on its size allow classifying

BMP antagonists (Avsian-Kretchmer and Hsueh 2004). Noggin is probably one of the most known BMP antagonists. It is expressed in the notochord, dorsal somite and condensing cartilage among others, and its presence is vital for a correct patterning of the neural tube and somites, as well as to regulate the stem cell niche during neurogenesis (Yanagita 2005). So, a correct regulation of the spatio-temporal expression of BMPs, their receptos and BMP antagonists is vital to control multiple developmental functions exerted by BMPs.

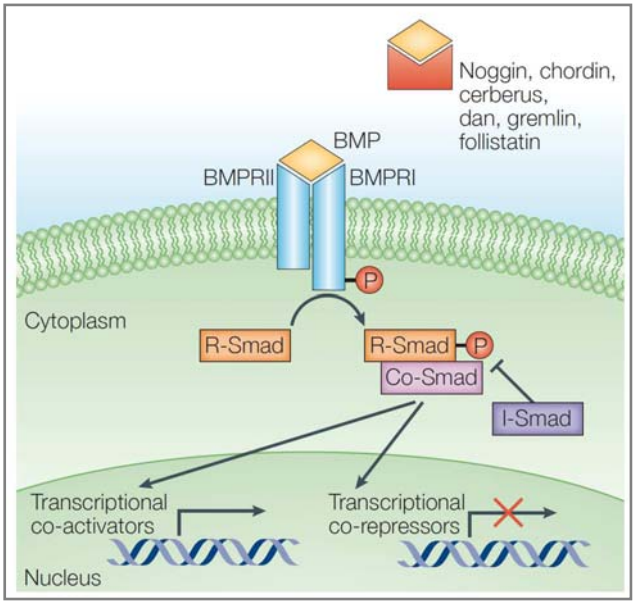

Fig.32. The canonical Smad-depedent pathway activated by BMPs. BMP ligands bind to BMPRI and BMPRII receptors activating Smad transcription factors, which transactivate a large amount of genes. BMP signal can be blocked by intracellular inhibitory Smads that repress the transcriptional action of activator Smads, while extracellularly many inhibitors can avoid the binding of BMP ligands to the receptor. Adapted from *Liu et al., 2005.*

3.2.3. ROLE OF BMP7 DURING DEVELOPMENT

BMP7 is firstly expressed in the notochord, surface ectoderm, and yolk sac endoderm (Lyons, Hogan et al. 1995). At later stages, BMP7 transcripts are detected in many developing organs, including the eye, kidney, heart, brain, and bone. Moreover, BMP receptors BMPR-II, ActR-I, and ActR-II are highly expressed in the brain indicating that BMPs may exert important functions in neurons during development and in response

to brain stimulus (Ebendal, Bengtsson et al. 1998; Hall and Miller 2004). However, defects in BMP7 mutant embryos are largely restricted to the developing eye and kidney (Dudley, Lyons et al. 1995; Luo, Hofmann et al. 1995; Dudley and Robertson 1997; Jena, Martin-Seisdedos et al. 1997; Godin, Takaesu et al. 1998). Although the loss of BMP7 seems to be essential during early developmental steps of the eye or kidney, the absence of BMP7 becomes progressively more severe with time, suggesting that BMP7 is required for continued organogenesis.

One possible explanation for the initial restricted defects caused by BMP7 is that other BMP family members functionally substitute BMP7 in tissues unaffected by loss of BMP7 signalling. Considerable data suggest that several members of the BMP subfamily possess overlapping functional activities. Dudley and Robertson showed that the organ systems that normally express BMP7 and that appear unaffected by a null mutation in BMP7, express one or more additional BMP family members. On the contrary, both the kidney and eye, which contain cell populations that exclusively express BMP7, show dramatic developmental defects in the absence of this morphogen (Dudley and Robertson 1997).

BMP members in mammals are expressed in the growing nervous system where emerged as crucial regulators of dorsoventral patterning of the neural tube, neural cell fate determination, and cell death as well as terminal neural cell differentiation. During gastrulation, BMPs are secreted by ectodermal cells and specify epidermal fate (Zimmerman, De Jesus-Escobar et al. 1996). Later, they are implicated in the decision of the embryonic cells to form the neural and non-neural ectoderm. The CNS initially develops from the dorsomedial region of the embryonic ectoderm, a process that requires the active repression of BMP signals (Liu and Niswander 2005). The following step of defining the neural crest cell region is also trigger by BMPs. BMPs keep controlling neural crest cells promoting neural crest cell migration, mediating neural crest apoptosis in the hindbrain, and directing these cells to differentiate into sympathetic neurons (Varley, McPherson et al. 1998; Sela-Donenfeld and Kalcheim 1999; Smith and Graham 2001).

There is evidence from spinal cord experiments that BMP7 produced by the dorsal surface ectoderm acts as dorsalizing signal inducing expression of dorsal neural markers (Liem, Tremml et al. 1995). As the neural tube closes, BMP proteins are secreted from the roof plate, a new-formed signalling centre at the dorsal-most region of the CNS. Shh, secreted from the ventral floor plate, together with BMPs are the sources of powerful signalling molecules that direct the patterning of the neural tube along the dorsoventral axis. The on/off BMP signalling stage along dorsoventral axis allows a gradual expression pattern of different transcription factors, such as Pax, Nkx, Math, Mash and neurogenin, that controls the differentiation of motorneurons, GABAergic neurons and laterly oligodendrocytes, in the proper region of the spinal cord (Liem, Jessell et al. 2000; Zhou, Wang et al. 2000; Liu and Niswander 2005).

The cerebellum is another area of the CNS that is under BMP modulation during development. Cerebellar granule neurons are crucial for the function of the cerebellum and they arise next to the roof plate, a source of BMP signals. Recent studies has pointed out that: (1) BMP2 is expressed in both external germinal layer and internal granular layer in the cerebellum, and (2) BMP signalling is required for cerebellar granule neuron development at both early and late developmental stages (Ming, Elkan et al. 2002; Angley, Kumar et al. 2003; Rios, Alvarez-Rodriguez et al. 2004).

Similar to spinal cord and cerebellum, BMP signalling is necessary for development of the dorsal forebrain whereas the development of the ventral forebrain is dependent on the inhibition of BMP signalling. During murine development, BMP7 is highly expressed in meninges and choroid plexus giving place to the possibility that the protein is secreted into the cortical parenquima and into the cerebrospinal fluid respectively (Helder, Ozkaynak et al. 1995; Furuta, Piston et al. 1997; Charytoniuk, Traiffort et al. 2000; Dattatreyamurty, Roux et al. 2001). Furthermore, expression of BMP2 and BMP4 is observed along cortical ventricular region during neurogenesis (Li, Cogswell et al. 1998), which makes them suitable factors for controlling the neural fate of ventricular and subventricular progenitors. Depending on the time of development, BMPs can promote either neuronal or astroglial differentiation of cortical progenitors

(Gross, Mehler et al. 1996; Li, Cogswell et al. 1998). In earlier neural development (E13) BMPs predominantly induce cell death and inhibit the proliferation, as a mechanism for the regulation of cell number and phenotype within the developing cortex (Furuta, Piston et al. 1997). Subsequently they exert sequential actions promoting neuronal differentiation at E16 and increasingly with time, they promote astrocytic differentiation (Li, Cogswell et al. 1998; Mabie, Mehler et al. 1999). BMP proteins participate in gliogenesis inhibiting the proliferation of neural progenitors at the same time that induce the expression of genes that are specific for astrocytes (Gross, Mehler et al. 1996; Nakashima, Takizawa et al. 2001; Yanagisawa, Takizawa et al. 2001). In fact, BMP signalling blocks the action of proneural basic helix-loop-helix (bHLH) transcriptional activators expressed in the cortex such as Neurogenin 1 and 2, and Mash1. Activated bHLH dimerize with E proteins that binds specific 6-bp DNA motifs, called E boxes, that are present in the promoter of a neural gene which transcription will be activated (Guillemot 2005). BMPs up-regulates Id and Hes, inhibitory bHLH factors, that binds to E proteins and so blocking the binding of the proneural transcriptional complex (bHLH-E protein) to the DNA (Yanagisawa, Takizawa et al. 2001; Ross, Greenberg et al. 2003; Vinals, Reiriz et al. 2004). Another astroglial fate inductive mechanism involves a complex formed by Smad1 and Stat3. BMPs in synergistic cooperation with IL-6 family of cytokines, promote the binding of Smad1-Stat3 with the transcriptional coactivator p300, which transactivates target genes related with astrocytogenesis and avoids the transcriptional inhibitory action of proneural bHLH at the same time (Yanagisawa, Takizawa et al. 2001; Guillemot 2005).

In the ventral forebrain BMP and noggin (BMP inhibitor) potentiate the elaboration of GABAergic neurons and oligodendrocytes respectively, from MGE and LGE progenitors after tangential cortical migration (Yung, Gokhan et al. 2002). BMP ligands are expressed during early embryonic period in the cerebral cortex at the time of GABAergic neuronal lineage specification, whereas noggin is selectively expressed later, in the perinatal subcortical white matter, where oligodendrocyte lineage specification and maturation is carried out (Yung, Gokhan et al. 2002; Samanta, Burke et al. 2007).

At later developmental stages BMPs promote astroglial fate and inhibit oligodendroglial fate in the subventricular progenitor cells (Mabie, Mehler et al. 1997; Gomes, Mehler et al. 2003; Samanta and Kessler 2004). At postnatal stages, bipotential O2-A that give rise to both oligodendrocytes and astrocytes, respond to BMP treatment by turning into astroglial cells in place of oligodendroglial cells. This effect might be also mediated by the BMP-induced ID factors Id2 and Id4 (Ross, Greenberg et al. 2003; Samanta and Kessler 2004).

4. CHEMOKINES AND DEVELOPMENTAL PATHOLOGIES

Abnormal development of the brain during fetal life is thought to contribute to the aetiology of many functional and behavioural disorders that manifest throughout life. Cerebral cortex development involves a set of highly complex and organized events, including neural stem cell proliferation, migration, and neuronal differentiation. Anomalies in these processes as well as possible insults produced during embryonic and perinatal developmental period can lead to an imbalance between neuronal excitatory and inhibitory system that has been associated with distinct neurological syndromes such as epilepsy, autism and intellectual or developmental disabilities (Gleeson 2001). It is expected that such imbalance can be directly linked to an aberrant development of glutamatergic and/or GABAergic populations in the cerebral cortex. One possible cause is the alteration of the ratio between cell proliferation and cell death that determines the ultimate number of neurons of both cell population in the developed brain. Perturbations in this delicate balance or perturbations occurring very early in development affecting subsequent cell progeny result in focal cortical dysplasia (Pang, Atefy et al. 2008). Another cause of abnormal cortical function involves defects in neuronal migration provoked by abnormal actin and microtubule cytoskeleton dynamics that also lead to aberrant corticogenesis. Mutation in proteins associated to cytoskeleton such as Lis1 and DCX provoke abnormal neuronal migration and severe cortical dysfunction (Guerrini and Parrini 2010). Cortical dysplasias associated to neurogenic and neuronal migration disorders have been reported to be the cause of different types of mental retardation and epilepsy (Pang, Atefy et al. 2008).

Disorders of synaptic plasticity also contribute to learning and behavioural difficulties associated to some types of epilepsy and many developmental neuropathologies. Abnormal synaptic plasticity may be due to alterations in receptors, signalling molecules, or neurotrophins (Brooks-Kayal 2011). Cortical networks are especially highly dependent on neurotrophic factors that are essential for the differentiation and survival of neurons as well as for the establishment of neuronal connections (Hu and

Russek 2008). A clear example is BDNF, one of the main regulators of synaptic plasticity that has emerged as key target in neurological diseases studies.

Genetic aetiologies are not always underlying functional and behavioural neuronal disorders. Several types of insults during pregnancy such as hypoxia, seizures, and infection, are known to be major causes of fetal brain damage and dysfunction. Our group is especially interested in perinatal pathologies associated with changes in BDNF levels and abnormal brain functionality. One pathological condition that fits into this context is hypoxic-ischemic (H-I) injury associated with preterm infants and distinct fetal anomalies. The causes of H-I injury are multiple. During embryonic development the fetus totally depends on the oxygen and nutrients supplied by placenta. Reduction of fetal cerebral blood flow by acute umbilical cord occlusion or by impaired placental function due to maternal hypertension, partial placental detachment or uterine artery occlusion reduce oxygen and/or nutrients delivery necessary for a correct brain development (Rees, Harding et al. 2011). In preterm infants with very low birth weight, a failure in oxygen delivery to the developing brain is often caused by immature lung development.

Even though the survival rate of affected individuals has improved, these patients at still high percentages exhibit mental retardation or borderline ranges at school, and cerebral palsy. The most common lesion observed in preterm ischemic brain is periventricular leukomalacia (PVL), characterized by cystic infarcts adjacent to the lateral ventricles and gliosis extending throughout the cerebral white matter. Oxygenation problems in these infants mainly lead to oligodendroglial/white matter damage despite cerebral cortex grey matter, cerebellum and hippocampus is also altered. Excessive apoptosis produced by low oxygen levels during perinatal period alters neuronal differentiation, synaptogenesis and loss of neurons, glia, and their progenitor cells that finally generate dramatic consequences (Curristin, Cao et al. 2002; Madri 2009).

The physiopathology of H-I injury is characterized by two phases: the first dominated by necrotic processes in the ischemic areas (ischemic phase) and the second dominated by apoptotic processes produced by oxygen reactive especies formed after reperfusion and reoxygenation (reperfusion phase). Distefano and Praticó (Distefano and Pratico 2010) summarized the complex cellular mechanisms implicated in both H-I phases in the following two tables:

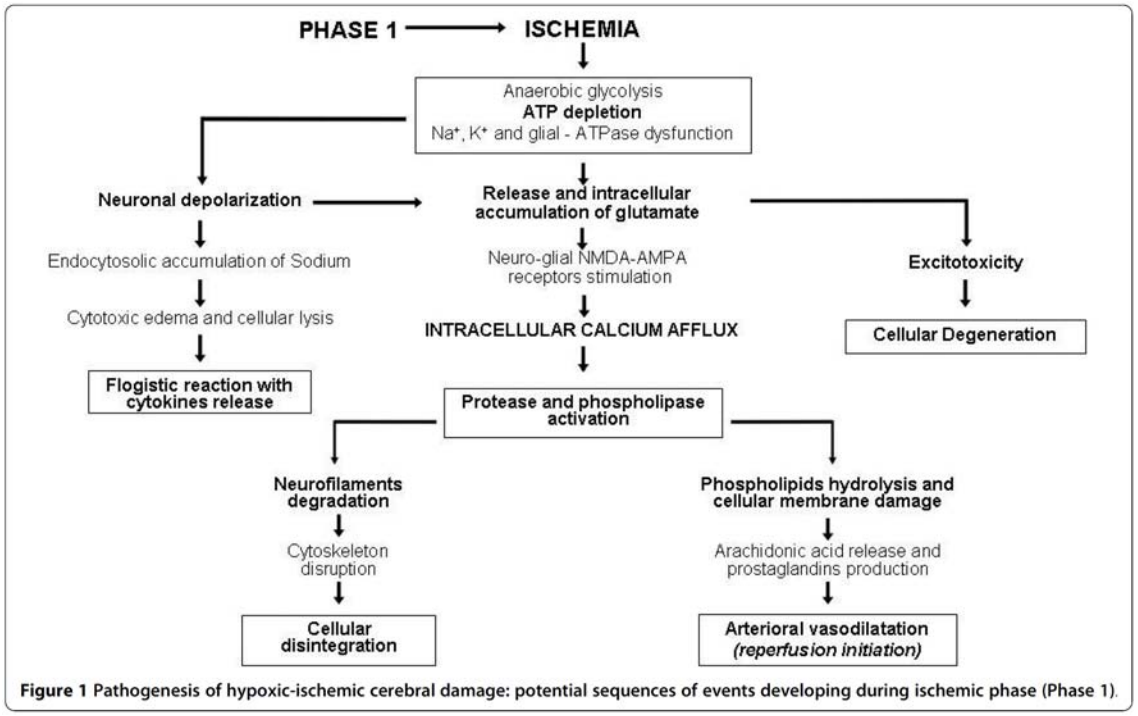

Figure 1 Pathogenesis of hypoxic-ischemic cerebral damage: potential sequences of events developing during ischemic phase (Phase 1).

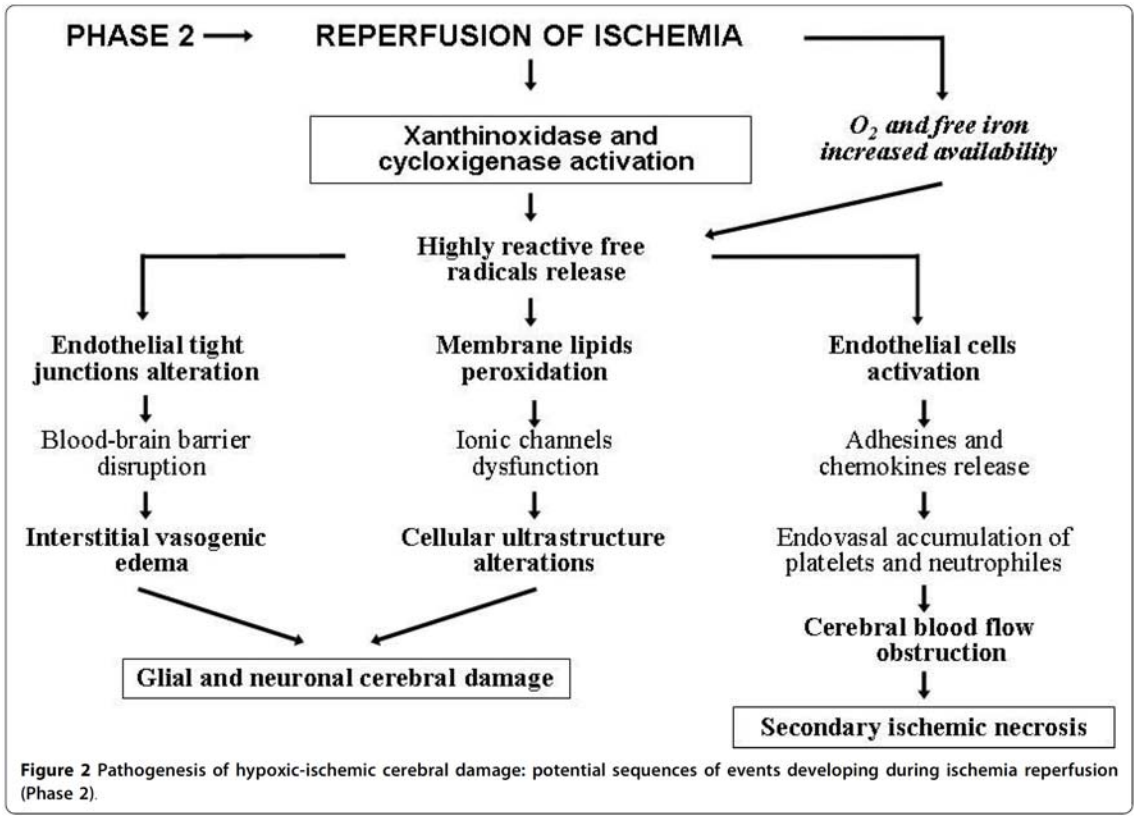

Figure 2 Pathogenesis of hypoxic-ischemic cerebral damage: potential sequences of events developing during ischemia reperfusion (Phase 2).

In preterm infants PVL is in part promoted by factors associated to prematurity such as metabolic hyperactivity of periventricular regions, where has been observed intense proliferation, differentiation and migration of glial and neuronal cells following hypoxia (Ong, Ling et al. 2005; Chou, Harvey et al. 2006; Yang, Covey et al. 2007). These processes require high levels of oxygen and nutrients, producing high amount of reactive oxygen species. Oligodendrocytes are particularly vulnerable to oxidative stress due to their poor content of anti-oxidizing enzymes (Volpe 2001), a plausible reason that may explain the severe white matter affectation caused by hypoxic injuries.

Milder hypoxic lesions have been also observed to produce gray and white matter damage, indicating that developing brain is especially sensitive to oxygen levels deviations (Rees and Inder 2005; Sizonenko, Camm et al. 2008). Indeed, moderate sub-lethal hypoxia is enough to alter key developmental programs of the newborn brain, compromising its developmental and cognitive potential (Curristin, Cao et al. 2002). Chronic mild placental insufficiency can result in long-term deficits in neural connectivity as well as effects on postnatal function related to mental disorders such as schizophrenia (Rees and Inder 2005). In addition, a milder hypoxic model than the classic carotid artery occlusion, such as perinatal hypobaric hypoxia in rodents is enough to alter morphological and functional maturation of the corpus callosum, neocortex and hippocampus (Langmeier, Pokorny et al. 1987; Maresova, Valkounova et al. 2001; Langmeier and Maresova 2005) and impair learning capacities (Simonova, Sterbova et al. 2003). So, the timing and severity of perinatal insults are critical in determining the outcome in terms of the severity of the damage and the regions of the brain affected.

An important handicap associated to hypoxic injuries is the imbalance produced in neurotrophins and morphogens levels that, as has been described above, are essential factors for morphogenesis and neuronal survival, as well as for the functional maintenance of the neural circuits (Cheng, Gidday et al. 1997; Galvin and Oorschot 2003; Chou, Harvey et al. 2006; Sun, Zhou et al. 2008). In the first phases of pregnancy, neurotrophins are received by the fetus from the mother through the placenta, and at

the end of the embryonic development they are synthesized by the same fetus. Preterm infants and infants that have suffered intrauterine growth restriction show very low availability of neurotrophins (Malamitsi-Puchner, Economou et al. 2004; Malamitsi-Puchner, Nikolaou et al. 2007), a condition associated with learning and behavioural disabilities.

Positive effects of BDNF after maternal hypoxic insults in the CNS has been broadly reported (Cheng, Gidday et al. 1997; Galvin and Oorschot 2003; Golan, Kashtuzki et al. 2004; Sun, Zhou et al. 2008). H-I injury is known to induce increased BDNF levels although is observed a drop in BDNF concentrations during the following normoxia (Lindvall, Ernfors et al. 1992; Korhonen, Riikonen et al. 1998; Hubold, Lang et al. 2009). Exposure to brief periods of moderate hypoxia confers relative protection against subsequent ischemic brain damage, a neuroprotective phenomenon of ischemic tolerance named preconditioning (Ran, Xu et al. 2005; Madri 2009). Hubold and colleagues observed that preconditioning treatment maintaining high levels of BDNF after acute H-I insult, preserving the neuroprotective properties of this neurotrophin (Hubold, Lang et al. 2009). A part from *in vitro* and *in vivo* results that demonstrated the neuroprotective role of BDNF (Husson, Rangon et al. 2005; Guo, Kim et al. 2008), it has been observed that cortical expression of BDNF after perinatal H-I injury is accompanied by angiogenesis and vessel stabilization as well as subventricular progenitors proliferation and neurogenesis (Ong, Ling et al. 2005; Yang, Covey et al. 2007; Madri 2009). Hypoxic Induced Factor (HIF) produced in response to reduced levels of oxygen, might promote BDNF expression in SVZ, a “neurovascular niche” bordered by ependymal cells and comprised of neuroblasts, astrocytes and neural stem cells/neuronal precursor cells (Madri 2009). Complex cross-talk between cells of this niche is known to control angiogenesis and different steps of neurogenesis (Fig.33). Endothelial cells are known to modulate neurogenesis in part through the secretion of soluble factors including BDNF, which are also known to be synthesized by neuronal cells which in turn modulate endothelial cells (Madri 2009). Thus, BDNF is a vital element to understand neurodevelopment abnormalities associated to chronic sub-lethal hypoxia.

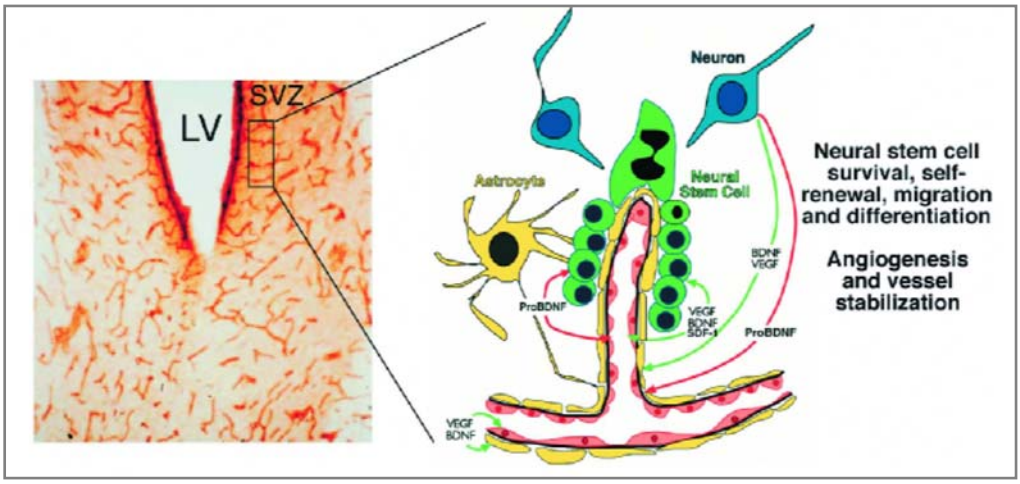

Fig.33. Scheme of the neurovascular niche located in the SVZ and the co-regulation mechanisms between endothelial and neural stem cells. Adapted from *Madri, 2009.*

Apart from its critical role in neuroprotection and in the maintenance and plasticity of the neural circuit, BDNF also has an important influence on the human mind and consciousness (Numakawa, Suzuki et al. 2010). Different works have demonstrated that BDNF expression is decreased in several mental disorders such as schizophrenia, bipolar disorder, and major depression (Knable, Barci et al. 2004; Gervasoni, Aubry et al. 2005; Karege, Bondolfi et al. 2005). On the contrary, BDNF elevated levels in serum and cephaloraquidium liquid have been observed in children with epilepsy and pervasive development disorders (PDD) (Tsai 2005; Connolly, Chez et al. 2006). Autism is an example of PDD, and is characterized by deficits in interpersonal social behaviours, abnormal language development and restricted interests with sterotypic/ritualistic behaviours. The increase in transcripts observed during pathological conditions may either be an aberrant mechanism contributing to the pathology or could act as a protective mechanism linked to the trophic action of BDNF (Kuczewski, Porcher et al. 2009). These data suggest that multiple diseases are associated with the potential deregulation of BDNF-driven signal transduction programs. Identifying the battery of gene products that respond to the release of BDNF will be especially important to the success of molecular therapeutics for disease intervention.

In this thesis I show that BDNF modulates BMP7 expression, and how both cytokines seem to be physiologically close linked. As BDNF, BMP7 has a neuroprotective and neuroregenerative role after injury (Tsai, Weng et al. 2007; Sabo, Kilpatrick et al. 2009; Tsai, Pan et al. 2010). BMP7 promotes regeneration and functional recovery through neurogenesis induction as well as improving blood flow and local cerebral glucose utilization after ischemia (Chang, Lin et al. 2003; Harvey, Hoffer et al. 2005; Chou, Harvey et al. 2006). After H-I lesion, both chemokines are also important for establishing new synapses by promoting dendritic growth and remodelling (Esquenazi, Monnerie et al. 2002; Deumens, Koopmans et al. 2006). However the neuroprotective action of both factors depends on the dose and on the spatio-temporal conditions (Zhu, Mehler et al. 1999; Schallert, Fleming et al. 2000; Husson, Rangon et al. 2005; Hubold, Lang et al. 2009). Husson and colleagues described that different doses of BDNF, at different postnatal days, show completely different white matter neuroprotective response in front brain insult (Husson, Rangon et al. 2005). Similarly, BMPs have neuroprotective and neuroregenerative properties depending on the treatment administration timing. While BMP administration is broadly accepted as a neuroprotective therapy in front of CNS injury, it has been also reported that the inhibition of BMP signalling may favour white matter regeneration (Hampton, Asher et al. 2007; Jablonska, Aguirre et al. 2010; Dizon, Maa et al. 2011).

Thus, understanding the roles that neurotrophins and morphogens play during development and during brain injury is critical to determine how their modulation promotes repair responses, and which strategies could be followed in a future for better recovering after CNS injury.

OBJECTIVES

OBJECTIVES:

1. Characterize the signalling pathways involved in BDNF-dependent BMP7 expression during cortical development *in vivo* and *in vitro*.

2. Analyze the function of BMP7 on neuronal and glial cortical progenitors differentiation.

3. Determine the effect of BMP7 on neuronal migration during cerebral cortex development.

4. Study the role of BMP7 on midline cell populations involved in corpus callosum formation.

5. Analyze changes in neural progenitor fate and differentiation induced by modified BMP7 levels in response to sublethal hypoxia.

MATERIALS AND METHODS

MATERIALS AND METHODS

Plasmid vectors construction

BDNF and BMP7 DNA sequences were amplified by Polymerase Chain Reaction (PCR) from genomic mouse DNA using specific primers with a restriction enzyme target inserted (Invitrogen, Paisley, UK):
BMP7-BamHI-Forward (5'to 3'): CGG GAT CCT TAA CCA TGC ACG TGC GCT CGC TGC G
BMP7-XhoI-Reverse (5'to 3'): CCG CTC GAG CTA GTG GCA GCC ACA GGC CC
BDNF-BamHI-Forward (5'to 3'): CGG GAT CCT TAA CCA TGA CCA TCC TTT TCC TTA C
BDNF-EcoRI-Reverse (5'to 3'): GGG AAT TCT ATC TTC CCC TTT TAA TGG
We digested the amplified DNA sequences of BDNF and BMP7 as well as the respective transcription vectors pBluescript II Ks (Clontech, Saint-Germain-en-Laye, France) and pENTR^{TM}1A (Invitrogen, Paisley, UK) with the respective restriction enzymes (Takara, Saint-Germain-en-Laye, France). Subsequently, BDNF sequence was sub-cloned from pBluescript II Ks to the expression vector pEF1 (Constructed and provided by H. Tabata) using the EcoRI and BamHI retriction enzymes. BMP7 was sub-cloned from pENTR^{TM}1A to the expression vector pDEST-EF1 (Constructed and provided by J.L. Rosa) using Gateway^{TM} Cloning Tecnology (Invitrogen, Paisley, UK).

Bacteria transformation was performed on competent DH5α E.coli strain. Sequencing was carried with the BigDye® Terminator v3.1 Cycle Sequencing Kit (TaqMan® Gene Expression Assay, Applied Biosystems, Foster city, CA) to confirm the correct sequence of the cloned gene.

In utero injection and electroporation in mice

Experiments were designed to minimize the number of animals used in the procedure. All animal protocols were approved by the Institutional Animal Care and Use Committee in accordance with Spanish and EU regulations.

Overexpression of BDNF and BMP7 was obtained in mice by expression vector injection and electroporation, following the protocol previously described by Tabata H and K Nakajima, 2001, or directly injecting the recombinant proteins. pEF1-GFP vector, or a mixture of pEF1-GFP + pEF1-BDNF or pEF1-BMP7 vectors at a 4:1 ratio were injected in the lateral ventricle of E14 and P0 mice depending on the experiment. Before surgery, P0 animals were anesthetized in ice while for the *in uterus* injection in embryonic murine brains, pregnant OF1 females carrying embryonic day 14 (E14) embryos (considering E0 the day where vaginal plug was observed) were anesthetized with Ketamine/Valium (150 µg/g, 5 µg/g, i.p.). Two microliters of recombinant human BMP7 (1 µg, R&D, Abingdon, UK), recombinant human BDNF (1,5 µg, Peprotech, London, UK), vehicle, or of DNA expression vectors (6-10 µg) were delivered into the lateral ventricles of the animals followed by electroporation in the case of vectors. In the *in utero* experiments the uterine horns were exposed before injection and they next were returned to the abdominal cavity allowing them to develop normally. Embryos were sacrificed at E15, E16 or E18, and used for protein or mRNA extraction, for cell culture, or for histological or immunocytochemical analysis.

BrdU birthdating

Thymidine analog 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich, Saint Louis, MO) was injected intraperitoneally into pregnant females at E14 at a concentration of 50 mg/kg body weight, 3h after BMP7 injection to the embryos. At E18, embryos were perfused and processed as described above. Incorporated BrdU was then detected by immunohistochemistry.

Genotyping protocol

Tiny piece of mouse tail (2-3mm) were cut to genotype BMP7 transgenic mice progeny. For tissue digestion we incubated tails with Proteinase K (Sigma-Aldrich, Saint Louis, MO) overnight at 55^{o}C. Subsequent DNA precipitation and PCR reaction was carried out to detect BMP7 and Lac Z loci in our mice. We used de following primers for PCR reaction:

BMP7- Forward (5'to 3'): GCC CGG GCC AGA ACT GAG TAA A

BMP7- Reverse (5'to 3'): CGT CCA CGA CCC GAG GTC ACT T

Lac Z-Forward (5'to 3'): CCG TCG TTT TAC AAC GTC GTG AC

Lac Z-Reverse (5'to 3'): CGG ATT GAC CGT AAT GGG ATA G

Amplified DNA was separated by electrophoresis in 2% agarose gels and detected by Etidium Bromide (Boehringer Ingelheim, Sant Cugat, Spain) DNA staining.

Prenatal hypobaric hypoxia protocol

E16 pregnant OF1 females were placed into hypobaric chamber simulating conditions altitudes of 5000 meters overnight (12 hours), with a pressure of 405 mmHg and 11 % oxygen levels. At these altitudes arterial oxygen pressure in human is reduced from 90 mmHg, in altitudes of 0 m, to 38 mmHg. We directly extract the embryos once hypoxic treatment is finished or we let the female get birth her litters and just at birth (P0) the animals are processed for the posterior RT-PCR and immunohistochemical (IHC) analysis.

Primary cell cultures

Primary cultures were obtained from E16 mice neocortex. Briefly, embryonic cortices were dissected out and dissociated by trypsin-EDTA (Biological Industries, Kibbutz Beit Haemek, Israel) and DNAse I (Sigma-Aldrich, Saint Louis, MO) treatment for 10 minutes, followed by mechanical disruption. To obtain enriched neuronal cultures the

dissociate was pre-plated in a 10 cm culture dish for 1 hour at 37ºC in DMEM supplemented with 10% normal horse serum (NHS) (Gibco, Auckland, NZ). Embryonic cortical cells were seeded in serum-free Neurobasal medium (Gibco, Paisley, UK) supplemented with B27 (Gibco, Paisley, UK) on 6- and 24-well plates containing slides coated with poly-D-Lysine (Sigma-Aldrich, Saint Louis, MO). Serum-free neuronal cultures were initially serum starved for 24h. To obtain primary glial cultures we used a similar protocol, in which P0-P1 cortices cell suspensions were plated directly onto uncoated 6- and 24-well plates in DMEM with 10% NHS. Once the cells reached confluence, they were dissociated and plated again in order to eliminate remanent neurons. We only used passages 1 to 3. Three days after plating, serum-free neuronal cultures or glial cultures were treated.

Cortical explant cultures

Cortical organotypic cultures were performed using 300 µm thick slices obtained by McIlwain Tissue Chopper (Campden Instruments, Leicestershire, UK) from E17 embryonic cortex. Sections were fixed after 48 h of exposition to agarose beads pre-absorbed with BMP7, BDNF or BSA. In each case, 4–5 explants were cultured in quadruplicates. At least 26 experiments were performed for each condition.

Pharmacological and hypoxic treatments *in vitro*

E16 primary neuronal cultures grown in serum-free medium were treated after 4-5 days *in vitro* (DIV) with pharmacological inhibitors of the BDNF-TrKB signalling pathway. We treated neuronal cultures with either the TrkB inhibidor K252a (0.6 µM Sigma-Aldrich, Saint Louis, MO), the MEK1-2 specific inhibitor UO126 (10 µM, Calbiochem, San Diego), or the PI 3-Kinase inhibitor Wortmannin (0.1 µM Sigma-Aldrich, Saint Louis, MO). We also used the p53 transcriptional inhibitor cyclic pifithrin-α (10 µM), and the activator Nutlin-3 (10 µM Cayman, Tallin, Estonia), which inhibits the binding of the inhibitor MDM2 to p53. All inhibitors were applied one hour

before applying 100 ng/ml BDNF (Peprotech, London, UK) for 1h or 6h. All experiments were carried out at least three times and BMP7 mRNA levels were analyzed by Real Time – PCR (RT-PCR).

