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I N S T I T U T O D E B I O T E C N O L O G I A

DEPARTAMENTO DE GENETICA DEL DESARROLLO Y FISIOLOGIA MOLECULAR

¨Determinación del Potencial Diferenciativo de las Células Precursoras Neurales”

Tesis que para obtener el grado de
Doctor en Ciencias Bioquímicas presenta:

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RESUMEN

La vasta diversidad celular en el Sistema Nervioso se origina a partir de las Células Precursoras Neurales (CPNs). Durante la formación del cerebro, las CPNs restringen progresivemente el número de subtipos neuronales y gliales que pueden generar según su posición en el tubo neural. Algunas poblaciones de CPNs se han identificado y expandido en cultivo, mientras que otras se han generado completamente *in vitro* a partir de las Células Troncales Embriónicas (CTEs). Sin embargo, aún se desconoce cuales poblaciones de CPNs tienen mayor susceptibilidad para responder a señales inductivas y diferenciar a diversos linajes neuronales.

En este estudio implementamos un sistema de cultivo en explante de la región del mesencéfalo (cerebro medio embrionario), el cual recapitula la neurogénesis y el desarrollo de las neuronas dopaminérgicas como ocurre *in vivo*. Nuestro enfoque consistió en transplantar diversas poblaciones de CPNs al mesencéfalo en cultivo, con la finalidad de evidenciar a las poblaciones con mayor susceptibilidad para responder a su entorno. Encontramos que el nicho neurogénico en el mesencéfalo ventral (MV) es necesario para la diferenciación dopaminérgica de las CPNs mesencefálicas. No obstante, esta capacidad de responder al entorno de diferenciación se pierde al expandir in vitro como neuroesferas a las CPNs mesencefálicas. Por otro lado, el nicho del MV fue también capaz de especificar hacia el linaje dopaminérgico mesencefálico a las CPNs aisladas del telencéfalo (cerebro anterior embrionario). La plasticidad de las CPNs telencéfalicas para producir linajes ectópicos fue una propiedad efímera presente entre los 8.5/10.5 días de desarrollo. La restricción temprana en el potencial diferenciativo de las CPNs fue también observada al transplantar al mesencéfalo diversas poblaciones celulares derivadas de las CTEs. Exclusivamente durante la etapa de formación de Cuerpos Embrioides (CEs), estas células fueron capaces de generar eficientemente múltiples linajes neuronales (incluyendo el dopaminérgico) en respuesta al nicho donde se integran en el explante. En contraste, la diferenciación en cultivo de los CEs a CPNs reduce significativamente la capacidad para responder al entorno del mesencéfalo. En conjunto, nuestros datos indican que la interpretación correcta de los nichos neurogénicos en el embrión es una propiedad distintiva de las CPNs primitivas presentes en estadios tempranos del desarrollo o en las etapas iniciales de la diferenciación de las CTEs al linaje neural.

ABREVIATURAS

AT Area tegmental.

AR Acido retinoico.

BMP "Bone morphogenetic protein". Proteina morfogenética de hueso.

BO Bulbo olfatorio.

CE Cuerpo embrioide.

CMR Corriente migratoria rostral.

CPN Célula precursora neural.

CTE Célula troncal embriónica.

CTN Célula troncal neural.

DAm Dopaminérgica mesencefálica.

EGL Eminencia gangliónica lateral.

Egf "Epidermal growth factor". Factor de crecimiento epidérmico.

EGM Eminencia gangliónica media.

FACS "Fluorescent Activated Cell Sorting". Separación celular activada por fluorescencia.

Fgf "Fibroblast growth factor". Factor de crecimiento fibroblástico.

GD Giro dentado.

GFP "Green Fluorescent Protein". Proteína verde fluorescente.

GR Glia radial.

HSA "Heat Stable Antigen". Antígeno termoestable.

MACS "Magnetic Activated Cell Sorting". Separación celular activada por magnetismo.

NR Núcleo rojo.

PNA "Peanut agglutinin". Aglutinina de cacahuate.

Shh	“Sonic hedgehog”
RSG	Región subgranular.
RSV	Región subventricular.
SNc	Substancia nigra pars compacta.
SNC	Sistema nervioso central.
TH	Tirosina hidroxilasa.

CONTENIDO

INTRODUCCION

1. Generalidades sobre el desarrollo del sistema nervioso central.

1.1 Inducción de la placa neural.

Durante el desarrollo embrionario, la gastrulación es el proceso responsable de establecer las tres principales capas germinales: ectodermo, mesodermo y endodermo, de las cuales derivan todos los linajes celulares del organismo adulto. El ectodermo es instruido para generar la epidermis y el Sistema Nerioso Central (SNC). El desarrollo del SNC a partir del ectodermo inicia con la formación del neuroectodermo o placa neural (Fig. 1). Este proceso es mediado por señales provenientes del mesodermo, las cuales instruyen al ectodermo medio para que se convierta en un epitelio con células columnares, las cuales difieren de las células aplanadas alrededor que darán origen a la epidermis. Una vez inducida la placa neural esta empieza a doblarse para formar el tubo neural. Este proceso origina un surco neural con dos pliegues laterales, lo cuales eventualmente se fusionan para terminar de cerrar la estructura en forma de tubo (Colas and Schoenwolf, 2001; Smith and Schoenwolf, 1997) (Fig. 1). La morfogénesis del tubo neural depende de los cambios en la forma celular dentro del neuroepitelio y de fuerzas extrínsecas derivadas del ectodermo epidermal adyacente a la placa neural (Colas and Schoenwolf, 2001; Smith and Schoenwolf, 1997).

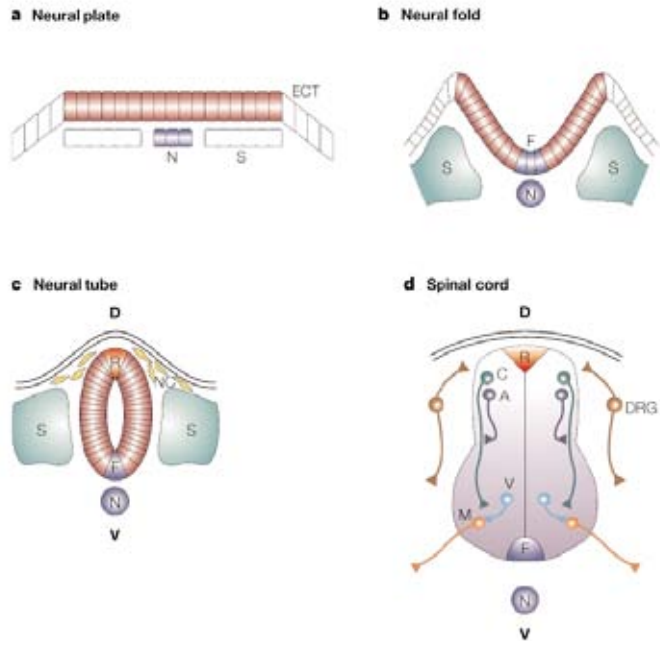

Fig. 1. Desarrollo del tubo neural. (A) Después de la gastrulación, la parte media del ectodermo (ECT) es inducida a diferenciar a placa neural (en rosa) a partir de señales derivadas de estructuras mesodérmicas subyacentes como la notocorda (N) y los somitas (S). **(B)** La placa neural comienza a doblarse a partir de la línea media para formar el surco neural. A su vez, la parte más ventral diferencia a

la placa del piso (“floor plate”; F) al recibir señales como Shh (ver texto) proveniente de la notocorda. **(C)** Al formarse el tubo neural, la parte más dorsal diferencia a la placa del techo (“roof plate”; R), mientras que las células suprayacentes producen la cresta neural (“neural crest”; NC). **(D)** Las Células Precursoras Neurales a lo largo del eje dorso-ventral producen neuronas especializadas en posiciones esterotípicas. En la región ventral se producen neuronas motoras (M) e interneuronas ventrales (V), mientras que en la parte dorsal se producen neuronas comisurales (C) y de asociasión (A). Las células de la cresta neural migran a la periferia para producir las neuronas del ganglio de la raíz dorsal (“dorsal root ganglion”; DRG). V, ventral; D, dorsal. (Jessell, 2000).

1.2 La organización del tubo neural.

Una vez formado el tubo neural, diferentes regiones muestran patrones específicos de expansión y constricción del neuroepitelio, lo que eventualmente lleva al establecimiento de 5 vesículas que dan origen a diferentes estructuras del cerebro adulto (Fig. 2). En orden antero-posterior estas vesículas son: el telencéfalo, que en su región dorsal da origen a la corteza, hipocampo y lóbulos olfatorios, mientras que de la zona ventral derivan la eminencia gangliónica lateral (EGL, la cual genera el estriado), la eminencia gangliónica media (EGM) y la eminencia gangliónica caudal (EGC); el diencéfalo, que produce el tálamo e hipotálamo; el mesencéfalo, que da origen a estructuras involucradas en la visión y el control del movimiento (ver adelante); el metencéfalo, del cual deriva el cerebelo y el mielencéfalo, este último forma la médula oblonga. El metencéfalo y mielencéfalo a su vez están organizados en compartimentos más pequeños conocidos como rombómeros, los cuales dan origen a diversos linajes celulares. En la parte posterior del mielencéfalo se desarrolla la médula espinal.

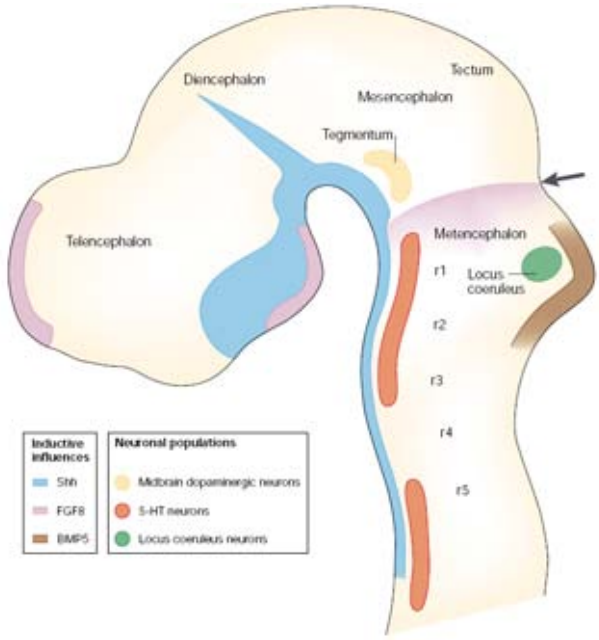

Fig. 2. Organización antero-posterior del tubo neural. Vista lateral del tubo neural donde se muestran las estructuras principales organizadas en 5 vesículas. De anterior a posterior se indica el Telencéfalo, el Diencéfalo, el Mesencéfalo y el Metencéfalo; este último formado por los rombómeros 1 y 2 (r1-2). En la parte más posterior se encuentra el Mielencéfalo (no indicado) formado por los rombómeros 3 al 8 (r3-r8). La constricción en la frontera del mesencéfalo y el metencéfalo (flecha) se conoce como el organizador del Istmo. La presencia de morfógenos como Shh (azul), Fgf8 (morado) y BMP5 (café) determina la posición de diversos núcleos neuronales especializados. En el mesencéfalo ventral, las neuronas dopaminérgicas (amarillo) se inducen por la presencia de Shh y Fgf8. Estas mismas señales en combinación con Fgf4 (no indicado) inducen las neuronas serotoninérgicas (rojo) en el metencéfalo. En la parte dorsal del metencéfalo, la presencia de BMP5 especifica a las neuronas noradrenérgicas del Locus coeruleus (verde). (Goridis and Rohrer, 2002).

2. El desarrollo del mesencéfalo.

2.1 La especificación antero-posterior de la vesícula mesencefálica: el organizador del Istmo.

Durante etapas tempranas del desarrollo del tubo neural, la expresión de *Otx2, En1* y *Pax2* define la región del mesencéfalo a lo largo del eje antero-posterior (Matsunaga et al., 2000) (Fig. 3). La presencia de estos factores transcripcionales es inducida por el organizador del Istmo, constituido por el tejido presente en la frontera del mesencéfalo con el metencéfalo (Nakamura and Watanabe, 2005) (Fig. 3). En la región más caudal del mesencéfalo, la expresión de *Otx2* se reprime mutuamente por Gbx2, el cual es un factor transcripcional involucrado en especificar el cerebro posterior (Millet et al., 1999). La zona donde la expresión de *Otx2* y *Gbx2* se encuentran adyacentes define molecularmente al organizador del Istmo (Katahira et al., 2000) (Fig. 3). Diversos estudios de transplantes demostraron que el Istmo puede generar un mesencéfalo ectópico al inducir la expresión de *En1*, *Pax2* y *Wnt1* en el diencéfalo (Bally-Cuif et al., 1992; Martinez et al., 1991). Estudios posteriores revelaron que Fgf ("Fibroblast Growth Factor") 8, un morfógeno generado en la región del Istmo (Fig. 3), era capaz de inducir por sí solo la expresión ectópica de genes mesencefálicos (Crossley et al., 1996; Liu and Joyner, 2001; Martinez et al., 1999).

2.2 La especificación dorso-ventral del mesencéfalo: la placa del piso y la placa del techo.

La organización dorso-ventral del mesencéfalo también esta determinada por centros

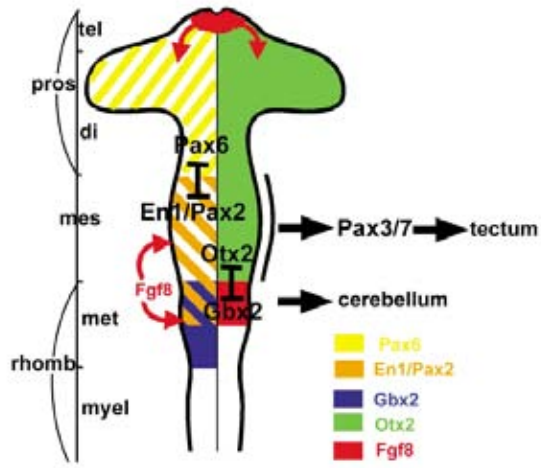

Fig. 3. Determinantes moleculares del mesencéfalo. Esquema del tubo neural visto desde la parte superior. La frontera posterior del mesencéfalo ("mesencephalon"; mes) la establece el límite de expresión de *Otx2* (verde). La represión recíproca entre *Otx2* y *Gbx2* (morado) establece la posición del Istmo, el cual produce Fgf8 (rojo). La inducción y el mantenimiento de genes como *En1* y *Pax2* (anaranjado), responsables del desarrollo temprano del mesencéfalo, depende de la presencia de Fgf8. En1 y Pax2 definen la frontera anterior del mesencéfalo al reprimir la expresión de *Pax6* (amarillo), un gen expresado en el diencéfalo ("diencephalon"; di). A su vez la región dorsal del mesencéfalo, conocida como el tectum óptico en aves, depende de la expresión de *Pax3/7*. Pros, prosencéfalo; rhomb, romboencéfalo; tel, telencéfalo; met, metencéfalo; myel, mielencéfalo. (Nakamura and Watanabe, 2005).

organizadores que secretan morfógenos. El organizador conocido como la placa del techo se origina alrededor de la línea media dorsal (Fig. 1). La placa del techo secreta miembros de la familia de BMP ("Bone Morphogenetic Proteins") y Wnt (Chizhikov and Millen, 2004b; Lee and Jessell, 1999). Estos morfógenos especifican a las neuronas dorsales a lo largo del eje AP del tubo neural (Chizhikov and Millen, 2005). En el mesencéfalo dorsal, la señal de BMP y Wnt participan en la formación del tectum óptico y el colliculus superior; el centro visual primario en el cerebro de vertebrados inferiores y mamíferos, respectivamente (Plas et al., 2008; Schmitt et al., 2006). Adicionalmente, la expresión en el tectum óptico de un gradiente de ligandos de guiamiento axonal por repulsión, como *Ephrin-A2* y *Ephrin-A5*, dirige la inervación esterotípica de neuronas del ganglio retinal (Cheng et al., 1995; Nakamoto et al., 1996). Este proceso es responsable de generar un mapa topográfico del espacio visual.

Alrededor de la línea media ventral del mesencéfalo, la formación de la placa del piso es inducida por la señal de Shh ("Sonic hedgehog") y Nodal (miembro de la familia de los TGFβ) generadas a partir del mesodermo precordal y la notocorda (Placzek and

Briscoe, 2005) (Fig. 1). La placa del piso a su vez produce Shh, el cual especifica diversas poblaciones neuronales ventrales dependiendo de su concentración local (Machold and Fishell, 2002; Placzek and Briscoe, 2005). Los mecanismos que permiten a las células del tubo neural integrar espacial y temporalmente el gradiente de Shh han comenzado a entederse (Dessaud et al., 2008). En el mesencéfalo, las poblaciones más ventrales estan expuestas a concentraciones elevadas de Shh, el cual induce la formación de los núcleos dopaminérgicos alrededor de la línea media (Andersson et al., 2006b). También en la región ventral, la señalización de Shh establece el núcleo ocular motor, contituído por neuronas motoras (Blaess et al., 2006; Fedtsova and Turner, 2001). Estas células inervan directamente los músculos extraoculares, controlando así el movimiento de los ojos.

2.3 La neurogénesis durante el desarrollo embrionario.

La organización del epitelio mesencefálico a lo largo del eje radial determina la ubicación de las células progenitoras y sus derivados neuronales durante el desarrollo (Fig. 4). Previo al inicio de la neurogénesis en el mesencéfalo (E9.5/10), el neuroepitelio se encuentra formado esencialmente por Células Troncales Neurales (CTNs) y otras células progenitoras indiferenciadas. Todas las células indiferenciadas (incluidas las CTNs) se conocen en su conjunto como Células Precursoras Neurales (CPNs). En el neuroepitelio, las CPNs mantienen contacto con la región ventrícular (zona apical) y la región pial (zona basal), mientras que el núcleo de estas células muestra migración intercinética entre las dos regiones (Zhong and Chia, 2008). La posición del núcleo a lo largo del eje radial del tubo neural indica la etapa del ciclo celular en el que se encuentra la CPN. De esta forma, la célula entra en fase G1 al iniciar el núcleo la migración desde el ventrículo, llegando a la fase S en el extremo más basal del recorrido para posteriormente regresar hacia la capa apical durante la fase G2 y alcanzar la mitosis en esa zona (Baye and Link, 2008). Entre mayor sea la distancia que recorre el núcleo hacia la región pial, existe una mayor probabilidad que la CPN salga del ciclo celular y diferencie a neurona (Baye and Link, 2007). La migración nuclear intercinética podría ser el mecanismo por el cual las CPNs interpretan el gradiente de Notch (ver abajo) a lo largo del eje radial (Del Bene et al., 2008). Durante la neurogenesis, algunas CPNs se mantienen indiferenciadas y proliferando, mientras que otras pierden sus contactos apico-basales y migran hacia la zona pial donde diferencian terminalmente a neuronas (Gotz and Huttner, 2005; Zhong and Chia, 2008)

(Fig. 4). Este fenómeno define a la región ventricular del tubo neural como el nicho de las CPNs. El mantenimiento de este nicho y el crecimiento simúltaneo del neuroepitelio es posible mediante el control del número de diviones simétricas y asimétricas de las CPNs (Anderson, 2001). Conforme el epitelio del cerebro se va engrosando, las CPNs mantienen sus contactos apico-basales al adquirir la identidad de glia radial (GR; Fig. 4) (Merkle and Alvarez-Buylla, 2006).

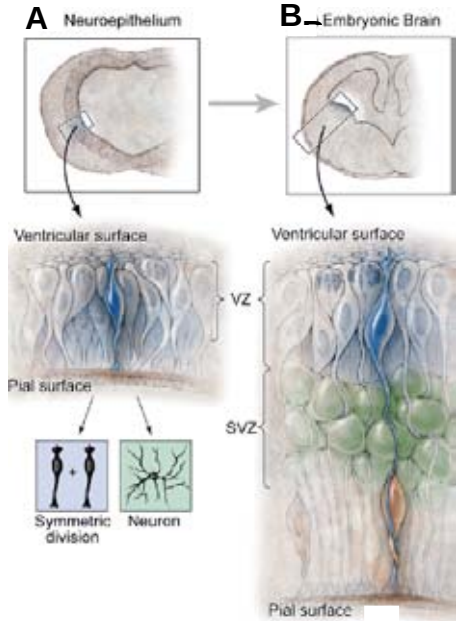

Fig. 4. Neurogénesis embrionaria. (A) En etapas tempranas del desarrollo del tubo neural, las Células Precursoras Neurales (CPNs) en el neuroepitelio mantienen contacto con la superficie ventricular y pial (ejemplo en azul), mientras proliferan por divisiones simétricas o diferencian a neuronas. **(B)** Al engrosarse el neuroepitelio a causa de la proliferación de las CPNs, se definen la región ventricular ("ventricular zone"; VZ) y la región subventricular ("subventricular zone"; SVZ) a lo largo del eje radial del tubo neural. En esta etapa, las CPNs mantienen contacto con la superficie ventricular y pial al diferenciar a glia radial (ejemplo en azul), la cual continua la neurogénesis además de funcionar como andamiaje para la migración de células diferenciadas hacia la capa pial (en anaranjado). Modificado de (Merkle and Alvarez-Buylla, 2006).

2.4 La organización celular del mesencéfalo.

La expresión combinada de genes que codifican para factores transcripcionales con homeodominios establece un código que determina la identidad posicional de las CPNs y su capacidad para diferenciar hacía linajes neuronales específicos (Briscoe et al., 2000). La expresión de estos genes en la región ventral es regulada por Shh, el cual se produce en la placa del piso. En la región cercana a la línea media ventral, la expresión de *Lmx1a* define al linaje de CPNs que producirá neuronas dopaminérgicas

mesencefálicas (Fig. 5) (Andersson et al., 2006b; Ono et al., 2007). Adyacente a la expresión de *Lmx1a* en la línea media, Nkx6.1 define bilateralmente los dominios de CPNs que dan origen a las neuronas glutamatérgicas del núcleo rojo (NR), las cuales mantienen la expresión de *Nkx6.1* y adquieren la expresión de *Brn3a* (Fig. 5) (Fedtsova and Turner, 2001; Nakatani et al., 2007). Dentro de estos dominios, un grupo de CPNs diferencia a neuronas motoras que expresan *Islet1* (Fedtsova and Turner, 2001). Este grupo de neuronas motoras conforma el núcleo ocular motor. A su vez, las CPNs que expresan *Nkx6.1* en regiones más dorsales producen exclusivamente neuronas gabaérgicas (Fig. 5) (Nakatani et al., 2007). Otro grupo de neuronas gabaérgicas es establecido en la región ventral por CPNs que expresan *Nkx2.2* pero no *Nkx6.1* (Fig. 5) (Nakatani et al., 2007). Este grupo de CPNs mantiene la expresión de *Nkx2.2* al diferenciar a interneuronas inhibitorias (Fig. 5) (Nakatani et al., 2007). Por otro lado, la expresión de *Pax7* en CPNs y neuronas dorsales (Fig. 5), define la región que eventualmente dará origen al tectum óptico en aves o al colliculus superior en mamíferos (Thomas et al., 2006; Thompson et al., 2004).