For hypoxic experiments E16 neuronal enriched cultures were 12 hours incubated under 2-3% oxygen levels, and subsequently treated under normoxic conditions with 25 ng/ml BMP7 (R&D, Abingdon, UK) or 200 ng/ml Follistatin (R&D, Abingdon, UK) for the indicated time periods (1 hour to 4 days).

mRNA isolation, cDNA synthesis and RT-PCR

Dissected cerebral cortices of mice were collected and individually frozen in RNAlater and stored at -80ºC until use. Both, mRNA from cerebral cortices and from treated cellular cultures was purified with the RNeasy Protect Mini Kit (Qiagen, Alameda, CA.) and was treated with DNase I to eliminate genomic DNA traces. The RNA concentration and integrity were analyzed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Synthesis of cDNA was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster city, CA). For real time PCR, TaqMan PCR assays (TaqMan® Gene Expression Assay, Applied Biosystems, Foster city, CA) for mouse BMP7 and GADPH (as the endogenous reference) were performed from the cDNA obtained from 6 ng of RNA, in triplicate, on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster city, CA). Standards were prepared using cDNA from control E18 mouse RNA. Finally, fluorescent signal was captured using the Sequence Detector Software (SDS version 1:9; Applied Biosystems, Foster city, CA).

Immunofluorescence

To collect tissue for immunohistochemical analysis, embryos were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.3, and their brains were post-fixed for 8-12h, cryoprotected and kept frozen. Coronal sections of 40 µm thickness were collected in a cryoprotective solution, and stored at -20ºC for

further use. *In vitro* assays performed in cortical cultures from E16 embryos were fixed also using 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer for 30 minutes at room temperature.

For immunofluorescence of primary cultures or tissue sections, sections that had been blocked with 10% serum for 2h at room temperature and were incubated with primary antibodies at 4ºC overnight. Subsequently we incubated the samples with secondary antibodies conjugated to phluorophores: Alexa488, Alexa555 (1:500, Molecular Probes, Eugene, Oregon). TO-PRO-3 iodide (1:500, Molecular Probes, Eugene, Oregon) and Hoechst pentahydrate bis-benzimide (20µg/ml, Molecular Probes, Eugene, Oregon) were used to stain nuclei. Cells and sections were coversliped with Mowiol (Calbiochem, San Diego).

Western blot analysis

For Western blot analysis, protein extracts were obtained from primary cultures or from cerebral cortex and collected in lysis buffer (150 mM NaCl, 1% TritonX-100, 0,1% SDS, 0,5% Sodium Deoxicolate, 50 mM Tris-HCl pH 7,5, 1mM PMSF, proteinase and phosphatase inhibitors) and proteins in total extracts were separated by SDS-PAGE and electro-transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were checked by Ponceau Red (Sigma-Aldrich, Saint Louis, MO) staining and next blocked 1 h at room temperature with 5% unfatty milk in tris saline buffer and next incubated with first primary antibodies overnight at 4ºC, and then with their corresponding secondary HRP-conjugated antibodies (1:3000, Santa Cruz Biotechnology, San Diego). Protein signal was detected using the ECL chemiluminescent system (Amersham, Buckinghamshire,UK). Densitometry analysis, standardized to actin as a control for protein loading, was performed using ImageJ software (National Institutes of Health, Bethesda, Maryland).

Fluorescent and confocal microscopy

Micrographs were captured with a light microscope Nikon Eclipse 800 (Nikon, Tokyo, Japan), or with a Spectral confocal microscope Leica TCS-SL (Leica Microsystems, Mannheim, Germany). Images were assembled in Adobe Photoshop (v. 7.0), with adjustments for contrast, brightness and color balance to obtain optimum visual reproduction of data.

Quantitative and morphologic cell analysis

Images of distinct neural populations stained with different cellular markers as well as Hoechst nuclei labeling were captured by fluorescent and confocal microscope and analyzed by Image J software (National Institutes of Health, Bethesda, Maryland). Fisher's least significant difference (LSD) procedure was used to discriminate between the means. At least three samples and nine fields per sample were analyzed per condition.

The quantification of cellular morphological properties was carried out by the aid of ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2009.). All the pictures were converted to black and white, adjusted for brightness and contrast and converted to binary images. Because of the varying exposition of pictures and in order to ensure proper calculation of binary images, these steps were done manually. Each cell was then processed in order to derive a broad number of morphological features, including: area, Feret's diameter, overall complexity, number of extremities and main branches. Area and Feret's diameter were measured on binary images of cells. Feret's diameter is defined as the longest distance between any two points along the selection boundary. Cell complexity was defined by the number of pixels in the cell skeleton divided by the diameter of the cell (Pouria Moshyedi et al, J. Phis.: Condens. Matter 22 (2010) 19411 (11pp)). A simple bipolar cell will have a complexity index close to 1, being its skeleton similar to its diameter, while highly ramified irregular cells will have

higher values. Cell skeleton was obtained using the process “skeletonize”, while cell diameter is the major axis of the best fitting ellipse. The number of extremities and main branches, a meaningful way to express cell maturation, was obtained counting manually. For the analysis three pictured from three different samples were considered and well defined number of cells was selected.

Quantitative analysis of cell position in the cerebral cortex

We used a general linear model (GLM) which is similar to an ANOVA model (Searle, 1987) to compare the position of labeled neurons in the cerebral cortex. Fisher’s least significant difference (LSD) procedure was used to discriminate between the means. Three to eight mice were analyzed per condition (untreated mice and injected with vehicle, BDNF, or BMP7). The position of Calb+, BrdU+, Ki-67+, Tbr2+Ki-67 and BRN1+BrdU double labeled cells was analyzed at E18 in three to four coronal sections (spaced by 200 µm) from the parietal cortex of each mouse. Images from immunostained sections were captured and then imported into Photoshop. A 1665 µm wide vertical strip along the radial axis of the cerebral cortex was divided into ten bins of equal size arranged in the following orientation: bin 1 at the pial surface and bin 10 at the ventricle. For representative purposes bins were grouped into cortical plate (CP, bins 1-3), corresponding to the marginal zone and developing layers II-IV, layers V-VI (bins 4-6), subplate/intermediate zone (SP-IZ, bins 7-8) and ventricular/subventricular zone (VZ/SVZ, bins 9-10), or VZ (bin10) and cortical parenchyma (bins 1-9). The number of labeled cells in each zone was determined as the average percentage of labeled cells with respect to the total strip.

RESULTS

1. BDNF/MAPK/ERK-INDUCED BMP7 EXPRESSION IN THE DEVELOPING CEREBRAL CORTEX INDUCES PREMATURE RADIAL GLIA DIFFERENTIATION AND IMPAIRS NEURONAL MIGRATION

Brain derived neurotrophic factor (BDNF) is a chemokine which levels are regulated by neuronal activity and could act as a sensor in front of distinct physiologic stimulus, activating the transcription of specific group of genes. In this work we show that BDNF induces the expression of BMP7, a TGF-β superfamily protein, in both *in vitro* and *in vivo* conditions.

We initially observed that BDNF injection in the lateral ventricle of E14 mice embryos produce an increase in BMP7 expression in the embryonic cerebral cortex. We carried out studies *in vitro* using neuronal and glial cultures obtained from E16 and P0 cerebral cortices respectively to deeper analyze the BDNF-induced BMP7 expression. Real Time-PCR analysis showed that 6 h of BDNF treatment promotes the increase of BMP7 mRNA levels in neuronal cultures but not in glial cultures. We used different inhibitors of BDNF/TrKB signalling pathway to identify the mechanism implicated in the induction of BMP7 expression in neuronal cultures. The inhibition of PI3K/AKT pathway with wortmanin does not affect BDNF-induced BMP7 expression, whereas the MAPK/MEK pathway inhibitor UO126 completely blocks the induction of BMP7 by BDNF. We also analyzed the transcriptional action of p53 family members on BMP7 expression induced by BDNF. We used pifithrin-α as an inhibitor of p53, and Nutlin 3 as an indirect activator of p53 pathway. Our results indicate that p53 transcription factor family members might act as co-repressors of BDNF-induced BMP7 expression and that BDNF induces BMP7 expression in part by releasing this repression.

To assess the function of BMP7 during cerebral cortex development we inject the recombinant protein in the lateral ventricles of E14 mice embryos. Analysis at E18 shows that BMP7 injection alters the laminar distribution of pyramidal neurons, which accumulates in the intermediate zone under cortical plate without affecting GABAergic neurons distribution. We next analyzed the effect of BMP7 on radial glial since it is the

main substrate for pyramidal neurons migration. Immunostaining assays with different radial glia and distinct astrocyte maturity stage markers showed that BMP7 induces radial glia differentiation prematurely. In addition, in BMP7 injected animals the decrease in radial glia maintenance is accompanied by and increase in the expression of SPARC like 1, an antiadhesive protein associated with the termination of radial migration.

Finally, based on these results is proposed a physiologic model where the expression of BDNF induced by the initial electrical activity in the perinatal period would induce in turn, an increase in BMP7 expression. Both chemokines may act coordinately maturating neurons and glial cells at the end of neurogenic period.

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BDNF/MAPK/ERK–Induced BMP7 Expression in the Developing Cerebral Cortex Induces Premature Radial Glia Differentiation and Impairs Neuronal Migration

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During development of the mammalian nervous system, a combination of genetic and environmental factors governs the sequential generation of neurons and glia and the initial establishment of the neural circuitry. Here, we demonstrate that brain-derived neurotrophic factor (BDNF), one of those local acting factors, induces Bone Morphogenetic Protein 7 (BMP7) expression in embryonic neurons by activating Mitogen-Activated Protein Kinase/Extracellular signal-Regulated Kinase signaling and by the negative regulation of p53/p73 function. We also show that intraventricular injection of BMP7 at midgestation induces the early differentiation of radial glia into glial precursors and astrocytes and the expression of mature glial markers such as the antiadhesive protein SC1. As a result of this precocious radial glia maturation, the laminar distribution of late-born pyramidal neurons is altered, most likely by the termination of radial glia ability to support neuronal migration and the early neuronal detachment from the glial rail. We propose a mechanism for BDNF regulation of BMP7 in which local activity–driven BDNF-induced BMP7 expression at the end of neurogenesis instructs competent precursors to generate astrocytes. Such a mechanism might ensure synchronic neuronal and glial maturation at the beginning of cortical activity.

Keywords: astrocytogenesis, cortical development, neurotrophins

Introduction

In the developing cerebral cortex, radial glial cells act both as precursors of excitatory pyramidal glutamatergic neurons (Gotz and Huttner 2005; Guillemot 2005) and as migratory scaffolds for the radial migration of newly generated neurons (Rakic 1990; Nadarajah and Parnavelas 2002). After the completion of neurogenesis, radial glia transform into cortical astrocytes (Hunter and Hatten 1995; Hartfuss et al. 2001). γ-aminobutyric acid (GABAergic) inhibitory interneurons originate at ganglionic eminences and migrate tangentially to the cortex (Anderson et al. 2001; Ang et al. 2003). Independently of their origins, neurons generated at the same time roughly converge in the same cortical layer, following an inside-out sequence of positioning.

Genetic programs regulate the early steps of mammalian cortical development, and, as development proceeds, sensory experience and electrical activity are the driving forces that match glial and neuronal numbers and finely tune the structural and functional refinement of cortical circuits (Zhang and Poo 2001; Fox and Wong 2005; Spitzer 2006).

It is well established that transcription of brain-derived neurotrophic factor (BDNF) mRNA is robustly induced by neuronal activity in late stages of cortical development, and this activity-regulated production of BDNF is needed for postnatal neuronal survival and to balance excitatory and inhibitory synapses in cortical networks (Lu 2003; Nagappan and Lu 2005; Pattabiraman et al. 2005). BDNF and its receptor TrkB play key roles in neural development and plasticity (Huang and Reichardt 2001; Lu et al. 2005). BDNF expression is subjected to fine temporal and spatial regulation, and some of its functions rely on its ability to act as a sensor of activity. For instance, activity-dependent regulation of BDNF is required for the development of cortical inhibition but not for the survival or differentiation of GABAergic neurons (Hong et al. 2008). Loss-of-function studies on animal models have shown subtle BDNF requirements during embryonic central nervous system (CNS) development that increase postnatally (Alcántara et al. 1997; Gorski et al. 2003). However, early embryonic exposure to increased BDNF alters cell fate, neuronal migration, and synaptic function in the cerebral cortex (Brunstrom et al. 1997; Ringstedt et al. 1998; Aguado et al. 2003; Alcántara et al. 2006). Therefore, altered BDNF expression during critical developmental periods may result in cortical malformations and excitatory/inhibitory imbalance and compromise cognitive function in the adult. In support of this notion, aberrant levels of BDNF are associated with neurodevelopmental disorders (Tsai 2005; Chang et al. 2006; Lu and Martinowich 2008) and epilepsy (Scharfman 2005).

The mechanism of activity-dependent induction of BDNF has been extensively investigated. However, less is known about the genes that are targets of BDNF regulation during late embryonic cortical development. In order to identify such genes, we injected BDNF into the brain of mice at defined times during embryonic development, and we monitored changes in the expression level of a selected group of genes that were represented in a customary DNA microarray. By using this approach, we found that expression of bone morphogenetic protein 7 (BMP7) was upregulated by BDNF.

Here, we demonstrate that BDNF induces neuronal BMP7 expression during embryonic development, both in vivo and in vitro, through the Mitogen-Activated Protein Kinase/Extracellular signal-Regulated Kinase (MAPK/ERK) pathway and that this expression is partially mediated by blockage of the transcriptional activity of the p53 family of transcription factors. Exposure to increased BMP7 induced a premature transformation of radial glia into astrocytes that altered neuronal radial migration. Finally, we propose a physiological role for BDNF regulation of BMP7 during corticogenesis.

Materials and Methods

Animals and Injection in Uterus

Experiments were design to minimize the number of animals used in the procedure. All animal protocols were approved by the Institutional Animal Care and Use Committee in accordance with Spanish and European Union regulations.

For the injection in murine brains in uterus, pregnant OF1 females carrying embryonic day 14 (E14) embryos (with E0 being the day the vaginal plug) were anesthetized with Ketamine/Valium (150 μg/g, 5 μg/g, intraperitoneal), and the uterine horns were exposed. Two microliters of recombinant human BMP7 (1 μg, R&D, Abingdon, UK), recombinant human BDNF (1 μg, PeproTech, London, UK), vehicle, or DNA expression vectors (6–10 μg) were delivered into the lateral ventricles of the embryos via intrauterine injection, followed by electroporation in the case of vectors. The uterus was returned to the abdominal cavity, and the embryos were allowed to develop normally. Embryos were sacrificed at E15, E16, or E18 and used for protein or mRNA extraction, cell culture, or Immunohistochemistry (IHC).

To collect tissue for IHC analysis, embryos were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3, and their brains were postfixed for 8–12 h, cryoprotected, and kept frozen. Coronal sections of 40-μm thickness were collected in a cryoprotective solution and stored at −30 °C for further use.

In Uterus Electroporation

Electroporation was performed in uterus as previously described (Tabata and Nakajima 2001). pEF1-GFP vector or a mixture of pEF1-BDNF (mBDNF cDNA inserted into pEF1 vector) and pEF1-GFP vectors at a 4:1 ratio were injected in the lateral ventricle of E14 mouse embryos. The head of the in uterus embryo was held by a tweezers-type electrode (CUY650-5; Nepagene, Ichikawa, Japan), and electronic pulses (34 V for 50 ms) were discharged 4 times at 950-ms intervals with a CUY21E electroporator (Nepagene). The embryos returned to the abdominal cavity to allow normal development.

5-Bromo-2-deoxyuridine Birthdating

Thymidine analog 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich, St Louis, MO) was injected intraperitoneally into pregnant females at E14 at a concentration of 50 mg/kg body weight, 3 h after BMP7 injection to the embryos. At E18, embryos were perfused and processed as described above. Incorporated BrdU was then detected by IHC.

mRNA Isolation, cDNA Synthesis, and Real-Time Polymerase Chain Reaction

Dissected cerebral cortices of E18 mice were collected and individually frozen in RNA later and stored at −80 °C until use. mRNA was purified with the RNeasy Protect Mini Kit (Qiagen, Alameda, CA) and was treated with DNase I to eliminate genomic DNA traces. The RNA concentration and integrity were analyzed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Synthesis of cDNA was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster city, CA). For real-time polymerase chain reaction (RT-PCR), TaqMan PCR assays (TaqMan Gene Expression Assay, Applied Biosystems) for mouse BMP7 and glyceraldehyde-3-phosphate dehydrogenase (as the endogenous reference) were performed from the cDNA obtained from 6 ng of RNA, in triplicate, on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Standards were prepared using cDNA from control E18 mouse RNA. Finally, fluorescent signal was captured using the Sequence Detector Software (SDS version 1:9; Applied Biosystems)

Cell Culture

Primary cultures were prepared from E15–E16 mice neocortex. Briefly, embryonic cortices were dissected out and dissociated by trypsin–ethylenediaminetetraacetic acid (Biological Industries, Kibbutz Beit Haemek, Israel) and DNAse I (Sigma-Aldrich) treatment for 10 min, followed by mechanical disruption. To obtain enriched neuronal cultures, the dissociate was preplated in a 10-cm culture dish for 1 h at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% normal horse serum (NHS) (Gibco, Auckland, New Zealand). Embryonic cortical cells were then recovered from the supernatant and seeded on 6- and 24-well plates containing slides coated with poly-D-lysine (Sigma-Aldrich) in serum-free Neurobasal medium (Gibco, Paisley, UK) supplemented with B27 (Gibco, Paisley, UK). By using these conditions, we obtained a neuron-enriched culture, in which few glial cells were retained. To obtain primary glial cultures, we used a similar protocol, in which P0–P1 cortical cell suspensions were plated directly onto uncoated 6- and 24-well plates in DMEM with 10% NHS. Once the cells reached confluence, they were dissociated and plated again in order to eliminate remnant neurons. We only used passages 1–3. Three to 4 days after plating, serum-free neuronal cultures or glial cultures that were serum starved for 24 h were treated with 75 ng/mL BMP7 (R&D) or 10–200 ng/mL BDNF (PeproTech, London, UK) for the indicated time periods (1 h–4 days).

Cortical organotypic cultures were performed using 300-μm thick slices from E17 embryonic cortex exposed to agarose beads preabsorbed with BMP7, BDNF, or bovine serum albumin (BSA) and cultured for 2 days.

Pharmacological Treatments

In some experiments, E15–E16 primary neuronal cultures grown in serum-free medium were treated after 4–5 days in vitro with pharmacological inhibitors of the BDNF-TrkB signaling pathway. We treated neuronal cultures with the TrkB inhibitor K252a (0.6 μM Sigma-Aldrich), the MAPK/ERK Kinase 1-2 (MEK1-2)-specific inhibitor UO126 (10 μM; Calbiochem, San Diego, CA), or the PI3-kinase inhibitor wortmannin (0.1 μM, Sigma-Aldrich). We also used the p53 transcriptional inhibitor cyclic pifithrin-α (10 μM) and the activator nutlin-3 (10 μM Cayman, Tallin, Estonia), which inhibits the binding of the inhibitor MDM2 to p53. All inhibitors were applied 1 h before applying 100 ng/mL BDNF (PeproTech, London, UK) for 1 or 6 h. All experiments were carried out at least 3 times and BMP7 mRNA levels were analyzed by RT-PCR.

Immunofluorescence of Culture Cells and Tissues and Western Blot Analysis

For immunofluorescence of primary cultures or tissue sections, sections that had been blocked for 1 h were incubated with primary antibodies at 4 °C overnight and subsequently with secondary antibodies conjugated to phluorophores: Alexa488, Alexa555, or Alexa647 (1:500; Molecular Probes, Eugene, Oregon). In some cases, sections were incubated with biotinylated secondary antibodies (1:200, Vector, Burlingame, CA) and subsequently with a streptoavidin-peroxidase complex (1:400, Amersham, Buckinghamshire, UK), and the enzymatic reaction was developed with diaminobenzidine (DAB, Sigma-Aldrich) and H_2O_2. TO-PRO-3 iodide (1:500, Molecular Probes, Eugene, Oregon) was used to stain nuclei. Cells and sections were coverslipped with Mowiol (Calbiochem).

For western blot analysis, protein extracts were obtained from primary cultures or from cerebral cortex and proteins in total extracts were separated by SDS-PAGE and electro-transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were blocked and incubated firstly with primary antibodies overnight at 4°C, and then with their corresponding secondary HRP-conjugated antibodies (1:3000; Santa Cruz Biotechnology, San Diego, CA). Protein signal was detected using the ECL chemiluminescent system (Amersham, Buckinghamshire,UK). Densitometric analysis, standardized to actin as a control for protein loading, was performed using ImageJ software.

Primary antibodies against the following proteins were used: actin (1:2000, Santa Cruz Biotechnology), BLBP (1:3000; Chemicon, Hampshire, UK), Brn1 (1:100, Santa Cruz Biotechnology), BrdU (1:200; GE Healthcare, Buckinghamshire, UK), calbindin (1:3000; Swant, Bellinzona, Switzerland), glial–fibrilary acidic protein (GFAP) (1:3000; Dako, Glostrup, Denmark), nestin (1:500; BD Pharmingen, Franklin Lakes, NJ), Tuj1 (βIII tubulin, 1:3000; Covance, Berkeley, CA), SC1 (Secreted Protein, Acidic and Rich in Cysteines-like 1 [SPARC-like 1], 1:100, Santa Cruz Biotechnology), calretinin (1:2000 Swant), reelin (1: 400, Chemicon), Ki-67 (1:400; Abcam, Cambridge, UK), phospho-AKT 308 (1:500; Cell Signalling, Danvers, MA), phospo-ERK 1/2 (1:1000, Sigma-Aldrich), TBR2/eomes (1: 500, Abcam).

For BMP7, we used 3 different polyclonal antibodies: BMP7 N19 and L19 antibodies (1:100; Santa Cruz Biotechnology) gave stronger ICC staining, and BMP7 antibody from PeproTech, London (1:1000) gave a clearer signal in western blots. F9 cell lysate (Santa Cruz Biotechnology) and recombinant BMP7 protein were used as positive controls, and blocking peptides were used for negative controls.

Light and Confocal Microscopy

Micrographs were captured with a light microscope Nikon Eclipse 800 (Nikon, Tokyo, Japan) or with a spectral confocal microscope Leica TCS-SL (Leica Microsystems, Mannheim, Germany). Images were assembled in Adobe Photoshop (v. 7.0), with adjustments for contrast, brightness, and color balance to obtain optimum visual reproduction of data.

Quantitative Analysis of Cell Position in the Cerebral Cortex

We used a general linear model that is similar to an analysis of variance model to compare the position of labeled neurons in the cerebral cortex. Fisher's least significant difference (LSD) procedure was used to discriminate between the means. Three to 8 mice were analyzed per condition (untreated mice and injected with vehicle, BDNF, or BMP7). The position of Calb+, BrdU+, Ki-67+, Tbr2 + Ki-67, and BRN1 + BrdU double-labeled cells was analyzed at E18 in 3–4 coronal sections (spaced by 200 μm) from the parietal cortex of each mouse. Images from immunostained sections were captured and then imported into Photoshop. A 1665-μm wide vertical strip along the radial axis of the cerebral cortex was divided into 10 bins of equal size arranged in the following orientation: bin 1 at the pial surface and bin 10 at the ventricle. For representative purposes, bins were grouped into cortical plate (CP, bins 1–3), corresponding to the marginal zone (MZ) and developing layers II–IV, layers V–VI (bins 4–6), subplate/intermediate zone (SP-IZ, bins 7–8) and ventricular/subventricular zone (VZ/SVZ, bins 9–10), or VZ (bin 10) and cortical parenchyma (bins 1–9). The number of labeled cells in each zone was determined as the average percentage of labeled cells with respect to the total strip. Error bars reflect the standard deviation of the means.

Results

BDNF Induces BMP7 Expression during Cerebral Cortex Development in Vivo

To identify BDNF-regulated genes during cortical morphogenesis, we injected BDNF into the lateral ventricle of E14 mouse embryos in uterus and collected cerebral cortex tissue at E18 for gene expression analysis. A low-density microarray was designed containing 25 gene members of the transforming growth factor (TGFβ) signaling cascade. As a control for selectivity in the BDNF injection assays, we also injected Neurotrophin 4 (NT4), the second neurotrophin that preferentially acts through TrkB receptor (Reichardt 2006), and SDF1α, a chemoquine not related to TrkB signaling pathway. As negative controls, we used noninjected animals and animals injected with vehicle (sham). The microarray results revealed that out of the 25 members of the TGFβ family, only BMP7 expression was significantly increased at E18 in the cerebral cortex of BDNF-injected mice (1.49-fold increase) as compared with intact, sham and SDF1α-injected mice. BMP7 was also increased in NT4-injected cortices although to a lesser extent (1.22-fold increase) (Supplementary Fig. 1).

To corroborate this finding, we performed RT-PCR analysis on a different group of E18 cerebral cortices obtained under the same conditions. Consistent with our microarray data, 4 days after direct intraventricular injection, BDNF and NT4 elicited a significant increase in BMP7 mRNA in the cerebral cortex compared with intact and sham operated animals (Fig. 1*A*). To determine whether the rise in BMP7 mRNA was correlated with increased protein levels, a third group of embryos treated similarly with BDNF were harvested 24 or 48 h after injection and analyzed by western blot. A significant increase in the 17-kDa mature form of BMP7 protein was found after 24 (not shown) and 48 h (Fig. 1*B*). Taken together, these results indicate that TrkB activation mediated by BDNF or NT4 elicits long-lasting BMP7 mRNA and protein expression changes in the embryonic cerebral cortex in vivo.

To further define the mechanism by which BDNF induces BMP7 expression, we used a model of focal BDNF overexpression. E14 cortices were focally transfected with a murine BDNF expression vector or a GFP control plasmid by electroporation in uterus. As vector incorporation was restricted to one cerebral hemisphere, we used the contralateral hemisphere as an untransfected control. We analyzed the extent of BDNF overexpression in the transfected cortices by IHC. GFP-transfected and the GFP-untransfected hemispheres showed the normal pattern of BDNF expression at E18, characterized by low intensity in the VZ, lower CP (layers VI–V) and the upper CP, and weak expression in IZ (Fig. 1*C*). BDNF expression was stronger in the areas transfected with the BDNF vector (Fig. 1*D*). In transfected areas, intensely labeled individual BDNF-positive cells were found scattered throughout the cortex, particularly in the VZ and deeper regions. In general, BDNF-transfected cells accounted for a small percentage of the total cellular content of the affected area. We next analyzed the expression of BMP7 protein in adjacent sections to those immunostained with BDNF. Control areas expressed low levels of BMP7, mainly localized to the most mature cortical layers and the MZ (Fig. 1*E*). In contrast, in the region transfected with BDNF vector, BMP7 labeling increased dramatically in the upper CP (Fig. 1*F*). BMP7 was not induced in the VZ or IZ despite increased BDNF expression there. The overwhelming number of BMP7-overexpressing cells in the CP compared with BDNF-transfected cells indicates that BMP7 expression is induced in a paracrine fashion in cortical postmigratory neurons.

BDNF Induce Neuronal but not Glial BMP7 Expression in Vitro

To identify the cell type responsible for BDNF-dependent BMP7 expression, we cultured cerebral cortices from E15–E16 embryonic mice in serum-free medium. E15–E16 cortical cultures were mainly composed of neurons, neural progenitors, and a few mature glial cells (Supplementary Fig. 2). Primary cortical cultures were harvested at different times after treatment with 100 ng/mL of BDNF. Analysis of BMP7 mRNA expression by RT-PCR showed an early rise in BMP7 mRNA levels 6 h after BDNF treatment (Fig. 2*A*) that correlated with an increase in protein levels (Fig. 2*B*). Moreover, BMP7 induction by BDNF is dose dependent (Supplementary Fig. 3), showing a linear relation at BDNF concentrations up to 50 ng/mL and reaching a plateau at 50 ng/mL that is maintained up to 200 ng/mL. These data would be consistent with a direct effect of BDNF/TrkB signaling on BMP7 transcription. The reduced glial content of E15–E16 cortical cultures indicates that neurons were the most likely source of this increase in BMP7 mRNA in response to BDNF.

To examine whether BDNF also induced BMP7 expression in glial cells, we performed pure glial cultures from newborn mice and analyzed their BMP7 expression by RT-PCR. BMP7 mRNA was expressed at similar levels in serum-starved glial cultures and neuronal cortical cultures. However, BDNF treatment did not induce an increase in BMP7 mRNA in pure glial cultures (Fig. 2*C*).

BDNF Induces BMP7 Expression through the MAPK/ERK Signaling Cascade and p53 Signaling

Neurons mainly express the full-length catalytic form of the BDNF receptor TrkB. Thus, we investigate pharmacologically whether BDNF-dependent BMP7 induction was mediated by TrkB protein tyrosine kinase activity. Figure 3 shows the effect

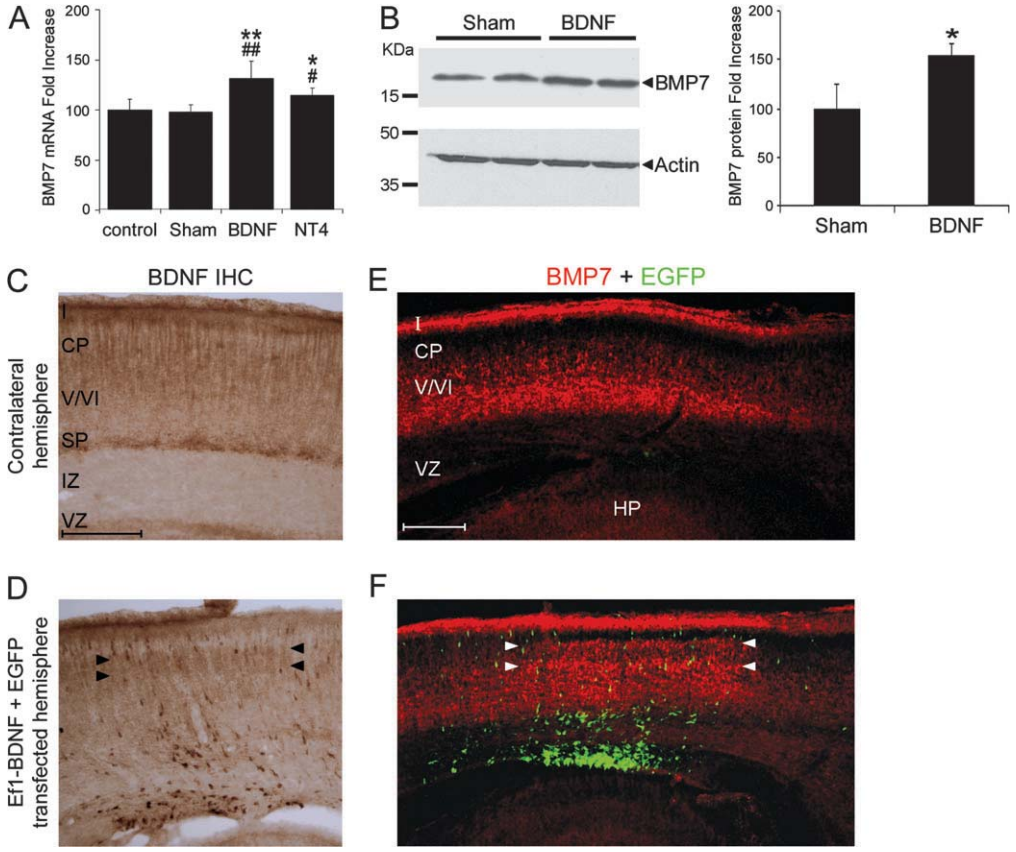

Figure 1. BDNF induces BMP7 expression. (*A*) Quantification of BMP7 mRNA levels by RT-PCR in E18 cerebral cortices from intact animals (control) or injected at E14 with vehicle (sham), BDNF, or NT4. (*B*) Representative western blot for BMP7 and actin as loading control in E16 cortical tissue from animals injected at E14 with vehicle (sham) or BDNF. Molecular weight markers are indicated to the left. The graph represents the quantification of BMP7 protein from 3 animals at each condition. (*C*) BDNF immunostaining in a coronal cortical section of a mouse electroporated with BDNF and GFP vectors (Ef1-BDNF + GFP) showing the normal protein distribution in the contralateral control hemisphere (Ef1-BDNF + GFPcontra) with respect to the transfected hemisphere (Ef1-BDNF + GFP) (*D*), where intense BDNF-expressing cells are seen through the cortical wall. (*E, F*) Double immunostaining for BMP7 (red) and EGFP (green) was performed in an adjacent section to that showed in (*C, D*). Arrowheads indicate BDNF overexpression in the upper CP in D and the area of strong BMP7 induction in (*F*). Error bars indicates the standard deviation. Scale bars, 200 μm. *Significant difference with respect to sham-operated animals (*$P < 0.05$, **$P < 0.01$, LSD test). #Significant differences with respect to control (#$P < 0.05$, ##$P < 0.01$, LSD test). I, layer I/MZ; V/VI, layers V/VI; HP, hippocampus.

of pretreating serum-free cortical cultures with the selected inhibitors for 1 h immediately preceding BDNF 1- or 6-h incubation. BMP7 mRNA levels were determined by RT-PCR. K252a compound is a potent protein kinase blocker that prefers Trk receptors. Pretreatment with K252a completely abolished BMP7 induction in cortical cultures treated with BDNF (Fig. 3*B*). To further dissect the TrkB signaling cascade involved in BDNF-dependent BMP7 expression, we focused on the PI3K/AKT pathway, mainly related to neuronal survival, and the MAPK/ERK pathway, which is involved in neuronal differentiation and synaptic plasticity (Chao 2003; Reichardt 2006). In order to test the involvement of these pathways in BDNF-mediated BMP7 upregulation, we used the specific inhibitors wortmannin (inhibitor of PI3K) and U0126 (that selectively inhibits MEK) in cortical primary cultures (Fig. 3*D,E*). Each inhibitor, individually or in combination, slightly reduced the basal levels of BMP7 mRNA in neuronal cultures. However, while PI3K inhibitor did not significantly affect BDNF-dependent BMP7 expression, MEK inhibitor completely abolished BMP7 induction by BDNF (Fig. 3*D*). These results indicate that BDNF-dependent BMP7 induction is mediated by direct activation of TrkB and MAPK/ERK signaling.

A recent study identified the p53 family of transcription factors (p53, p63, and p73) as transcriptional corepressors of BMP7 (Laurikkala et al. 2006). Furthermore, neurotrophins and ERK promote neuronal survival in part by decreasing p53 activation (Wade et al. 1999; Wu 2004; Miller and Kaplan 2007). Thus, we examined whether BDNF-dependent BMP7 induction involves a reduction in the transcriptional activity of p53. If so, pharmacological blockage of p53/p73–dependent transcription with pifithrin-α (Davidson et al. 2008) would induce BMP7 expression. Otherwise, pharmacological activation of p53/p63/p73 with nutlin-3, which blocks their binding to MDM2 (Vassilev et al. 2004), would reduce BMP7 expression. Pretreatment with 10 μM cyclic pifithrin-α or 10 μM nutlin-3 did not affect the basal levels of BMP7 mRNA, but, as expected, they modulated BDNF-dependent BMP7 expression in opposite ways (Fig. 3*E,F*). Pretreatment with pifithrin-α induced a 26% increase, whereas nutlin-3 decreased BMP7 expression by 30% after 6 h of BDNF treatment. These results indicate that the p53 family of transcription factors corepresses BMP7 transcription and that BDNF activation of the ERK pathway induced BMP7 expression in part by releasing this repression (Fig. 8*A*).