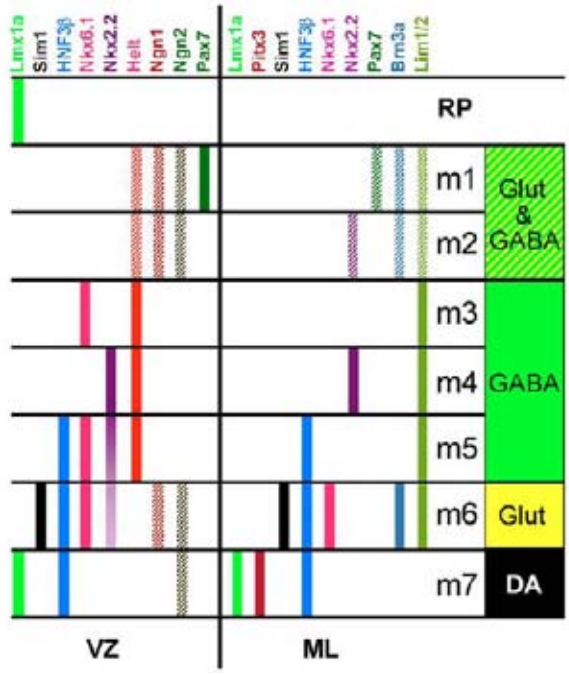

Fig. 5. Un código molecular determina la identidad neuronal en el mesencéfalo. El diagrama indica el patrón de expresión de diversos genes en diferentes dominios dorso-ventrales del mesencéfalo anterior a los 12.5 días de desarrollo. La parte inferior indica la región más ventral (m7 o placa del piso) y la superior la región más dorsal ("roof plate"; RP o placa del techo). Las barras en color indican las zonas de expresión en la región ventricular ("ventricular zone"; VZ) y en la capa del manto ("mantle layer"; ML). La columna derecha indica los subtipos neuronales que aparecen en la capa del manto; se indican las neuronas dopaminérgicas (DA, negro), las neuronas glutamatérgicas (Glut, amarillo), las neuronas

gabaérgicas (GABA, verde) y una región dorsal donde se producen tanto neuronas glutamatérgicas como gabaérgicas (Glut & GABA, verde rayado). Modificado de (Nakatani et al., 2007).

2.4.1 Las neuronas dopaminérgicas mesencefálicas. Organización y función.

Diversos tipos de neuronas dopaminérgicas se producen en diferentes regiones del SNC. Estas neuronas tienen en común la producción de enzimas que generan el neurotransmisor dopamina. La enzima más empleada como marcador dopaminérgico es la Tirosina Hidroxilasa (TH), la cual cataliza la reacción limitante en la síntesis de dopamina (Fig. 6). La TH también esta involucrada en la síntesis de otras catecolaminas como la noradrenalina y adrenalina (Fig. 6); por lo tanto, marcadores adicionales son necesarios para identificar a las neuronas dopaminérgicas.

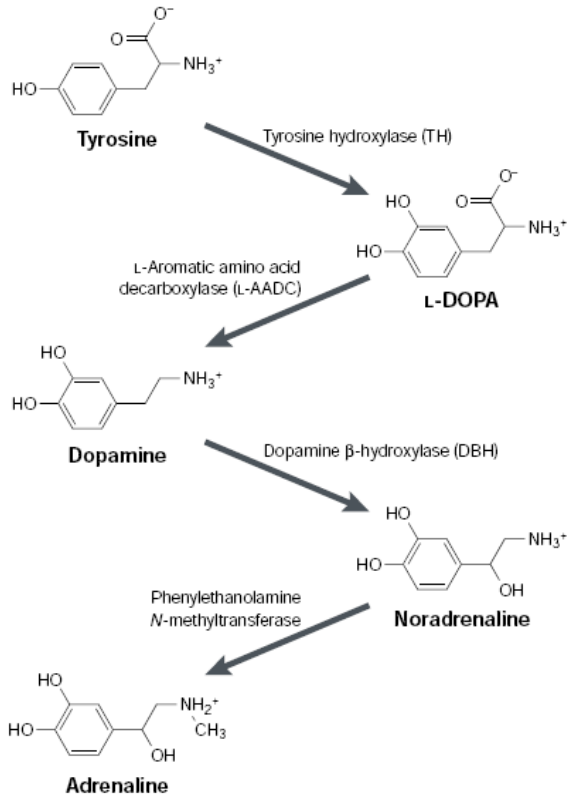

Fig. 6. Ruta enzimática para la síntesis de las catecolaminas. La Tirosina es el precursor metabólico a partir del cual se producen las tres catecolaminas: dopamina, noradrenalina y adrenalina. Modificado de (Goridis and Rohrer, 2002).

Diversos núcleos de neuronas dopaminérgicas se producen en diferentes regiones del cerebro (Fig. 7). En el bulbo olfatorio, las interneuronas dendríticas periglomerulares producen dopamina (Lledo et al., 2008). En el hipotálamo y en la región ventral del tálamo también se producen neuronas dopaminérgicas (Smidt and Burbach, 2007). Algunos de los grupos dopaminérgicos en el hipotálamo están

involucrados en el control neuroendócrino (Goridis and Rohrer, 2002). En regiones más posteriores, las neuronas dopaminérgicas mesencefálicas (DAm) conforman el principal núcleo productor de dopamina en el cerebro. Estas neuronas están organizadas en tres regiones: el área tegmental (AT), la substancia nigra pars compacta (SNc) y el campo retrorubral (Fig. 7). Las neuronas DAm presentes en la SNc inervan hacia el estriado dorsal formando la ruta nigroestriatal, involucrada en el control del movimiento voluntario (Abeliovich and Hammond, 2007). La degeneración de estas neuronas en el organismo adulto produce la enfermedad de Parkinson (Dauer and Przedborski, 2003). Las neuronas DAm del área tegmental forman parte del sistema mesolimbocortical donde regulan mecanismos cerebrales de recompensa, novedad y adicción (Abeliovich and Hammond, 2007). Finalmente, las DAm del campo retrorubral están involucradas en formar circuitos mesencefálicos locales regulando la actividad de la SNc (Abeliovich and Hammond, 2007).

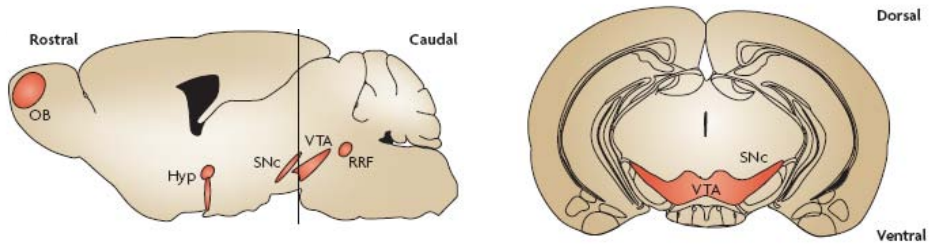

Fig. 7. Las neuronas dopaminérgicas en el cerebro adulto. El esquema izquierdo muestra un corte sagital del cerebro adulto donde se muestran en rojo las neuronas dopaminérgicas del bulbo olfatorio ("olfactory bulb"; OB), del hipotálamo ("Hypothalamus"; Hyp) y del cerebro medio, formado por la substancia nigra compacta (SNc), el área tegmental ventral ("ventral tegmental area"; VTA) y el campo retrorubral ("retrorubral field"; RRF). El esquema derecho muestra un corte coronal a nivel del cerebro medio (línea recta en dibujo izquierdo) donde se indica la distribución dorso-ventral de los dos principales núcleos dopaminérgicos mesencefálicos. Modificado de (Smidt and Burbach, 2007).

2.4.2 La especificación molecular del linaje dopaminérgico mesencefálico.

Diversos factores trancripcionales se han identificado como relevantes en diferentes etapas del proceso de diferenciación del linaje dopaminérgio mesencefálico (Fig. 8) (Ang, 2006). En etapas tempranas del desarrollo, los genes *Otx1/2* establecen la región prospectiva del cerebro anterior y medio (ver atrás). En la frontera del mesencéfalo-metencéfalo, la expresión de *Otx1/2* define las zonas en las que los morfógenos Shh y Fgf8 difunden y establecen diversos linajes neuronales, incluyendo el dopaminérgico

(Puelles et al., 2003). La ausencia de *Otx2* a niveles ventrolaterales del mesencéfalo provoca la difusión de Shh hacia regiones más dorsales, disminuyendo dramáticamente el número de neuronas DAm y del NR (Puelles et al., 2004). Esto es producto de la expansión ventral de *Nkx2.2*, el cual al co-expresarse con *Shh* induce la aparición ectópica de neuronas serotoninérgicas en el mesencéfalo (Puelles et al., 2004). Este efecto no es consecuencia de un incorrecto desarrollo del Istmo, ya que la deleción de *Otx2* exclusivamente en las células progenitoras Nestina^{+}, también redirige a las células mesencefálicas ventrales al linaje serotoninérgico (Vernay et al., 2005). Datos más recientes han demostrado que la sobre-expresión de *Otx2* promueve la proliferación de los progenitores DAm, mientras que la ausencia de *Otx2* induce una salida precoz del ciclo celular de estos progenitores (Omodei et al., 2008). En ausencia de *Otx2*, la expresión de determinantes del linaje dopaminérgico como *Lmx1a*, *Msx1* y los factores proneurales *Ngn2* y *Mash1* (ver adelante) no es activada en las células progenitoras (Omodei et al., 2008). Además, la expresión ectópica de *Otx2* en la placa del piso del metencéfalo, inicia la expresión de *Lmx1a* y de marcadores neuronales en esta región donde en condiciones normales no hay neurogénesis (Ono et al., 2007). Todas estas evidencias demuestran que *Otx2* controla la especificación y propagación del linaje dopaminérgico mesencefálico.

Lmx1a un miembro de la familia de factores transcripcionales con homeo-dominios LIM, especifica el linaje DAm. La expresión temprana de *Lmx1a* (E9.0) en la línea media ventral del mesencéfalo establece a la población de CPNs que eventualmente producirá a las neuronas DAm (Andersson et al., 2006b; Ono et al., 2007). Como la producción de Lmx1a se mantiene en las neuronas DAm, este factor transcripcional marca específicamente a todo el linaje dopaminérgico en el mesencéfalo ventral (Andersson et al., 2006b; Ang, 2006; Ono et al., 2007). La expresión de *Lmx1a* es inducida por Shh en explantes de mesencéfalo de forma concomitante a la supresión de Pax7, un marcador dorsal (Andersson et al., 2006b). El dominio de expresión de *Lmx1a* parece definir la zona DAm prospectiva al inhibir la expresión de *Nkx6.1* en la misma región (Andersson et al., 2006b). En el pollo, el bloqueo de la expresión de *Lmx1a* en la región ventral abate la producción de neuronas DAm, mientras que la expresión ectópica de *Lmx1a* en el mesencéfalo dorsal induce la formación de neuronas DAm (Andersson et al., 2006b). En el ratón, la mutación "*dreher J*" (drJ) se ha identificado como el cambio de una cisteína esencial para la función del "zinc finger" en el dominio LIM de *Lmx1a* (Millonig et al., 2000). Sin embargo, aún es controversial si

la mutación drJ es hipomórfica o nula (Chizhikov et al., 2006; Ono et al., 2007). En el mesencéfalo, la mutación drj ocasiona una reducción de alrededor del 30% en las neuronas DAm (Ono et al., 2007). Este efecto podría deberse a una inducción reducida en la expresión de los genes proneurales *Mash1* y *Ngn* (Ono et al., 2007). Además, la mutación drJ provoca la expresión aberrante de *Lim1/2* en la línea media ventral, indicando que Lmx1a es también necesario en el ratón para reprimir subtipos neuronales alternativos en esa región (Ono et al., 2007). El análisis de ratones nulos para *Lmx1a* permitirá esclarecer el papel de este gen en la especificación del linaje DAm.