BMP7 Affects Radial Neuronal Migration

We then explored the physiological consequences of the rise in BMP7 levels. First, we analyzed the effect of BMP7 exposure on the laminar organization of the cerebral cortex. E14 progenitors

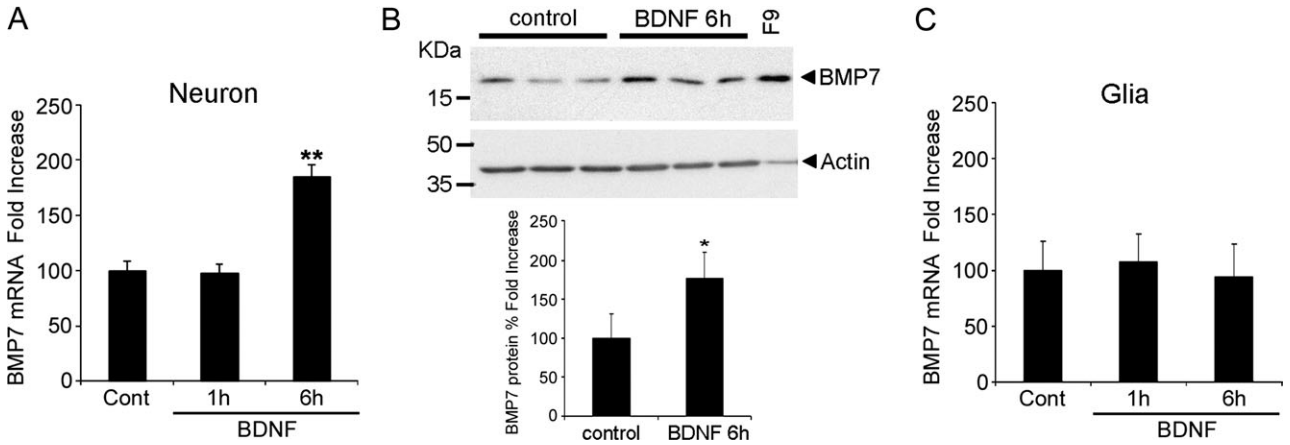

Figure 2. BDNF upregulates BMP7 in vitro. (*A*) Quantification of BMP7 mRNA levels by RT-PCR in primary neuronal E16 cortical cultures treated with BDNF (100 ng/mL) for 1 and 6 h. (*B*) Western blot for BMP7 and actin as loading control in neuronal cultures treated for 6 h with BNDF (100 ng/mL). F9 cells (embryonic carcinoma cells that overexpress BMP7) were used as a positive control. The graph summarizing quantification of western blots demonstrates that neurons treated with BDNF for 6 h express higher BMP7 protein levels. (*C*) BMP7 mRNA was quantified in pure neonatal glial cultures treated with BDNF (100 ng/mL) for 1 or 6 h. BMP7 mRNA levels increased after 6 h of BDNF treatment in neuronal cultures (*A*) but not in glial cultures (*C*).

were labeled by BrdU administration 3 h after the intraventricular injection of BMP7 or vehicle. The number and position of labeled cells were examined at E18. In vehicle-injected animals, the greatest number of BrdU+ cells was found in the CP corresponding to developing layers IV–II. In contrast, mice injected with BMP7 showed altered distribution of labeled cells, with a significant increase in the percentage of BrdU+ cells in the IZ, together with a lower percentage of labeled cells in the upper CP (Fig. 4*A–C*). Despite their altered laminar distribution, the total number of BrdU+ cells was not significantly different in BMP7-treated (130 ± 22 cells) and sham-operated animals (149 ± 26 cells per 1665-μm wide strip), indicating that neurogenesis was not affected by BMP7 treatment.

Alterations in the radial distribution of birthdated neurons as described here might be caused by a change in the laminar fate of late-generated neurons or by a defect in the machinery of neuronal migration. To investigate whether BMP7 affects the laminar fate or the migratory machinery, we analyzed the number and position of neurons double-labeled with BrdU and BRN1, a protein specific to layer II–V glutamatergic neurons (McEvilly et al. 2002). This colocalization experiment revealed that at E18 substantial numbers of E14 labeled BrdU+ cells also express BRN1 both in vehicle- and BMP7-treated cortices (Fig. 4*E–H*). In vehicle-injected animals, most double-labeled neurons were in the CP and in the VZ and SVZ. In contrast, in BMP7-injected animals, double-labeled neurons accumulated in the SP and IZ, with a marked reduction in the number of double-labeled neurons in the CP (Fig. 4*D–H*). The laminar distribution of BrdU-labeled neurons expressing BRN1 was identical to that of single BrdU-labeled cells. No significant differences in total number of double-labeled cells were found between BMP7-treated (41 ± 12 cells) and vehicle-treated (46 ± 14 cells per 1665-μm wide strip) cortices or in the total number of BRN1+ neurons (257 ± 40 in sham vs. 254 ± 43 BMP7) suggesting that the migratory machinery rather than the laminar fate was altered by BMP7.

Cortical glutamatergic neurons migrate on radial glia fibers, whereas GABAergic neurons use different substrates for migration (Rakic 1990; Ang et al. 2003; Kriegstein and Noctor 2004). To address whether BMP7 also affects the laminar position of GABAergic neurons, we used calbindin immunostaining. The number and laminar distribution of calbindin-positive GABAergic neurons in the cerebral cortex was analyzed at E18 in animals injected at E14 with vehicle or BMP7. BDNF-injected animals were used as a positive control for altered interneuron migration (Fig. 5*A–D*). The number and laminar position of GABAergic neurons remained unaltered after vehicle (74 ± 18 cells) or BMP7 injection (80 ± 10 cells per 1665-μm wide strip). In contrast, the total number of calbindin-positive neurons increased significantly in BDNF-treated cortices (92 ± 17 cells per 1665-μm wide strip, 99% LSD test), and their laminar position had shifted to the deeper layers V–VI. Furthermore, BMP7 did not show any attractive or repulsive effect on GABAergic neurons when agarose beads preabsorbed with BMP7 were placed on E17 cortical organotypic cultures (Fig. 5*E–G*). Taken together, these results indicate that early overexposure to BMP7 impairs the radial migration of pyramidal neurons but not that of GABAergic interneurons.

BMP7 Affects Radial Glia Organization

Radial migration in the cerebral cortex is dependent on the integrity of radial glia and the expression of several cell surface or extracellular factors that regulate neuron–glial adhesion. A frequent cause of defective radial migration involves reelin, an extracellular matrix protein secreted by Cajal–Retzius cells in the MZ. The lack of reelin gives rise to the reeler phenotype of inverted lamination (D'Arcangelo et al. 1995), in part by affecting radial glia integrity (Hartfuss et al. 2003). Using calretinin to identify Cajal–Retzius cells, we found that they were similarly arranged in the MZ of vehicle- and BMP7-injected mice (Fig. 6*A,B*). Reelin immunostaining in BMP7-injected mice also showed normal distribution (Fig. 6*C,D*). These results suggest that the impaired migration observed in these mice cannot be explained by defects in the organization of Cajal–Retzius cells or deficits of reelin.

A second possibility is that BMP7 directly affects radial glia phenotype or integrity, as BMPs promote astrocytogenesis from neural progenitors (Yanagisawa et al. 2001). We then analyzed the expression of several markers of radial glia and astrocytic maturation. Nestin is an intermediate filament expressed in neural progenitors and radial glia (Hartfuss et al. 2001). At E18, nestin staining was intense in the VZ lining the ventricle,

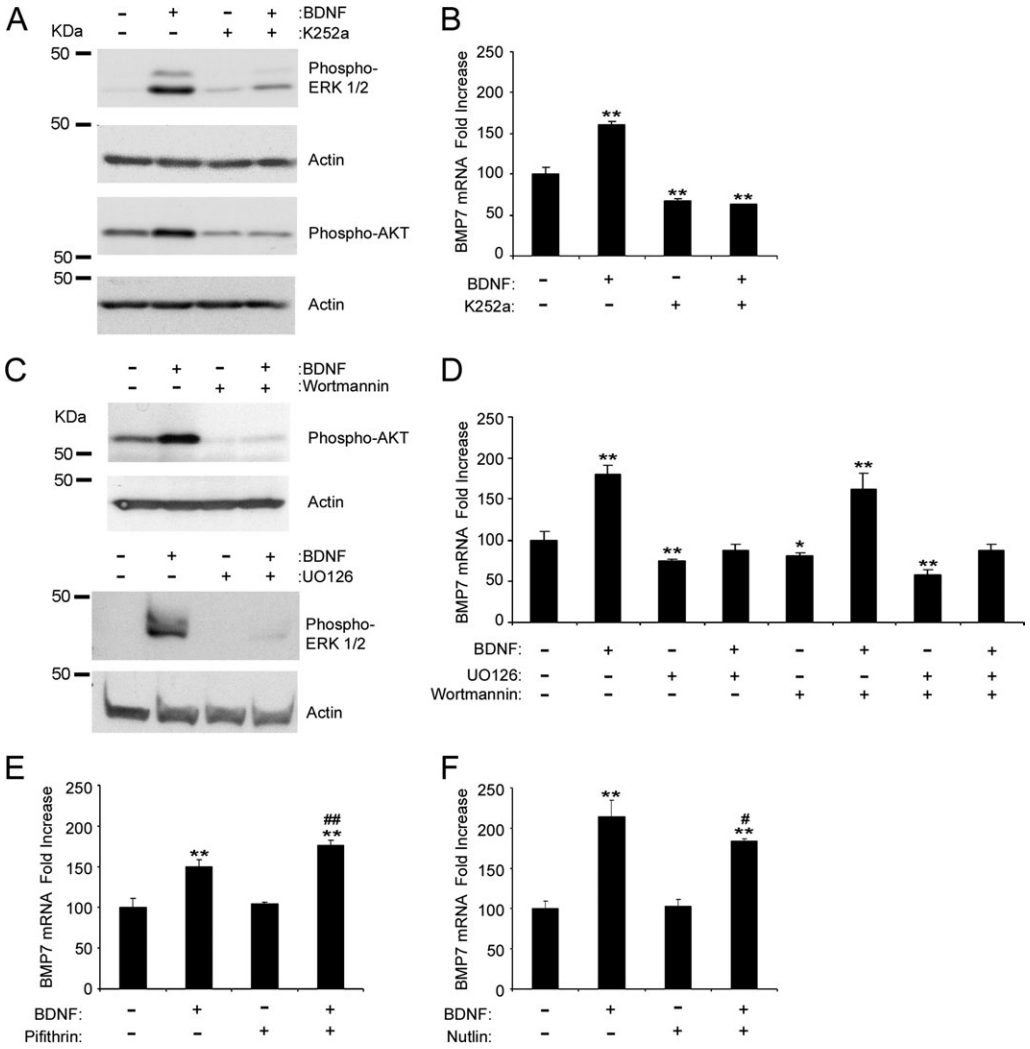

Figure 3. BDNF induces BMP7 expression through TrkB receptor and MAP kinase/ERK pathway. (*A*) Western blots of E16 neuronal cultures exposed to the TrkB inhibitor K252a (0.6 μM) or dimethyl sulfoxide (DMSO) as control, 1 h before BDNF treatment for 1h. (*B*) RT-PCR results showing K252a blockage of the BMP7 mRNA expression induced by 6-h BDNF treatment. (*C*) Immunoblots showing the effect on BDNF-dependent phosphorylation of ERK1/2 and AKT, respectively, of MEK inhibitor UO126 (10 μM), PI3-kinase inhibitor wortmaninn (0.1 μM), or DMSO as control on E16 neuronal cultures exposed to them 1 h before BDNF treatment for 1 h. (*D*) Histogram summarizing the effect of the different inhibitors of TrkB downstream pathways on BMP7 mRNA expression analyzed by RT-PCR after 6 h of BDNF treatment. (*E*) Histogram summarizing the effect of p53 transcriptional activity inhibitor pifithrin-α on BMP7 mRNA expression analyzed by RT-PCR. A 10 μM cyclic pifithrin-α or DMSO as control was administered 1-h before BDNF treatment for 6 h. (*F*) Real-time results showing the effect of p53 activation through nutlin-3 (10 μM), which inhibits MDM-2, a p53 inhibitor. Error bars reflect the standard deviation. *Significant differences with respect to controls, and #differences between BDNF and BDNF + inhibitor treatments ($^{\#/*}P < 0.05$, $^{\#\#/**}P < 0.01$, LSD test).

where it strongly labels radial glia cell bodies and other progenitors located in this area. In addition, nestin-positive fibers spanning the cortical wall from the VZ to the pia lined the entire radial glial palisade (Fig. 6*E*). Mice injected with BMP7 showed reduced nestin immunoreactivity in the VZ, where radial glia somas are located. Distorted positive fibers and isolated nestin-positive cell bodies were also frequent in the SVZ and IZ (Fig. 6*F*).

We next analyzed the expression of brain lipid-binding protein (BLBP), also a marker for subsets of radial glia and differentiating astrocytes (Feng et al. 1994; Feng and Heintz 1995; Hartfuss et al. 2001). In vehicle-injected cortices, BLBP labeled radial glia with a pattern that closely resembled the nestin distribution. In addition, a few ramified BLBP-positive cells were found scattered throughout the cortical wall (Fig. 6*G*). As occurs with nestin, BMP7 injection also reduced BLBP staining in radial fibers in the IZ and deep cortical layers and increased the number of BLBP-labeled cells scattered throughout the cortex (Fig. 6*H*). The changes in nestin and BLBP distribution observed in BMP7-injected cortices are consistent with an early transformation of radial glia to the astrocytic lineage.

We used IHC to detect the expression of astrocytic maturity markers as SPARC-like 1 (SC1) and GFAP. SC1 is an extracellular protein that is involved in the final neuronal detachment from radial glia at destination and is also expressed in mature astrocytes (Mendis et al. 1996; Lively and Brown 2007). In control animals, SC1 labeling was found in the entire CP (Fig. 6*I*). In the animals treated with BMP7, SC1 immunoreactivity was similarly distributed through the cortex but was increased, especially in layers VI–V (Fig. 6*J*). Similarly, SC1 protein content increases in primary cortical cultures treated with BMP7 (Supplementary Fig. 4). On the other hand, GFAP is a final marker for astrocyte maturation that is weakly expressed in the developing rodent cerebral cortex (Sancho-Tello et al. 1995). Agarose beads preabsorbed with BSA or BMP7 were deposited on organotypic cultures from E17 cortices. After

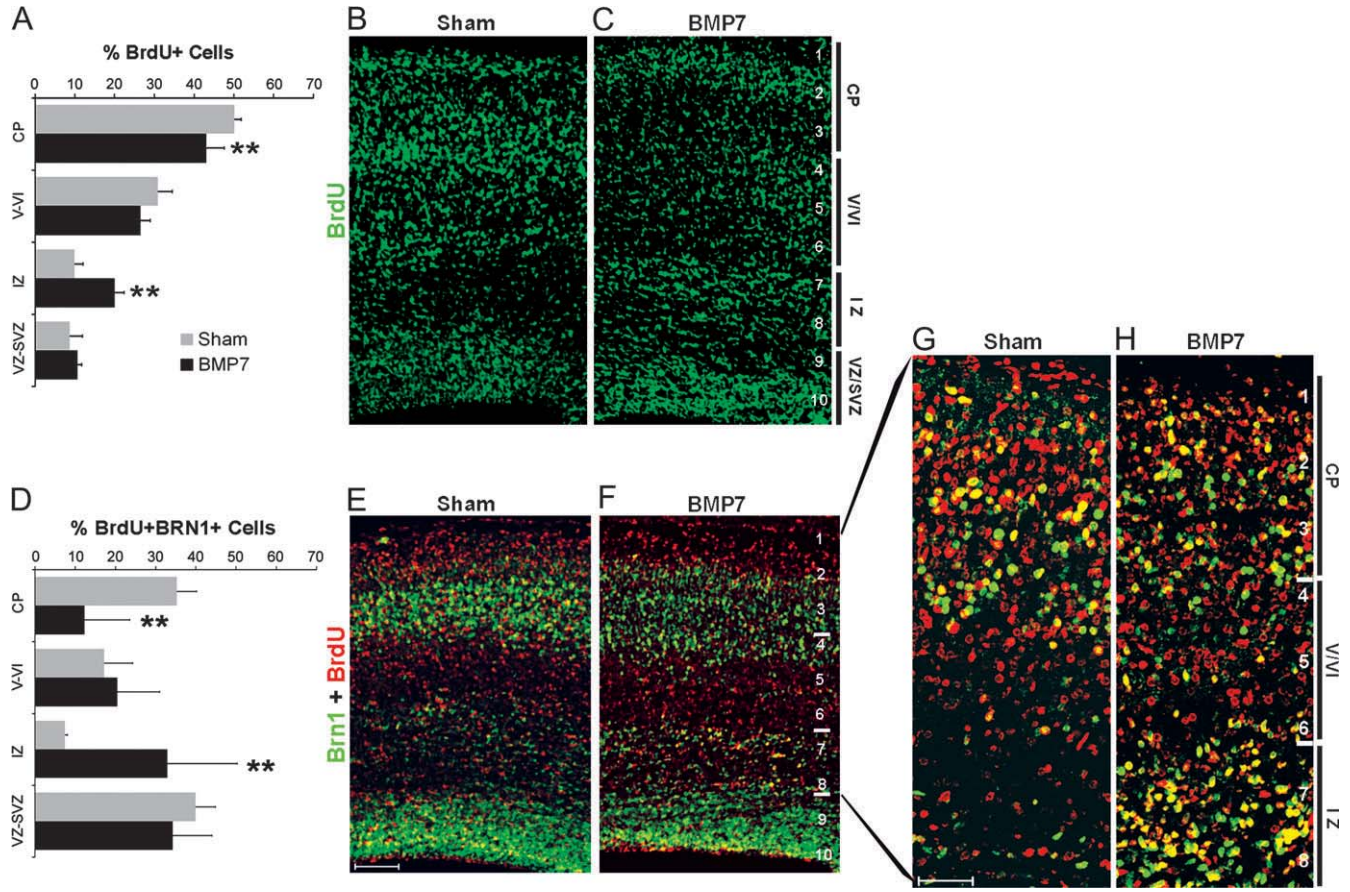

Figure 4. BMP7 treatment in E14 embryos impairs radial migration. (*A*) Graph of the laminar distribution of BrdU-positive cells in E18 cerebral cortex of embryos injected with vehicle (sham) (*B*) or BMP7 (*C*) at E14. BrdU was injected to the E14 pregnant females 3 h after BMP7 injection. The graph shows significant reduction in the percentage of labeled cells in the upper CP and a parallel increase in the percentage of labeled cells in the SP and IZ in BMP7-treated mice. (*D*) Laminar distribution of double-labeled neurons for BrdU (red) and Brn1 (green) at E18 in sham-operated (*E*) and BMP7-treated (*F*) animals. (*G, H*) Higher magnifications showing that ectopic BrdU-positive cells in the IZ of BMP7-treated mice (*H*) expressed Brn1 and had stopped migrating. **Significant difference $P < 0.01$, LSD test. Error bars reflect the standard deviation. Scale bar, 80 μm.

2 days in culture, GFAP expression was not affected by BSA beads, whereas BMP7 beads showed more intense GFAP staining and the presence of ramified astroglia in their vicinity (Fig. 6*K,L*). Similarly, the number of GFAP-positive cells increased in primary cortical cultures treated with BMP7 (Supplementary Fig. 2).

Taken together, these results indicate that BMP7 induces a precocious radial glia-to-astrocyte transformation and increased expression of SC1 protein in the embryonic cerebral cortex.

BMP7 Effects on VZ and SVZ Progenitors

SVZ progenitors constitute a second proliferating population mostly derived from radial glia that appears at E13 and increases at the end of neurogenesis (Malatesta et al. 2003; Noctor et al. 2004). To determine if BMP7 alters the distribution of progenitors in this secondary germinal region, we determined the position of all progenitor cells at E18 using antibodies against Ki-67 nuclear antigen, a protein that is present during all active phases of the cell cycle but absent from resting cells (Scholzen and Gerdes 2000). This is also a good way to estimate the persistence of radial glia in the VZ, as in rodents all radial glial cells are cycling and express Ki-67 (Hartfuss et al. 2001). The total number of proliferating cells was similar in sham- and in BMP7-injected cortices (from 77 ± 15 to 116 ± 24 cells per 1665-μm wide strip). However, we found significant differences in the laminar distribution of cycling cells. In E18 sham-operated cortices, Ki-67-positive cycling progenitors were mainly found in the VZ (58%), while in BMP7-injected cortices, this percentage was reduced to 44% (99% LSD test) (Fig. 7*A–C*).

To determine if BMP7 treatment affects progenitor subtypes, we performed a double immunofluorescence with Ki-67 and T-brain gene-2 (TBR2) that is specifically expressed intermediate (basal) progenitor cells (IPCs), a type of neurogenic progenitors (Englund et al. 2005). We calculated the ratio of IPCs respect to the total progenitor pool by dividing the number of Ki-67 + TBR2 cells into the total number of Ki-67 cells. What we found was that in E18 sham-operated cortices 84 ± 15% of Ki-67 cells were double-labeled with TBR2 while in BMP7-injected cortices the percentage of Ki-67 + TBR2 double-labeled cells was significantly reduced (55 ± 9%, 99% LSD test). Attending to their laminar distribution, Ki-67 + TBR2 progenitors were present in roughly normal proportions in the VZ (bin 10) while reduced through the SVZ and cortical parenchyma (bins 1–9) (Fig. 7*D–F*).

Taking together, our data suggest that BMP7 does not affect the total number of cortical progenitors but accelerates the transformation of radial glia into SVZ progenitors. Moreover,

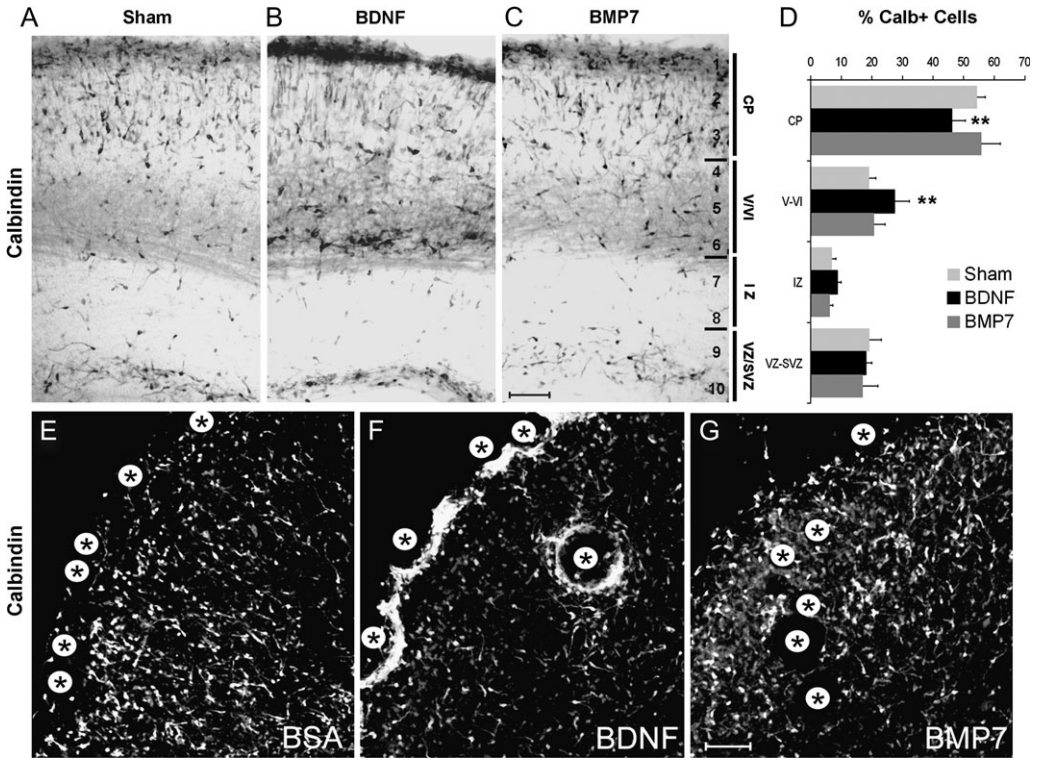

Figure 5. BMP7 do not affect the migration of GABAergic neurons identified by calbindin immunostaining at E18 in cortical coronal sections of mice injected at E14 with vehicle (sham) (*A*), BDNF (*B*), or BMP7 (*C*). (*D*) Graph of the laminar distribution of calbindin-positive neurons in control and BDNF- and BMP7-injected animals showing increased percentage of GABAergic neurons in layers V–VI of BDNF-treated animals and normal GABAergic cell distribution in BMP7- and vehicle-treated cortices. Calbindin-positive GABAergic neurons in E17 cortical organotypic cultures exposed to agarose beads (*) preadsorbed with BSA as negative control (*E*), BDNF as positive control (*F*), or BMP7 (*G*) for 48 h. BDNF exerted a dramatic attractive response in GABAergic neurons, while BMP7 had no effect. **Significant difference ($P < 0.01$, LSD test). Error bars reflect the standard deviation. Scale bar in *A–C*, 100 μm; *E–G*, 40 μm.

the reduction of Ki-67 + TBR2 intermediate neurogenic progenitors respect to the total progenitor pool is suggestive of a bias from neurogenesis to gliogenesis. Although due the complexity of this process, further work will be needed to confirm this hypothesis.

Discussion

Our results in vivo and in vitro support 3 main conclusions. First, in the developing cerebral cortex, TrkB ligands BDNF and NT4 induce BMP7 expression in neurons through MAPK/ERK signaling, probably involving blockage of repressor activity from p53/p63/p73 transcription factors. Second, the rise in BMP7 at midgestation induces radial glia to begin their transformation into astrocytes. Third, as a result of this precocious radial glia transformation, radial neuronal migration is impaired, and cortical lamination is altered. Together, these findings support a developmental mechanism by which, at the end of corticogenesis, activity-driven rises in BDNF induce BMP7 expression in cortical neurons that in turn locally instructs competent precursors to generate astrocytes. Such a mechanism might ensure simultaneous neuronal and glial maturation at the beginning of cortical activity (Fig. 8*B*).

Our results indicate that neurons are the main factors responsible for BDNF-dependent BMP7 expression in vitro. Neuronal pattern of BMP7 expression was also observed in vivo in the cerebral cortex after BDNF transfection at E14 or at P0 (not shown). However, we cannot rule out the possibility that in vivo some glial cells or other cell types such as capillary endothelial cells, a recently identified source of BMP7 in the cerebral cortex (Imura et al. 2008), might also account for their upregulation, as cerebral endothelium also expresses and responds to BDNF (Guo et al. 2008). The differences in the induction of BMP7 by BDNF in neurons and glia might rely on the distinct TrkB isoforms that they express. Differential splicing of TrkB mRNA generates the full-length TrkB, which is mainly expressed in neurons, and several truncated isoforms (TrkB-t) predominant in glial cells (Cheng et al. 2007). Signaling is also different and TrkB activates PI3K/AKT, MAPK/ERK and PKC signaling pathways, whereas TrkB-t isoforms that lack kinase activity do not (Chao 2003; Reichardt 2006). By analyzing the activation of TrkB signaling pathways, we have shown that BDNF-dependent BMP7 expression requires the activation of TrkB and MAPK/ERK pathway but not that of PI3K/AKT, as the Trk inhibitor K252a and the ERK1/2 and ERK5 inhibitor U0126 but not the PI3K inhibitor wortmannin blocked BMP7 induction by BDNF.

Activated ERK phosphorylates a number of transcription factors, including p53, which in turn induce or repress the transcription of downstream genes (Chang et al. 2003; Wu 2004). A recent study has identified a p53-responsive element in intron 1 of the BMP7 gene (Yan and Chen 2007). Mutations in p53 that abrogate its DNA binding or N-terminally truncated isoforms of p63 (Δp63) and p73 (Δp73) that fail to transactivate p53-dependent gene expression induce BMP7 expression in several systems (Laurikkala et al. 2006; Yan and Chen 2007). This indicates that full-length p53 family members repress transcription of BMP7. In agreement with these

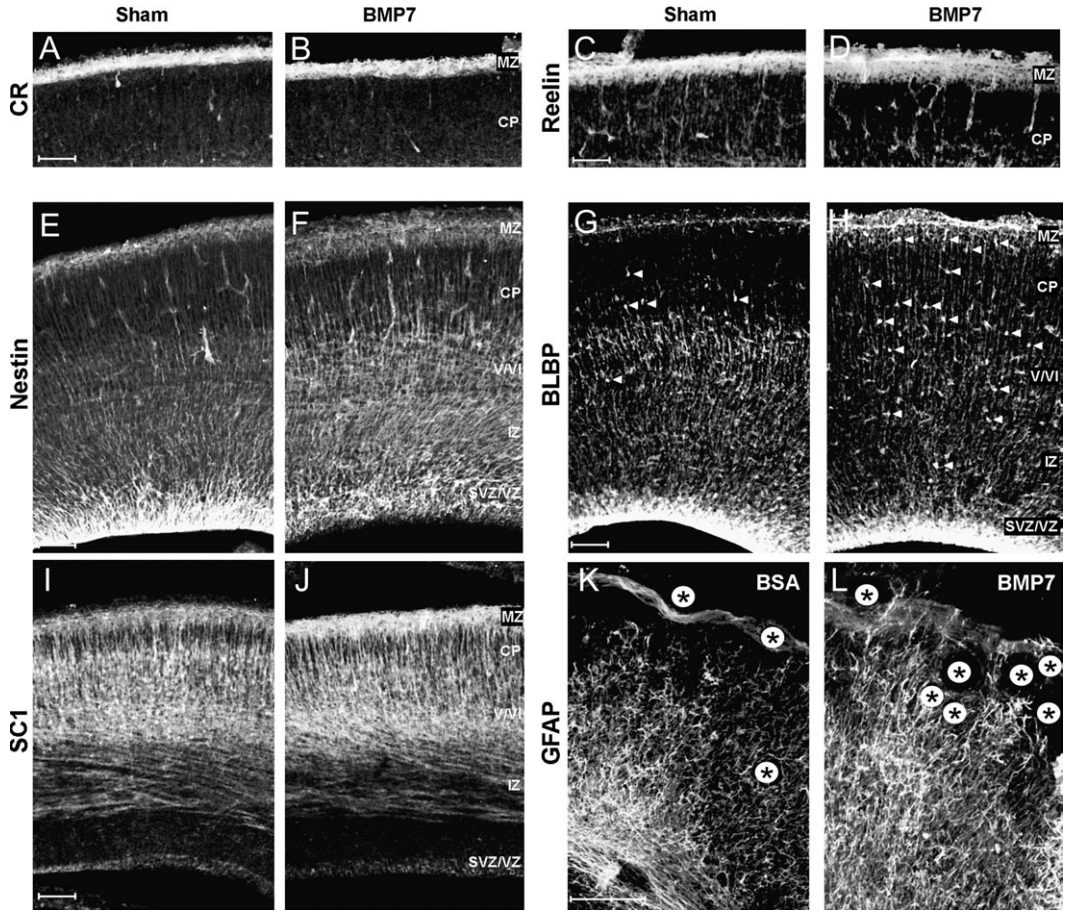

Figure 6. BMP7 effects on radial glia organization in developing mouse cerebral cortex. Cajal–Retzius cells identified by antibodies for calretinin (*A, B*) and reelin (*C, D*) showing their normal distribution in E18 cortical coronal sections from mice sham operated (*A, C*) or injected with BMP7 (*B, D*) at E14. (*E, F*) Staining for the progenitor marker nestin showing less intensity and a marked decrease in the radiality of labeled structures in the VZ of BMP7-treated brains (*F*) with respect to sham-operated brains (*E*). (*G, H*) BLBP protein staining showing reduced expression in deeper layers and more BLBP-positive cells in the CP of BMP7-treated animals (*H*) with respect to controls (*G*). (*I, J*) Staining for the antiadhesive protein SC1 showing a marked increase in BMP7-treated cortex (*J*) with respect to controls (*I*). (*K, L*) Organotypic cortical cultures exposed for 48 h to agarose beads preadsorbed with BSA as negative control (*K*) or BMP7 (*L*), showing increased GFAP expression and ramification in glial cells in the vicinity of BMP7 beads. V/VI, cortical layers V and VI. Scale bar, 80 μm.

findings, our results showed that pharmacological blockage of p53/73 transcriptional activity synergizes with BDNF in the induction of BMP7 transcription, whereas pharmacological activation of p53/73 partially reverted it. Our results also point to a basal and a regulated mechanism for BMP7 transcription, as basal BMP7 expression was not completely abolished by any of our pharmacological manipulations. Additional transcriptional activators may be required for regulation by BDNF, and p53 family members might contribute to repression.

Trk-mediated MAPK/ERK activation contributes to neuronal survival and differentiation by decreasing activation of the p53 pathway (Wade et al. 1999; McCubrey et al. 2007). Moreover, Δp73 and Δp63 isoforms are induced in the developing nervous system by Trk (Pozniak et al. 2000) and BMP7 (Laurikkala et al. 2006) signaling, respectively. This induction facilitates a regulatory loop between TrkB signaling and BMP7 transcriptional regulation by blocking the activation of p53 family members and by inducing the expression of their dominant negative truncated forms.

Our findings indicate that increased BMP7 levels at midgestation arrests the migration of glutamatergic neurons destined for the upper cortical layers. BDNF alters the laminar fate of glutamatergic neurons (Fukumitsu et al. 2006) and impairs radial neuronal migration by reducing reelin expression in Cajal–Retzius cells and cortical interneurons (Ringstedt et al. 1998; Alcántara et al. 2006). Our data indicate that BMP7 mainly affects the machinery for gliophilic radial migration, as E14-labeled ectopic neurons maintained the expression of transcription factors characteristic of their birthdates, and the laminar fate and tangential migration of cortical interneurons was preserved, at least at the early ages we studied.

Defective radial migration is caused by alteration of radial glia morphology or cell adhesion and adhesion-modulating proteins. Reelin and SC1 are extracellular matrix proteins controlling gliophilic migration. Although the mode of reelin action in neuronal migration is still controversial, a "detach-and-go" model in which reelin regulates detachment from radial glia and somal translocation has recently been proposed (Cooper 2008). In the present study, we found preserved cellular organization of the MZ after the intraventricular injection of BMP7, including reelin expression and distribution. We also failed to detect a local effect of BMP7 on Cajal–Retzius cells when applying BMP7-preabsorbed beads directly to the MZ in organotypic cultures (not shown). Our results indicate that alterations in Cajal–Retzius cell organization or in reelin expression are not the principal responsible of the migration

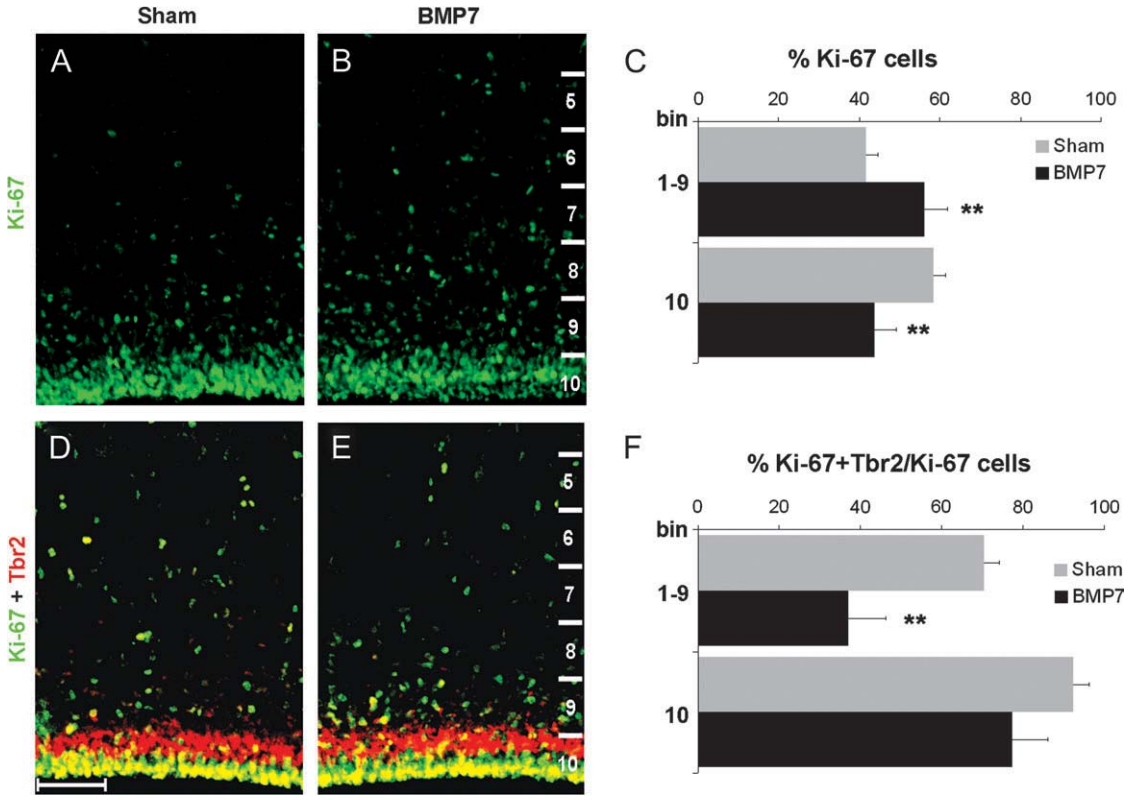

Figure 7. BMP7 effects in VZ and SVZ progenitors Ki-67 immunostaining showing the distribution of the cycling progenitor pool in sham-operated (*A*) and BMP7-injected animals (*B*). (*C*) Histogram showing the displacement of Ki-67 progenitors from the VZ (bin 10) to more basal positions (bin 1–9). (*D, E*) Figures show the distribution of IPCs double-stained with Ki-67 and TBR2 in sham-operated (*D*) and BMP7-injected (*E*) animals. (*F*) Histogram showing different distribution of IPCs between sham-operated and BMP7-injected animals. **Significant difference $P < 0.01$, LSD test. Error bars reflect the standard deviation. Scale bar, 80 μm.

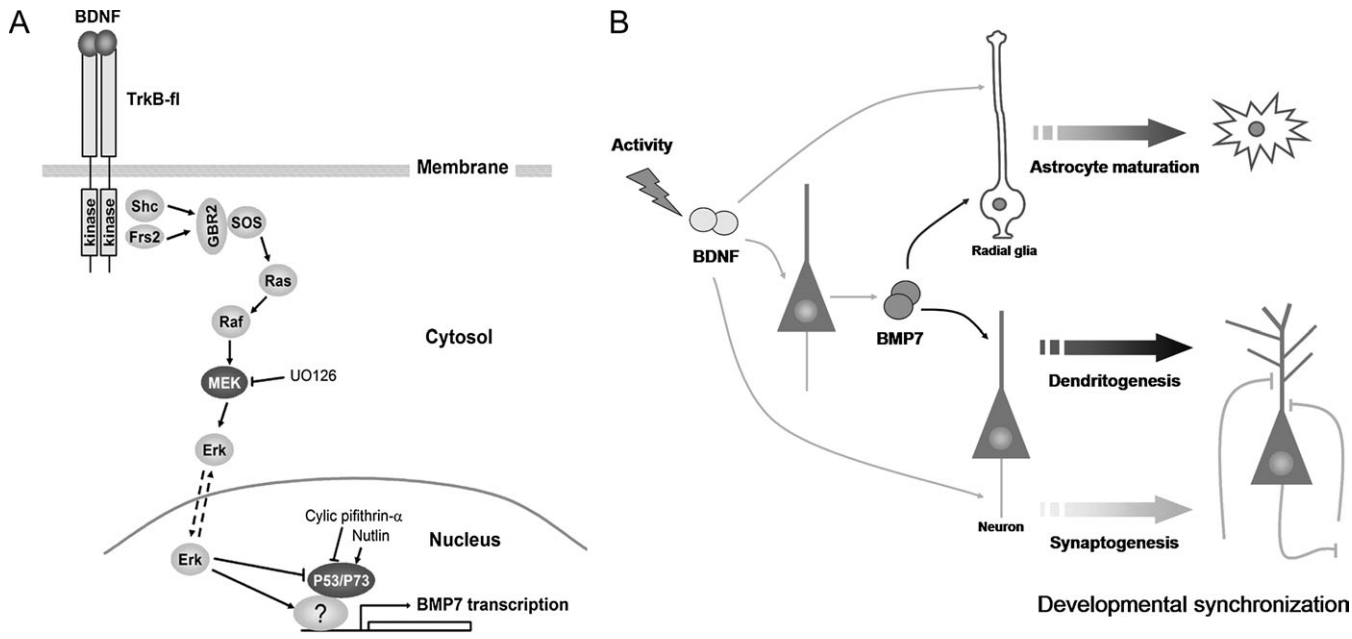

Figure 8. Model of BMP7 activation by BDNF. (*A*) Pathway of activation. (*B*) Model of physiological role for BDNF-dependent BMP7 expression during development.

arrest caused by BMP7, although we cannot completely rule out their involvement. On the other hand, SC1 is an antiadhesive protein of the SPARC-related family that regulates the interaction of cells with the ECM and that has been implicated in neuronal detachment at the end of migration (Gongidi et al. 2004). BMP7-dependent increases of SC1 expression in the CP as shown here might induce the early detachment of migrating neurons from the glial rail as they approach the CP, resulting in ectopic accumulation in the IZ similar to that observed in BMP7-treated cortices.

In addition to serving as a radial scaffold for neuronal migration, radial glia originate neurons, IPCs and glial restricted progenitors of the SVZ, and postnatally evolve into astrocytes with a precise although overlapping temporal sequence (Ihrie and Alvarez-Buylla 2008; Malatesta et al. 2008), a dynamic process regulated by crosstalk with embryonic neurons (Hatten 1985; Miller and Gauthier 2007). This process occurs in 2 sequential steps: First, notch ligands secreted by young neurons induce the expression of nuclear factor I, which promotes the demethylation of astrocyte-specific genes in neural precursors (Namihira et al. 2009), and second, glyogenic cytokines secreted by subsequent neuronal waves might then act on these demethylated glial promoters, committing competent neural precursors to the astrocyte lineage (Barnabe-Heider et al. 2005). Secreted BMPs induce astrocytogenesis and astroglial maturation from competent neural progenitors through the induction of inhibitory transcription factors of the Inhibitor of DNA binding (ID) family. ID factors antagonize proneural basic Helix-Loop-Helix protein function and induce GFAP promoter in late embryos (Yanagisawa et al. 2001; Miller and Gauthier 2007). Our data are consistent with a precocious radial-glia-to-astrocyte transformation induced by BMP7. The loss of radiality and reduction of nestin expression in radial glia, together with the greater number of BLBP-positive cells in the cortical parenchyma and the rise in SC1 expression, indicate that glioblasts increase at expenses of radial glia after BMP7 treatment. In addition, GFAP and ID1 transcription factor expression were also induced in E16 cortical cultures after BMP7 treatment (Supplementary Fig. 4).

The total number of cycling progenitors at E18 is not significantly altered by BMP7 treatment; however, progenitors are displaced from the VZ to more basal positions. Double-labeling experiments using TBR2, a specific marker for IPCs (Englund et al. 2005), showed a reduction of TBR2 progenitors in the SVZ respect to the total progenitor pool. IPCs are considered neurogenic transit amplifying progenitors; thus, our data are compatible with the notion of an early radial glia transformation to progenitors of the glial linage induced by BMP7. However, due to the complex dynamics of germinal matrices during corticogenesis, further work and long-term analysis of changes in neuronal and glial populations will be needed to sustain this assumption.

Taken together, our results indicate that the migration arrest observed in BMP7-treated animals is the result of precocious transformation of radial glia to astrocyte and a change in the extracellular matrix composition that promotes neuron–glia detachment by increasing the expression of antiadhesive factors such as SC1.

At the end of neurogenesis, sensory experience and electrical activity shape the functional and structural architecture of the CNS by regulating the transcription of a large set of genes including BDNF and its receptor TrkB (Hughes et al. 1999; Nagappan and Lu 2005; Hong et al. 2008). Activity-driven BDNF expression promotes neuritogenesis, the formation of new synapses, and the development of cortical inhibition (Bonhoeffer 1996; Aakalu et al. 2001; Kohara et al. 2007), and by binding to TrkB-t isoforms, BDNF also directs cortical progenitors to a glial cell fate (Cheng et al. 2007).

On the basis of our results, we propose a model in which BDNF-dependent BMP7 expression constitutes a local regulatory system. At the end of corticogenesis, BMP7 induction by BDNF through the TrkB and MAPK/ERK/p53 pathway might synchronize neuronal survival and differentiation with astocytic maturation on the arrival of incoming axons and the beginning of cortical activity.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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Notes

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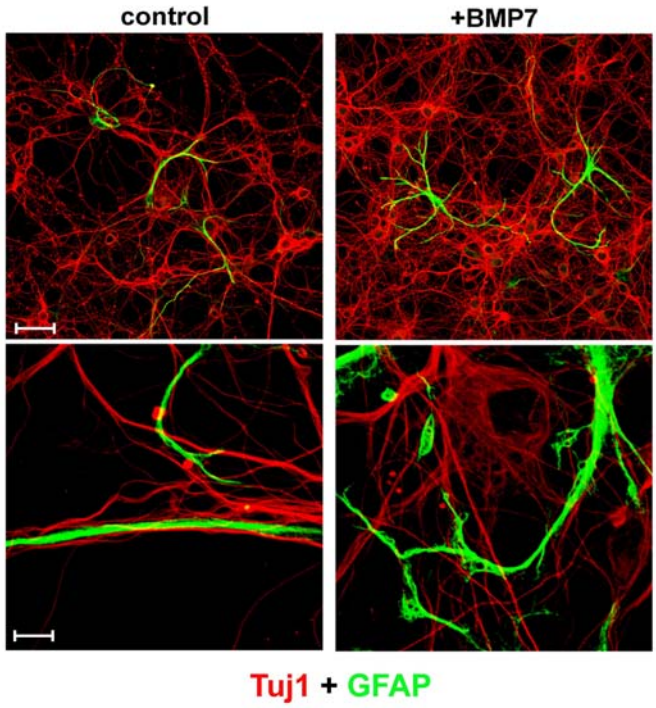
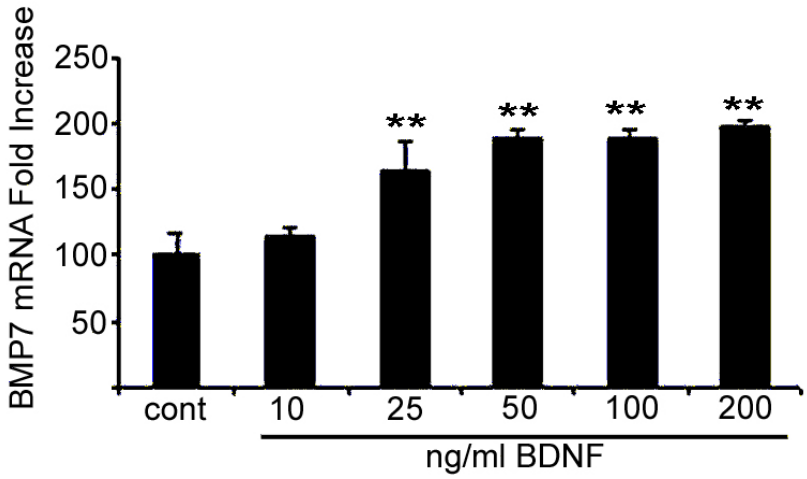
SUPPLEMENTARY FIGURE LEGENDS

BMP/TGFβ, receptors and inhibitors	Cx E18 Sham +1,25/-1,25	Cx E18 BDNF +1,39/-1,37	Cx E18 NT4 +1,30/-1,31	Cx E18 SDF1 +1,27/-1,29
Acvr1	-1.01	-1.28	-1.00	1.02
Acvr1b	1.09	1.13	1.12	1.05
Acvr1c	1.04	1.18	1.03	-1.04
Acvrl1	1.04	1.12	1.24	-1.01
Bmper	-1.05	-1.17	-1.06	1.17
Bmpr1a	-1.10	-1.02	-1.16	-1.15
Bmpr1b	1.01	1.02	-1.00	-1.01
Bmpr2	-1.04	1.12	1.12	1.02
Tgfbr1	-1.02	1.01	1.04	1.02
Tgfbr2	-1.05	1.00	1.04	-1.04
Tgfbra1	-1.03	-1.14	-1.10	-1.13
Tgfbra3	-1.19	-1.12	-1.05	-1.27
TGFβ superfamily of ligands				
Bmp1	-1.10	-1.03	-1.06	-1.09
Bmp2	1.06	1.10	-1.01	-1.03
Bmp3	-1.04	-1.16	-1.02	1.11
Bmp4	-1.24	-1.31	-1.25	-1.25
Bmp5	-1.01	1.02	-1.16	-1.03
Bmp7	1.08	1.49	1.22	1.11
Bmp8b	-1.01	1.07	-1.02	-1.07
Bmp15	1.07	1.13	1.09	1.16
Gdf1	1.14	1.19	1.10	1.07
Gdf2	1.06	1.03	1.06	1.02
Gdf3	1.04	1.19	-1.01	-1.04
Gdf5	1.03	1.09	1.03	1.06
Gdf8	-1.09	1.15	-1.04	-1.21
Gdf10	1.03	-1.05	-1.04	1.03
Gdnf	1.03	-1.02	1.03	1.03
Tgfb1	-1.06	1.09	1.14	1.03
Tgfb2	-1.08	-1.10	-1.05	-1.14
Tgfb3	-1.16	-1.08	-1.07	-1.13
Ligand inhibitors				
Chrd	1.14	1.22	1.18	1.18
Grem1	-1.01	-1.01	1.08	1.05
Grem2	-1.01	-1.04	1.06	-1.01
Nog	1.10	-1.04	1.07	1.00
Thbs1	-1.20	-1.06	-1.22	-1.19

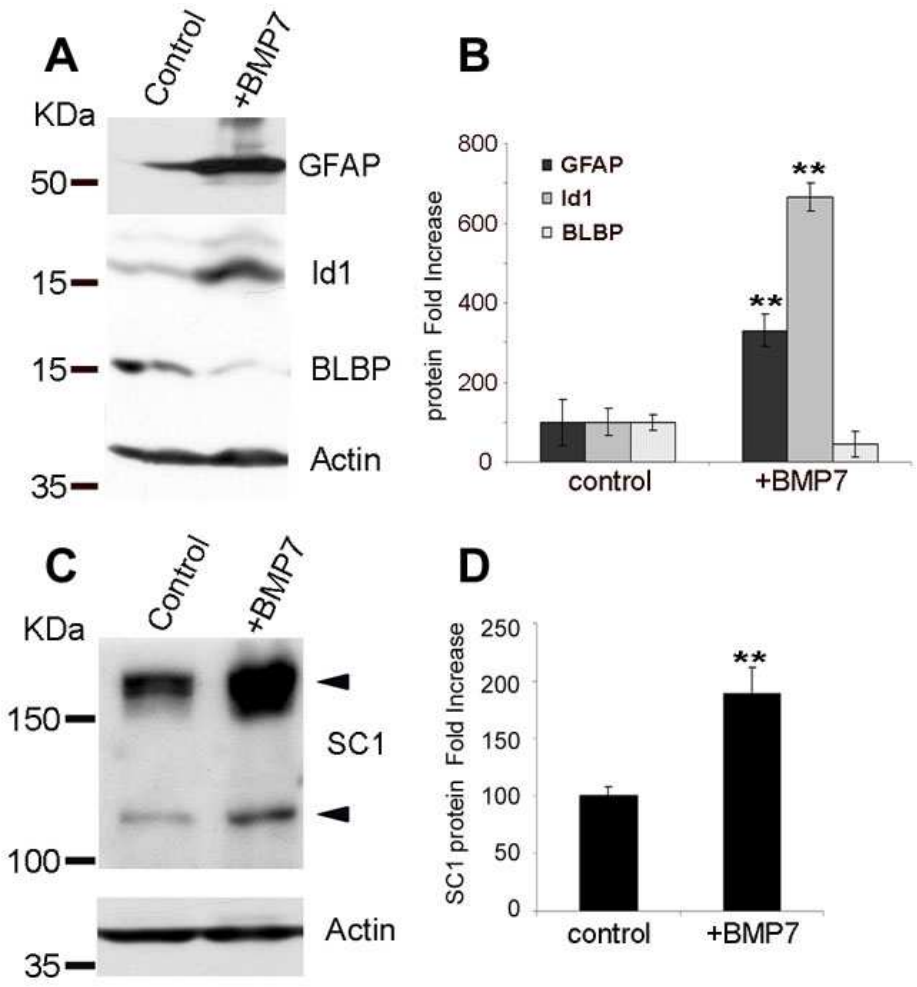
Supplementary Figure 1. Table summarizing the results from the low-density cDNA microarray of E18 cortices from mice injected at E14. In the microarray were represented 25 genes members of the TGFβ superfamily, including receptors, inhibitors and several other related genes. In addition to BDNF, as a control for selectivity we also injected NT4, the second neurotrophin that preferentially acts through TrkB receptor, and SDF1α, a chemoquine not related to TrkB signaling pathway. As negative controls, we used intact animals, or animals injected with vehicle (sham). Data were normalized to intact animals and only differences higher than 3 standard deviations (confidence intervals) respect intact and sham operated mice were considered statistically significant.



Supplementary Figure 2. BMP7 induced astroglial maturation *in vitro*. **A-D,** Immunostaining for the neuronal marker Tuj1 (red) and the astroglial marker GFAP (green) in neuron-enriched cultures treated with vehicle and BMP7 for 96h. **C-D,** Higher magnification images showing an increase in branching and morphological complexity of astrocytes treated with BMP7. Scale bar, 50µm in **A**, **B**; 8 µm in **C**, **D**.



Supplementary Figure 3. Histogram summarizing the effect of BDNF dose on bmp7 expression in serum-free neuron enriched cultures analyzed by real time PCR after 6h treatment. BDNF treatment increases bmp7 mRNA expression to reach a plateau from 50 to 200 ng/ml of BDNF. ** Significant difference respect controls $p<0.01$, LSD test.



Supplementary Figure 4. BMP7 treatment induces astrocytic maturation. **A,** Western blots for GFAP, Id1, BLBP and actin as loading control in neuron-enriched cultures treated for 96h with BMP7 (75ng/ml) showing higher levels of astroglial marker GFAP, and lower levels of the radial glia marker BLBP in BMP7 treatment, which also presents a dramatic increase in Id1, an anti-neurogenic transcription factor. **B,** Graph summarizing densitometry quantification of Western blots demonstrating significantly increases in two astroglial maturation markers: GFAP and the anti-neurogenic transcription factor Id1, in neuron-enriched cultures treated with BMP7. **C**, Western blots displaying increased levels of SC1 in the neuron-enriched cultures treated with BMP7 respect the control. **D**, Densitometry analysis histogram showing significant increases in SC1 levels in BMP7 treated cultures. Arrowheads indicate the two species of SC1, the normal form around 100 kDa, and a modified form of 200 kDa approximately (described by Gongidi et al., 2004). ** Significant difference $p<0.01$, LSD test. Error bars reflect the standard deviation.

2. APPROPRIATE BMP7 LEVELS ARE REQUIRED FOR THE DIFFERENTIATION OF MIDLINE GUIDEPOST CELLS INVOLVED IN CORPUS CALLOSUM FORMATION

The corpus callosum (CC) is the largest commissural tract in the vertebrate brain formed by the axons of a subtype of cortical pyramidal neurons mainly located in layers 2/3 and 5. It connects neurons in the left and right cerebral hemispheres and coordinates the transfer of information between them. Axonal crossing process and the resulting corpus callosum formation is directed by different glial populations located in the midline region, which act as guidepost cells. They participate in the formation of the CC guiding callosal axons to cross the midline into their final target region. These populations include the glial wedge (GW), the indusium griseum (IG), the midline zipper glia (MZG), and the subcallosal sling cells. The IG, dorsal to the CC, is formed by a group of neurons and astrocytes located underneath the pial membrane of the dorsomedial pallium, whereas the bilateral GW is composed by radial glial cells that reside ventral to the CC at the cortico-septal boundary. Finally sling cells are distributed forming an "U" shape structure that ventrally limits the CC (Shu et al., 2003).

Agenesis of the CC is a developmental defect that can result from the disruption of multiple steps of CC development. CC agenesis is often associated with alterations of its midline guidepost cells (Richards et al 2004; Paul et al., 2007; Donahoo and Richards, 2009), but the precise mechanisms that control their specification are still poorly defined. Here, we have investigated whether signalling activated by Bone Morphogenetic Proteins (BMP) might be one of these mechanisms since these cytokines are known to promote glial differentiation in the CNS (Gross et al., 1996; Li et al., 1998; Mabie et al., 1999). We have focused in the study of BMP7, an astrocyte fate inducing factor (Mabie et al., 1997; Nakashima and Taga, 2002; Ortega and Alcántara, 2010), using a transgenic animal model with the mutated $Bmp7^{lacZ/Neo}$ allele. We observed the expression of BMP7 in midline guidepost cells of GW, IG and sling cells.

Studies with knock out animals showed that the absence of BMP7 produces CC agenesis. In these animals we observed that axons did not cross the midline but remained in the ispilateral side of the brain forming Probst-like bundles. We analyzed if this effect was due to a possible ability of BMP7 to modify the cortical fate of neurons that project their axons towards CC. However only mild differences were observed in cortical lamination between knock out and control animals. BMP7 tropism activity on cortical projecting neurons was also tested but changes in axonal outgrowth were not observed in presence of BMP7. Finally, changes in BMP7 knock out animals were found in midline glial populations (GW, IG) as well as in sling cells population. GW and IG were disorganized and abnormally distributed across the midline, while the number of sling cells was dramatically reduced in the cortico-septal boundary from the lateral ventricles to the midline region.

Surprisingly, a gain of function model where mice embryos were injected with the recombinant BMP7 protein also showed CC agenesis and abnormal GW and IG development, marked by a premature high expression of the astroglial marker GFAP. Subcallosal sling cells were also reduced in number as we observed in BMP7 deficient mice. Altogether those results demonstrate that Bmp7 is required for CC formation, and that appropriate levels of this factor are necessary for timely differentiation of CC associated midline glial and neuronal guidepost cells.

Appropriate *Bmp7* Levels are Required for the Differentiation of Midline Guidepost Cells Involved in Corpus Callosum Formation

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ABSTRACT: **Guidepost cells are essential structures for the establishment of major axonal tracts. How these structures are specified and acquire their axon guidance properties is still poorly understood. Here, we show that in mouse embryos appropriate levels of Bone Morphogenetic Protein 7 (*Bmp7*), a member of the TGF-β superfamily of secreted proteins, are required for the correct development of the glial wedge, the indusium griseum, and the subcallosal sling, three groups of cells that act as guidepost cells for growing callosal axons. *Bmp7* is expressed in the region occupied by these structures and its genetic inactivation in mouse embryos caused a marked reduction and disorganization of these cell populations. On the contrary, infusion of recombinant *Bmp7* in the developing forebrain induced their premature differentiation. In both cases, changes were associated with the disruption of callosal axon growth and, in most animals fibers did not cross the midline forming typical Probst bundles. Addition of *Bmp7* to cortical explants did not modify the extent of their outgrowth nor their directionality, when explants were exposed to a focalized source of the protein. Together, these results indicate that *Bmp7* is indirectly required for corpus callosum formation by controlling the timely differentiation of its guidepost cells.** © 2010 Wiley Periodicals, Inc. Develop Neurobiol 71: 337–350, 2011

Keywords: **morphogen; cerebral cortex; axon guidance; glial cells; neuronal migration**

INTRODUCTION

Long-distance axon outgrowth relies on multiple navigational mechanisms. Seminal observations in the grasshopper embryos suggested that pioneer axons use cells strategically positioned along their trajectories as landmarks for pathfinding (Bate, 1976). Cells with the same characteristics of these so called "guidepost cells" (Bentley and Keshishian, 1982) have been rarely found in other organisms. However, a broader definition of the term can be easily applied to many structures of invertebrate and vertebrate organisms. Thus, guideposts are discrete groups of glial or neuronal cells that provide discontinuous

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information in intermediate positions along the path of growing axons (Palka et al., 1992). Guidepost cells have also been defined as "intermediate targets" when the information they provide determine sharp changes in the axon trajectory or its sorting in different directions (Bovolenta and Dodd, 1990). In this context, good examples of guidepost structures in vertebrates are the optic disc and chiasm for visual axons (Bovolenta and Mason, 1987; Godement et al., 1990; Stuermer and Bastmeyer, 2000; Morcillo et al., 2006), the floor plate for commissural axons of the spinal cord (Bovolenta and Dodd, 1990; Bovolenta and Dodd, 1991) or the basal ganglia primordium for thalamo-cortical projections (Garel et al., 2002; Dufour et al., 2003; Seibt et al., 2003; Lopez-Bendito et al., 2006). Guidepost cells secrete or express on their surface guidance cues that confer their properties and their surgical or genetic removal causes severe alterations in the associated axon trajectory (Learte and Hidalgo, 2007). Therefore, establishing how guidepost cells are specified is fundamental for the complete understanding of brain wiring. This question, however, is far from being fully resolved, particularly in the case of the corpus callosum (CC), a mayor axon tract that heavily relies on the integrity of guidepost cells for its development (Paul et al., 2007).

The CC is the largest commissural tract in the vertebrate brain and is devoted to coordinate information between the left and right brain. This commissure is formed by the axons of a subtype of cortical pyramidal neurons located in layers 2/3 and 5 (Yorke and Caviness, 1975; Alcamo et al., 2008; Britanova et al., 2008; Molyneaux et al., 2009). Callosal neurons project their axons to the intermediate zone of the cortex where axons turn toward the midline. After a steep ventral growth, callosal fibers abruptly turn to cross the midline at the cortico-septal boundary to follow an inverse trajectory in the opposite hemisphere of the brain [Fig. 1(a); Richards et al., 2004)]. The midline path of callosal axons is surrounded by multiple cellular structures that act as guidepost cells. These include the glial wedge (GW), indusium griseum (IG), midline zipper glia (MZG), and subcallosal sling cells [SCS, Fig. 1(A)]. The IG, dorsal to the CC, is formed by a group of neurons and astrocytes located underneath the pial membrane of the dorsomedial pallium, whereas the bilateral GW is composed of radial glial cells that reside ventral to the CC at the cortico-septal boundary (Shu et al., 2003). Both the structures express *Slit2*, a potent chemorepellent that restricts the site of callosal axons cross at the midline (Shu and Richards, 2001; Bagri et al., 2002; Shu et al., 2003). The MZG, which might

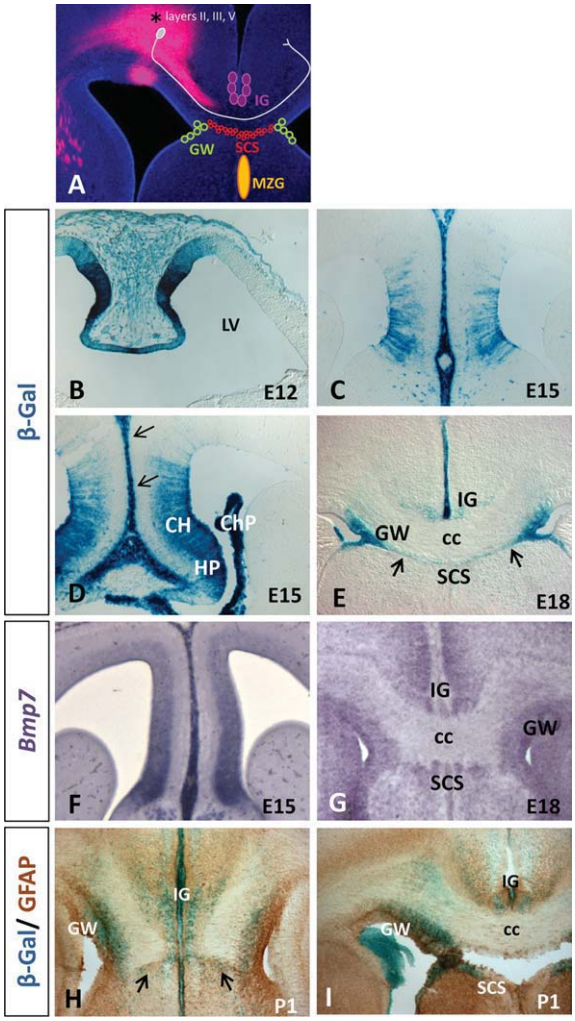

Figure 1 *Bmp7* is expressed in cortical midline guidepost cells. (A) Semi-schematic representation of the callosal pathway and the associated midline guidepost populations. The position of the GW (green circles), IG (purple dots), SCS (red circles), and MZG (yellow oval) are indicated in a frontal Hoestch-stained section at the level of the septum, where pioneer callosal axons have been traced with a DiI crystal placed in the cerebral cortex (black asterisk). The trajectory of callosal axons has been highlighted in white. B–E) β-Gal staining of coronal sections from $Bmp7^{lacZ/+}$ embryos at E12, E15 and E18. Reporter expression localized to the dorsal telencephalic midline and meninges (B, C, arrows in D), in the choroid plexus (ChP), cortical hem (CH) and hippocampal primordium (HP) and in the GW, IG, and SCS (arrows in E) surrounding the CC. F–G) Coronal sections from wt embryos hybridizes with a *Bmp7* specific probe. H–I) Coronal sections from $Bmp7^{lacZ/+}$ pups double labeled with β-Gal staining and antibodies against GFAP.

be involved in telencephalic fusion, and the SCS [Fig. 1(A)], an array of glial cells and neuronal that migrate from the lateral ventricle, provide a substratum over which CC pioneer axons extend (Silver

et al., 1982; Silver and Ogawa, 1983; Silver et al., 1993; Shu et al., 2003; Shu et al., 2003). A recent study has also demonstrated the existence of additional transient glutamatergic and GABAergic neuronal populations, which intermingle with the nascent callosal axons and contribute to their guidance by expressing the chemoattractant Sema3C (Niquille et al., 2009; Piper et al., 2009).

Agenesis of the CC is a developmental defect, which can result from the disruption of multiple steps of CC development. CC agenesis is often associated with alterations of its midline guidepost cells (Paul et al., 2007), but the precise mechanisms that control their specification are only poorly defined. Genetic inactivation of *Nfia* and *Nfib,* two transcription factors of the nuclear factor I (NFI) family, results in an acallosal phenotype due to reduced formation of cortical midline glia (das Neves et al., 1999; Shu et al., 2003; Steele-Perkins et al., 2005). Similarly, disruption of Fgf signaling prevents CC formation because *Fgf receptor 1* is required to form the IG, whereas other guidepost structures are normal (Smith et al., 2006; Tole et al., 2006), suggesting that their development requires other yet undefined signaling mechanisms. Here we have investigated whether signaling activated by Bone Morphogenetic Proteins (BMP), members of the TGFβ super-family of signaling factors (Chen et al., 2004), might be one of these mechanisms. The choice of BMP and in particular of BMP7-mediated signaling seemed particularly adequate because these cytokines are well known glial-inducing factors (Nakashima and Taga, 2002). Furthermore, BMP7, acting ahead of Sonic hedgehog, promotes the specification of optic disc glial cells, which guide visual axons out of the eye (Morcillo et al., 2006). In line with this hypothesis, herein we demonstrate that *Bmp7* is required for CC formation because appropriate levels of this factor are necessary for timely differentiation of its associated midline glial and neuronal guidepost cells.

MATERIALS AND METHODS

Animals

Bmp7-deficient mice, generated and genotyped as described (Godin et al., 1998), were kindly provided by Prof. E. Robertson (University of Oxford) and maintained in a C57/Bl6 genetic background by backcrossing (at least seven times). Wild type (wt) embryos from C57BL/6J pregnant mice were collected between E16.5 and E18.5 (E0.5 corresponds to the day of the vaginal plug). All animals were used according to the Spanish (RD 223/88) and European (86/609/ECC) regulations.

In Utero Injection of BMP7 Protein

Pregnant dams at 14.5 were anesthetized with intraperitoneal injection of Ketamine/Valium (150 μg/g; 5 μg/g). After exposure of the uterine horns, 2 μL of vehicle or of human recombinant BMP7 (0.5 μg/μL, R&D, Abingdon, UK) were delivered into the lateral ventricles of the embryos by intrauterine injection as described (Ortega and Alcantara, 2010). The uterus was returned to the abdominal cavity to allow four additional days of development (E18.5). Embryos were thereafter processed for immunohistochemistry.

BrdU Incorporation

Wt or *Bmp7* null pregnant females at 14.5 or 15.5 days of gestation were injected intraperitoneally with 5-bromo-2-deoxyuridine (BrdU; 50 mg/kg body weight; Sigma-Aldrich). In the case of vehicle or BMP7-injected animals, BrdU was administered at E14.5 3h after BMP7 injection or at E15.5. In all cases, embryos were sacrificed at E18.5 and processed for immunohistochemistry.

In Situ Hybridisation and Immunohistochemical Procedures

Mouse embryos were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer pH 7.3 (PB). Brains were dissected and postfixed for 8–12 h, cryoprotected in 30% sucrose solution in PB, and sectioned with a cryostat (Leica). Coronal sections of 20–40-μm thickness were collected and then processed for *in situ* hybridization or histochemical procedures. Immunostaining of cryostat sections or explant cultures were performed with standard protocols using antibodies against the following antigens: Glial Fibrillary Acidic Protein (GFAP; rabbit antiserum, 1:3000; Dako), tubulin-βIII (mAb, 1:1000 Promega;), Cux1 (1: 1000; Santa Cruz Biotechnology), anti-Tbr1 (1: 2000; Chemicon), anti-TBR2 (mAb, 1:500; abcam), Beta 3 (mAb, 1:500; Santa Cruz Biotechnology), Ctip2 (mAb, 1:100; abcam), L1 (mAb, 1:1000; Chemicon), Sox5 (rabbit antiserum 1:3000; a gift from A. Morales), Nestin (mAb, 1:500; BD Pharmingen), BrdU (mAb, 1:200; GE Healthcare), Brn1(1:100, Santa Cruz Biotechnology, San Diego), and 488 or 594-Alexa™-conjugated fluorescent secondary antibodies (1:500; Molecular Probes). Sections were counterstained with Hoechst 33258 (Molecular Probes). *In situ* hybridisations were performed with digoxigenin-labeled probes designed against *Clim1* (a gift from P. Arlotta) and *Slit2*, using standard protocols. *Bmp7* distribution was determined by X-gal histochemistry, anti-βgal immunostaining in $Bmp7^{lacZ/+}$ embryos and *in situ* hybridisation with a *Bmp7* digoxigenin-labeled specific probe.

DiI Labeling

The trajectory of callosal axons in E18.5 and P0 wt and *Bmp7* null animals was determined by unilateral antero-

grade labeling with a small DiI crystal (Molecular Probes) placed onto the surface of the fixed cerebral cortex. Brains were stored for 15 days at 37°C in Phosphate Buffer Saline (PBS)/PFA to allow dye diffusion and thereafter embedded in agar/agarose (2%, 2%) solution and sectioned at 50-μm thickness using a vibratome (Leica).

Cortical Explant Cultures

The brains of E16.5 and E18.5 embryos were removed and embedded in 4% low-melting agarose and sectioned in the coronal plane at 200-μm thickness using a vibratome. The cerebral cortex was separated from the rest of the slice, divided in cubes of about 200 μm, and embedded in collagen gel matrix in the presence or absence of soluble or bead-immobilised BMP7 (100 ng/μL; R&D) as described (Trousse et al., 2001). After 48 h, explants were fixed in 4% PFA and stained with antitubulin-βIII antibody. In each case, 4–5 explants were cultured in quadruplicates. At least 26 experiments were performed for each condition.

Western Blot Analysis

The cortico-septal region of Wt, *Bmp7* null, Sham-operated and BMP7-injected neonatal animals was dissected and collected in lysis buffer (150 mM NaCl, 1% TritonX-100, 50 mM Tris pH 8; 1mM PMSF, proteinase inhibitors). Lysates were fractionated by ultracentrifugation, and the pellets were resuspended in 5× loading buffer and separated by 12% SDS-PAGE. After electrophoresis, proteins were transferred to PDVF membranes (Hybond-P, Amersham), checked by Ponceau Red staining, and probed with mAb against GFAP (1:10.000, Dako); Nestin (1:5000, Abnova Corporation), and α-tubulin (1:50.000). Primary antibodies were detected with peroxidase-conjugated secondary antibodies and detected with Enhanced chemiluminescence (ECL) Advanced Western Blotting Detection Kit (Amersham). Densitometric analysis, standardized to α-tubulin, was performed using ImageJ software (National Institutes of Health).

Statistical Analysis

The data were collected using the ImageJ software (NIH) and quantified with the GraphPad software. The extent of neurite outgrowth in collagen gel experiments was determined subtracting the area occupied by the explants from the total Tuj1-positive area, normalising for the explant area. The quadrants proximal and distal to the position of the soaked beads were analysed. Neurite length was determined by measuring the distance from the edge of the explant to the tip of the longest immunopositive fibre. Data are presented as means ± SEM in pixels. The number of Tbr2-positive cells presents in the subcallosal sling of wt, *Bmp7*$^{-/-}$, BMP7-injected or sham-operated embryos were quantified with ImageJ software in confocal images (Leica TCS LS). In each case, positive cells were counted in an area of 450 μm^2 in a number of sections (wt, 10; *Bmp7*$^{-/-}$, 11; sham, 18; BMP7, 17) from different embryos (wt, 3; *Bmp7*$^{-/-}$, 3; sham, 8; BMP7, 5). Statistical significance was calculated using unpaired Student's *t*-test.