Lmx1b, otro factor transcriptional con dominio LIM tiene una amplia expresión temprana en el mesencéfalo ventral y solo hasta E9.5 se restringe a la población dopaminérgica (Smidt et al., 2000). Mutantes nulos para *Lmx1b* presentan neuronas TH^+ hasta E16, aunque estas carecen de la expresión de *Ptx3* (Smidt et al., 2000). Datos más recientes indican que estos defectos se deben exclusivamente a la ausencia de *Lmx1b* en el Istmo y no en las neuronas DAm (Guo et al., 2008). Sin embargo, esta evidencia no es del todo concluyente porque en el fondo nulo para *Lmx1b* la expresión de este gen se restableció bajo el promotor de *Wnt1*, el cual se expresa en el Istmo pero también en la población DAm (Guo et al., 2008; Joksimovic et al., 2009). Interesantemente, la eliminación de *Wnt1* o *Lmx1b* en las células $Nestina^+$ no parece tener un efecto significativo en las neuronas DAm (Guo et al., 2008; Joksimovic et al., 2009). Estos datos indican que la expresión de *Wnt1* y *Lmx1b* en las neuronas DAm sería necesaria antes de E10.5/11.5 (Guo et al., 2008; Joksimovic et al., 2009). De forma similar a su función en el Istmo (Guo et al., 2007), Lmx1b podría ser responsable de la expresión de *Wnt1* en la población DAm. Datos adicionales son necesarios para dilucidar si Lmx1b tiene un papel autónomo en el linaje DAm, así como la redundancia funcional de ese gen con *Lmx1a*.

La expresión de *Msx1/2* en el mesencéfalo ventral se encuentra restringida a la población de CPNs que eventualmente producirá las neuronas DAm (Andersson et al., 2006b; Ono et al., 2007). La aparición de *Msx1/2* depende de la expresión de *Lmx1a*, aunque la expresión ectópica de *Msx1/2* en el mesencéfalo dorsal es insuficiente para inducir neuronas DAm (Andersson et al., 2006b). Al parecer la función de *Msx1/2* es reprimir transcripcionalmente a *Nkx6.1* en la región ventral, definiendo así un dominio permisivo para el desarrollo dopaminérgico (Andersson et al., 2006b). En sustento a estos datos, la mutación nula de *Msx1* produce una reducción del 40% en las neuronas DAm (Andersson et al., 2006b).

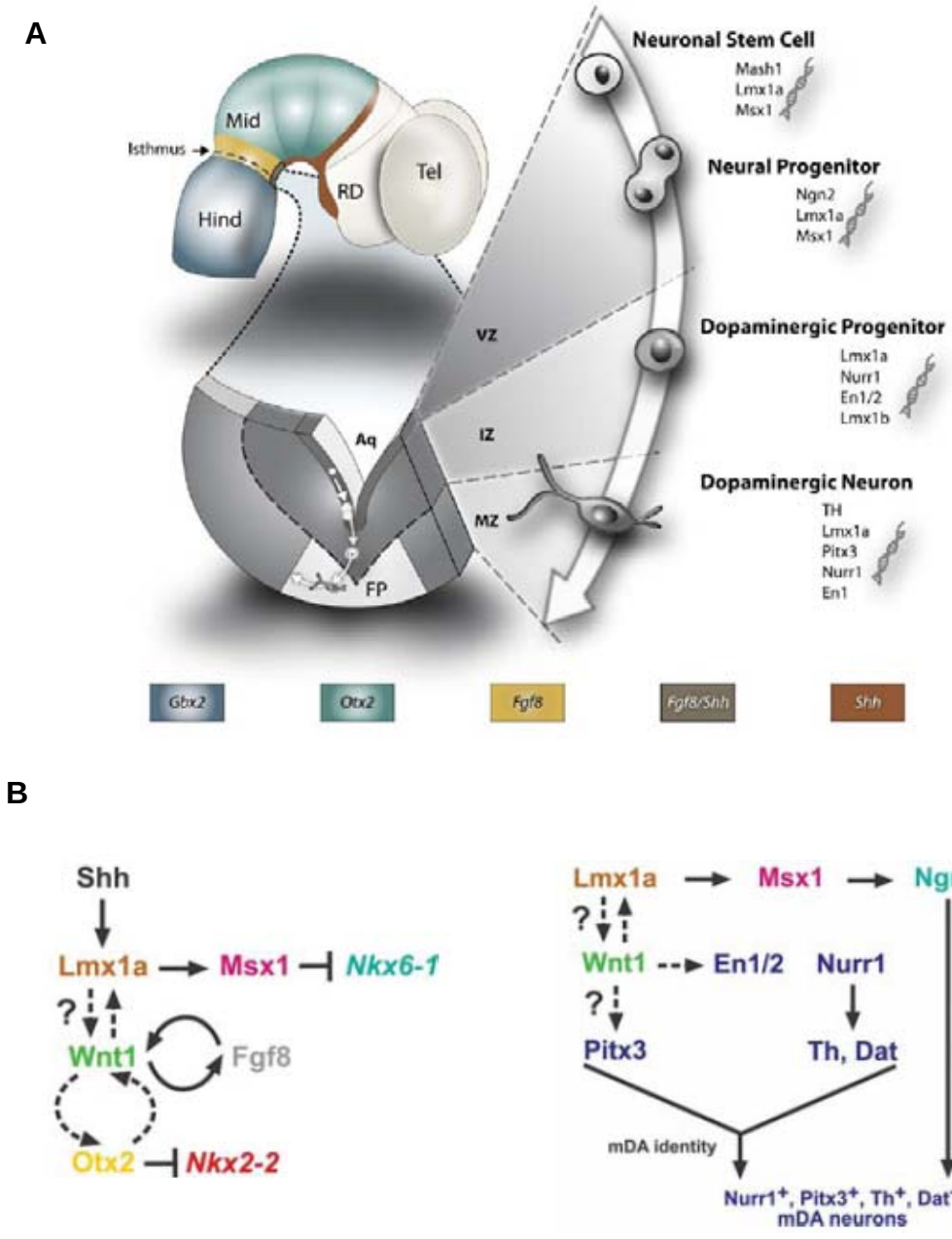

Fig. 8. El linaje celular y los determinantes moleculares de las neuronas dopaminérgicas mesencefálicas. (A) Vista lateral del tubo neural indicando el patrón de expresión de algunos genes. El sitio en el cual Shh y Fgf8 convergen (gris) cerca del Istmo (amarillo) es la región ventral del mesencéfalo donde se originan las neuronas dopaminérgicas. Las líneas punteadas delimitan la región del corte coronal donde se indican la zona ventricular ("ventricular zone"; VZ), la zona intermedia ("intermediate zone"; IZ) y la zona del manto ("mantle zone"; MZ) a lo largo del eje radial del mesencéfalo ventral. A la derecha se indican las poblaciones celulares que constituyen el linaje

dopaminérgico, así como algunos de los genes relevantes para determinar dichas poblaciones; la flecha indica la dirección de la migración y la diferenciación. Hind ("hindbrain"; cerebro posterior); Mid ("midbrain"; cerebro medio); RD ("rostral diencephalon"; diencéfalo rostral); Tel ("telencephalon"; telencéfalo); Aq ("aqueduct"; acueducto); FP ("floor plate"; placa del piso). Modificado de (Deierborg et al., 2008). **(B)** Interacciones entre factores transcripcionales y morfógenos que determinan la identidad de las neuronas DAm. Durante la especificación dopaminérgica (E9/10.5; diagrama izquierdo) Shh induce la expresión de *Lmx1a* en la línea media ventral. A su vez, Lmx1a regula positivamente a *Msx1*, el cual apaga la expresión de *Nkx6.1*, definiéndose así el dominio dopaminérgico. Wnt1 induce la expresión de *Lmx1a* y *Otx2*, además de ser necesario para la proliferación de los progenitores dopaminérgicos. Otx2 es requerido para regular negativamente la expresión de *Nkx2.2* en la línea media. Por otro lado, Fgf8 podría activar y mantener la expresión de *Wnt1*, tal como ocurre durante la especificación del territorio mesencefálico. Durante la neurogénesis dopaminérgica (E11/14.5; diagrama derecho), *Lmx1a* activa la expresión de *Msx1* y este a su vez la expresión del gen proneural *Ngn2*, confiriendo de esta forma potencial neurogénico a la placa del piso mesencefálica. Wnt1 podría además activar genes como *En1/2* y *Ptx3*. La activación de Nurr 1 en las neuronas DAm llevaría a la inducción de genes como *Th* y *Dat*. Ver detalles en el texto. Modificado de (Prakash and Wurst, 2006).

Marcadores de la placa del piso como FP4, Foxa2 y Shh tienen un dominio de expresión que traslapa con el de *Lmx1a* (Ono et al., 2007). Recientemente, experimentos de marcaje de linaje, demostraron que la mayor parte de las neuronas TH^+ en el mesencéfalo derivan de las células Shh^+ (Kittappa et al., 2007). Además, explantes mesencefálicos nulos para *Foxa2* no desarrollan neuronas DAm (Kittappa et al., 2007). En conjunto, estos datos evidenciaron que la población de neuronas DAm desciende directamente de la placa del piso. Este caso es particular, porque en otras regiones del cerebro embrionario, las células de la placa del piso no diferencian a neuronas (Ono et al., 2007). La actividad neurogénica de la placa del piso en el mesencéfalo es mediada por Neurogenina 2 (Ngn2), un factor proneural con dominios hélice-asa-hélice. La presencia de Ngn2 es requerida para la diferenciación y quizás para la maduración de las neuronas DAm (Andersson et al., 2006a; Kele et al., 2006). Además, aunque otro factor proneural conocido como Mash1 es dispensable en la generación de las neuronas DAm, puede compensar los defectos en este linaje neuronal ocasionados por la mutación nula de *Ngn2* (Andersson et al., 2006a; Kele et al., 2006).

Los represores transcripcionales *En1* y *En2* también están involucrados en el desarrollo de las neuronas mDA (Simon et al., 2003; Simon et al., 2004). En1 y En2 se encuentran en gran parte del linaje de DAm, aunque el primero tiene mayores niveles de expresión (Simon et al., 2001). Los dobles mutantes nulos para *En1* y *En2* muestran una

disminución inicial en el número de neuronas DAm, las cuales posteriormente mueren por apoptosis (Simon et al., 2001). Además, mutantes postnatales *En1+/- En2-/-* desarrollan síntomas de Parkinson como consecuencia de la degeneración progresiva de las neuronas de la SNc (Sgado et al., 2006). Estos datos demuestran el papel de *En1* y *En2* en la especificación y en el mantenimiento de las neuronas DAm. La función de los genes *En1/2* se requiere de forma autónoma en las neuronas DAm (Alberi et al., 2004).