RESULTS

Bmp7 is Expressed in the Region Occupied by CC Guidepost Cells

A number of BMPs, including *Bmp7*, are crucial for early dorsal telencephalic development (Furuta et al., 1997; Hebert et al., 2002; Hebert et al., 2003) but their precise expression during CC development has not been reported. We focused on *Bmp7* and determined its expression using the activity of the *LacZ* transgene of the mutated *Bmp7*$^{lacZ/Neo}$ allele (Dudley et al., 1995). β-Gal staining of coronal sections from E12.5, E13.5 and E15.5 *Bmp7*$^{lacZ/+}$ embryos revealed high expression of *Bmp7* in the meninges, choroid plexus, cortical hem, hippocampal primordial, and cortico-septal boundary before pioneer CC axons reach the midline [Fig. 1(B–D), not shown]. When callosal axons begin to form a visible tract between E16.5 and E18.5, *Bmp7* expression was localized to the regions occupied by the GW, IG and in scattered cells of the SCS [Figs. 1(E) and 2(A,B,E,F)]. This distribution was confirmed by *in situ* hybridization analysis [Fig. 1(F,G)] Immnunocolocalization of β-Gal and anti-GFAP signal confirmed the glial nature of a proportion of the *Bmp7*-positive cells at the cortico-septal boundary at E16.5, when the first commissural axons begin to elongate [Fig. 2(A,B)]. GFAP-positive cells were localized in the GW, IG, and SCS. However, in the latter structure, many β-Gal-positive cells did not express GFAP [Fig. 1(H,I)], suggesting that *Bmp7* positive cells could also be neuronal in nature. To confirm this possibility, we analyzed the expression of the transcription factors T-brain-2 and 1 (Tbr2, Tbr1), which are respectively expressed in the intermediate (basal) progenitor cells and in postmitotic neurons of the developing cerebral cortex (Englund et al., 2005). Many double-labeled Tbr2 and $\beta-$Gal positive cells were observed at the cortico-septal boundary. From E16.5 onward, an increasing number of Tbr2 positive cells appear to migrate towards the developing callosal region [Figs. 2(E,F) and 7(A)], delineating the SCS at E18 [Fig. 7(B,G)]. As reported (Niquille et al., 2009), Tbr1-positive neurons were mostly observed within the developing CC [Fig. 6(E)], where $\beta-$Gal and *Bmp7* mRNA was hardly detected [Figs. 1(E,G,C) and 2]. Together, these results indicate that *Bmp7* is expressed by most glial and neuronal midline guidepost cells of the CC.

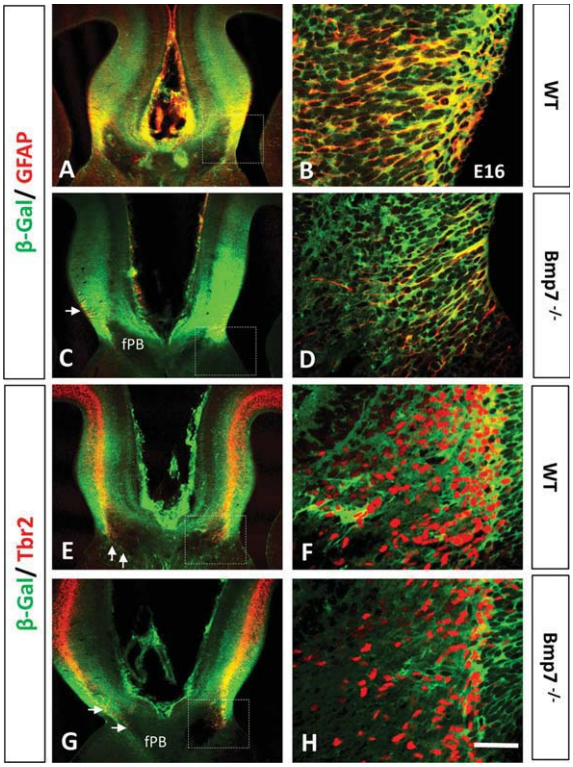

Figure 2 *Bmp7* is expressed in glial and neuronal cells surrounding the nascent CC. Confocal images of frontal cryostat sections from E16.5 wt and *Bmp7* null embryos at the level of the CC immunostained with antibodies against *β*-Gal (green) and GFAP (A–D) or Tbr2 (E–H). Note the decrease of both *β*-Gal/GFAP and *β*-Gal/Tbr2 double stained cells (arrows in C, E, G) in the mutants, where callosal axons form incipient Probst-like bundles (fPB).

Bmp7 is Required for Proper CC Formation

If *Bmp7* expression at the telencephalic midline is directly or indirectly involved in CC formation, *Bmp7* null mice should present callosal commissural defects. Indeed, abnormal bundling of commissural axons at the cortico-septal boundary was already observed in *Bmp7* null embryos at E16.5 [Fig. 2(C,G)]. These abnormalities were clearly visualized when frontal sections of wt and *Bmp7* null embryonic or postnatal brains were immunolabelled with antibodies against L1 [Fig. 3(A,B)], a cell adhesion protein abundantly expressed by callosal axons (Fujimori et al., 2000), or traced with a crystal of DiI [Fig. 3(C,D)]. At E18.5, wt callosal fibers crossed the cortical midline and grow dorsally to the contralateral hemisphere [$n = 10$; Fig. 3(A,C)]. While heterozygous $Bmp7^{+/-}$ embryos ($n = 6$) were indistinguishable from wt littermates, $Bmp7^{-/-}$ embryos presented an abnormal CC, albeit with a variable penetrance. In half of the analyzed homozygous embryos ($n = 13$) callosal axons did not cross the midline but remained in the ispilateral side of the brain forming Probst-like bundles [Fig. 3(B)], although hindlimb polydactyly, a landmark for *Bmp7* null embryos (Dudley et al., 1995), was observed in all homozygous embryos. Photo-converted DiI tracing revealed that in less penetrant phenotypes, many defasciculated cortical fibers reached the midline, whereas the remaining axons formed bundles in the ipsilateral side of the cortex [Fig. 3(D)].

Callosal axon guidance defects of *Bmp7* null embryos could result from abnormal pyramidal neuron specification or alterations in layer formation. To explore these possibilities, we analyzed the organization of the cerebral cortex using specific markers. Immnohistochemical localization of Sox5 and Ctip2, two transcription factors expressed by subcortical projection neurons of layers V and VI, and Beta 3, a marker for cortical plate and layer V neurons, showed no significant differences between wt and $Bmp7^{-/-}$ E18.5 cerebral cortex [Fig. 4(A–D,H–K)]. Similarly, the mRNA of *Clim1*, a marker of layer V callosal neurons, was distributed with a similar pattern in both

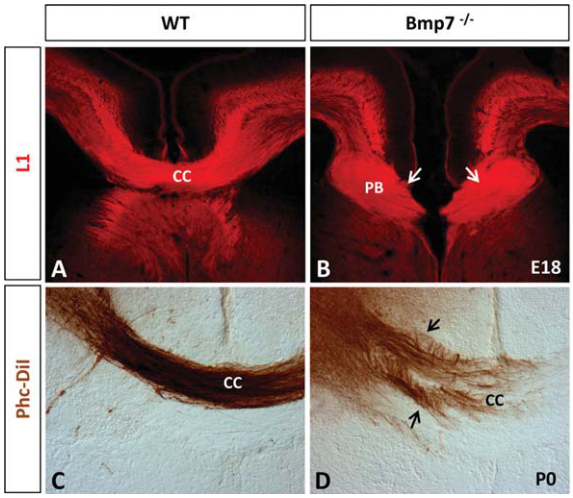

Figure 3 The CC does not form properly in *Bmp7* null embryos. A–B) Confocal images of coronal sections from E18.5 wild-type (A) and $Bmp7^{-/-}$ (B) brains immunostained for L1, a marker for the CC. C–D) Photo-converted DiI tracing of cortical axons from P0 wt (C) and $Bmp7^{-/-}$ brains. In wt many callosal axons have crossed the midline, entering the contralateral hemisphere between E18 and P0 (A, C). In severely affected $Bmp7^{-/-}$ embryos, callosal fibers do not cross the midline and stall at the cortical midline, forming Probst-like bundles (PB, arrows in B). In less penetrant phenotypes, a proportion of cortical fibers reaches the midline but in a defasciculated manner, whereas the remaining fibers form bundles in the ipsilateral side of the cortex (arrows in D).

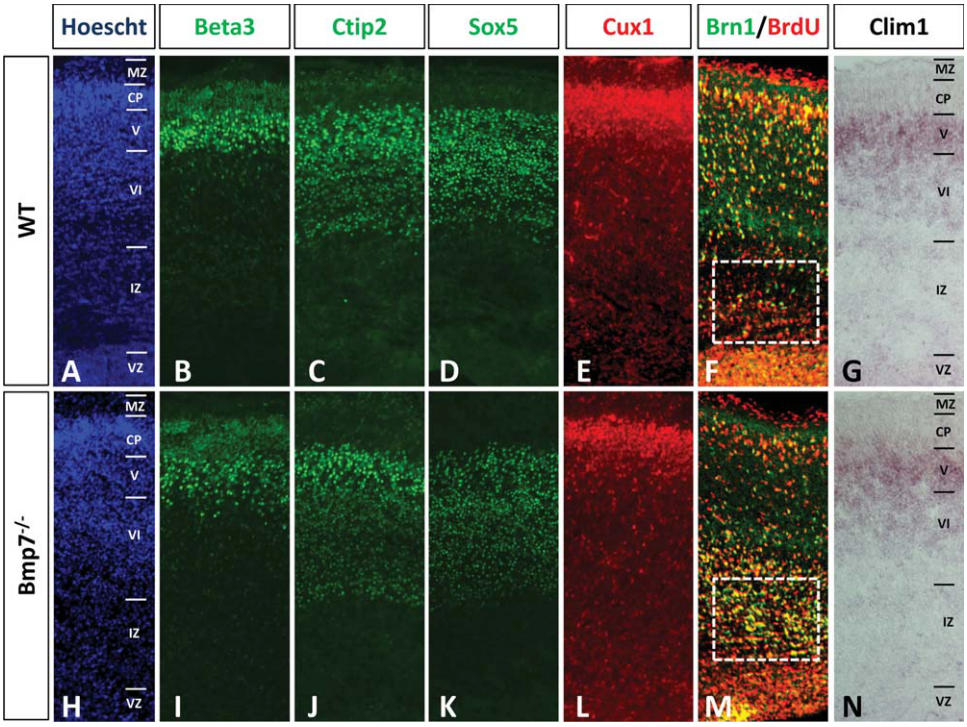

Figure 4 Cortical neurons are normally specified but upper layer neuron migration is impaired in *Bmp7*$^{-/-}$ embryos. Confocal images of coronal sections from E18.5 wt and *Bmp7* null embryos stained with Hoescht (A, H), immunostained with antibodies against Beta 3 (B, I), Ctip2 (C, J), Sox5 (D, K), Cux1 (E, L), Brn1 and BrdU (F, M) or hybridized with a probe for *Clim1* (G, N). Note that upper layer neurons are normally specified but accumulates (dotted square in M) in the IZ in the cortex of *Bmp7* null embryos. Abbreviations: CP, cortical plate; IZ, intermediate zone; MZ, marginal zone; VZ, ventricular zone, V, layer V; VI, layer VI.

wt and *Bmp7* null embryos [Fig. 4(G,N)]. In contrast, the distribution of Brn1, a transcription factor expressed by glutamatergic neurons of layers II-V (McEvilly et al., 2002; Sugitani et al., 2002), and Cux1, a marker for upper layer cortical neurons (Cubelos et al., 2008), revealed a decrease in the density of the upper layers and an increase in Cux1 and Brn1 positive cells in the lower layers of the *Bmp7* null cortex as compared with wt [Fig. 4(E,F,L,M)]. This decrease was further confirmed by examining the generation of upper layer neurons in embryos injected with BrdU at E14.5. When examined at E18.5 acallosal *Bmp7* null embryos, many BrdU and Brn1 double labeled neurons accumulated in the IZ below the cortical plate, suggesting that a proportion of late generated neurons, although normally specified, fail to migrate to their proper layers [Fig. 4(F,M)].

Together these data indicate that, despite some layering defects, cortical projection neurons are normally specified in absence of *Bmp7*, which, instead, might be directly or indirectly required for callosal axon pathfinding across the cortical midline.

BMP7 Does Not Act as a Guidance Cue for Callosal Axons

BMP-mediated signaling controls axons' movements in different contexts (Bovolenta, 2005; Sanchez-Camacho and Bovolenta, 2009) and regulates cortical dendrite-genesis (Lee-Hoeflich et al., 2004). Therefore, we tested the possibility that BMP7, likely released by the GW, IG, and SCS could act as an axon guidance cue for callosal axons as they cross the midline. To address this hypothesis, E16.5-E18.5 cortical explants were grown in collagen gel for 48h in the presence or absence of BMP7 (100 ng/μL) provided either directly in the culture medium or in soaked beads as a focal source. Cortical explants extended numerous radially oriented neurites and this pattern of outgrowth was not modified by the presence of beads soaked in PBS [Fig. 5(A,B)]. In none of the two experimental paradigms the addition of BMP7 had any apparent effect [Fig. 5(C,D)]. There was no significant difference between control and BMP7-treated explants when the total area of outgrowth (26.4 $\pm$ 0.95 vs. 30.41 $\pm$ 2.98, respec-

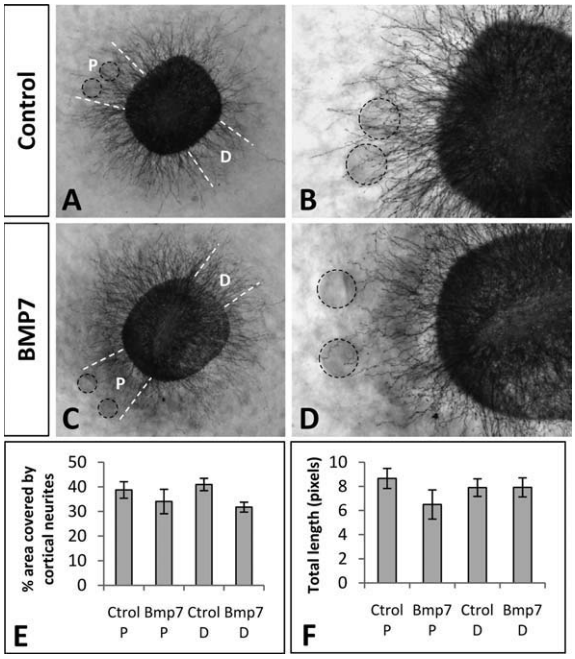

Figure 5 *Bmp7* does not modify the outgrowth of cortical axons. A–D) E17.5 cortical explants from wt cerebral cortices were cultured in collagen gel in the presence of beads soaked with PBS or BMP7 (100 ng/μL) and immunostained with antitubulin-βIII antibody to visualise the extent of neurite outgrowth. E–F) Quantification of the area covered by cortical neurites (E) and the axonal length (F, in pixels) in the proximal (P) or distal (D) quadrants of cortical explants grown in the presence ($n = 26$) or absence ($n = 30$) of BMP7-impregnated beads. No significant differences were observed in the presence of BMP7 compared with the controls.

tively) and neurite length (8.01 $\pm$ 0.71 vs. 8.82 $\pm$ 0.42, respectively) was quantified. Similar neurite density and length was also observed in the distal and proximal quadrants when explants were challenged with PBS or BMP7-soaked beads [Fig. 5(E,F)].

The Development of the GW, IG, and SCS is Impaired in *Bmp7* Null Embryos

Because BMP7 did not appear to directly control the outgrowth and directionality of callosal axons, we finally tested whether *Bmp7* expression in the surroundings of the GW, IG, and SCS was actually needed for their specification and/or differentiation.

In wt embryos, the first radially oriented glial cells of the GW begin to differentiate at E16.5 and express the astrocytic marker GFAP [Fig. 2(A,B)]. These populations gradually increases at E18.5 when GFAP-positive cells are observed in the GW as well

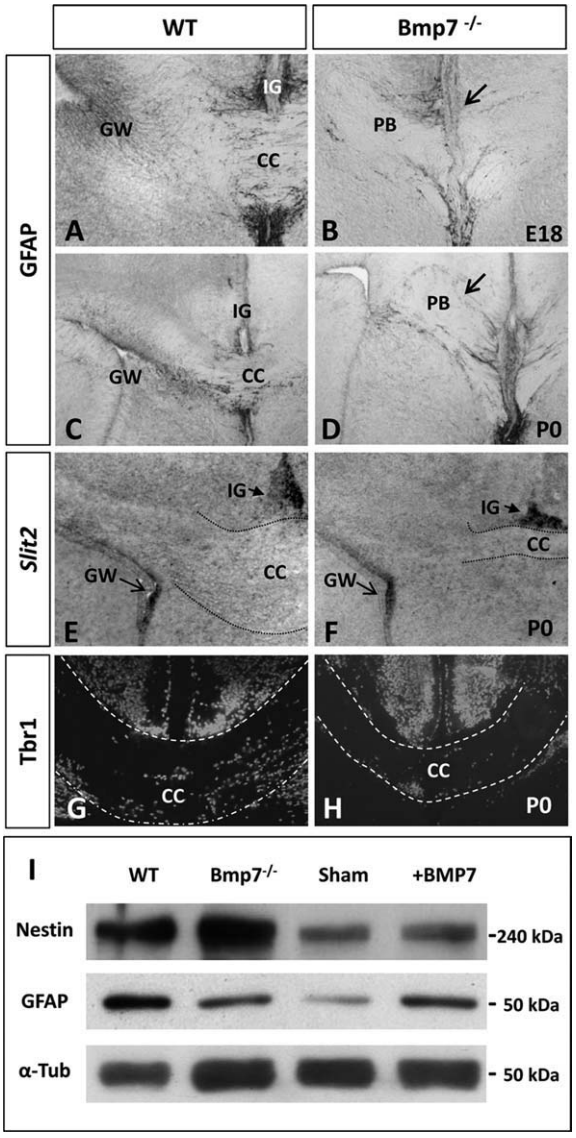

Figure 6 Cortical midline glia is altered in *Bmp7*$^{-/-}$ embryos but the levels of Slit2 expression are normal. A–D) Coronal sections from E18.5 (A, B) and P0 (C, D) wt and *Bmp7*$^{-/-}$ brains were immunolabeled with antibodies against GFAP (A–D) or Tbr1 (G, H). GFAP-positive cells in the GW and IG are strongly reduced while those intermingled with the callosal axons in the Probst bundles (PB) are misoriented in *Bmp7* null animals when compared with wt brains. Less Tbr1-positive cells are observed within the CC of *Bmp7*$^{-/-}$ newborns (H). E, F) Coronal sections from P0 wt and *Bmp7*$^{-/-}$ brains were hybridised with a *Slit2* probe. The levels of *Slit2* expression in the GW and IG regions are roughly similar in *Bmp7*$^{-/-}$ and wt brains. Note however the strong reduction of the CC and IG size in the mutants. I) Western blot analysis of GFAP and Nestin levels in the CC region in wt, *Bmp7* null newborns and Sham or BMP7 injected E18.5 embryos. αTubulin was used as load control. Note that GFAP levels are decreased in the *Bmp7* mutant while increased after BMP7 addition as compared with their respective controls.

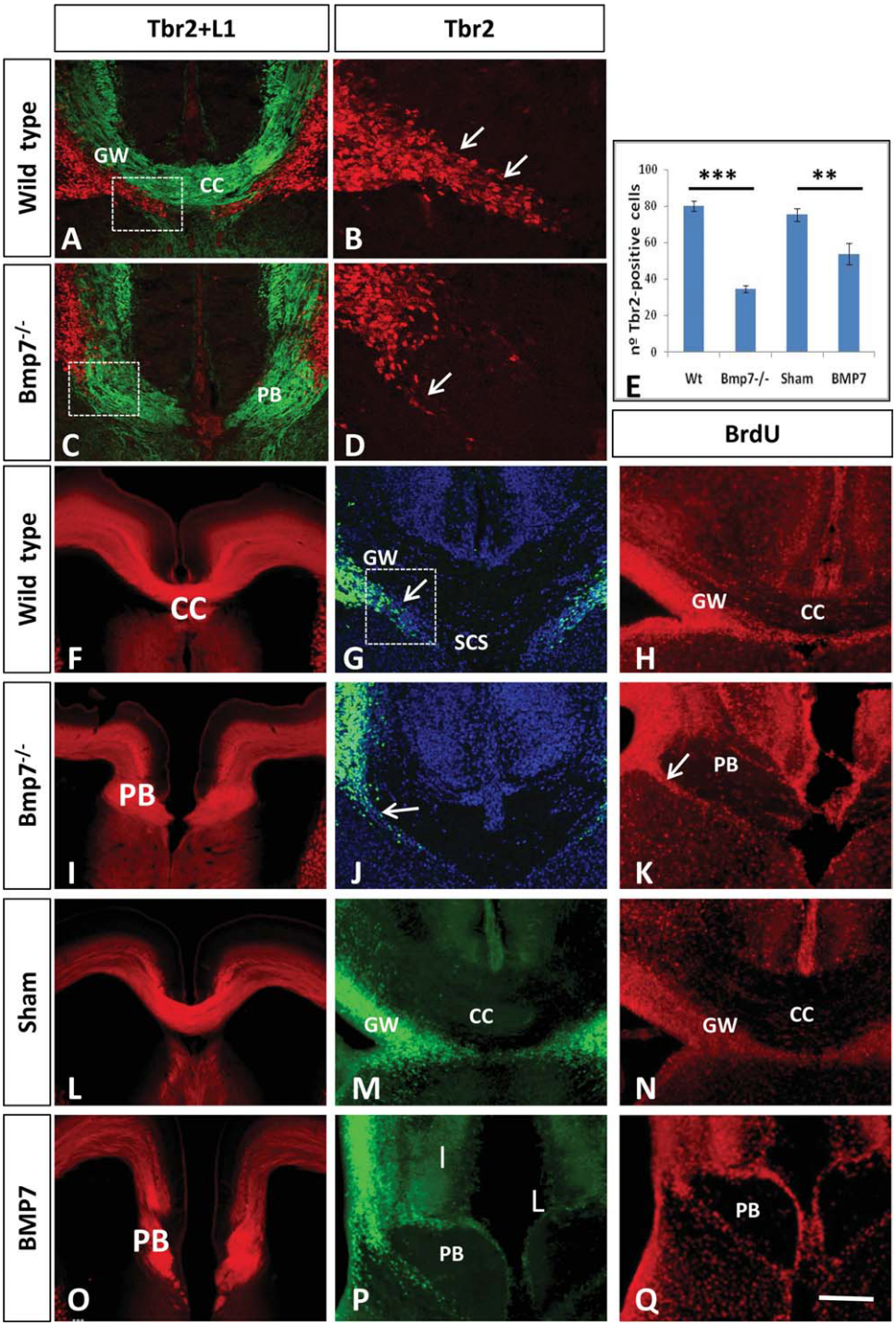

Figure 7 Overexpression and loss of *Bmp7* function disrupts the formation of the subcallosal sling and correlates with abnormal callosal axon growth. A–L) Confocal images of coronal sections from E17.5 (A–D) and E18.5 (F–Q) BrdU-treated wt, $Bmp7^{-/-}$, sham or BMP7 (1μg) injected brains immunostained with antibodies against BrdU, Tbr2 and L1. BrdU was injected at E15.5. Tbr2- (A, B) and Tbr2 and BrdU-positive immature neurons accumulate in the subcallosal sling of wt (G, H) and sham injected embryos (M, N). In *Bmp7* null and in BMP7-injected embryos, Tbr2- (C, D) and Tbr2 and BrdU-positive cells are reduced in the subcallosal sling (SCS) but seem to accumulate (arrows in J, K) at the lateral ventricle in correspondence of the GW. This decrease correlates with the formation of Probst-like bundles (PB in I, K, Q). E) Quantification of the number of Tbr2-positive cells within the subcallosal sling. Both gain- and loss-of *Bmp7* function causes a statistically significant reduction in the number of Tbr2-positive neurons. (**$p < 0.01$, ***$p < 0.001$; Student's unpaired *t*-test). Scale bar, 200 μm.

as in the IG and MZG [Fig. 6(A); (Shu et al., 2003)]. In contrast, the number of GFAP-positive cells in the GW *Bmp7* null embryos was strongly reduced from E16.5 onward [Figs. 2(C,D) and 6(B)]. At E18.5, some GFAP-positive staining was detected at the level of the IG and MZG but cells were disorganized and abnormally positioned across the midline [Fig. 6(B)]. These defects did not reflect a simple developmental delay because in newborn animals the number of GFAP-positive cells at the GW was still reduced and staining at the midline was abnormal [Fig. 6(C,D)]. Western blots analysis of the GFAP and Nestin levels present in the septo-callosal region of wt and $Bmp7^{-/-}$ newborn pups confirmed these results [Fig. 6(I)]. Densitometric quantification revealed a roughly fivefold decrease of GFP protein levels in *Bmp7* null tissue (wt; 1.1 arbitrary units, a.u.; $Bmp7^{-/-}$, 5.7 a.u normalized to α-tubulin) with no significant variations of Nestin levels (wt; 1.0 a.u.; $Bmp7^{-/-}$, 1.3 a.u). Despite this difference, the mRNA of *Slit2*, one of the guidance cues required for callosal axon extension at the midline (Bagri et al., 2002; Shu et al., 2003) was expressed in the lateral ventricle and in the reduced IG of $Bmp7^{-/-}$ newborn brains at levels similar to those observed in wt [Fig. 6(E,F)], suggesting that other factors may explain the failure of axon growth across the midline.

Integrity of the SCS is among the factors required for successful callosal axon growth through the midline (Silver et al., 1982; Shu et al., 2003; Niquille et al., 2009). In addition to glial cells, this structure contains dividing immature neuronal cells (Shu et al., 2003). Immunohistochemical analysis revealed that fewer Tbr2-positive immature neurons were present in the lateral ventricle at the septo-cortical boundary of *Bmp7* null embryos at E16.5 as compared with wt littermates [Fig. 2(E–H)]. Similarly, fewer Tbr2-positive cells than those observed in wt were present in the developing SCS of $Bmp7^{-/-}$ embryos [Fig. 7(A–E,G,J)] and, at E18.5, positive cells appeared to accumulate at the ventricle edges from where they migrate [Fig. 7(G,J)]. This decrease was always associated with evident CC defects [Fig. 7(C,I)]. Injections of BrdU into pregnant dams at E15.5, when the SCS begins to form (Shu et al., 2003), confirmed a decrease of the migrating BrdU-positive SCS neuronal cells in *Bmp7* null embryos whereas many BrdU-positive cells seemed to accumulate at the edges of the lateral ventricles [Fig. 7(H,K)], suggesting that *Bmp7* might be required for the proper migration of Tbr2-positive cells. Notably, the number of transient Tbr1-positive neurons intermingled with nascent callosal axons (Niquille et al., 2009) was also diminished in $Bmp7^{-/-}$ newborns as compared with controls, even in cases of mild callosal reduction [Fig. 6(G,H)], although these cells did not seem to express *Bmp7*.

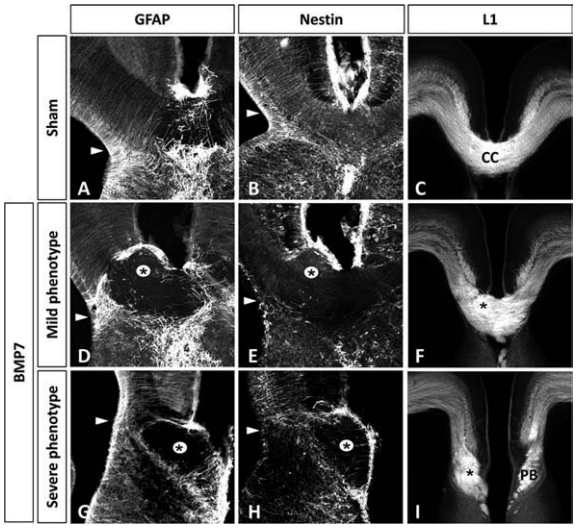

Figure 8 Cortical midline glia is altered in BMP7 injected animals. A–D) GFAP, Nestin and L1 immunostaining in coronal sections from E18.5 embryos injected in the lateral ventricle at E14.5 with vehicle (A–C) or 1μg of BMP7 (D–I). BMP7-treated embryos show an increase in GFAP immunostaining and a mild decrease in that of Nestin. These defects were associated to mild callosal axon bundling (asterisk in F) or to a complete acallosal phenotype with formation of Probst-like bundles (PB, asterisk in I).

Together these results support the idea that *Bmp7* is needed for the differentiation of the glial cells that compose the GW, IG, and SCS as well as for the positioning of sufficient numbers of neurons that contribute to SCS formation.

BMP7 Injections Accelerates Guidepost Cell Development and Cause an Acallosal Phenotype

Intraventricular injections of BMP7 induce a precocious transformation of cerebral cortex radial glial cells into astrocyte (Ortega and Alcantara, 2010). To confirm that *Bmp7* is involved in the differentiation and migration of CC glial cells, we hypothesized that addition of BMP7 could have a similar effect on the GW and SCS. Indeed, intraventricular injection of recombinant *Bmp7* in embryos at E14.5, when the GW and SCS are forming, resulted in a strong increase in GFAP staining of these structures at E18.5 as compared with sham injected littermates [Fig. 8(A,D,G)]. This increase was paralleled by an apparent down-regulation of the radial progenitors' marker Nestin in the GW, IG, and MZ [Fig. 8(B,E,H)]. Western blots

of the GFAP and Nestin levels [Fig. 6(I)] from the septo-callosal region of E18.5 sham-operated and BMP7-injected embryos confirmed a fourfold increase of GFAP protein levels following *Bmp7* addition (sham; 0.45 arbitrary units, a.u.; +*Bmp7*, 1.9 a.u., normalized to α-tubulin) but no significant variations in Nestin levels could be appreciated (sham; 1.0 a.u.; +*Bmp7*, 0.7 a.u). These results support that BMP7 controls the differentiation of glial cells in the surrounding of the CC. In notable contrast, BMP7 injections strongly reduced the number of proliferating Tbr2-positive neuronal cells in the SCS [Fig. 7(E,M,P)], which did not populate the midline [Fig. 7(P)].

Notably, these changes in the callosal guidepost cells were associated with variable defects in callosal axons, which formed small tangles at the ipsilateral side of the brain [Fig. 7(F)] or developed an acallosal phenotype in 75% of the cases [$n = 9$; Figs. 7(O) and 8(I)] as compared with sham-operated littermates [$n = 10$; Figs. 7(L) and 8(C)].

Together these data suggest that appropriate BMP7 levels are required for CC development.

DISCUSSION

BMPs signal through serine-threonine kinase receptors composed of Type I and Type II receptor subunits. Compound inactivation of the two BMP type I receptor genes, *Bmpr1a* and *Bmpr1b,* impairs astroglial differentiation although cells are normally generated (See et al., 2007). Conversely transgenic overexpression of *Bmp4* enhances the generation of astrocytes and accelerates their differentiation from radial glial cells (Gross et al., 1996; Kan et al., 2004), as also observed in the cortex upon intraventricular injections of BMP7 into the lateral ventricles (Ortega and Alcantara, 2010). In line with these findings, we have demonstrated that physiological levels of BMP7 are required for the timely differentiation of the GW, IG, and SCS that support CC formation. These structures are mostly composed of glial cells although the SCS contains also immature neurons. Notably, appropriate levels of BMP7 are also required for the migration of these neurons, indicating that *Bmp7* has a dual role in the development of the guidepost cells that support CC formation.

Alterations in CC formation were frequently but not always observed upon BMP7 injections and characterized approximately half of the *Bmp7*$^{-/-}$ embryos, whereas the remaining showed milder defects. The reasons for the incomplete penetrance in *Bmp7* null embryos are unclear but they might be simply linked to the genetic background of the mouse line we used. Indeed, this strain has an incidence of milder (microphthalmia) versus extreme (anophthalmia) eye defects (Godin et al., 1998; Morcillo et al., 2006), which are higher than those reported in other genetic backgrounds (Dudley et al., 1995; Wawersik et al., 1999). Alternately, there might be functional redundancy of *Bmp7* with other BMP family members, such as, for example, *Bmp4*, which is expressed in the subependymal zone (Peretto et al., 2004). Mild or no defects have also been observed in tissues other than the brain where *Bmp7* colocalizes with *Bmp2*, *Bmp4*, and *Bmp5* during early embryonic development (Dudley and Robertson, 1997; Solloway and Robertson, 1999).

In the affected embryos, alteration in BMP7 levels caused an axon guidance phenotype characterized by midline defasciculation of callosal fibers and failure of callosal axons to cross the midline with the formation of typical Probst bundles. Although we cannot exclude that cell autonomous defects in cortical neurons may contribute to this phenotype, we believe that noncell autonomous causes might better explain these defects. Indeed, the laminar organization of deep projection neurons appeared largely preserved in *Bmp7* null embryos and genetic defects causing migration-related cortical laminar disorganization, as observed in the upper cortical layers of acallosal *Bmp7* null mice have been only rarely associated to CC dysgenesis in mammals (Gressens, 2006; Kerjan and Gleeson, 2007; Paul et al., 2007; Donahoo and Richards, 2009). Furthermore, the dispersion of Cux1- and Brn1-positive neurons was also observed in *Bmp7* null embryos with mild or no callosal defects (not shown), suggesting no clear correlation between neuronal migration defects and the acallosal phenotype. Moreover, our studies did not favor a direct effect of *Bmp7* on callosal axon outgrowth, although Bmp signaling has been shown to act as an axon guidance cue in different context (Sanchez-Camacho and Bovolenta, 2009). We did not find significant differences in the pattern of neurite outgrowth from cortical explants grown in the presence or absence of BMP7. In our assays, we could not specifically distinguish the behavior of callosal axons from that of other cortical neurites. Therefore, we cannot totally exclude that BMP7 might have subtle effects on callosal axons masked by the presence of other nonresponding fibers or that concentrations different from those we used might accentuate the slight tendency of BMP7 to repress cortical outgrowth in the proximity of a focalized BMP7 source [Fig. 5(E)]. Nonetheless, the marked defect in midline glial cell organization and the reduced number of migrating Tbr2 positive neurons found in the

SCS of *Bmp7* null embryos makes us favor the hypothesis of the secondary nature of the callosal axon defects.

Indeed, several studies have demonstrated the importance of midline telencephalic glial cell integrity for CC formation, in particular of the IG, GW, and SCS (Richards et al., 2004), which are altered in $Bmp7^{-/-}$ brains. More precisely, we show that timely controlled BMP7 levels are necessary for the proper generation and differentiation of telencephalic GFAP-positive midline cells. When *Bmp7* was absent, midline cortical cells of the IG, GW, and SCS were reduced in number with an aberrant morphology and organization, whereas increased BMP7 levels induced a premature astroglial differentiation. This effect is also observed after BDNF-induced *Bmp7* overexpression in the cortex, where a premature presence of differentiated astrocytes is associated with impaired neuronal migration and cortical lamination (Ortega and Alcantara, 2010). The simplest explanation for these observations is that normally BMP7 contributes to both the generation and differentiation of midline telencephalic astroglia, in line with the abundant *Bmp7* expression in the IG, GW, SCS and MZG observed from embryonic to postnatal stages. In *Bmp7* null embryos, Bmp signaling activation is reduced, although probably not absent owing to the presence of other BMP ligands (Peretto et al., 2004), and less glial cells are generated at the midline. Because glial cells of the IG, GW, and SCS act as guidepost cells, the molecular cues normally expressed by these cells should also be diminished impairing callosal axon outgrowth. On the contrary, increased levels of BMP7 accelerated glial differentiation, similarly interfering with the timely expression of guidance cues. Notably, the expression of *Slit2*, a main callosal axon repellent (Shu and Richards, 2001; Bagri et al., 2002; Shu et al., 2003), appeared normal in $Bmp7^{-/-}$ embryos. This result was somewhat surprising because *Slit2* is secreted by the GW and IG (Shu and Richards, 2001; Shu et al., 2003), making it the most appropriate candidate to explain our overall observations. Although not tested, the decreased expression of other factors (Paul et al., 2007) might therefore explain the *Bmp7* callosal phenotype. Among these, Netrin1 Wnt5a or Draxin might be particularly relevant. In fact, *Netrin1* and *Draxin* mutants are characterized by the formation of Probst bundles (Ren et al., 2007; Islam et al., 2009). Callosal axons of mice lacking Ryk, a receptor that mediates the axon guidance activity of many Wnt ligands (Bovolenta et al., 2006), cannot respond to Wnt5a expressed in the surrounding of the CC (Keeble et al., 2006). As a result, cortical axons grow through the CC in a defasciculated manner and stall at the controlateral side without reaching their targets (Keeble et al., 2006), resembling some of our observations [Fig. 3(D)].

We have shown that immature neurons in the SCS express Tbr2 and BMP7. In addition to impaired glial cell differentiation, both loss and gain of *Bmp7* function affected the migration of Tbr2-positive neurons, which, together with glial cells, contributed to the formation of the SCS (Shu et al., 2003; Ren et al., 2006). Surgical removal of the SCS strongly interfere with callosal axon midline crossing (Silver et al., 1982) and Tbr2 silencing in humans leads to CC agenesis (Baala et al., 2007). A reduced number of these migrating cells might thus be an additional cause of the acallosal phenotype observed upon alterations of *Bmp7* expression levels. Indeed, there is increasing evidence that discrete populations of migrating neurons have a fundamental role in axon guidance in both vertebrates and invertebrates (Chotard and Salecker, 2004; Lopez-Bendito et al., 2006; Learte and Hidalgo, 2007), including a very recent study which has identified two transient Tbr1-positive neuronal populations fundamental for CC formation (Niquille et al., 2009). These are GABAergic and calretinin-positive glutamatergic neurons, which form a complex meshwork that attract callosal axons, at least in part by secreting *Sema3C* (Niquille et al., 2009). Notably, this meshwork of Tbr1-positive cells appeared reduced in number in *Bmp7* null animals. However, it is unclear whether this is a direct consequence of *Bmp7* inactivation since β-Gal signal was almost undetectable within the CC. It is equally unclear whether Tbr1 and Tbr2 recognize partially overlapping neuronal populations. For example, the population of Tbr2-positive neurons impaired by altered *Bmp7* levels may correspond to the ventralmost calretinin-positive neurons described by Niquille and coworkers (2009). Because *Bmp7* is expressed in the lateral ventricle from where these neurons originates, it is tempting to speculate that BMP7 might normally act as a repellent cue, which forces Tbr2-neuron migration toward the midline. This hypothesis would explain why these cells appeared to accumulate in the lateral ventricle of *Bmp7* null mice and why increasing levels of *Bmp7* in the ventricle prevent Tbr2-positive neurons to reach the midline. To our knowledge, a role of *Bmp7* in the regulation of neuronal migration has not been reported before. However, BMP7-mediated chemotactic and inhibitory functions in cell migration have been reported in other systems, including melanomas, osteoblasts and monocytic cell lines (Lee et al., 2006; Na et al., 2009; Perron and Dodd, 2009).

Early in development, the specification of the telencephalic dorsal midline is regulated by two signalling centres, the anterior neural ridge rostrally and the cortical hem caudally. These two structures express FGF and BMP ligands, respectively. BMPs and FGFs regulate each others' expression and an altered BMP/FGF equilibrium in the rostro-caudal midline affects the specification of the commissural plate (Shimogori et al., 2004; Donahoo and Richards, 2009). Our data, together with the observation that inactivation of FGF signaling prevents the traslocation of the radial glia cells into mature astrocytes of the IG (Smith et al., 2006; Tole et al., 2006), suggest that Fgf and Bmp signaling might also cooperate, albeit with independent mechanisms, to generate CC guidepost cells at the appropriate time. Furthermore, the role of *Bmp7* signaling in CC guidepost development establishes an interesting parallel with the reported function of *Bmp7* in the formation of the optic disc. Similarly to the GW, IG, and SCS, the optic disc is composed of a specialized glial population indispensable for the growth of retinal ganglion cell axons out of the eye. In the absence of *Bmp7*, the optic disc does not form causing the abnormal accumulation of retinal ganglion cell axon in the subretinal space with a consequent optic nerve aplasia (Morcillo et al., 2006). Thus, together these studies suggest that *Bmp7* signaling might be a key factor in the formation of different CNS guidepost cells.

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3. OXYGEN TENSION MODULATES GLIAL LINEAGE COMMITMENT THROUGH MODIFICATIONS ON BMP7 EXPRESSION

Perinatal hypoxia is a major cause of chronic disabilities such as cerebral palsy, mental retardation or epilepsy in infants and children, being white matter and cortical damage two of the most prominent morphological features. Bone Morphogenetic Protein 7 (BMP7) treatment improves functional recovery after stroke in adult rodents and its expression is altered after hypoxic-ischemic (H-I) injury in brain. We used both *in vitro* and *in vivo* hypoxic models in order to analyze possible changes on BMP7 expression induced by hypoxia, and whether these changes are associated with modifications in cortical progenitors. BMPs are important regulation factors to maintain the equilibrium between astrocyte and oligodendrocyte populations. During perinatal development, BMPs promote astroglial differentiation in detrimental to oligodendroglial differentiation from cortical progenitors. Cortical cultures undergone to hypoxia showed and initial decrease of BMP7 expression followed by an increase in the number of NG2-positive (NG2+) oligodendroglial progenitors. Similarly than the results obtained *in vitro*, E16 animals subjected to hypobaric hypoxia showed an immediate decrease of BMP7 levels after the hypoxic treatment, followed by increased immunoreactivity for the oligodendroglial markers NG2 and Olig2. To corroborate the results obtained under hypoxic conditions we used gain and lose of function models, through treatments with BMP7 and its inhibitor follistatin (FST), respectively. As expected, FST treatments mimic the effects observed in hypoxic conditions, which show reduced BMP7 levels. FST treatments increase the immunoreactivity for oligodendroglial markers both *in vivo* and *in vitro*. On the contrary BMP7 treatments generate dramatic opposite changes in the correct development of glial populations, increasing the expression of the astroglial marker GFAP, and concomitantly decreasing the levels of the oligodendrocyte markers NG2 and Olig2.

Thus, BMP7 levels require a close regulation in order to properly control the differentiation of cortical progenitors during perinatal development. An imbalance between different cortical populations in the cerebral cortex caused by aberrant BMP7

expression might lead to brain circuitry dysfunction, underlying behavioural and cognitive disorders. A better understanding of BMP7 effects on the immature cerebral cortex might help to identify potential treatments to prevent neurological impairments produced by hypoxia during perinatal development.

OXYGEN TENSION MODULATES GLIAL LINEAGE COMMITMENT THROUGH MODIFICATIONS ON BMP7 EXPRESSION

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INTRODUCTION

Oxygen is a vital energy source for cell metabolism that is tightly regulated in the central nervous system (CNS) (Bruick 2003; Panchision 2009). PO_2 values in the CNS are ranged from 0.5 % (4.1 mmHg) in the midbrain to 8.0% (60mmHg) in the pia, while in the cortex the PO_2 values are between 2.53%-5.33% (19-40 mmHg) (Erecinska and Silver 2001; Ivanovic 2009). During embryonic development, fetus totally depends on the oxygen and nutrients supplied by the placenta. Perinatal hypoxia in term and preterm infants is a major cause of chronic neuropsychological handicaps, such as cerebral palsy and behavioural disabilities with or without associated learning disability or epilepsy (Vannucci and Vannucci 2005; Rees, Harding et al. 2011). A failure in oxygen delivery to the developing brain can be produced by numerous causes such as

umbilical cord occlusion, impaired placental function and in the case of preterm infants also by immature lung development. In rodents, acute periods of fetal hypoxia can significantly affect neural populations of cerebellum, hippocampus and cortex (Rees, Harding et al. 2011). Indeed, oxygen is integrated into the normal regulatory pathways of neurogenesis and angiogenesis in the neurovascular niches of the developing CNS. Hence, deviations from normal oxygen levels produced in many brain pathologies lead to severe modifications of endothelial cell and neural stem cell (NSC) survival, proliferation and differentiation (Madri 2009).

Oligodendrocyte progenitor cells (OPCs) are particularly vulnerable to hypoxic-ischemic (H-I) injury and they are the main cell type responsible for the white matter damage observed in preterm infants (Volpe 2001; Back 2006). This assertion is mainly based on results showing an increased vulnerability of oligodendrocyte progenitors to glutamate, free radicals as well as inflammatory products produced in H-I injury (Back, Gan et al. 1998; Back, Han et al. 2002). OPCs are characterized in part by the expression of particular markers such as the NG2 glycoprotein, a type I membrane proteoglycan expressed in the developing and adult CNS. Demyelinating lesions after H-I damage leads to increased NG2+ proliferative activity (Watanabe, Toyama et al. 2002; Sizonenko, Camm et al. 2008). The physiological relevance of the rapid response of the NG2+ population to brain damage still remains unclear. It has been widely speculated that NG2 negatively regulates axonal growth during regeneration processes (Dou and Levine 1994; Fawcett and Asher 1999; Levine, Reynolds et al. 2001). Otherwise recent studies contradict this theory, and postulate that NG2 does not repel but promote axonal growth regardless of the level of NG2 expression (Jones, Sajed et al. 2003; de Castro, Tajrishi et al. 2005; Yang, Suzuki et al. 2006). In addition NG2+ cells are well-known to react in front of injury generating new myelinating oligodendrocytes (Watanabe, Toyama et al. 2002; Sizonenko, Camm et al. 2008), that would moderate the effects of H-I in the vulnerable white matter. Recently, it has been also reported that NG2+ cells have the ability of differentiate into neurons (Dayer, Cleaver et al. 2005; Rivers, Young et al. 2008) showing the potentiality of these cells in regenerating processes.

Bone Morphogenetic Proteins (BMPs) are considered as therapeutic factors in front of oxygen restriction associated injuries. After stroke, BMP7 administration protects against various neurodegenerative processes and has neuroregenerative properties, improving motor function by genesis of new neural cells in different areas of the ischemic brain (Chang, Lin et al. 2003; Chou, Harvey et al. 2006). During the early development of the CNS, BMP activity covers enormous variety of functions including the initial differentiation of neural plate cells from epidermal ectoderm (Liem, Tremml et al. 1995), and the regulation of dorsoventral patterning (Mehler, Mabie et al. 1997) (Liem, Jessell et al. 2000; Liu and Niswander 2005). In the forebrain, BMPs are also implicated in the formation of choroid plexus epithelium, cortical hem, and dorsal cortex (Hebert, Mishina et al. 2002; Cheng, Hsu et al. 2006; Chen and Panchision 2007). In addition, BMPs are well-characterized to regulate NSC, inducing mitotic arrest and cell survival as well as their differentiation into astroglial fate in detrimental to oligodendroglial fate (Mehler, Mabie et al. 1997; Mabie, Mehler et al. 1999; Panchision, Pickel et al. 2001). Contrarily, the endogenously secreted BMP antagonist Noggin limits astroglial differentiation promoting the maintenance of NSC self-renewing and redirecting these progenitors to oligodendroglial or even neuronal lineage commitment (Santilli, Lamorte et al. 2010; Mabie, Mehler et al. 1999; Alvarez-Buylla and Lim 2004; Chen and Panchision 2007; Colak, Mori et al. 2008). Some recent studies suggest that the combination of oxygen levels and BMPs direct the balance between survival/apoptosis, and self-renewal/differentiation of the neural progenitors. Under hypoxic conditions there is a blockage of the canonical BMP Smad-pathway due to the over activity of hypoxia inducible factor 1α (HIF1α), the best-described mediator of oxygen response. Active HIF1α promotes survival and self-renewal of neural precursors, while under high oxygen levels HIF1α degradation occurs and BMP signalling is released, inducing depletion of precursors and astrocyte differentiation (Pistollato, Chen et al. 2007; Panchision 2009; Pistollato, Chen et al. 2009).

To analyze hypoxia-induced changes in BMP7 expression and if these changes can modify survival, proliferation or differentiation of the distinct neural progenitor populations, we decided to use the model of prenatal hypobaric hypoxia. Previous

experiments carried out in rats showed that hypobaric hypoxia severely affects morphological and functional maturation of the corpus callosum, neocortex and hippocampus of infant rats (Langmeier, Pokorny et al. 1987; Maresova, Valkounova et al. 2001; Langmeier and Maresova 2005). Exposition of pregnant females and they litters to conditions simulating altitude of 5000 m induce lower cortical thickness with higher neuronal density in layer I, II, V and VI in infant rats. Moreover, changes in astrocytes and oligodendrocytes after hypobaric hypoxia have been associated to alterations in animal behaviour and learning (Brichova 1984; Zimmer, Sampaolo et al. 1991). This work aims to describe the effect of a sublethal hypoxic insult on BMP7 expression pattern and assess whether BMP7 deregulation might underlie the changes in cellular survival and differentiation of neural progenitors associated to hypoxic damage.

MATERIALS AND METHODS

Culture conditions

Primary cultures were obtained from E16 mice neocortex. Embryonic cortices were dissected out and dissociated by trypsin-EDTA (Biological Industries, Kibbutz Beit Haemek, Israel) and DNAse I (Sigma-Aldrich, Saint Louis, MO) treatment for 10 minutes, followed by mechanical disruption. To obtain enriched neuronal cultures the dissociate was pre-plated in a 10 cm culture dish for 1 hour at 37ºC in DMEM supplemented with 10% normal horse serum (NHS) (Gibco, Auckland, NZ). Embryonic cortical cells were seeded in serum-free Neurobasal medium (Gibco, Paisley, UK) supplemented with B27 (Gibco, Paisley, UK) on 6- and 24-well plates containing slides coated with poly-D-Lysine (Sigma-Aldrich, Saint Louis, MO). After 24h the medium was changed for a serum-free medium in order to avoid interferences with our treatments. Three to four days after plating, neuronal enriched cultures were 12 hours incubated under hypoxic conditions, 2-3% oxygen, for 12h and subsequently treated under normoxic conditions with 25 ng/ml BMP7 (R&D, Abingdon, UK) or 200 ng/ml Follistatin (R&D, Abingdon, UK) for the indicated time periods (1 hour to 4 days).

In utero injection and postnatal electroporation in mice

Experiments were designed to minimize the number of animals used in the procedure. All animal protocols were approved by the Institutional Animal Care and Use Committee in accordance with Spanish and EU regulations.

For the injection in murine brains *in uterus*, pregnant OF1 females carrying embryonic day 14 (E14) embryos (with E0 being the day the vaginal plug) were anesthetized with Ketamine/Valium (150 µg/g, 5 µg/g, i.p.), and the uterine horns were exposed. Two microliters of recombinant human Follistatin (1 µg, R&D, Abingdon, UK), or vehicle were delivered into the lateral ventricles of the embryos via intrauterine injection. The uterus was returned to the abdominal cavity and the embryos were allowed to develop normally.

Focal overexpression of BMP7 was obtained by expression vector injection and electroporation in P0 mice, adding some modifications to the protocol previously described by Tabata H and K Nakajima, 2001. DNA expression vector pEF1-GFP or a mixture of pEF1-BMP7 and pEF1-GFP vectors at a 4:1 ratio (6-10 µg) were injected in the lateral ventricle of P0 mice which were previously anesthetized on ice. The head of the animal was held by a tweezers-type electrode (CUY650-5, Nepagene, Ichikawa, Japan) and electric pulses (100mV for 50 ms) were discharged five times at 950ms intervals with a CUY21E electroporator (Nepagene, Ichikawa, Japan).

Model of prenatal hypobaric hypoxia

E16 pregnant OF1 females were placed in a hypobaric chamber simulating altitudes of 5000 meters overnight (12 hours), with a pressure of 405 mmHg and 10-11 % oxygen levels. At these altitudes, arterial oxygen pressure in human is reduced from 90 mmHg, in altitudes of 0 m, to 38 mmHg. We directly extracted the embryos once hypoxic treatment is finished or we let the female get birth her litters and just at birth (P0) the

animals were processed for the posterior RT-PCR, Western Blot, and immunohistochemical analysis.

mRNA isolation, cDNA synthesis and real-time PCR (RT-PCR)

Cell cultures or dissected cerebral cortices of mice collected and individually frozen in RNAlater were stored at -80ºC until use. mRNA was purified with the RNeasy Protect Mini Kit (Qiagen, Alameda, CA.) and was treated with DNase I to eliminate genomic DNA traces. The RNA concentration and integrity were analyzed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Synthesis of cDNA was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster city, CA). For real time PCR, TaqMan PCR assays (TaqMan® Gene Expression Assay, Applied Biosystems, Foster city, CA) for mouse BMP7 and GADPH (as the endogenous reference) were performed from the cDNA obtained from 6 ng of RNA, in triplicate, on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster city, CA). Standards were prepared using cDNA from control E18 mouse RNA. Finally, fluorescent signal was captured using the Sequence Detector Software (SDS version 1:9; Applied Biosystems, Foster city, CA)

Immunofluorescence and Western blot analysis

To collect tissue for immunohistochemistry (IHC), embryos were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.3, and their brains were post-fixed for 8-12h, cryoprotected and kept frozen. Coronal sections of 40 µm thickness were collected in a cryoprotective solution, and stored at -30ºC for further use. *In vitro* assays performed in neuron-glial mixed cultures from E16 cultures were fixed also using 4% paraformaldehyde in 0.1 M phosphate buffer for 30 minutes at room temperature.

For immunofluorescence of fixed primary cultures (ICC) or tissue sections, fixed cultures were blocked for 1h or 2h respectively and subsequently incubated with primary antibodies at 4ºC overnight, and next with secondary antibodies conjugated to phluorophores: Alexa488, Alexa555 (1:500, Molecular Probes, Eugene, Oregon). TO-PRO-3 iodide (1:500, Molecular Probes, Eugene, Oregon) and Hoechst pentahydrate bis-benzimide (20µg/ml, Molecular Probes, Eugene, Oregon) were used to stain nuclei. Cells and sections were coversliped with Mowiol (Calbiochem, San Diego).

For Western blot analysis, protein extracts were obtained from primary cultures or from cerebral cortex, and proteins in total extracts were separated by SDS-PAGE and electro-transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were blocked and incubated first with primary antibodies overnight at 4ºC, and then with their corresponding secondary HRP-conjugated antibodies (1:3000, Santa Cruz Biotechnology, San Diego). Protein signal was detected using the ECL chemiluminescent system (Amersham, Buckinghamshire,UK). Densitometric analysis, standardized to actin as a control for protein loading, was performed using ImageJ software (National Institutes of Health, Bethesda, Maryland).

Primary antibodies against the following proteins were used: BMP7 (1:1000, Peprotech, London), Cleaved Caspase-3 (1:100 Cell Signalling, MA), GFAP (1:3000, Dako, Glostrup, Denmark), NG2 Chondroitin Sulfate Proteoglican (1:500, Chemicon, Hampshire, UK), Olig2 (1:500, Santa Cruz Biotechnology, San Diego), PDGFRα (1:200, BioLegend, San Diego), and Phospho-Smad1/5/8 (1:150, Abcam, Cambridge, UK).

Fluorescent and confocal microscopy

Micrographs were captured with a light microscope Nikon Eclipse 800 (Nikon, Tokyo, Japan), or with a Spectral confocal microscope Leica TCS-SL (Leica Microsystems, Mannheim, Germany). Images were assembled in Adobe Photoshop (v. 7.0), with adjustments for contrast, brightness and color balance to obtain optimum visual reproduction of data.

Quantitative and morphologic cell analysis

GFAP and NG2 positive stained cells as well as Hoechst nuclei images captured by fluorescent and confocal microscope were analyzed by Image J software (National Institutes of Health, Bethesda, Maryland). Fisher's least significant difference (LSD) procedure was used to discriminate between the means. At least three samples and nine fields per sample were analyzed per condition.

Quantification of the morphological properties of NG2 positive cells was carried out by the aid of ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2009.). All the pictures were converted to black and white, adjusted for brightness and contrast and converted to binary images. Because of the varying exposition of pictures and in order to ensure proper calculation of binary images, these steps were done manually. Each cell was then processed in order to derive a broad number of morphological features, including: area, Feret's diameter, overall complexity, number of extremities and main branches. Area and Feret's diameter were measured on binary images of cells. Feret's diameter is defined as the longest distance between any two points along the selection boundary. Cell complexity was defined by the number of pixels in the cell skeleton divided by the diameter of the cell (Pouria Moshyedi et al., 2010). A simple bipolar cell will have a complexity index close to 1, being its skeleton similar to its diameter, while highly ramified irregular cells will have higher values. Cell skeleton was obtained using the process "skeletonize", while cell diameter is the major axis of the best fitting ellipse. The number of extremities and main branches (Fig.3), a meaningful way to express cell maturation, was obtained counting manually. For the analysis, three pictures from three different samples were considered and well number of defined cells was selected.

RESULTS

Mild hypoxic exposure induces changes in BMP7 expression *in vitro*

To determine whether BMP7 levels might underlay some of the alterations induced by perinatal hypoxia we used an *in vitro* model of sublethal hypoxia. Neuron-enriched cortical cultures from E16 mouse embryos were exposed to 2-3% oxygen concentration during 12 hours and then returned to normoxia (20% oxygen). E16 cortical cultures were mainly composed of neurons, neural progenitors and a few mature glial cells. Our hypoxic conditions do not induce cell death and maintain unaltered the number of Ki67 positive cycling progenitors, the total number of cells and the number of neurons (NeuN stained) when analyzed at 48 h or 4 days after the insult (Fig. 1A, B).

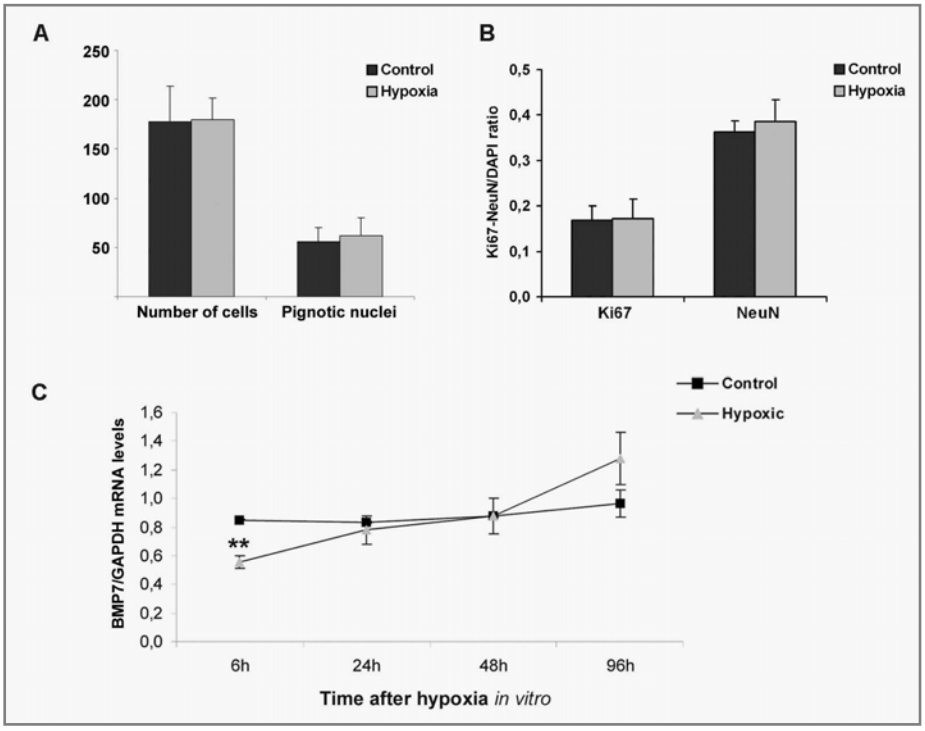


Figure 1. BMP7 expression in response to hypoxia *in vitro.*
A, Graph chart showing no differences in cell proliferation or apoptosis 48 h after hypoxic treatment, according to the similar number of cells and pignotic nuclei in normoxic and hypoxic cultures stained with Hoechst. **B,** Graphic chart showing similar number of neurons (NeuN+ cells) and proliferating cells (Ki67+ cells) in hypoxic cultures respect to control 4 days after hypoxic treatment. **C**, Quantification of BMP7 mRNA levels by RT-PCR in hypoxic cortical cultures 6, 24, 48 and 96 hours after returning to normoxic conditions. BMP7 mRNA levels decreased 6 h after hypoxic treatment and then increase to control levels or even higher levels onwards. Significant difference (** $p<0.01$, LSD test). Error bars reflect the standard deviation.

BMP7 mRNA was analyzed by RT-PCR at different time points after hypoxia. BMP7 expression was reduced 6h after the end of the hypoxic stimulus and gradually increased thereafter to reach control levels between 24-48h. This increase is sustained on time and 96h post hypoxia BMP7 mRNA remains slightly higher than in controls (Fig. 1C).

Mild hypoxia induce BMP7-dependent changes in glial progenitors fate *in vitro*

As BMP7 regulates astroglial fate and differentiation, we analyzed by ICC the expression of different glial cell markers 4 days after the hypoxic treatment. The number of GFAP (Glial Fibrilar Astrocytic Protein) positive astrocytes (GFAP+) was not changed in hypoxic respect normoxic cultures, but there was a significant increase in the number of NG2 positive (NG2+) cells (OPCs) in the hypoxic condition (Fig. 2A, D, G, J).

To determine if this bias to the OPC progenitor phenotype is related with the reduced BMP7 signalling in hypoxic cultures, we treated E16 cortical cultures with BMP7 (25 ng/ml) or the BMP7 inhibitor follistatin (FST, 200 ng/ml) in serum-free medium for 4 days, in control conditions or starting immediately after the hypoxic period. BMP7 treated cultures exhibit higher number of GFAP+ astrocytic cells and a dramatic decrease in the number of NG2+ OPCs (Fig. 2C, F, I, L). FST treatment exerts the opposite effect, increasing significantly the number of NG2+ OPCs but without altering the number of GFAP+ astrocytes (Fig. 2B, E, H, K). As expected, BMP7 treatment of hypoxic cultures completely blocks the increase in OPC progenitors. In contrast, BMP blocking treatment with FST under hypoxic conditions produces the same effect as hypoxia or FST alone in the induction of the OPC phenotype (Fig. 2M). The observation that our hypoxic conditions do not affect the total number of cells or neurons suggests that hypoxia modifies the fate of glial progenitors but not their proliferation rate.

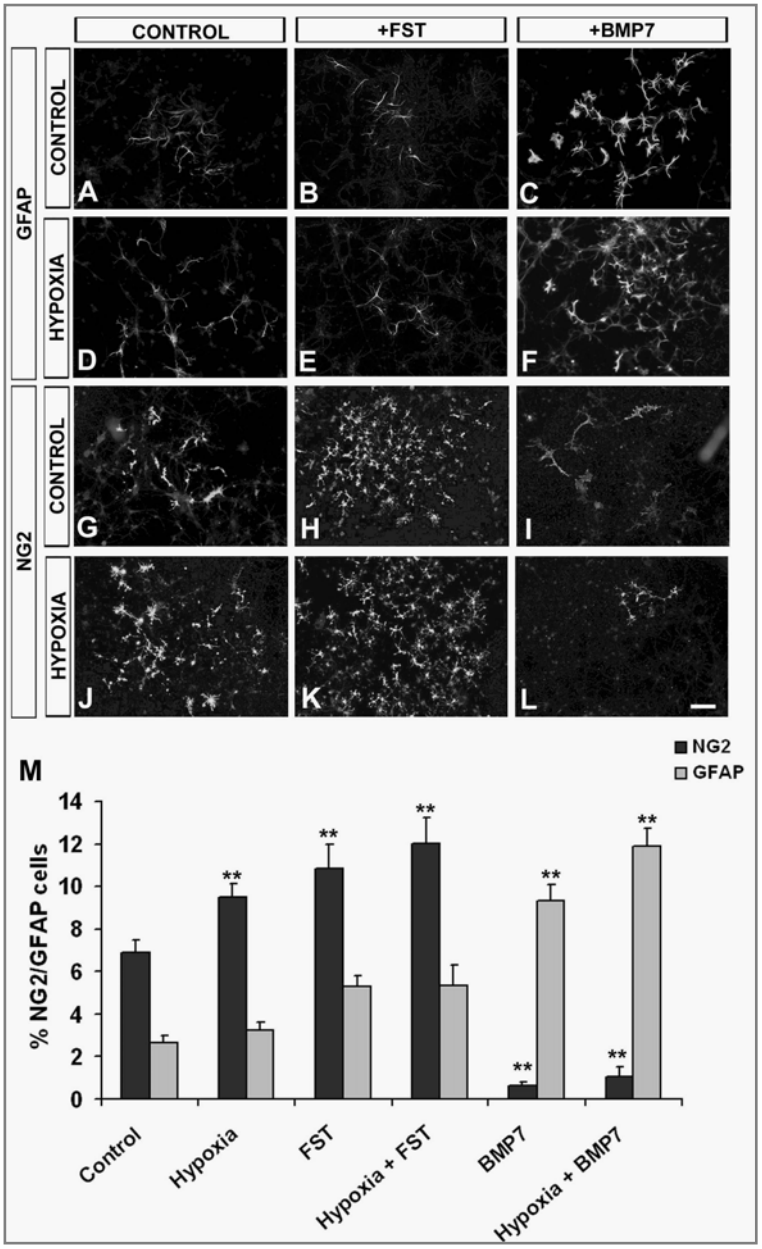


Figure 2. Hypoxia increases the number of NG2+ cells in cortical cultures.
Immunostaining for the astroglial (GFAP) (**A-F**) and O-2A progenitor (NG2) markers (**G-L)** in control and hypoxic cortical cultures treated with vehicle, BMP7 (25 ng/ml) or follistatin (FST, 200 ng/ml) during 4 days in normoxia. (**M**) Histogram summarizing the effect of FST, BMP7 and the hypoxic treatment in cortical cultures. Higher numbers of NG2+ cells are observed in FST and hypoxic cultures, while BMP7 treatment produces a dramatic decrease in NG2+/OPC cells and a clear increase in number of GFAP+ astrocytes. Scale bars: 100 µm. Significant difference (** $p<0.01$, LSD test). Error bars reflect the standard error of the mean.

OPCs can originate type-2 astrocytes and oligodendrocytes (Rao, 1999; Liu and Rao, 2004). Therefore, we wanted to determine if the increase of NG2+/OPC progenitor number is associated with the promotion of the oligodendroglial fate determination. We analyzed the morphology of NG2+ cells in the different culture conditions, as unipolar/ bipolar NG2+ cells represent the more immature or progenitor states whereas complex multipolar morphologies are associated with premyelinating oligodendrocytes (reviewed by Mallon, Shick et al. 2002; Karram, Chatterjee et al. 2005; Nishiyama, Komitova et al. 2009). We measured area and Feret's diameter on binary images of cells, defining Feret's diameter as the longest distance between any two points along the selection boundary (Fig. 3A-F). Cell complexity value was

obtained by dividing the number of pixels in the cell skeleton by the diameter of the cell (Pouria Moshyedi et al., 2010). Morphological analysis revealed higher percentages of NG2+ cells with higher values of area and complexity in hypoxic and FST treated cultures (Fig. 3G, H). In conclusion, lower BMP7 availability induced by either hypoxia or FST exposure seems to induce OPC/NG2+ cells to advance into the oligodendrocyte lineage commitment.

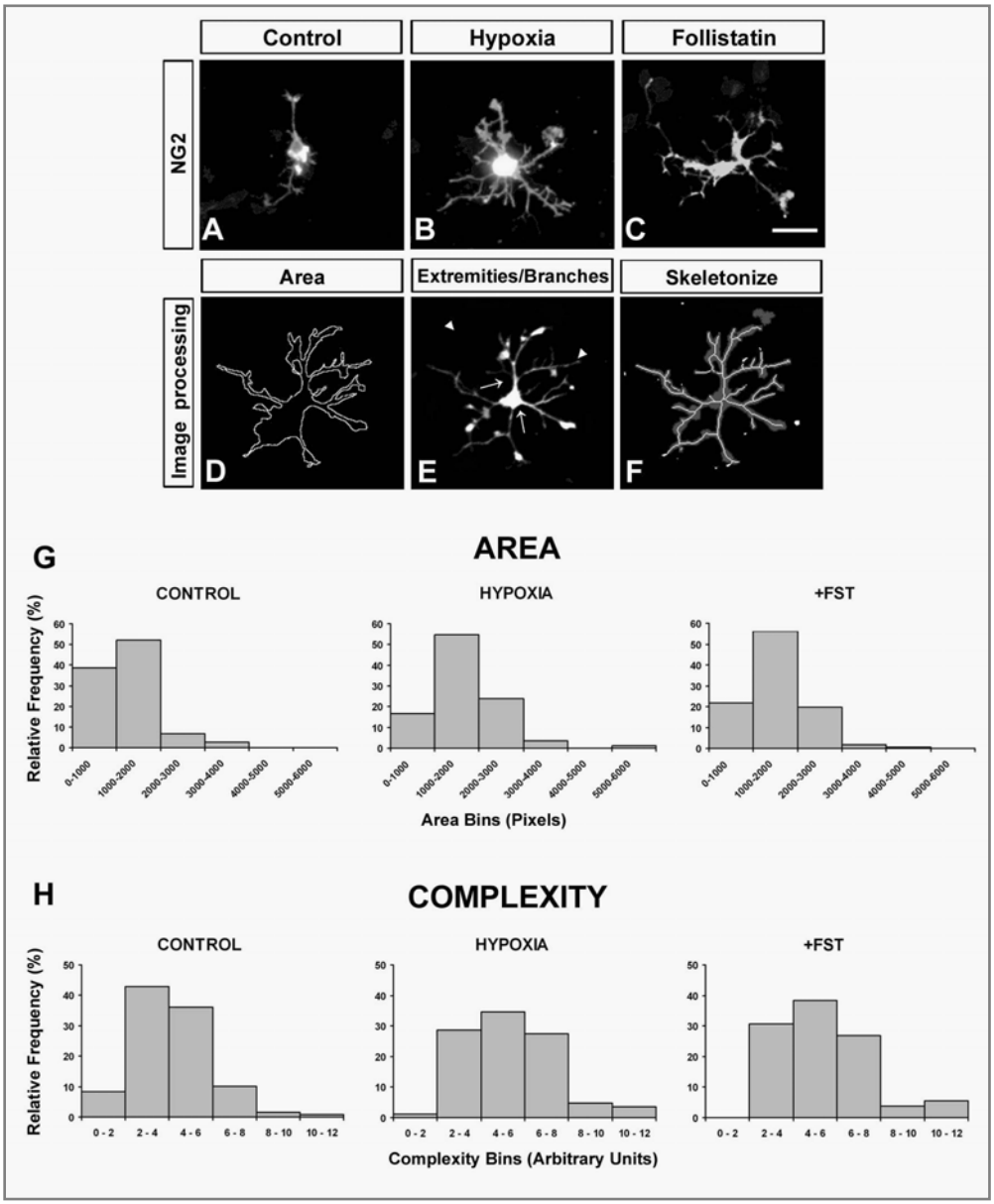


Figure 3. Hypoxia induce NG2+ cell maturation in cortical cultures.
A-F Illustration of NG2+ cell morphometry. To analyze cell morphology, fluorescent pictures were taken **(A-C)**, and the number of extremities (►) and main branches (→) was counted manually **(E)**. A sample cell is represented in terms of area (D) and skeleton (F). **G, H** Relative frequency charts show similar behaviour of FST-treated and hypoxic cultures related to NG2+ cell morphology. Hypoxia and FST show a significant displacement to higher area **(G)** and complexity **(H)** values respect control cultures. More complex NG2+ morphologies are associated with a pre-myelinating oligodendrocyte phenotype. Scale bars: 25 µm. (ANOVA $p<0.01$, LSD test).

Mild hypobaric hypoxia reduces BMP7 expression *in vivo*

To analyze the effect of sublethal reductions in oxygen availability *in vivo*, we used an animal model of hypobaric hypoxia. E16 OF1 pregnant females were kept overnight (12h) in a hypobaric chamber simulating 5000 meters of altitude, where oxygen levels were cut down to 10-11%. The pregnant dams did not reveal any problem to give birth their litters, which were normal in number and body weight (data not shown). Under these conditions, apoptosis (identified by cleaved caspase-3) was not induced (Fig. 4A) and the total number of Ki67+ progenitors or neuron restricted Tbr2 progenitors were not altered in the cerebral cortex of the exposed pups (Fig. 4B).