Finalmente, los factores transcriptionales Nurr1 y Ptx3 actúan en etapas más tardías de la diferenciación de las neuronas DAm. Nurr1 es un receptor nuclear huérfano (no se ha identificado un ligando) necesario para la diferenciación y el mantenimiento de las neuronas DAm (Saucedo-Cardenas et al., 1998). Nurr1 actúa como activador transcripcional directo de la *Th*, del transportador vesicular de monoaminas 2 ("vesicular monoamine transporter 2" o vmat2), del transportador de dopamina DAT ("dopamine transporter" o DAT) y de Ret (el receptor del "glial-derived neurotrophic factor" o GDNF) (Smits et al., 2003; Wallen et al., 2001). Por otro lado, el factor transcripcional Ptx3 también se encuentra en todas las neuronas DAm (Smidt et al., 2000; Smidt et al., 1997). Ratones "aphakia" nulos para *Ptx3* muestran una disminución de las neuronas DAm de la SNc; demostrando que *Ptx3* tiene un papel en el mantenimiento de este linaje (Hwang et al., 2003; Nunes et al., 2003). Actualmente, se desconoce la razón por la cual la SNc es más susceptible que el AT a la pérdida de *Ptx3* (Smidt and Burbach, 2007). Ptx3 es necesario en las neuronas DAm para la activación transcripcional de la aldehído deshidrogenada 1 (Aldh1a1) una enzima involucrada en la síntesis de ácido retinoico (AR) (Jacobs et al., 2007). El restablecimiento de la señal del AR incrementa el número de neuronas TH^+ en ratones nulos para *Ptx3* (Jacobs et al., 2007). Ptx3 es también necesario para activar la transcripción de genes regulados por Nurr1 (Jacobs et al., 2009). El mecanismo mediado por Ptx3 involucra la disociación de Nurr1 de su represor SMRT, permitiendo que el primero pueda activar a sus genes blancos (Jacobs et al., 2009).

2.4.3 Factores solubles involucrados en el desarrollo de las neuronas dopaminérgicas mesencefálicas.

Estudios con explantes de la placa neural han demostrado que Shh, secretado por la placa del piso y Fgf8, secretado por el organizador del Istmo, especifican a las neuronas DAm en el eje dorso-ventral y antero-posterior del mesencéfalo, respectivamente (Hynes et al., 1995; Hynes and Rosenthal, 1999; Ye et al., 1998). Shh y Fgf8 pueden

inducir la expresión de genes relevantes para el desarrollo del linaje DAm. Por ejemplo, Shh es capaz de establecer ectópicamente la expresión de *Lmx1a* y *Nurr1* (Andersson et al., 2006b) mientras que Fgf8 induce la expresión de los genes *En* en la región del Istmo (Crossley et al., 1996; Liu and Joyner, 2001; Martinez et al., 1999). Sin embargo, aún se desconoce la función de Fgf8 específicamente en el linaje DAm.

Estudios de ganancia y pérdida de función han implicado a Wnt1 en el desarrollo de las neuronas DAm (Prakash et al., 2006). Estudios de marcaje de linaje indican que gran parte de la población DAm deriva de células que en etapas tempranas expresaron *Wnt1* (Zervas et al., 2004). En esta etapa (alrededor de E9.5) Wnt1 mantiene la expresión de *Otx2*, evitando indirectamente una expansión del dominio de expresión de *Nkx2.2*, lo que llevaría a su vez a una pérdida del dominio de expresión de *Wnt1* y de la población DAm (Prakash et al., 2006). En etapas más tardías (E10.5/11.5), Wnt1 antagoniza la señal de Shh en la placa del piso mesencefálica, permitiendo la proliferación y neurogénesis de los progenitores DAm de forma autócrina (Joksimovic et al., 2009). La pérdida de *Wnt1* resulta en la producción de pocas neuronas DAm que no expresan *Ptx3*, *Lmx1a* y *Otx1/2* (Joksimovic et al., 2009; Prakash et al., 2006). Además, la inducción ectópica de neuronas DAm por Shh y Fgf8 requiere la presencia de la señal de Wnt1 (Prakash et al., 2006). A su vez, Wnt5a, el cual señaliza por la vía no canónica de Wnt (independiente de la beta-catenina) es también necesario para la diferenciación dopaminérgica (Castelo-Branco et al., 2003).

Finalmente, las moléculas "Transforming growth factors" (TGF) α y β, producidos en la placa del piso, son necesarios para el desarrollo de las neuronas DAm en murinos y en aves, quizás a través de un efecto mitogénico o de supervivencia sobre esta población (Blum, 1998; Farkas et al., 2003).

3. Las Células Precursoras Neurales durante el desarrollo del tubo neural y en el cerebro adulto.

3.1 Propiedades de las Células Troncales Neurales.

Las Células Troncales Neurales (CTNs) son aquellas que presentan elevada capacidad para autorenovarse a largo plazo y mantener la multipotencia, es decir, la habilidad para generar los tres principales linajes neurales: neuronas, astrocitos y oligodendrocitos (Fig. 9). Al inicio del desarrollo, las CTNs mantienen una alta tasa proliferativa al acortar la duración de su ciclo celular (Gotz and Huttner, 2005). En la región ventricular, las divisiones simétricas proliferativas producirán dos CTNs hijas, mientras

que las divisiones asimétricas diferenciativas generarán una CTN y un progenitor neuronal (Gotz and Huttner, 2005). Las células comprometidas al linaje neuronal mantienen una baja o nula tasa proliferativa en virtud de una mayor duración de su ciclo celular (Gotz and Huttner, 2005). Estas células migrarán fuera de la región ventricular mientras diferencian terminalmente a neuronas (Gotz and Huttner, 2005). Al final de la etapa neurogénica en el cerebro, las CTNs comienzan a generar glioblastos, que son progenitores comprometidos a linajes gliales (astrocitos y oligodendrocitos). Este proceso de especificación neuronal y glial a partir de precursores multipotentes se ha observado *in vitro* en tiempo real (Ravin et al., 2008). Durante el proceso de especificación y diferenciación, las CTNs multipotentes son capaces de producir neuronas, astrocitos y oligodendrocitos directamente o através de su progenie comprendida por células progenitoras bipotentes y unipotentes (Fig. 9) (Ravin et al., 2008). De forma interesante, el estado multipotente parece restablecerse en muchas células durante el subcultivo, sugiriendo la presencia de un "programa" interno de especificación y no un compromiso a cierto linaje (Ravin et al., 2008). Esto ha llevado a la propuesta de un "modelo secuencial" donde el mismo grupo de CTNs hace un cambio de potencial neurogénico a gliogénico (Qian et al., 1998; Qian et al., 2000). Sin embargo, datos recientes en el diencéfalo indican que para los progenitores que expresan la proteína proteolipídica (PLP), existe una segregación temprana (E9.5) de los progenitores neuronales y gliales (Delaunay et al., 2008). Al conjunto de todas las poblaciones de células indiferenciadas en el sistema nervioso, se le conoce como Células Precursoras Neurales (CPNs; Fig. 9).

3.2 El desarrollo de las CPNs: desde la placa neural hasta los nichos neurogénicos del cerebro adulto.

Las primeras CPNs aparecen durante la inducción de la placa neural. Estas células neuroepiteliales elongadas expresan marcadores como *Sox1/2* y mantienen contacto con la superficie ventricular y con la capa pial (Fig. 5) (Merkle and Alvarez-Buylla, 2006). Previo al inicio de la neurogenesis, estas CPNs generan progenitores proliferantes que comienzan a engrosar el neuroepitelio y adquieren la expresión de *Nestina*, una proteína de filamentos intermedio (Lendahl et al., 1990). Las CPNs además producen una proteína de unión a Ácido Ribonucleico conocida como Musashi (codificada por los genes *Msi1* y *Msi2*), necesaria para la proliferación y el mantenimiento de las CPNs (Sakakibara et al., 2002). El efecto de Musashi depende de su capacidad para activar la

vía de señalización de Notch, responsable de mantener la autorenovación de las CPNs (ver abajo) (Okano et al., 2005).

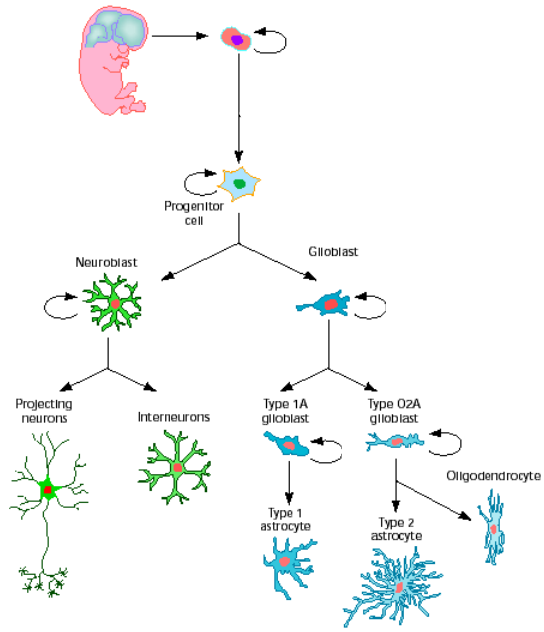

Fig. 9. El linaje de las Células Troncales Neurales. En la parte superior de la jerarquía se encuentra la célula troncal neural, la cual presenta la capacidad de autorenovarse a largo plazo. Estas células se dividen para dar origen a progenitores transitorios amplificantes con elevada capacidad proliferativa, los cuales a su vez generan progenitores comprometidos al linaje neuronal (neuroblastos) y glial (glioblastos). Los neuroblastos pueden diferenciar a neuronas de proyección o interneuronas, mientras que los glioblastos generan progenitores de astrocitos tipo 1, 2 y oligodendrocitos. Las flechas circulares indican las poblaciones capaces de autorenovarse. Modificado de (Zigova and Sanberg, 1998).

Al entrar en mitosis, las CPNs acercan su núcleo a la superficie ventricular, mientras que en la etapa de interfase el núcleo se aleja hacia la capa pial (Gotz and Huttner, 2005). Durante el inicio de la neurogénesis, las CPNs adquieren la identidad de Glia Radial (GR) alrededor de E9/10 (Misson et al., 1988). En similitud con las CPNs neuroepiteliales, las células de la GR también son alargadas y contactan la superficie ventricular y pial (Fig. 4). Además la GR expresa *Nestina* así como sus modificaciones post-traduccionales reconocidas por los anticuerpos RC1 y RC2 (Gotz and Barde, 2005; Merkle and Alvarez-Buylla, 2006). Aunque aún no ha sido confirmado experimentalmente, es esperado que la GR descienda directamente de las CPNs neuroepiteliales tempranas (Merkle and Alvarez-Buylla, 2006). Sin embargo, a diferencia de las CPNs neuroepiteliales, la GR expresa marcadores astrocíticos, tales como el transportador de glutamato específico de astrocitos (“Astrocyte-Specific

Glutamate Transporter" o GLAST), S100β, la glutamina sintetasa, la vimentina, la tenascina C y, en algunas especies como el humano, la GFAP ("Glial Fibrilary Acidic Protein") (Campbell and Gotz, 2002; Gotz and Barde, 2005). Estudios de marcaje de linaje *in vivo* han demostrado que la GR contribuye a la producción de nuevas neuronas en todas las regiones del cerebro durante la neurogénesis embrionaria (Anthony et al., 2004). La GR funciona además como una estructura de andamiaje para la migración neuronal hacia la capa pial durante etapas avanzadas del desarrollo, cuando el neuroepitelio es muy grueso (Fig. 4) (Nadarajah and Parnavelas, 2002). Posteriormente, desde el final de la neurogénesis embrionaria hasta la etapa postnatal, la GR produce oligodendrocitos y astrocitos (Anthony et al., 2004; Malatesta et al., 2003). Eventualmente, la GR producirá los astrocitos que representan la población de CTNs de la región subventricular del cerebro adulto (ver adelante) (Merkle et al., 2004).

Las CPNs en la región ventricular adquieren paulatinamente la expresión de un código de factores transcripcionales de acuerdo a su ubicación en los ejes dorso-ventral y antero-posterior del tubo neural. Estos factores transcripcionales con homeodominios determinan el subtipo neuronal y glial que se producirá durante la diferenciación (Briscoe et al., 2000; Hochstim et al., 2008; Zhou and Anderson, 2002). De esta manera, por ejemplo, se determina que en la parte dorsal del tubo neural se encuentren las neuronas sensoriales y que las neuronas motoras se especifiquen ventralmente (Lupo et al., 2006). En el caso del linaje glial, los oligodendrocitos aparecen en la región media ventral, mientras que los astrocitos se desarrollan principalmente en regiones más dorsales (Zhou and Anderson, 2002).

Las CPNs generan una enorme diversidad de subtipos neuronales y gliales en respuesta a una integración espacio-temporal de múltiples señales presentes en su nicho. Durante el proceso de diferenciación, las CPNs abandonan el nicho que las mantiene indiferenciadas y proliferando. Ese nicho está presente en la zona ventricular del tubo neural en el embrión y en la región subventricular en el cerebro adulto (Merkle and Alvarez-Buylla, 2006). Diversos estudios con ratones mutantes han demostrado que la señalización mediada por la familia de receptores Notch, principalmente Notch1, es necesaria para mantener el nicho de las CPNs (Mizutani et al., 2007). Al unirse a sus ligandos Delta y Jagged, la fracción intracitoplásmica de los receptores Notch sufre un corte proteolítico, mediado por la presenilina, transformándolo en NICD ("Notch Intracitoplasmic Domain") (Fig. 10). Al desplazarse al núcleo, NICD convierte el complejo represor CBF1 en un activador transcripcional (Fig. 10). El complejo NICD-

CBF1 induce la expresión de *Hes* y *Herp*, los cuales regulan negativamente genes proneurales como *Mash1* y las neurogeninas, involucrados en la diferenciación neuronal (Fig. 10). La actividad transcripcional de CBF1 es alta en las CTNs pero esta muy atenuada en las células progenitoras intermediarias que están más cerca de la diferenciación neuronal terminal (Mizutani et al., 2007). La pérdida de miembros de la señalización de Notch, incluyendo receptores, ligandos, moduladores y efectores, resulta en una neurogénesis precoz en el tubo neural, acompañada por una disminución de la población de CPNs (Bray, 2006; Yoon and Gaiano, 2005). En etapas más tardías, la señalización de Notch participa en la instrucción de las CPNs hacia el linaje glial (Lundkvist and Lendahl, 2001).

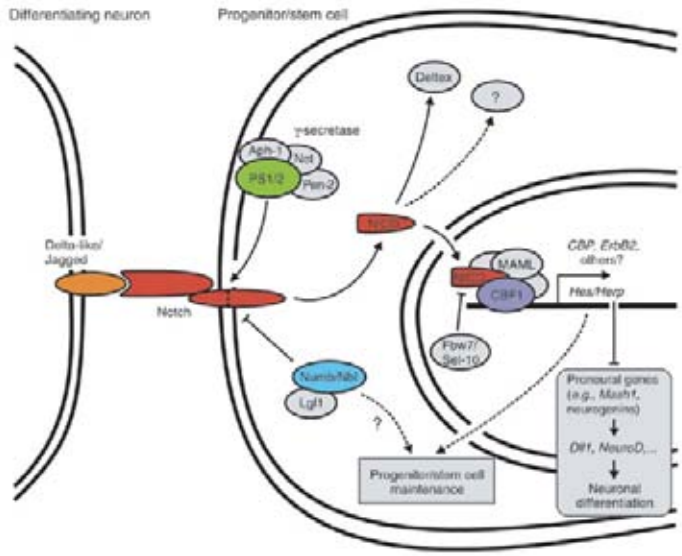

Fig. 10. La vía de señalización de Notch en mamíferos. En el sistema nervioso, Notch se encuentra presente en las CPNs, mientras que sus ligandos Dll ("Delta-like") y Jagged, se encuentran en las neuronas en diferenciación. La interacción de Notch con sus ligandos activa al complejo de la γ-secretasa, el cual realiza un corte proteolítico que libera el dominio intracelular de Notch (NICD; "notch intracelular domain"). NICD se transloca al núcleo donde interactúa con un complejo transcripcional que incluye a RBP-J/CBF1 y MAML, convirtiendo la actividad de este complejo de represora a activadora. De esta forma, se activa la transcripción de genes como *Hes*/*Herp* que antagonizan a su vez la expresión de genes proneurales como *Mash1* y las neurogeninas. Este mecanimo bloquea la expresión de genes neuronales (*Dll1*, *NeuroD*) y por lo tanto se inhibe la diferenciación. Por otro lado, Numb y Deltex parecen actuar como reguladores negativos de Notch en mamíferos, al promover su endocitosis y ubiquitinación, respectivamente (Yoon and Gaiano, 2005).

3.3 La neurogénesis en la región subventricular y el hipocampo del cerebro adulto de los mamíferos.

La neurogenesis en el cerebro adulto murino y humano continúa en algunos nichos debido a la presencia de las CTNs. Estos nichos se encuentran principalmente en la región subventricular (RSV) del ventrículo lateral anterior (Fig. 11) y en la región subgranular (RSG) del giro dentado (GD) del hipocampo (Alvarez-Buylla and Garcia-Verdugo, 2002; Zhao et al., 2008). La RSV en el cerebro adulto se encuentra subyacente a una delgada capa de células ependimales que forman la pared del ventrículo. Inicialmente, estas células fueron consideradas la población de CTNs en el cerebro adulto (Johansson et al., 1999). Aunque esta conclusión ha sido controversial (Spassky et al., 2005), evidencias recientes han confirmado que las células ependimales $CD133^{+}$ se comportan como CTNs, aunque mantienen un estado más quiescente que las CTNs en la RSV (Coskun et al., 2008). Al parecer, las CTNs de la RSV así como las del ependima, proliferan y dan origen a nuevas neuronas en el BO en condiciones normales (Fig. 11) (Coskun et al., 2008; Zhao et al., 2008). Aún se desconoce si estas dos poblaciones de CTNs tienen alguna relación de linaje (Nakafuku et al., 2008). En condiciones de daño, las células ependimales parecen ser más susceptibles de abandonar su estado quiescente para diferenciar a neuronas del BO y astrocitos (Carlen et al., 2009). Este proceso requiere la regulación negativa de la vía de señalización de Notch en las células ependimales (Carlen et al., 2009).

Las nuevas neuronas producidas en la RSV y el ependima migran a través de la corriente migratoria rostral (CMR) hasta el bulbo olfatorio (BO), donde diferencian terminalmente a neuronas granulares y periglomerulares (Fig. 11). En el hipocampo, las nuevas neuronas producidas en la RSG migran hacia la capa granular del GD donde terminan de diferenciar a neuronas granulares dentadas (Alvarez-Buylla and Garcia-Verdugo, 2002; Zhao et al., 2008). Diversos estudios han demostrado que las neuronas derivadas de la RSV y de la RSG se integran funcionalmente a los circuitos neuronales locales (Lledo and Saghatelyan, 2005; van Praag et al., 2002). Hasta el momento, diversas evidencias correlativas indican que además de una integración funcional, las nuevas neuronas participan en la discriminación de olores en el BO y en eventos de aprendizaje y memoria en el GD (Zhao et al., 2008).

3.3.1 La organización celular en la región subventricular en el cerebro adulto.

Tres poblaciones se han caracterizado en la RSV: las células tipo B que son la población de CTNs, las células tipo C formada por progenitores con elevada capacidad proliferativa y la célula tipo A representada por neuroblastos migratorios (Fig. 11)

(Zhao et al., 2008). La CTN o célula tipo B tiene múltiples características astrocíticas, tales como la expresión de *Gfap* (Doetsch et al., 1999). Aunque la mayoría de estos estudios se han realizado en el ratón, las CTNs en el cerebro humano adulto también tienen identidad astrocítica (Sanai et al., 2004). Las células tipo B constituyen la única población subventricular que puede restablecer la neurogénesis en el BO después de tratamientos cortos con AraC, los cuales eliminan a las células en mitosis (Doetsch et al., 1999). Esta propiedad demuestra el estado quiescente de la célula tipo B y su capacidad para autorenovarse a largo plazo y generar nuevas células tipo A y C (Doetsch et al., 1999). A su vez, la célula tipo B en la RSV no expresa CD133, un marcador de superficie de las células ependimales (Pfenninger et al., 2007).

Las CPNs presentes en la RSV derivan de diferentes poblaciones embrionarias (Lledo et al., 2008). La parte más dorsal de la RSV tiene como origen a las células $Emx1^+$ de la corteza embrionaria, mientras que la parte lateral de la RSV deriva principalmente de las células $Gsh2^+$ de la Eminencia Gangliónica Lateral (EGL) y Media (EGM) (Young et al., 2007). El análisis de la contribución de estas CTNs a poblaciones neuronales del BO reveló que la mayoría de las neuronas periglomerulares $calretinina^+$ derivan de las células de la corteza $Emx1^+$, mientras que las interneuronas $calbindina^+$ se originan principalmente de la población $Gsh2^+$ (Merkle et al., 2007; Young et al., 2007). En contraste, las interneuronas dopaminérgicas periglomerulares se originan a partir de las poblaciones $Emx1^+$ y $Gsh2^+$ (Willaime-Morawek and van der Kooy, 2008; Young et al., 2007). Estudios de transplante y de cultivos celulares demostraron que las CPNs de la RSV producen los mismos linajes neuronales de su región de origen al ser ubicadas en otras zonas de la RSV o al ser diferenciadas *in vitro* (Merkle et al., 2007). Estos datos sugieren que las CTNs a lo largo de la RSV estan comprometidas a ciertos linajes y por tanto son resistentes a la re-especificación.

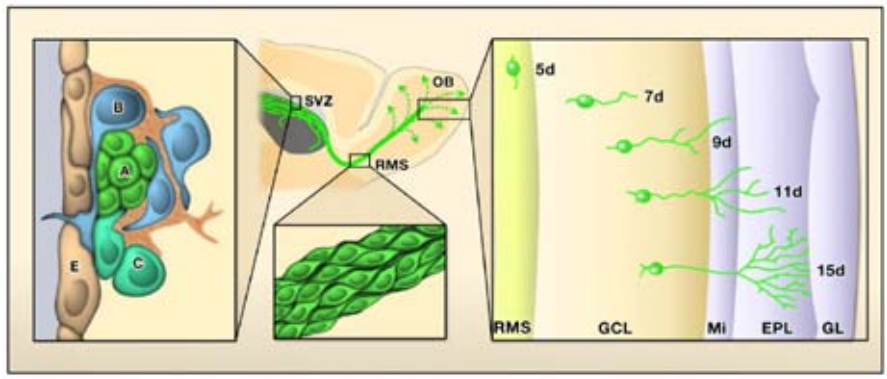

Fig. 11. Neurogenesis en la región subventricular anterior del cerebro adulto. A la izquierda se muestra la organización celular de la región subventricular. Las células ependimales (E) recubren la pared ventricular y están íntimamente asociadas con las células astrocíticas tipo B; juntas representan la población de células troncales en el cerebro adulto. Las células tipo B dan origen a las células progenitoras tipo C que se dividen rápidamente y generan neuroblastos o células tipo A comprometidas al linaje neuronal. En la imagen central se muestra un corte sagital del cerebro adulto donde se indica la ruta migratoria en cadena (recuadro) desde el ependima y la región subventricular (SVZ) a través de la corriente migratoria rostral (RMS) hasta alcanzar el bulbo olfatorio (OB) y diferenciar progresivamente a neuronas (recuadro derecho). GCL, capa celular granular; Mi, capa celular de la mitra; EPL, capa plexiforme externa; GL, capa glomerular. Modificado de (Zhao et al., 2008).