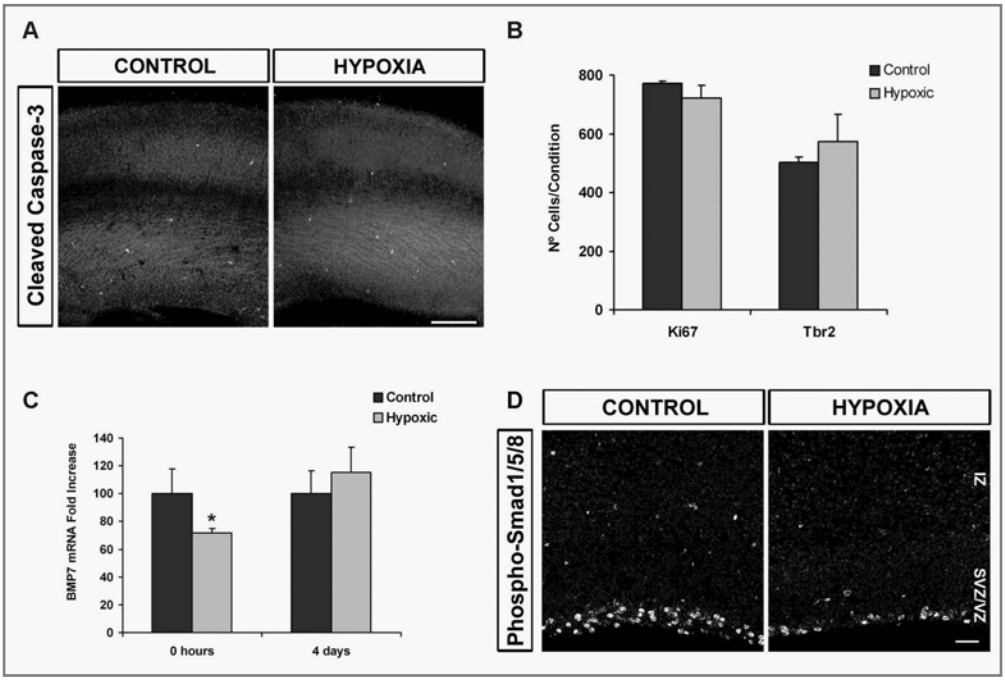


Figure 4. Sublethal hypobaric hypoxia reduces BMP7 expression and signalling.
A, Cleaved Caspase-3 immunostaining does not reveal cell death differences in P0 animals subjected to 12 hours of hypobaric hypoxia at E16, respect control mice. **B,** Graph reflects no significant changes in the number of Ki67+ (proliferating cells) and Tbr2+ (neuronal progenitors) cells counted in control and hypoxic P0 mice. **C,** Quantification of BMP7 mRNA by RT-PCR showing a significant reduction in BMP7 levels in the cerebral cortex of embryos exposed to prenatal hypobaric hypoxia for 12 hours at E16 .**D,** Immunolabeling for phospho-Smad1/5/8 at the end of the hypoxic period (E17) showing reduced number of SVZ/VZ cells with the smad pathway activated in hypoxic respect control animals. (IZ) intermediate zone, (SVZ/VZ) subventricular/ventricular zone. Significant difference (* $p<0.05$, LSD test). Error bars reflect the standard deviation. Scale bars: 160µm in A,B; 40 µm in C-F.

Nevertheless, RT-PCR analysis showed decreased BMP7 mRNA levels in the cerebral cortex of embryos processed just at the end of the hypoxic periods respect to controls. As occurs *in vitro*, BMP7 expression recovered normal levels after four days in normoxia (Fig. 4C). This reduction in BMP7 was accompanied by a concomitant reduction in the activation of BMP signalling pathway determined by reduced expression of phospho-smad1/5/8 in the hypoxic cortices (Fig. 4D). This reduction was concentrated in the VZ/SVZ germinal areas, suggesting that progenitor cells located in those regions were the main affected.

Mild hypobaric hypoxia induces oligodendrocyte specification *in vivo*

Newborn mice subjected to prenatal hypobaric hypoxia did not show evident alterations in the white matter or corpus callosum (Annex 1) or in the expression of the astrocytic marker GFAP (Fig. 5A). Nonetheless, those newborn mice showed higher NG2 immunoreactivity than control animals. NG2+ OPCs were mainly located in the cortical ventricular/subventricular and intermediate zones (Fig. 5A). To determine whether this increase was correlated with a bias to the oligodendrocytic fate we analyzed the expression of the transcription factor Olig2, an oligodendrocyte marker. We observed a marked increase in Olig2+ cells that was also restricted to the cortical ventricular/subventricular zone (Fig. 5A). For quantification, we performed Western blot analysis of GFAP and Olig2 protein content. As expected, prenatal hypobaric hypoxia did not affect GFAP protein expression whereas significantly increased Olig2 protein levels were observed (Fig. 5B).

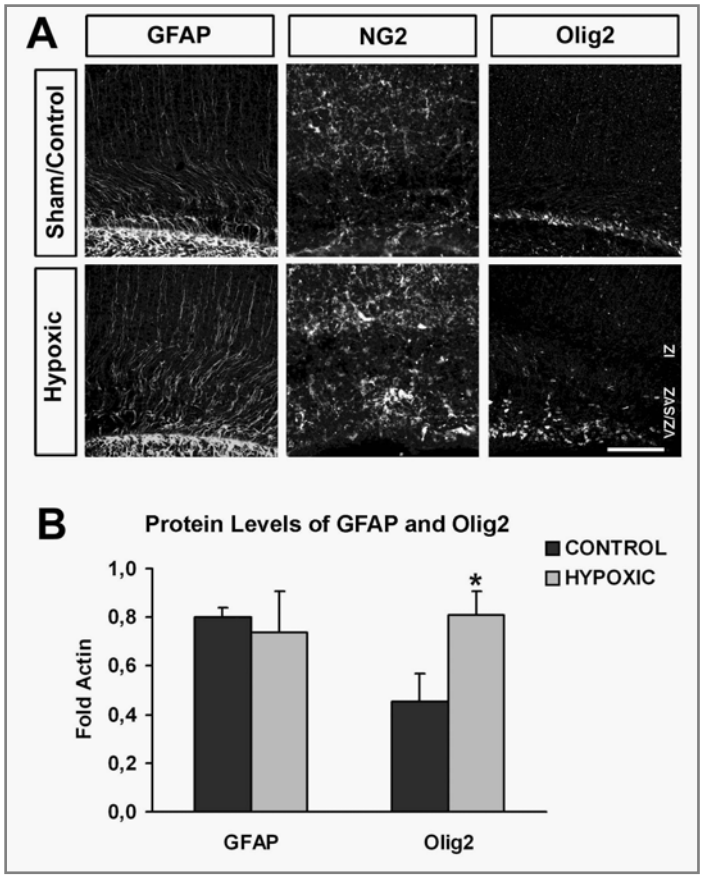


Figure 5. Sublethal hypobaric hypoxia induces oligodendroglial differentiation.
A, Immunoanalysis on of astroglial (GFAP+) and oligodendroglial (NG2+ and Olig2+) cells on coronal sections of P0 mice from pregnant females exposed to hypobaric hypoxia at E16. Images show higher immunoreactivity for NG2 and Olig2 on the SVZ/VZ of hypoxic mice than in control mice, whereas not changes were observed in GFAP immunostaining among both animal groups. **B,** Graph summarizing the quantification of Western Blot for GFAP and Olig2 protein levels in P0 cortical tissue from control and hypoxic animals. Hypobaric hypoxia induces a significant increase on the expression of the oligodendrocyte transcription factor Olig2. Not remarkable changes were observed for GFAP. Scale bars: 40 µm. Significant difference * $p<0.05$, LSD test. Error bars reflect the standard deviation.

Oligodendrocyte fate induction in prenatal sublethal hypoxia is mediated by BMP7 signalling.

We developed two *in vivo* lose and gain of function strategies to corroborate that the bias to oligodendrocyte fate induced by prenatal sublethal hypoxia was mediated by BMP7 signalling. To block BMP7 function prenatally we injected FST in the lateral ventricle of E14 embryos and analyzed them at E18. FST exposure causes a mild reduction of GFAP-immunoreactive radial glia fibers and an increase in Olig2+ cells in the VZ/SVZ (Fig. 6A-D). Those results are coincidental with those observed in the newborns exposed to prenatal hypoxia.

Then, to corroborate that local BMP7 overexpression in gliogenic progenitors blocks oligodendrocytic fate by favouring the astrocytic fate, we transfected newborn mice with a BMP7 expression vector by electroporation. 2 µL (4µg/µL) of a mixture 1:4 of pEF1-BMP7 and pEF1-EGFP vectors, or only pEF1-EGFP were injected in the lateral

ventricle of P0 mice. We immediately exposed injected animals to an electrical pulse of 100 mV with 5 repetitions and animals were sacrificed at P3 to analyze their cerebral cortex. GFP labelling delineated the entire radial glia processes at P3 in control and BMP7 transfected animals, and BMP7 was overexpressed in the soma of those radial glia progenitors, indicating a successful transfection (Fig. 6E-H). As expected, BMP7 overexpression induced a marked increase in GFAP immunoreactivity while almost abrogate Olig2 expression (Fig. 6I-L). Taking together, those results indicate that the modulation of BMP7 signalling is responsible of the glial fate choice in the perinatal cerebral cortex.

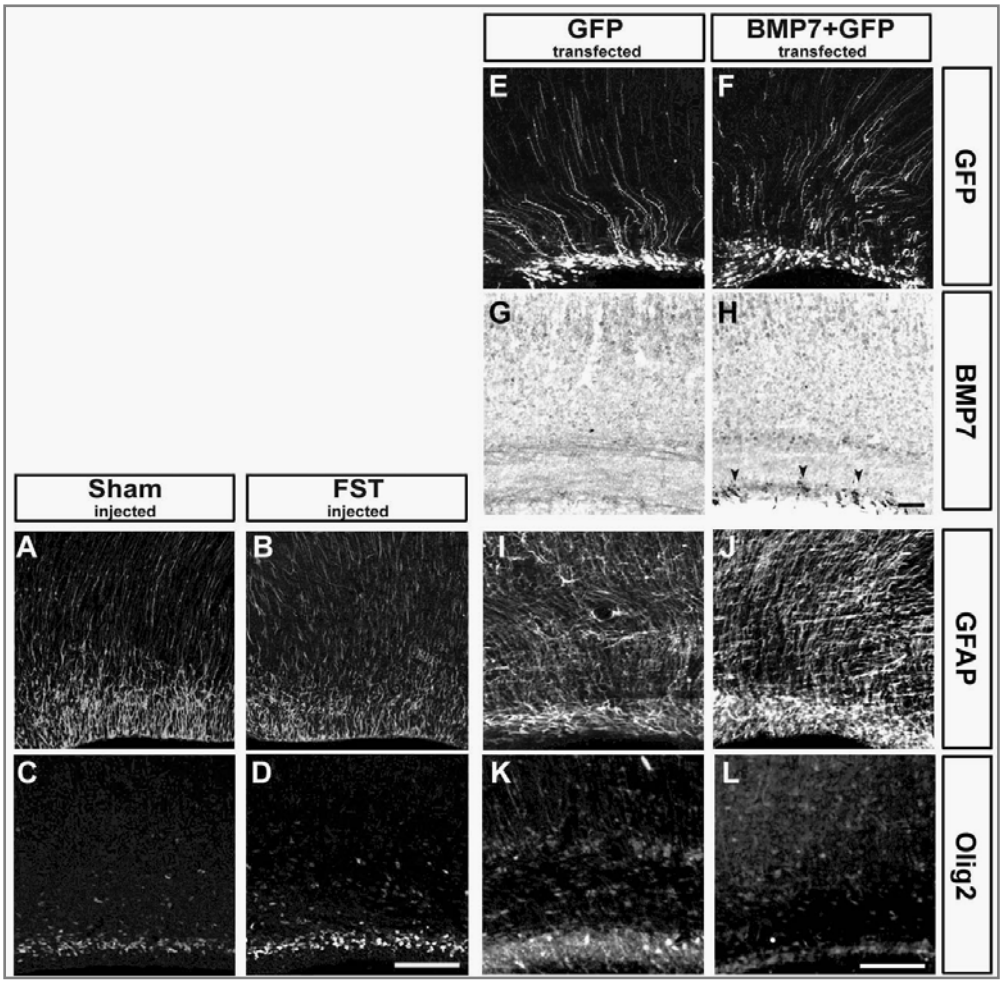


Figure 6. BMP7 signalling modulates Olig2 immunoreactivity.
(A-D) E18 cortical sections of intraventricular FST-injected animals at E14, showing reduced GFAP staining **(A,B)** and increased Olig2 immunoreactivity **(C,D)** in the VZ/SVZ respect sham-injected animals used as controls. **(E-L)** Cortical pictures of P3 mice that were transfected at P0 by electroporation with GFP or GFP+BMP7 construct. GFP fluorescence **(E, F**) and BMP7 immunolabelling **(G, H)** showing the effective transfection in postnatal radial glia progenitors. BMP7 overexpression increases the immunoreactivity for the astroglial marker GFAP **(I, J)** and reduces Olig2 + cells **(K, L)** in the VZ of transfected hemispheres. Not transfected contralateral hemispheres were used as controls. Scale bars for A-D, E-H and I-L: 40 µm. (▼) Indicates BMP7 stained cells in the VZ of the GFP/BMP7-transfected cortices.

DISCUSSION

Our findings demonstrate that the modulation of BMP7 levels exerted by hypoxia is sufficient to change the commitment of cortical progenitors *in vitro* and *in vivo*. BMPs secreted by both neurons and glia exert different action on CNS depending on the developmental period. In the earlier neural development (E13) BMPs predominantly induce cell death and inhibit stem cell proliferation, as a mechanism for the regulation of the cell number within the developing cortex (Furuta, Piston et al. 1997). Subsequently, they exert sequential actions promoting neuronal differentiation at E16 (at moderate doses) and as corticogenesis proceeds, they promote astrocytic differentiation (Li, Cogswell et al. 1998; Mabie, Mehler et al. 1999). The dual effect of BMPs was also observed by *in vitro* studies, that indicate that low secretion of BMPs induces cortical stem cells to differentiate into neurons, whereas high BMPs levels secreted by astrocytes cause cell differentiation into astrocytes (Chang, Son et al. 2003).

In this work we focused on BMP7, a TGF-β superfamily protein which pattern of expression is required to be minutely controlled for the correct cortical glia maturation (Ortega and Alcantara 2010; Sanchez-Camacho, Ortega et al. 2011). BMP7 is relatively low expressed at E16 (Annex 2), an embryonic developmental stage where neurogenesis predominates in front gliogenesis (Bayer and Altman 1991). However, its expression rapidly increases during perinatal period, reaching a peak during the first postnatal week, when astroglial maturation is really active (Suzuki and Goldman 2003; Mori, Buffo et al. 2005). During development and in the adulthood BMPs are expressed within the ventricular/subventricular germinal region of the cerebral cortex (Furuta, Piston et al. 1997; Li, Cogswell et al. 1998; Lim, Tramontin et al. 2000; Colak, Mori et al. 2008). Exogenous BMPs are known to decrease proliferation in mouse SVZ progenitor cells promoting astroglial differentiation in detrimental to oligodendrogenesis (Gross, Mehler et al. 1996; Mabie, Mehler et al. 1997; Mabie, Mehler et al. 1999; Grinspan, Edell et al. 2000). Actually BMPs have been shown to inhibit the differentiation of OPCs to premyelinating oligodendrocytes and to block the maturation of premyelinating oligodendrocytes (reviewed by Sabo, Kilpatrick et al.

2009). Altogether indicates that the brain seems to require a well organized BMPs pattern of expression to establish the different cell populations in their correct spatio-temporal timing.

Several studies have demonstrated that CNS injuries alter the expression of BMPs and/or their receptors (Lewen, Soderstrom et al. 1997; Charytoniuk, Traiffort et al. 2000; Setoguchi, Yone et al. 2001; Hampton, Asher et al. 2007). After stroke, BMP7 mRNA is increased in the ischemic hemisphere, where this morphogen would exert a neuroprotective and neuroregenerative role (Chang, Lin et al. 2003). In agreement with this hypothesis, it was observed that BMP7 (+/-) mice have an impaired recovery of the locomotor activity after an ischemic episode. We have focused on H-I encephalopathy, the most important cause of cerebral damage in the perinatal period both in term and preterm infant. Surviving infants suffer developmental or behavioural disabilities with or without associated cognitive defects and epilepsy (Vannucci and Vannucci 2005; Rees, Harding et al. 2008). Hypoxic deleterious effects in the perinatal period are thought to be consequence of altered neuronal differentiation, synaptogenesis and lose of neurons, glia, and their progenitors due to excessive apoptosis (Curristin, Cao et al. 2002). Moreover, hypoxia is known for promoting changes in BMPs expression and signalling (Martinez, Carnazza et al. 2001; Pistollato, Chen et al. 2007; Panchision 2009; Pistollato, Chen et al. 2009; Pistollato, Rampazzo et al. 2009).

Prenatal hypobaric hypoxia is a useful model that allows analyzing deleterious effects on cognitive function associated to reduced oxygen availability during perinatal period (Simonova, Sterbova et al. 2003; Maiti, Singh et al. 2006). We used a mild hypoxic model (10% oxygen) where we did not observe an increased apoptotic response and white matter defects (Annex 1), more characteristic of acute H-I insult. However, both *in vivo* and *in vitro* mild hypoxic treatments induced a rapid downregulation of BMP7 expression. Furthermore, in agreement with recent studies (Pistollato, Chen et al. 2007; Pistollato, Chen et al. 2009; Pistollato, Rampazzo et al. 2009), we observed less activation of the canonical BMP smad pathway in the germinal VZ/SVZ of the cerebral cortex in hypoxic animals. It has been demonstrated that low oxygen levels exert a

repressive effect on BMP signalling, blocking the activation of Smad1/5/8 that is essential for BMPs transduction signals (Pistollato, Chen et al. 2007; Panchision 2009; Pistollato, Chen et al. 2009; Pistollato, Rampazzo et al. 2009). This repression is thought to be due by the over activity of HIF1α, the best-described mediator of oxygen response. HIF1α transcript stabilization is inversely proportional to oxygen concentration, increasing HIF1α protelytic degradation at higher oxygen tensions (Bruick 2003; Guzy and Schumacker 2006). In turn, exogenous BMP treatment induces a rapid HIF1α downregulation, as happens in hiperoxic conditions (Pistollato, Chen et al. 2007). In fact, mild hypoxia (2.5-5% oxygen) highly approximates to physiological conditions (oxygen concentration in brain varies from 0.5% to 7%) that might favour the state of quiescence of NSC maintaining their self-renewing divisions (Santilli, Lamorte et al. 2010; Ivanovic 2009). Mild hypoxia promotes the HIF1α stabilization, which induce cell survival blocking p53, the major effector of mitotic arrest and apoptosis (Hammond and Giaccia 2005), and a blockage of Smad pathway that leads to survival and self-renewal of neural precursors retaining their pluripotency (De Filippis and Delia, 2010; Santilli, Lamorte et al. 2010; Chen and Panchision 2007) In mild hypoxia, stabilized-HIF1α also interacts with the activated transmembrane receptor Notch, repressing the differentiation of neural precursors (Gustafsson, Zheng et al. 2005). In our hypoxic cultures we neither observed significant changes in the number of proliferative cells (Ki67 positive cells) nor in neuronal precursors (NeuN labeled cells) as well as in the number of apoptotic cells, indicating that although under our conditions neurogenesis is not induced the low oxygen levels used here are not harmful for precursor cells. On the contrary, under conditions with high oxygen levels, HIF1α degradation occurs and then BMP signalling through smad 1/5/8 is regained, inducing depletion of precursors and astrocyte differentiation (Pistollato, Chen et al. 2007; Panchision 2009; Pistollato, Chen et al. 2009; Pistollato, Rampazzo et al. 2009).

Mild hypoxia is an ideal environment for yield oligodendroglial cells (Santilli, Lamorte et al. 2010). Our results perfectly fit into this hypothesis, because under mild hypoxic conditions we observed a decrease in BMP7 expression accompanied by a concomitant increase in oligodendrocyte progenitor markers such as Olig 2 and NG2+ both *in vivo* and *in vitro*. On the contrary, higher levels of BMP7 decrease Olig2 immunoreactivity *in*

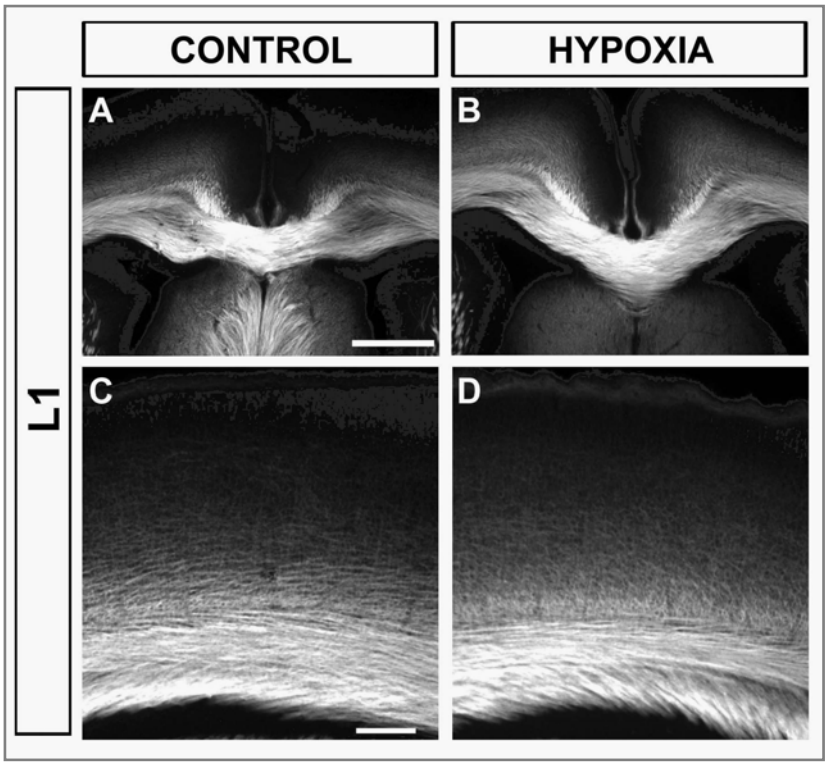
vivo, and dramatically reduce the number of NG2+ cells *in vitro*. NG2+ cells were already identified by Raff and colleagues in 1983 as progenitor cells able to generate type 2 astrocytes and myelinating oligodendrocytes (O-2A progenitor cells) (Diers-Fenger, Kirchhoff et al. 2001; Mallon, Shick et al. 2002). It has been described that NG2+ cells have two distinct morphologies: the first morphology resembles a protoplasmic astrocyte containing an oblong nucleus and limited cytoplasm with bipolar or unipolar processes. The second morphology resembles a microglia cell or a premyelinating oligodendrocyte with multipolar stellate processes (Horner and Palmer 2003). We observed a change in the morphology of NG2+ cells in our hypoxic cultures. In control cultures predominates unipolar/bipolar NG2+ cells, whereas in hypoxic cultures we mainly distinguished multipolar NG2+/OPCs. Co-staining of NG2+ cells with PDGFRα, a pre-oligodendrocyte specific marker confirms their oligodendroglial identity (Annex 3). However, deeper immuno-analysis with more mature oligodendrocyte specific antibodies would ratify the oligodendroglial maturation mechanism induced by hypoxia. The increase in NG2+ cell progenitors in hypoxic cultures occurs without change in the total percentage of neuronal and non neuronal cells, or in the number of proliferative cells (Ki67+ cells). Those data suggest that mild hypoxia favours the differentiation of cortical glial progenitors into premyelinating oligodendrocytes instead of to astrocytes. Treatments with BMP7 recombinant protein and its inhibitor, FST, indicate that the switch effect of hypoxia on glial fate might be due, at least in part, to the downregulation of BMP7.

Increased number of NG2+ cells is a suitable condition for white matter recovery after H-I insult (Rhodes, Moon et al. 2003). NG2 molecule is a single membrane-spanning chondroitin sulphate proteoglycan with a large extracellular domain and a short cytoplasmic tail expressed in the developing and adult central nervous system (CNS), which role is still not well understood (Dawson, Levine et al. 2000). There are theories that support that NG2 has growth-inhibiting properties that may contribute to the creation of an environment no longer supportive of nerve regeneration and repair (Dou and Levine 1994; Fawcett and Asher 1999; Levine, Reynolds et al. 2001). Even though there are some studies that argue against the concept of a purely inhibitory role of NG2 on neurite outgrowth, and support that NG2+ cells are intimately

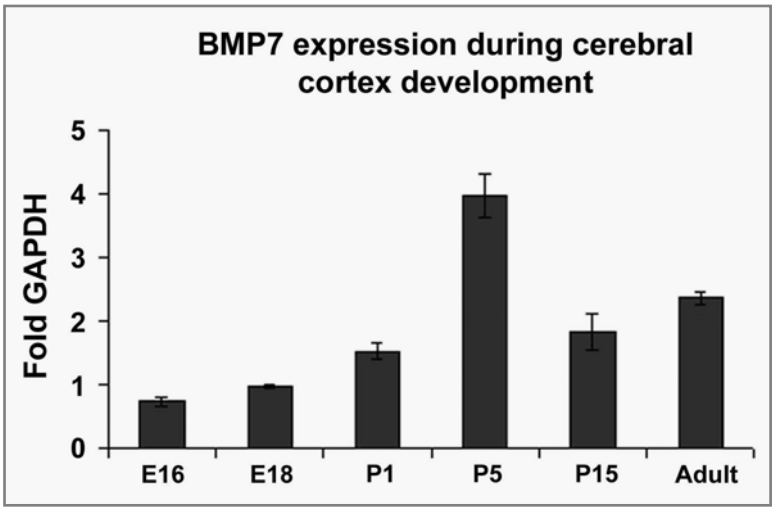
associated with axonal growth cones and promote axonal outgrowth (Jones, Sajed et al. 2003; de Castro, Tajrishi et al. 2005; Karram, Chatterjee et al. 2005; Yang, Suzuki et al. 2006). In addition, in demyelinating models it has been observed around lesions elevated proliferation rates of NG2+ cells in the primary response to demyelination. Some weeks later, NG2+ cells decreased when oligodendrocytes appeared and remyelinate the lesions (Keirstead, Levine et al. 1998; Watanabe, Toyama et al. 2002; Sizonenko, Camm et al. 2008). In the neonatal period, NG2+ cells have been shown to favour outgrowth from developing neurons, facilitating axonal growth during development and regeneration (Yang, Suzuki et al. 2006). In addition, recent studies suggest that NG2-positive cells in normal developing or pathologic brains are also involved in the genesis of neurons (Dayer, Cleaver et al. 2005; Yokoyama, Sakamoto et al. 2006; Rivers, Young et al. 2008). Finally, some studies suggest that high levels of chondroitin sulphate proteoglycans, including NG2, may have a neuroprotective effect after CNS damage (Nishiyama 2007).

In conclusion, our results propose that oxygen and BMP7 are important factors in the modulation of the lineage commitment of cortical precursors. We have demonstrated that increased BMP7 levels promote astroglial differentiation, while reduced BMP7 expression, as occurs after mild hypoxia, potentiates oligodendrocyte fate determination in the cerebral cortex. Different studies have shown that BMP7 treatment has neuroprotective effects in front brain ischemia and spinal cord injury (Chang, Lin et al. 2003; Chou, Harvey et al. 2006; de Rivero Vaccari, Marcillo et al. 2009). The present work, in agreement with Sabo and colleagues (2009), reinforces the theory that depending on the spatio-temporal context and the origin of the progenitor cell, BMPs can promote or inhibit the production of different neural lineage cells, which are more or less willing to participate in the CNS recovery. Understanding the developmental roles played by BMP7, as well as their regulation in front of an insult in the immature brain, is essential to identify future strategies to prevent neurological impairments produced by perinatal encephalopathologies.

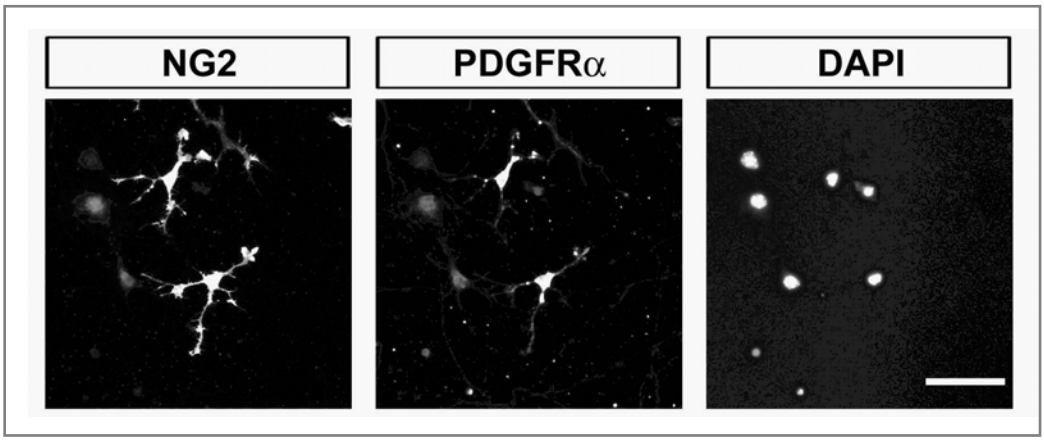
ANNEX FIGURES



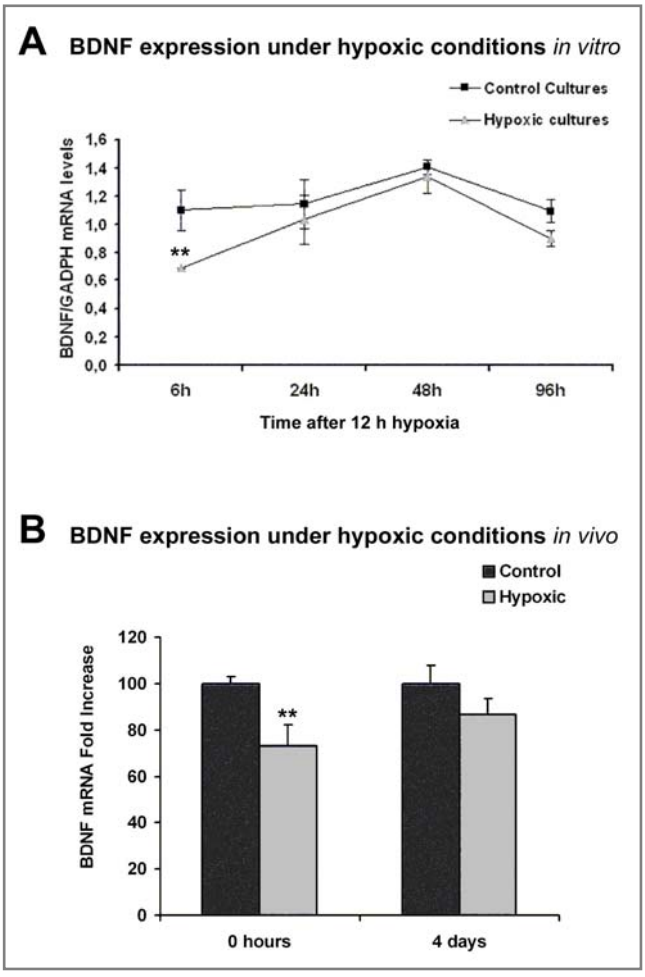
Annex 1. Sublethal hypobaric hypoxia does not affect cortical white matter.
L1 immunostaining show that P0 mice from pregnant females subjected to hypobaric hypoxia did not display white matter anomalies neither in corpus callosum **(A,B)** nor in cerebral cortex **(C,D)**. Scale bars: 500µm in B, C; 100 µm in D,E.



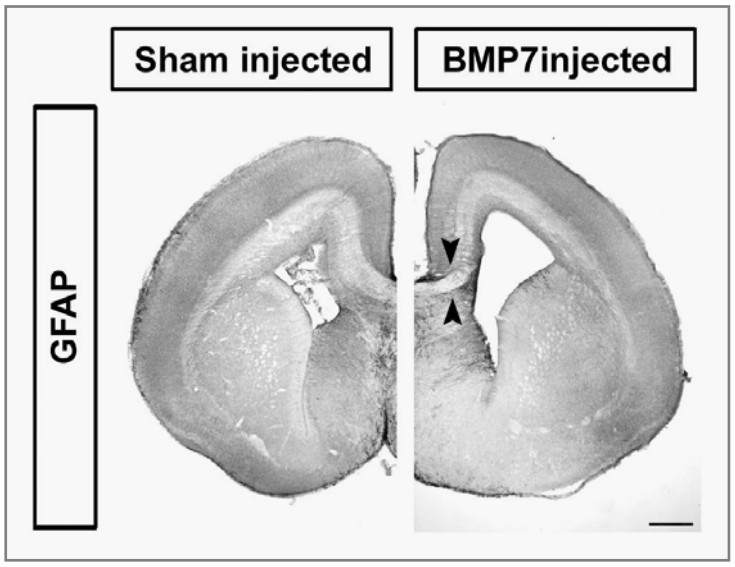
Annex 2. BMP7 pattern of expression during the cerebral cortex development.
Quantification of BMP7 mRNA levels by RT-PCR in cerebral cortices from E16, E18, P1, P5, P15 and adult mice. Error bars reflect the standard deviation.



Annex 3. Doble immunostaining of immature oligodendrocytes with antibodies against NG2 and PDGFRα antigens. DAPI labelling was used to stain the nuclei. Scale bars: 100µm.



Annex 4. BDNF expression in response to hypoxia.
A. BDNF mRNA was quantified by real time PCR in cortical cultures incubated under hypoxic conditions for 12 hours. Cultures were next returned to normoxic conditions for 6, 24, 48 and 96 hours. As occurs with BMP7, BDNF mRNA levels are decreased 6 h after hypoxic treatment, recovering the normal levels at 24 hours and onwards. **B.** Quantification of BDNF mRNA levels by RT-PCR showing a significant reduction in BDNF levels in the cerebral cortex of embryos exposed to prenatal hypobaric hypoxia for 12 hours. Significant difference (** $p<0.01$, LSD test). Error bars reflect the standard deviation.



Annex 5. E18 mice injected at E14 with BMP7 exhibiting enlarged ventricles in cortical coronal sections stained with GFAP antibodies. Arrowheads indicate the reduced width of the callosal comissure in the BMP7-injected animals. Scale bars: 500µm.

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DISCUSSION

During the late embryonic development, the neuroepithelium undergoes a switch from neurogenesis to gliogenesis as part of the constitutive maturation program. BMPs and their receptors are expressed within the VZ/SVZ of the cerebral cortex, where the neural cortical progenitors are located (Furuta, Piston et al. 1997; Li, Cogswell et al. 1998; Lim, Tramontin et al. 2000; Colak, Mori et al. 2008). In this thesis, it is shown the importance of the regulation of BMP7 levels to modulate the cell fate determination of cortical precursors. BMPs can promote the differentiation of neural progenitors into either neurons or astrocytes, depending on time of exposure and on whether their action is directed to the neural precursor population located within the VZ or SVZ respectively (Gross, Mehler et al. 1996; Li, Cogswell et al. 1998). Some studies associate this dual effect with a dosis-dependent BMP action. Low secretion of BMPs by neurons induces cortical precursors to differentiate into neurons, whereas high BMPs levels secreted by astrocytes cause differentiation into astrocytes (Chang, Son et al. 2003). Our observations show that at midgestation, when neurogenesis prevails over gliogenesis, BMP7 expression levels are rather low. Otherwise, there is a rapid increase in BMP7 expression during perinatal period reaching a peak during the first postnatal week, when astroglial maturation is really active (Annex 2) (Suzuki and Goldman 2003; Mori, Buffo et al. 2005). BMPs exert a vital function modulating glial lineages commitment. Many *in vitro* assays have demonstrated that BMPs induce astroglial differentiation concurrent to the suppression of neuronal and oligodendroglial lineages (Gross, Mehler et al. 1996; Mabie, Mehler et al. 1997; Mabie, Mehler et al. 1999; Yanagisawa, Takizawa et al. 2001), being BMP7 the most effective BMP exerting this action (Gross, Mehler et al. 1996). These data point to a sequential action of BMP along the cerebral cortex development, promoting neuronal differentiation at E13 (at moderate doses) and later, from E16 to postnatal development, promoting astrocytic differentiation and inhibiting oligodendrocyte generation (Mabie, Mehler et al. 1997; Li, Cogswell et al. 1998; Mabie, Mehler et al. 1999). Conversely, treatments based on BMP inhibitors, such as noggin, promote glial progenitors differentiation into oligodendrocytes (Mabie, Mehler et al. 1999; Samanta, Alden et al. 2010). Altogether seems to indicate the importance of having a well organized BMP expression pattern to define the lineage commitment of different cell

populations in their correct spatio-temporal axis within the developing cerebral cortex program.