3.4 Evidencias sobre posible neurogénesis en el cerebro medio adulto.

Además de la RSV y el hipocampo, diversos reportes se han publicado sobre la generación de nuevas neuronas en la neocorteza, el estriado, el hipotálamo, la amígdala y la SNc del cerebro medio (Gould, 2007). Debido a que en estas zonas la neurogénesis es poco abundante, estos reportes son aún controversiales, ya que en gran parte dependen de la sensibilidad del método empleado para evaluar la generación de novo de las neuronas (Gould, 2007).

En el caso del cerebro medio adulto, estudios iniciales demostraron que en esta región existen CPNs que producen exclusivamente células gliales in situ, aunque presentan capacidad neurogénica al ser aisladas y diferenciadas *in vitro* (Lie et al., 2002). Estos datos apuntaban a que podría ser el nicho adecuado de diferenciación dopaminérgica, más que una población de CPNs, lo que estaba ausente en el cerebro adulto. Sin embargo, estudios posteriores reportaron que la neurogénesis dopaminérgica continuaba en el cerebro adulto (Zhao et al., 2003). La tasa de recambio estimada permitiría una renovación completa de la población de neuronas DA durante la vida adulta del ratón (Zhao et al., 2003). Usando la misma estrategia experimental, estos hallazgos no fueron observados por otros grupos (Cooper and Isacson, 2004; Frielingsdorf et al., 2004), manteniéndose la controversia sobre la existencia de neurogénesis dopaminérgica en la SNc del adulto. Por otro lado, recientemente se demostró que la salamandra adulta es capaz de regenerar toda la población dopaminérgica después del daño con 6-hidroxidopamina (Parish et al., 2007). Estos hallazgos en otros organismos modelos podrían facilitar la identificación del nicho dopaminérgico adecuado para la regeneración en mamíferos adultos.

4. Las Células Precursoras Neurales en cultivo.

4.1 La expansión in vitro por formación de neuroesferas.

Diversos sistemas de cultivo permiten la expansión de las CPNs del embrión y del cerebro adulto. Uno de los ensayos más comunes es la formación de neuroesferas, las cuales son agregados celulares enriquecidos en CPNs (Fig. 12). Este procedimiento consiste en la disociación del tejido nervioso en una suspensión celular, la cual se expone a los mitógenos Fgf2 y Egf ("Epidermal Growth Factor"). La proporción de células generadoras de neuroesferas es baja en el tejido nervioso, siendo alrededor de 1% en el embrión (Reynolds and Weiss, 1996b). Sin embargo, está población se enriquece durante la formación de las neuroesferas primarias, las cuales una vez disociadas y vueltas a sembrar muestran que hasta el 20% de esa población forma neuroesferas secundarias (Reynolds and Weiss, 1996b). Se ha propuesto que la capacidad para generar neuroesferas por varios pases en cultivo (>10) permite distingir a las CTNs de su progenie proliferativa (Golmohammadi et al., 2008; Louis et al., 2008). En condiciones adherentes y ausencia de los mitógenos, las neuroesferas pueden inducirse a diferenciar hacia neuronas, astrocitos y oligodendrocitos, demostrando la expansión *in vitro* de una población multipotente (Fig. 12) (Reynolds and Weiss, 1996b). Entre las poblaciones con capacidad para generar neuroesferas se encuentran las CPNs neuroepiteliales, la glia radial y las CPNs adultas de la RSV (ver Figura 1, publicación 3).

La expansión *in vitro* de las CPNs del cerebro adulto se ha realizado principalmente mediante la formación de neuroesferas o creciendo a las CPNs en monocapa. Este último sistema de cultivo se ha utilizado principalmente para expandir progenitores del hipocampo, mientras que las neuroesferas se han logrado obtener de la RSV, el estriado, el hipocampo, el cerebro medio y la médula espinal (Baizabal et al., 2003).

Un análisis detallado de las propiedades de las CPNs obtenidas del giro dentado del hipocampo sugirió que esta población no estaba compuesta por CTNs, ya que presentaban una capacidad de autorenovación limitada en cultivo (Bull and Bartlett, 2005; Seaberg and van der Kooy, 2002). En contraste, las neuroesferas generadas de CTNs se propagan a partir de la pared lateral del ventrículo que rodea al hipocampo (Bull and Bartlett, 2005; Seaberg and van der Kooy, 2002). Adicionalmente, la administración de Fgf2 en cultivos organotípicos del hipocampo induce una respuesta neurogénica corta en el GD y prolongada en la región periventricular (Chechneva et al.,

2005). En conjunto, estas evidencias sugieren que las CTNs que residen cerca del ventrículo generarían los progenitores más comprometidos del giro dentado del hipocampo. En contra de este modelo, el marcaje del linaje de las CPNs $Sox2^+$ en el GD demostró que algunas de estas células tienen la capacidad de autorenovarse y mantenerse multipotentes *in vivo* (Suh et al., 2007). En cultivo, las células $Sox2^+$ son capaces de expandirse hasta por 30 pases en forma de monocapa celular (Suh et al., 2007). Por lo tanto, existe la posibilidad que las CTNs $Sox2^+$ en el GD sea un reservorio derivado de las células periventriculares.

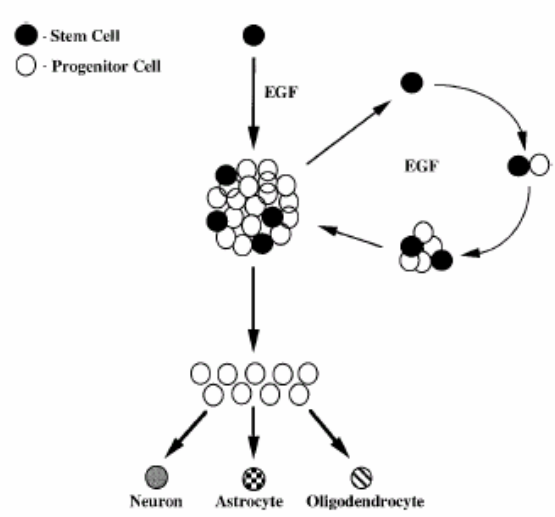

Fig. 12. Expansión *in vitro* de las Células Precursoras Neurales. Las Células Precursoras Neurales con elevada capacidad proliferativa (círculos negros) se expanden *in vitro* en presencia de mitógenos como Egf y Fgf2 (no mostrado) mediante la formación de agregados celulares conocidos como neuroesferas (centro). Durante la formación de la neuroesfera, las células proliferantes generan progenitores más comprometidos con limitada capacidad proliferativa (círculos blancos). En condiciones de diferenciación, estas células generan los tres principales linajes neurales: neuronal, astrocítico y oligodendrocítico (abajo). Al disociar las "neuroesferas primarias" y resembrar en cultivo, las células proliferantes pueden producir "neuroesferas secundarias" en presencia de los mitógenos (lateral). Modificado de (Reynolds and Weiss, 1996b).

4.1.1 La especificación y diferenciación de las CPNs expandidas como neuroesferas.

La identidad posicional de una CPN está definida por el código de factores transcripcionales que expresa esa célula en relación con su ubicación en el tubo neural. Algunos estudios han demostrado que las CPNs de neuroesferas murinas y humanas mantienen solo parcialmente la identidad posicional de su región de origen. Las neuroesferas murinas derivadas de la corteza, EGM, mesencéfalo y cerebro posterior, expresan los genes de su región de origen *Emx1*, *Dlx2*, *En1* y *Hoxb1*, respectivamente

(Hitoshi et al., 2002). A su vez, las neuroesferas humanas generadas de diferentes niveles antero-posteriores del cerebro, retienen ciertos marcadores posicionales, aunque la expresión de estos decrementa significativamente con el número de pases en cultivo (Kim et al., 2006b). Esta especificación regional se mantiene durante la etapa de diferenciación; así, las neuroesferas obtenidas de la EGM y EGL producen neuronas que expresan *Meis2*, *Pbx* y *Dlx*; marcadores específicos de estas regiones (Parmar et al., 2002; Skogh et al., 2001). Además, las neuronas Nkx2.1^{+} derivaron exclusivamente de neuroesferas de la EGM pero no de la EGL, de acuerdo con la restricción espacial de este gen *in vivo* (Parmar et al., 2002). Solo algunos genes posicionales se mantienen en cultivo, por ejemplo, la expresión de *Dlx1*, *Dlx5* y *Vax1* se regula negativamente en neuroesferas derivadas de la EGL y EGM (Parmar et al., 2002). De forma similar, *HoxB9* y *Hoxb1* no se expresan en neuroesferas del cerebro posterior y medula espinal, mientras que el marcador dorsal *Dbx1* no se detecta en neuroesferas de ninguna región antero-posterior (Santa-Olalla et al., 2003b). La expansión *in vitro* de las CPNs también puede promover la activación transcripcional "ectópica" de algunos genes. De esta forma, las neuroesferas obtenidas a lo largo del eje antero-posterior del tubo neural, expresan *Emx2*, *En2* y *Krox20*, los cuales *in vivo* se localizan exclusivamente en el telencéfalo, mesencéfalo-metencéfalo y mielencéfalo, respectivamente (Hitoshi et al., 2002; Santa-Olalla et al., 2003b). En conjunto estos datos indican que señales del entorno en el tubo neural son necesarias para preservar completamente la identidad de las CPNs.

La expresión de algunos genes de identidad en las CPNs puede cambiarse al colocar las neuroesferas en rebanadas del cerebro embrionario. Por ejemplo, las neuroesferas mesencefálicas adquieren la expresión de *Dlx2* cuando son colocadas sobre rebanadas de la EGM, mientras que las neuroesferas humanas aumentan la expresión de *En1* al colocarse en rebanadas del cerebro medio (Hitoshi et al., 2002; Kim et al., 2006b). Estos datos sugieren que las neuroesferas mantienen cierto grado de plasticidad que les permite cambiar su identidad en presencia de señales extrínsecas. No obstante, la tinción con beta-galactosidasa y la técnica de RT-PCR ("Reverse Transcriptase Polymerase Chain Reaction") no permitieron analizar a nivel celular la supuesta re-especificación posicional (Hitoshi et al., 2002; Kim et al., 2006b), así que resultados más convincentes y cuantitativos son necesarios en este campo.

Las CPNs de las neuroesferas se mantienen multipotentes durante muchos pases en cultivo. En condiciones de diferenciación, la mayoría de estas células genera

astrocitos (50-80%) y solo unas pocas producen neuronas (5-20%) u oligodendrocitos (2-15%) (Grandbarbe et al., 2003a; Ostenfeld et al., 2002; Reynolds and Weiss, 1996a). Dependiendo de su región de origen, las neuroesferas generan diferentes proporciones de neuronas, siendo las corticales las más neurogénicas (Ostenfeld et al., 2002).