BMP7 EXPRESSION IS MODULATED BY BDNF-DEPENDENT SIGNALLING PATHWAY

This thesis demonstrates that BDNF is one of the factors that modulate BMP7 expression during corticogenesis. BDNF induces BMP7 expression basically in neurons but not in glial cells. Nonetheless, we cannot rule out glial cells or capillary endothelial cells as a source of BMP7 in response to BDNF *in vivo*. Although vascular endothelial cells have not been analyzed in this work, they are a very interesting target because they have recently been identified as a source of BMP7 in the cerebral cortex (Imura T et al., 2008) and they also express and respond to BDNF (Guo, Kim et al. 2008; Madri 2009). These recent studies propose a close interaction between vessels and neural precursor cells that would lead to a coordinated development between vessels, glial and neuronal cells leaded by endothelial produced factors.

The neuronal release of BMP7 in response to BDNF might be explained because neurons predominantly express the full-length TrkB receptor, whereas glial cells express a truncated isoform that lacks the intracellular tyrosine kinase domain (Cheng, Coksaygan et al. 2007; Islam, Loo et al. 2009). The binding of BDNF to the full-length TrkB receptor produces the receptor dimerization and the subsequent receptor autophosphorylation, which engages different intracellular transducing signal pathways, including Ras-Raf-ERK (extracellular signal-regulated kinase) protein kinase pathway, the phosphatidylinositol-3-kinase (PI-3kinase)/Akt kinase pathway, and phospholipase C (PLC)-γ1 (Kaplan and Miller 2000). Blocking TrkB signalling pathways using specific inhibitors we demonstrated that BDNF-dependent BMP7 expression requires the activation of TrkB and the MAPK/ERK pathway (Fig.D1).

Looking for transcription factors involved in this induction mechanism we pointed to a recent work of Yan and Chen (Yan and Chen 2007) which demonstrated that p53 family proteins (p53, p63 and p73) are able to bind to a responsive element located in the

intron 1 of the BMP7 gene. Mutational analysis of p53 family members have showed increased BMP7 expression levels when p53-dependent transactivation activity is diminished. Moreover, p53 activity is modulated by the phosphorilation of active ERK (Chang, Steelman et al. 2003; Wu 2004) and BDNF induces the nuclear translocation of p53 (Jordan-Sciutto, Murray Fenner et al. 2001). Thus we analyzed whether p53 family member are involved in BDNF-dependent BMP7 expression. Inhibiting p53/73 transcriptional activity BDNF-dependent BMP7 upregulation was enhanced in a 26 %, while the induction of p53/73 activity generates a proportional reduction of BMP7 expression of around 30%. These data confirm that p53 family members have a co-repressor activity on BMP7 expression and that BDNF induces BMP7 expression in part by releasing this transcriptional repression (Fig.D1).

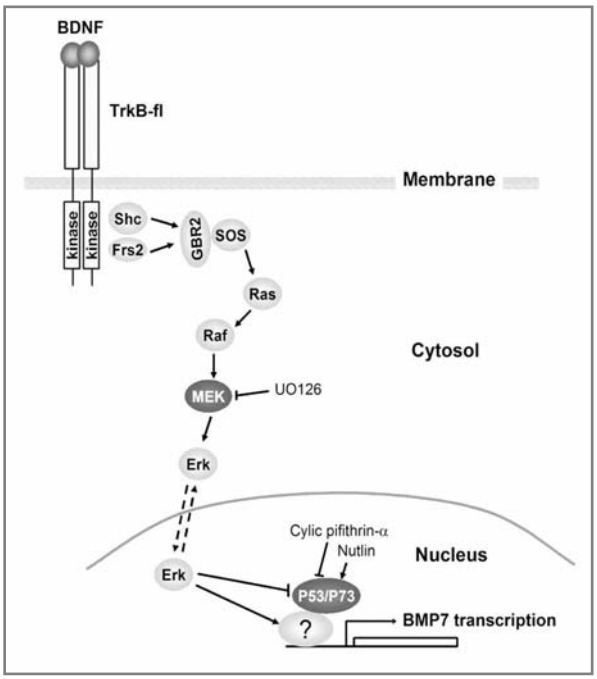

Fig.D1. Intracellular pathway involved in BDNF-induced BMP7 expression.

Other regulatory elements might be involved in the regulatory mechanism of BMP7 transcription, attending to the fact that any of our pharmacological approaches completely abolished basal BMP7 expression. BDNF is known to induce gene expression through distinct transcription factors such as CREB, NFAT and egr4 (Groth and Mermelstein 2003; Sun, Zhou et al. 2008; Ludwig, Uvarov et al. 2010). Although these regulatory elements have not been associated to genomic regulation of BMP7 gene, it would be interesting to check whether they are directly or indirectly

controlling BDNF-dependent BMP7 expression. Furthermore, BMP7 expression is modulated by epigenetic mechanisms involving histone acetylation (Wang, Lin et al. 2007; Yoshikawa, Hishikawa et al. 2007; Marumo, Hishikawa et al. 2008; Nott, Watson et al. 2008). Increase in histone acetylation generally stimulates gene transcription by relaxing chromatin structure, a process that is determined by the balance between the activities of histone acetyltransferase (HAT) and histone deacetylase (HDAC). Different studies have demonstrated that reducing the inhibitory activity of different HDACs promotes BMP7 expression (Wang, Lin et al. 2007; Marumo, Hishikawa et al. 2008; Oxburgh 2009). BDNF was found to induce a rapid and sustained S-nitrosylation of HDAC2, which promotes its release from chromatin and a concomitant increase of histones acetylation (Nott, Watson et al. 2008). Hence, all these data indicate that further studies are necessary to better understand the regulation of BMP7 gene expression during embryonic development.

BMP7 FUNCTIONS ON CEREBRAL CORTEX DEVELOPMENT

The main goal of the thesis is to identify the roles of BMP7 in neuronal and glial proliferation and differentiation, and its possible effect on corticogenesis. Our findings demonstrate that changes in glial maturation induced by increased BMP7 levels at midgestation disturb the migration of projecting neurons destined for the upper cortical layers. Immunostaining with antibodies for specific transcription factors of upper cortical layers helped us to discard that abnormal glutamatergic neurons distribution was due to an effect of BMP7 on neuronal fate determination. Upper cortical neurons were generated normally but they failed to reach their proper cortical position. The main defect observed in BMP7 injected mice was a clear loss of RGC processes produced by an abnormal higher maturation of RGC into astrocytes. The morphology and distribution of RGC along cerebral cortex are essential for being the main support of locomoting neurons (Rakic 1978). Appart from serve as a scaffold for neuronal migration, RGC originate neurons, subventricular IPCs and GRPs.

During development, RGC undergo a transformation into astrocytes (Ihrie and Alvarez-Buylla 2008; Malatesta, Appolloni et al. 2008), a dynamic process close regulated by migrating cortical neurons (Hatten 1985; Miller and Gauthier 2007). It has been proposed a model that describes a sequential signalling induced by migrating neurons in close contact with RGC which maintain the radial glia scaffold (Patten, Peyrin et al. 2003). When neurogenesis is almost complete and radial scaffold are no longer required for neuronal migration, cytokines secreted by newly born neurons promote that radial glia transformation into astrocytes (Barnabe-Heider, Wasylnka et al. 2005). Secreted BMPs induce astrocytogenesis and astroglial maturation at the same time that they inhibit the proliferation of cortical progenitors (Gross, Mehler et al. 1996; Nakashima, Takizawa et al. 2001; Yanagisawa, Takizawa et al. 2001). Through the induction of inhibitory transcription factors of the ID family, BMPs block pro-neural bHLH protein function and so enhancing astroglial promoters in late embryos (Yanagisawa, Takizawa et al. 2001; Ross, Greenberg et al. 2003; Vinals, Reiriz et al. 2004; Miller and Gauthier 2007). The administration of exogenous BMP7 at midgestation promotes a premature transformation of RGC into astrocytes. Moreover, we observed possible symptoms of premature cortical maturation in BMP7-injected animals within the SVZ, a proliferative region that expands from midgestation onwards in detrimental of VZ. The subventricular progenitors are produced during late neurogenesis from RGC which give rise to neurons but also to a second pool of progenitor cells, which migrate away from the ventricular zone to populate the external surface of lateral ventricles and so generating the SVZ (Noctor, Martinez-Cerdeno et al. 2004). Subventricular progenitors are considered neurogenic transit amplifying progenitors which would exponentially increase the neuronal pool in the cortex (Reillo, de Juan Romero et al. 2010). Although during the early postnatal development, this region is also a source of astroglial and oligodendroglial cells (Levison and Goldman 1993). Immunolabeling experiments showed that progenitor cells are displaced from the VZ to more external locations in BMP7 treated animals. In addition, we observed a reduction of subventricular progenitors destined to give rise to neurons (IPC/Tbr2+cells), corroborating that BMP7 induces an early RGC transformation into glial linage precursors.

We further examined cell adhesion-modulating proteins, surface and extracellular matrix signals that play a critical role in the process of neuronal migration. These signals direct the stages of gliophilic migration, instructing migrating neurons the time and the place where they may stop, detach and differentiate. Reelin and SC1 are extracellular matrix proteins that control different steps of radial migration. The action of reelin on radial migration and cortical neurons adhesion is still matter of discussion. The recently proposed "detach and go" model suggests that reelin may have a dual effect acting as an attractive signal for migrating neurons, while it is also known that through alpha 3-integrin signalling, it directs neurons to detach from RGC when they arrive to their final position (Myant; Dulabon, Olson et al. 2000; Luque 2004; Cooper 2008; Huang 2009). In BMP7 injected animals we neither observed changes in reelin expression nor in the distribution of Cajal-Retzius cells, the neurons located in the marginal zone and the responsible for reelin expression in the cerebral cortex. Nonetheless, we observed in BMP7-injected animals an increase in SC1 expression, an anti-adhesive extracellular matrix protein distributed along the upper cortical plate and expressed by radial glia fibers passing through the upper cortical layers and by mature astroglia. Overexpression of SC1 dramatically disrupts neuronal migration (Gongidi, Ring et al. 2004). The abnormal high SC1 levels expressed by prematurely formed astroglia might induce the early detachment of migrating neurons from the glial scaffold, producing an ectopic accumulation in the IZ of neurons destined to the upper cortical layers. In summary, our results indicate that BMP7 treatment during midgestation generates a migration arrest of projecting neurons as a result of the precocious transformation of RGC into astrocytes.

The effect of BMP7 on glial maturation is also crucial for the correct development of distinct intermediate glial populations that participate in the genesis of the corpus callosum (CC). Animals with aberrant levels of BMP7 display CC agenesis characterized by defasciculation of callosal fibers in the midline, and the formation of typical Probst bundles, generated by callosal axons that have failed to cross the midline. BMP7 null embryos showed a marked decrease of mature astroglia markers in GW, IG and MZ while the injection of BMP7 severely induces astroglia differentiation in these midline structures. The midline SCS population was also altered by abnormal levels of BMP7

during CC formation period. We observed a decreased number of migrating SCS neurons in the midline region in both BMP7-injected and BMP7 knock out animals. So, dose-controlled BMP7 expression is required for the correct generation and differentiation of midline cell populations (Fig.D2).

Nevertheless, CC agenesis could be due to multiple errors in some of the diverse developmental steps responsible of CC formation (Paul, Brown et al. 2007). CC formation depends in part on the correct formation of callosal neurons, including their migration and differentiation in their proper cortical layer. The study of the laminar distribution of callosal projecting neurons indicates that knock out animals, as happened with BMP7 injected animals, showed less populated upper layers than control animals. However we observed that animals with abnormal BMP7 levels can display different rate of CC agenesis but always have a similar cortical distribution of callosal projecting neurons, suggesting that abnormal cortical distribution of these neurons might not be directly associated with callosal defects in our models.

Another factor involved in CC development is the modulation of the cytoskeleton that mediates neuronal migration and axon growth (Lindwall, Fothergill et al. 2007). BMPs are known to activate LIM Kinase 1, a key downstream effector of Rho GTPases that can regulate important CNS cellular mechanisms such as neurite outgrowth and synapse formation (Lee-Hoeflich, Causing et al. 2004). In addition, BMP signalling has been observed to act as an axon guidance cue in different context (Sanchez-Camacho and Bovolenta 2009). However, our *in vitro* studies did not reveal a direct effect of BMP7 on axonal outgrowth.

So, we considered that the modifications in the integrity of IG, GW and SCS induced by aberrant levels of BMP7 are the most plausible cause of CC malformations observed. It is expected that alterations in midline structures might also lead to modifications in the release of molecular cues that modulate callosal axon outgrowth. We initially focused on Slit2, a chemorepellent ligand expressed by IG and GW cells, that assists the guidance of callosal axons across the midline avoiding their entrance into the septum (Shu and Richards 2001; Shu, Sundaresan et al. 2003). Although IG and GW

populations are severely affected by abnormal BMP7 levels, Slit2 expression levels appeared rather normal. Abnormal cellular response to Slit2 can be due to alterations in the activity of their modulators. Chalasani and collaborators have demonstrated that SDF1 acts as a modulator of Slit2 reducing their repulsive action on retinal axons (Chalasani, Sabol et al. 2007). In addition, it is reported that SDF1 expression and secretion is stimulated by BMP2 and BMP7 (Yang, Pham et al. 2008). So, aberrant levels of BMP7 could indirectly alter the guide action of Slit2 on callosal axons through the regulation of SDF1 expression and secretion. It would be also really interesting the analysis of other secretable molecules such as Wnts, Netrins, Ephrins and Semaphorins and the recently described Draxin, that are essential for callosal axon guidance (Chedotal et al. 2011) as well as extracellular matrix components (i.e. heparan sulfate proteogycans and chondroitin sulfate proteoglycans) that attach these secreted pathfinding cues, playing a key role in establishing diffusible gradients (Conway, Howe et al.; Lindwall, Fothergill et al. 2007).

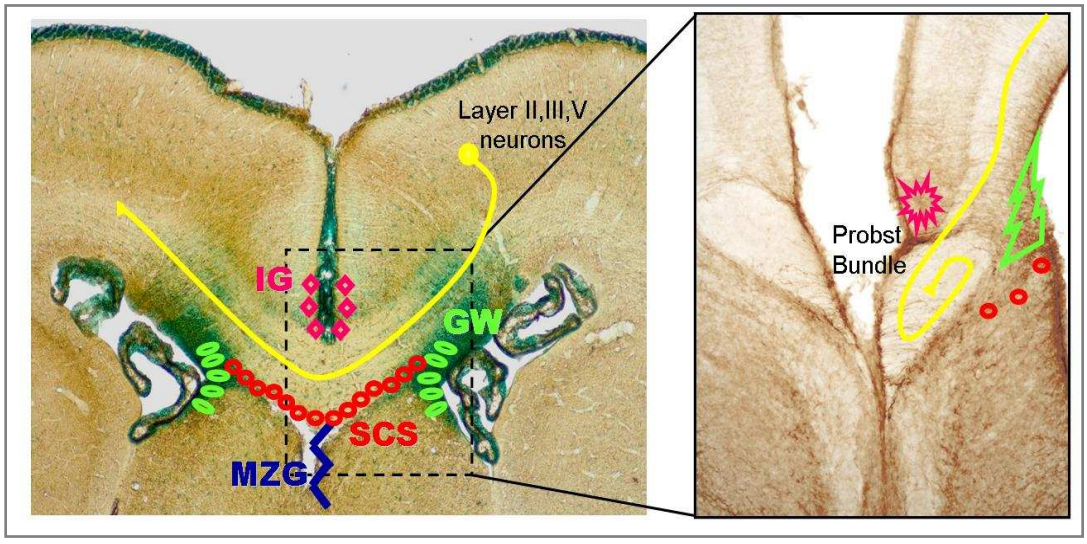

Fig.D2. B-Gal staining (green) indicates the pattern expression of BMP7 in the midline. The scheme also shows the cortical projecting neurons from layers II, III and V as well as midline cell populations: Indusium griseum (IG), Glial Wedge (GW), Subcallosal Sling (SCS), Midline Zipper Glia (MZG). A higher magnification of the midline region showed how in our BMP7 gain and loss of function model comissural axons fail to cross the midline generating the typical Probst Bundle. These animals mainly displayed anomalies in IG, GW and SCS midline populations.

Recent studies put forward the theory that discrete neural populations play a key role in axon guidance. Niquille and colleagues (Niquille, Garel et al. 2009) have identified two transient neuronal populations exclusive of embryonic ages within the white matter of the developing CC that form a complex cellular network with midline glial cells for the proper CC formation. In our BMP7 gain and loss of function model there is a marked reduction of the ventral most subpopulation characterized by Niquille and collaborators (2009) that corresponds to SCS. The expression of BMP7 along the path followed by migrating SCS neurons towards the midline region, and the reduction observed along callosal commissure of these cells in BMP7-injected and KO embryos, made us to hypothesize with a possible chemotactic effect exerted on this neuronal population by BMP7. In fact, BMP7-mediated chemotactic and inhibitory functions in cell migration have been reported in other systems (Lee, Park et al. 2006; Na, Seok et al. 2009; Perron and Dodd 2009). One intriguing possibility is that changes in the GW radial glia maturation induced by abnormal BMP7 levels can alter the migration of the SCS cells, as occurs in the cerebral cortex with pyramidal neurons, where BMP7 deregulation alters their radial migration.

Taking together, our results indicate that is necessary a strict control of BMP7 expression for the correct development of cortical structures. We propose that BMP7 together with BDNF might constitute a control system for the synchronization of the neuronal and glial maturation during CNS development. At the end of corticogenesis and during perinatal period, external inputs traduced in higher electrical activity might lead to increased BDNF expression in differentiated neurons. This BDNF increase would be one of the inductors of BMP7 expression in the cerebral cortex, which reach a peak during the first postnatal weeks, coinciding with the peak of BDNF expression and the gliogenic period. Secreted BDNF is known to promote neuronal survival, neuronal fate, neurite outgrowth, and synaptogenesis (Numakawa, Suzuki et al.; Huang 2009). In turn, BMP7 induces dendritogenesis (Lee-Hoeflich, Causing et al. 2004) and radial glia to astrocyte maturation (present work). We propose that the coordinated action of both chemokines might help to synchronize neuronal survival and differentiation with the astrocytic maturation on the arrival of incoming axons at the initial period of cortical activity (Fig.D3).

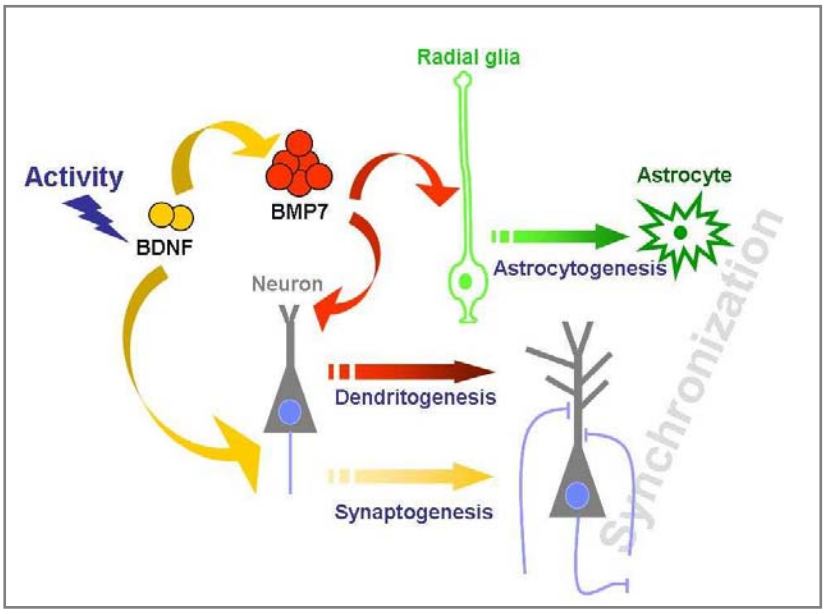

Fig.D3. Model of physiological role for BDNF-dependent BMP7 expression during cerebral cortex development.

Therefore the alteration of BDNF and BMP7 spatio-temporal expression patterns could dramatically affect the proper cerebral cytoarchitecture and consequently the cerebral functioning (Fig.D4). Indeed, different traumas occurred during embryonic and perinatal development are associated with an imbalance in BDNF and BMP7 levels. Several studies have demonstrated that both BDNF and BMP7, and their receptors are present in abnormal higher levels in distinct pathological conditions (Lindvall, Ernfors et al. 1992; Lewen, Soderstrom et al. 1997; Korhonen, Riikonen et al. 1998; Charytoniuk, Traiffort et al. 2000; Setoguchi, Yone et al. 2001; Hampton, Asher et al. 2007; Hubold, Lang et al. 2009; Madri 2009). On the other hand, both chemokines play a neuroprotective and neuroregenerative roles in response to distinct CNS injury (Galvin and Oorschot 2003; Meng, Zhiling et al. 2005; Chou, Harvey et al. 2006; Chen and Panchision 2007; Tsai, Weng et al. 2007; Guo, Kim et al. 2008; Sun, Zhou et al. 2008; Sabo, Kilpatrick et al. 2009) Tsai et al, 2010). However, in developing brains, the response of these chemokines in order to protect the CNS might have side effects on the highly regulated cerebral cortex developmental program.

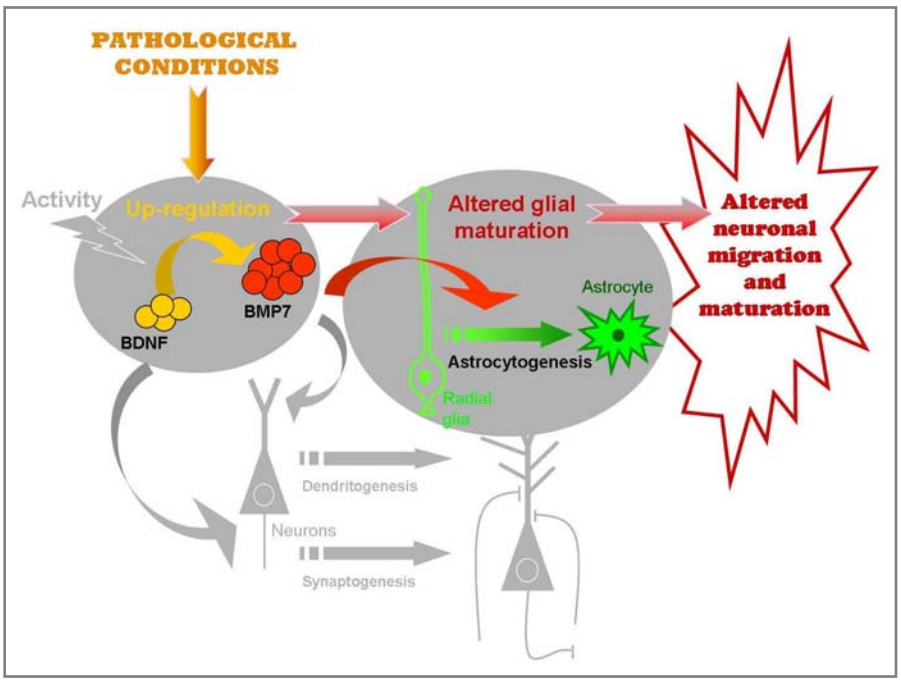

Fig.D4. Some SNC injuries during embryonic and perinatal period produce abnormal higher levels of BDNF and BMP7, an imbalance associated with distinct mental disorders. We proposed a possible explanation of the physiopathologycal mechanisms induced by BDNF underlying important cerebral disorders.

OXYGEN PRESSURE AND BMP7, TWO IMPORTANT FACTORS FOR COMMITMENT OF NEURAL PROGENITORS

Hypoxia is a pathological condition that can be associated to altered BDNF and BMP7 expression (Martinez, Carnazza et al. 2001; Schmidt-Kastner, Truettner et al. 2001). Hypoxic encephalopathy is the most important cause of cerebral damage in infants produced within the perinatal period. Hypoxic deleterious effects in the perinatal period are produced by alterations on neuronal differentiation and synaptogenesis as well as excessive apoptosis of neurons, glia, and their progenitors (Curristin, Cao et al. 2002). So, perinatal reduction of oxygen input can dramatically affect the cerebral cortex developmental program. As a result, many behavioural and learning disorders in infants have been associated to H-I injuries. Interestingly, it is thought that chemokine imbalance is underlying the dramatic defects observed in some of these disorders. Our observations *in vivo* and *in vitro* indicated that hypoxia decreases BDNF expression and

once normoxic conditions are re-established there is a progressive recovering of BDNF basal levels (Annex 4). Individuals that have suffered a fetal hypoxic trauma exhibit reduced levels of BDNF expression and signalling (Schmidt-Kastner, Truettner et al. 2001), and a high percentage of these patients show mental disorders such as schizophrenia (Buckley, Pillai et al. 2011; Cannon, Yolken et al. 2008). BDNF plays a critical role in neuronal survival and morphogenesis, as well as in the maintenance and plasticity of the neural circuit, having an important influence on the human mind and consciousness (Numakawa et al., 2010). It is also known that BDNF treatment has a prophylactic effect against hypoxic-ischemic insults in neonatal rodents (Cheng, Gidday et al. 1997; Galvin and Oorschot 2003; Meng, Zhiling et al. 2005; Sun, Zhou et al. 2008). Nevertheless, the modulation exerted by hypoxia on BDNF expression is far to be well-known. Depending on the degree of hypoxic insult aggressiveness or on the developmental period we can observe opposite responses (Golan, Kashtuzki et al. 2004; Cannon, Yolken et al. 2008). In adult, reduced levels of oxygen may promote BDNF expression in the germinal SVZ through hypoxic induced factor (HIF) (Madri 2009; Shi, Zhang et al. 2009). Within the subventricular region there are an active and complex cross-talk between astrocytes, neuroblast, NSC and vascular endothelial cells that control angiogenesis and different neurogenesis steps. Endothelial cells may modulate neurogenesis in part, through the secretion of soluble factors, including BDNF (Guo, Kim et al. 2008), which are also known to be synthesized by neuronal cells, which in turn modulate endothelial cells (Madri 2009). Thus, BDNF is a vital element to understand neurodevelopment abnormalities associated to chronic sublethal hypoxia.

Hypoxia is also reported to induce changes in BMPs expression and signalling (Martinez, Carnazza et al. 2001; Panchision 2009). In agreement with recent studies performed by Pistollato and colleagues (Pistollato, Chen et al. 2007; Pistollato, Chen et al. 2009; Pistollato, Rampazzo et al. 2009), in this thesis is shown that BMP7 expression and signalling is rapidly down-regulated after hypoxic treatment. This repression might be due to the over activity of HIF1α, which at low oxygen levels exerts a repressive effect on BMP signalling, blocking the activation of Smad1/5/8, an essential molecular complex for BMPs transduction signals (Fig.D5) (Panchision, Pickel et al. 2001; Pistollato, Chen et al. 2007; Pistollato, Chen et al. 2009).

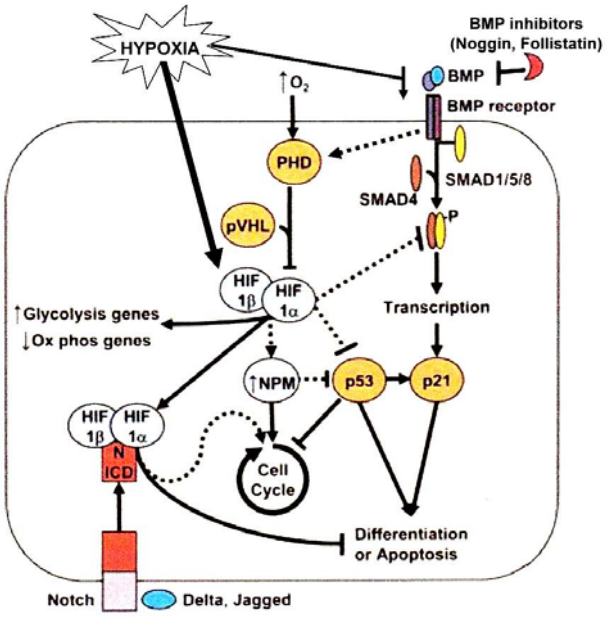

Fig.D5. Effect of hypoxia on BMP7 expression and signalling. Modified from *Panchision, 2009*.

Reduced oxygen levels as observed in the brain (0.5% to 7% of oxygen) favour the maintenance of the NSC niche, which are high sensitive to higher oxygen levels (Santilli, Lamorte et al. 2010; Ivanovic 2009). So, BMP signalling inhibition produced by our hypoxic conditions is likely to be associated with the maintenance of self-renewal state of neural progenitors. On the contrary, in hyperoxic conditions, NSC might save cellular death differentiating into more resistant cellular phenotypes, as happens with exogenous BMP treatment. It has been observed that high oxygen levels and BMPs induce a rapid depletion of neural precursors and astrocyte differentiation through the downregulation of HIF1α (Pistollato, Chen et al. 2007; Panchision 2009; Pistollato, Chen et al. 2009; Pistollato, Rampazzo et al. 2009).

It is widely known that BMPs enhance astroglial commitment and inhibit the differentiation of OPCs to premyelinating oligodendrocytes during embryonic and postnatal development (reviewed by Sabo et al., 2009). Oligodendrocytes derive from O-2A precursors found within the cerebral cortex, which are also able to generate type 2- astrocytes. The plastic behaviour of O-2A/OPCs is importantly influenced by local environmental factors. We have demonstrated that mild hypoxia (2.5-5% oxygen) induces a rapid reduction of BMP7 expression and signalling as well as an increased

oligodendroglial differentiation. On the contrary, hypoxic cultures treated with BMP7 showed a dramatic decrease of NG2+/OPCs cell number accompanied by an increase in GFAP+/astroglial cell number. The increase of NG2+ cells progenitors and the maintenance of the total number of glial cells in the hypoxic cultures, suggest that the reduction of BMP7 expression in response to low oxygen levels do not modify the proliferation of glial progenitors. So, mild hypoxia might favour OPCs initial differentiation into premyelinating oligodendrocytes instead of into astrocytes through the regulation of BMP7 expression.

BMP7 has been proposed as a treatment to attenuate the dramatic effects triggered by hypoxic damage (Chang, Lin et al. 2003; Chou, Harvey et al. 2006). The expression and effect of BMP7 after a lesion can be totally different depending on the lesion, the area affected and the developmental period. While perinatal hypoxia seems to down-regulate BMP7 levels, there is an increased BMP7 expression in adult ischemic brains after stroke, where it exerts a neuroprotective and neuroregenerative role (Chang, Lin et al. 2003). According to these data, BMP7 (+/-) mice have an impaired recovery of the locomotor activity after an ischemic episode, suggesting that BMP7 is important for the recovery process after stroke. However, abnormal high levels of BMP7 can also exert negative effects on cerebral development. BMP7-injected animals during midgestation show ventriculomegaly and white matter disturbances at corpus callosum level (Annex 5), a phenotype similar than the observed in rodents after severe H-I injury. On the other hand, high BMPs levels are associated with an increase in astrogliosis during the glial scar formation, as well as the inhibition of the remyelinization process (Fuller, DeChant et al. 2007). According to these data our *in vitro* and *in vivo* analysis indicate that radial glia progenitors respond to BMP7 promoting the premature astroglial differentiation, avoiding the possibility of new oligodendrocytes generation from cortical progenitors. Recent studies on demyelinating injury models propose a treatment blocking BMP signalling to promote the survival and the migration of OPCs from SVZ (Dizon, Maa et al. 2011; Jablonska, Aguirre et al. 2010; Hampton, Asher et al. 2007). The decreased levels of BMP7 induced by mild hypoxia observed in our experiments produce an increased number of NG2+ cells, a reaction that is associated with white matter recovery after H-I damage

(Rhodes, Moon et al. 2003). Many studies have observed elevated proliferation rates of NG2+ cells around injured regions in response to demyelinating stimulus, which are replaced weeks later by oligodendrocytes that remyelinate the affected area (Keirstead, Levine et al. 1998; Watanabe, Toyama et al. 2002; Sizonenko, Camm et al. 2008). Hence, BMP signalling might negatively affect the correct or at least more desirable CNS recovering after white matter lesion. Nevertheless, BMPs effects after injury are not restricted only to gliosis induction. Several evidences support that BMPs exert neuroprotective and neuroregenerative actions after a variety of different insults, facilitying functional recovery (Setoguchi, Yone et al. 2001; Chang, Lin et al. 2003; Harvey, Hoffer et al. 2005; Chou, Harvey et al. 2006). Following a H-I episode, BMP7 improves neurological function, the local cerebral blood flow and local cerebral glucose utilisation and the locomotor activity, as well as the proliferation and differentiation of adult neural stem cells (Liu, Belayev et al. 2001; Chang, Lin et al. 2003; Chou, Harvey et al. 2006; Sabo, Kilpatrick et al. 2009).

It has been broadly reported that more aggressive ischemic-hypoxia models, such as the classic carotid artery occlusion, induce proliferation of SVZ progenitors (Fagel, Ganat et al. 2006; Yang, Covey et al. 2007; Burgers, Schelshorn et al. 2008). In our hypoxic cultures we neither observed significant changes in the number of proliferative cells (Ki67 positive cells) nor in neuronal precursors (NeuN labelled cells) or in apoptotic cell number. These results indicate that although neurogenesis is not induced under our experimental conditions, the low oxygen levels used are not harmful for precursor cells. Respect SVZ progenitors, immunologically distinguished by the expression of the Tbr2 expression factor, display a general tendency to increase under hypobaric hypoxic conditions, although their number is not significantly different respect the controls. The fact that BMP7-treated cortical cultures and BMP7-injected animals showed the opposite effect than hypoxia, diminishing the number of Tbr2+ cells, lead us to speculate with the direct implication of BMP7 on IPC proliferation in response to hypoxia. The use of a more powerful hypoxic animal model, reducing even more the oxygen percentages, or the use of a more hypoxic sensitive mice strand, C57 Bl/6 instead of OF1 (Li, Liu et al. 2009), will probably help us to confirm our hypothesis.

The vast bibliography, often showing opposite point of views, indicates that BMP7 action and regulation after CNS injury is complex. Depending on the spatio-temporal context and the origin of the progenitor cell, BMPs can promote or inhibit the production of different neural lineage cell which are more or less willing to participate in CNS recovery (Sabo, Kilpatrick et al. 2009). Our findings indicate that changes on BMP7 expression in the tightly regulated developmental program might importantly modify the cellular fate choice of cortical progenitors. When this change occurs during the critic perinatal developmental period, it could compromise the normal brain functionality in the adult.

CONCLUSIONS

CONCLUSIONS:

1. BDNF induces the expression of BMP7 in neurons through TrkB receptor and MAPK/ERK pathway. An induction mechanism that is mediated in part by the release of the transcriptional repression exerted by p53 family proteins.

2. BMP7 promotes astroglial maturation *in vivo* and *in vitro*.

3. The physiological function of the BDNF-dependent BMP7 induction might be to establish a mechanism for synchronic neuronal and glial maturation in response to activity at the beginning of synaptogenesis.

4. Abnormal high levels of BMP7 during midgestation induce the premature radial glia maturation into astrocytes and impair the radial migration of upper layers pyramidal neurons, which improperly remain accumulated in the lower cortical layers.

5. The correct pattern of BMP7 expression is necessary for the proper maturation of intermediate structures such as the glial wedge, the induseum griseum and the subcallosal sling, that provide essential guidepost signals for the corpus callosum formation.

6. Sublethal hypoxia reduces BMP7 expression and signalling in the cerebral cortex. This reduction induces the differentiation of cortical progenitors into the oligodendroglial lineage in detrimental to the astroglial fate.

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