La diferenciación dirigida de las neuroesferas hacia subtipos neuronales específicos ha sido poco exitosa hasta el momento. Por ejemplo, las neuroesferas mesencefálicas humanas producen solo 1.2% de células TH^+ en condiciones de diferenciación (Horiguchi et al., 2004). Parte del problema radica indirectamente en la poca capacidad neurogénica de las neuroesferas (Horiguchi et al., 2004). Además, las pocas neuronas derivadas de neuroesferas no se especifican eficientemente en repuesta a la exposición a morfógenos *in vitro*. En el caso de la diferenciación hacia neuronas dopaminérgicas, la exposición de neuroesferas telencefálicas a Fgf2 y medio condicionado de una línea atrocítica, resulta en la generación de solo 5% de neuronas TH^+ (Daadi and Weiss, 1999). Estas neuronas además co-expresan el neurotransmisor GABA (ácido gamma-aminobutírico), indicando que no representan neuronas DAm, sino del cerebro anterior (Daadi and Weiss, 1999). En otro estudio, Wnt5a aumentó solo ligeramente la proporción de neuronas mDA derivadas de las neuroesferas (Parish et al., 2008). Tampoco la línea estromal PA6 tuvo un efecto en la diferenciación dopaminérgica de las neuroesferas (Roybon et al., 2005). Esto resulta particularmente interesante, porque la línea PA6 puede sustituir el efecto de Shh y Fgf8 al dirigir la diferenciación dopaminérgica de las Células Troncales Embriónicas (ver adelante) (Kim et al., 2006a). La exposición de neuroesferas mesencefálicas a TGFβ parece tener un efecto significativo en la inducción dopaminérgica (Roussa et al., 2006). Sin embargo, como en este estudio se emplearon neuroesferas primarias, no puede descartarse que el TGFβ afecte a progenitores dopaminérgicos comprometidos, los cuales se eliminarían al generar neuroesferas secundarias. Debido a que el entorno en cultivo difiere del nicho de las CPNs *in vivo*, existe la posibilidad que las neuroesferas mantengan la capacidad de interpretar adecuadamente las señales de diferenciación en el tubo neural. Para resolver este problema, es necesario un análisis detallado del potencial neurogénico de las neuroesferas al integrarse en entornos naturales *in vivo* o en cultivos de tejido.

La manipulación genética ofrece la posibilidad de modificar intrínsicamente el destino de la célula. En el caso de las neuroesferas, la sobre-expresión de factores de diferenciación dopaminérgica como *Lmx1a, Msx1/2, Ngn2,* y *Ptx3,* no aumentó la

generación de este linaje neuronal a partir de CPNs murinas, mientras que tuvo un efecto poco significativo en las CPNs humanas (Roybon et al., 2008). Por otro lado, neuroesferas adultas obtenidas de la materia blanca o de la RSV y que sobre-expresan *Nurr1*, muestran un aumento en su capacidad para producir neuronas dopaminérgicas (Shim et al., 2007). Sin embargo, en este estudio no se demostró que las neuronas dopaminérgicas generadas eran del linaje mesencefálico, lo cual es muy importante porque las CPNs de la RSV normalmente producen interneuronas dopaminérgicas (ver atrás). Estos hallazgos apuntan a que determinantes transcripcionales del linaje DAm son insuficientes para dirigir el destino de las neuroesferas *in vitro*.

4.1.2 La Diferenciación in vivo de las neuroesferas: transplantes al cerebro embrionario y adulto.

El potencial de diferenciación *in vivo* de las neuroesferas se ha probado mediante transplantes al cerebro embrionario, postnatal y adulto (Baizabal et al., 2003). Estos estudios revelaron que las CPNs de las neuroesferas murinas diferencian mayoritariamente a linajes gliales *in vivo* (Pluchino et al., 2003), incluso cuando son colocadas en ambientes neurogénicos (Klein et al., 2005). En particular las neuroesferas telencefálicas y mesencefálicas de embriones E14 mostraron solo fenotipos gliales al integrarse al cerebro anterior (Winkler et al., 1998). En otros estudios, se reportó que las CPNs de las neuroesferas produjeron neuronas en el cerebro huésped (Brustle et al., 1998; Fricker et al., 1999; Ogawa et al., 2002; Ourednik et al., 2001; Vitry et al., 2001). Además, las neuroesferas obtenidas del cerebelo embrionario adquieren la identidad neuronal correspondiente al sitio de integración en transplantes homotípicos (en la misma región de origen) en el cerebelo postnatal (Klein et al., 2005). No es claro, sin embargo, si esta diferenciación también se observaría al transplantar neuroesferas secundarias. Tampoco es claro si las neuronas fueron instruidas a tomar la identidad correcta por influencia del entorno o este solo promovió la supervivencia de progenitores comprometidos. Al igual que en los casos mencionados anteriormente, en este estudio las neuroesferas diferenciaron principalmente al linaje astrocítico, en especial después de transplantes heterotípicos, es decir, en diferente región a la de origen (Klein et al., 2005). Aún no han sido publicados datos contundentes que indiquen especificación neuronal de las neuroesferas secundarias en respuesta a nichos neurogénicos.

4.2 Purificación de las CPNs embrionarias y del cerebro adulto.

Diversos protocolos con la técnica de FACS ("Fluorescent Activated Cell Sorting") se han reportado para obtener poblaciones puras o enriquecidas de CPNs. En el cerebro embrionario, la selección positiva del marcador de superficie Lewis X (LeX), también conocido como SSEA1 o CD15, permite obtener una población donde un 13-25% de CPNs presenta elevada capacidad proliferativa (Capela and Temple, 2006). Esta propiedad es evidenciada mediante el ensayo de formación de neuroesferas *in vitro*. Al utilizar LeX como único criterio de purificación, también se produce una suspensión enriquecida en CPNs a partir de la RSV del cerebro adulto (Capela and Temple, 2002). Además, la selección simultánea de LeX con el receptor de quimiocinas CXCR4, genera un aumento relativo de alrededor del 50% en la frecuencia de CPNs embrionarias y adultas capaces de generar neuroesferas (Corti et al., 2005). Recientemente, se ha demostrado que la purificación de las CTNs de la RSV en base a su expresión de la *Gfap* y del receptor a EGF, incrementa en un 33% la frecuencia de células formadoras de neuroesferas (Pastrana et al., 2009). Por otro lado, la Prominina 1 (CD133) se ha identificado como un marcador de superficie de CPNs murinas y humanas (Corti et al., 2007; Uchida et al., 2000). El enriquecimiento de CPNs proliferativas por selección de CD133 es de alrededor de 3% (1/32) para embriones murinos y fetos humanos (Corti et al., 2007; Uchida et al., 2000). En las neuroesferas humanas, CD133 se encuentra en 90-95% de la población celular total (Uchida et al., 2000). En otro estudio, la selección de una elevada actividad de aldehído deshidrogenadas resulta en aproximadamente un 14% (1/7) de CPNs embrionarias generadoras de neuroesferas (Corti et al., 2006). Finalmente, la proteína de superficie Corin permite la selección positiva de progenitores dopaminérgicos del mesencéfalo (Ono et al., 2007).

La falta de marcadores específicos que identifiquen a las CPNs, ha promovido que otros grupos purifiquen estas células por selección negativa. En este caso, las CPNs se aíslan en base a marcadores (epítopes) que no expresan. Uno de los primeros reportes basados en esta técnica demostró un enriquecimiento del 80% en CPNs formadoras de neuroesferas a partir de una suspensión de la RSV del ratón adulto (Rietze et al., 2001). La selección consiste en separar a las células mayores a 12 micras que además presentan poca afinidad a la aglutina de cacahuate ("Peanut Agglutining" o PNA) y baja producción del antigeno termoestable ("Heat Stable Antigen" o HSA) (Rietze et al., 2001). Como variante de este procedimiento, la purificación de las células mayores a 12 micras con baja afinidad a PNA (Fig. 13) también produce un notable enriquecimiento

en CPNs y además permite una mayor recuperación celular para transplante (ver resultados). La selección negativa también se ha utilizado para separar CPNs embrionarias de su progenie comprometida a linajes neuronales o gliales (Maric et al., 2003). Incluso se ha propuesto que, al igual que la célula troncal hematocitopoyética, la CTN es aquella que no expresa ningún marcador de linaje (Lin-) (Maric et al., 2007). Sin embargo, esta población Lin- tiene un comportamiento variable en cultivo y solo en algunos casos se comporta como multipotente (Maric et al., 2007). El enriquecimiento de CPNs capaces de generar neuroesferas secundarias se ha logrado mediante la selección de células que presentan baja incorporación del colorante para DNA, Hoestch 33342 (Kim and Morshead, 2003). Esta población constituye solo el 3.6% de la células en las neuroesferas pero representa cerca del 99% de la actividad para mantener la autorenovación en cultivo (Kim and Morshead, 2003). Futuros protocolos de identificación y purificación de CTNs tendrían que estar basados en la expresión combinada de marcadores y en pruebas funcionales que indiquen que las células de la población purificada se comportan como CTNs

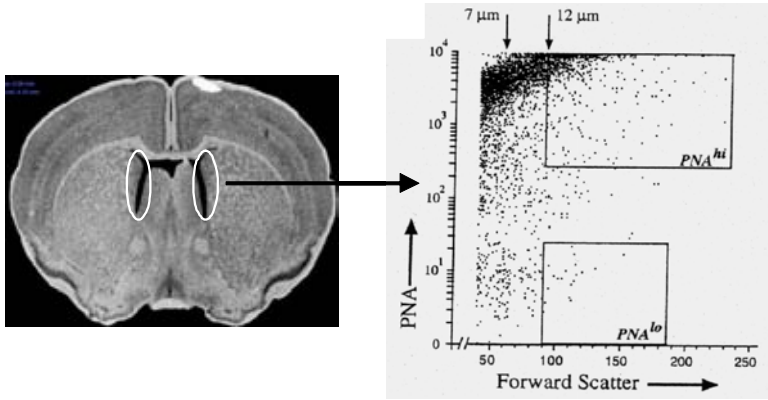

Figura 13. Purificación de las Células Precursoras Neurales del cerebro adulto. Mediante la técnica de FACS se separó la población de la RSV (marcada en círculos en imagen izquierda) que es mayor a 12 µm y presenta baja afinidad al PNA (recuadro inferior en el diagrama). Esta población se encuentra altamente enriquecida en CPNs y es libre de neuroblastos y neuronas.

5. Las Células Troncales Embriónicas.

5.1 Propiedades de autorenovación y pluripotencia.

Las Células Troncales Embriónicas (CTEs) han sido derivadas principalmente de la masa celular interna de embriones en la etapa de blastocistos. Más recientemente, se han logrado obtener CTEs humanas a partir de una sola blastómera (Chung et al., 2008; Klimanskaya et al., 2006). Las CTEs pueden mantenerse indiferenciadas y proliferando

indefinidamente *in vitro* en presencia de una capa de fibroblastos "alimentadores" o de LIF. Debido a su elevada capacidad de autorenovación, las CTEs pueden fácilmente formar tumores (conocidos como teratomas) al inyectarse en ratones suprimidos inmunológicamente. Al reintegrarse a la masa celular interna del blastocisto, las CTEs pueden contribuir a formar todos los tejidos somáticos del embrión y la línea germinal. Además, mediante diversos ensayos de diferenciación *in vitro* y por la formación de teratomas, también es posible generar células de las tres capas germinales embrionarias a partir de las CTEs (Keller, 2005; Murry and Keller, 2008). Esta capacidad para generar todos los linajes somáticos se conoce como pluripotencia. A diferencia de su contraparte en el ratón, las CTEs humanas pueden diferenciar a células del trofectodermo, que es el tejido extraembrionario que da origen a la placenta (Schulz et al., 2008). El estado pluripotente es conferido por un grupo de genes que codifican para factores transcripcionales tales como Oct4, Sox2 y Nanog. La sobre-expresión de estos genes en fibroblastos de ratón y humanos, puede reprogramarlos a un estado pluripotente similar al de las CTEs (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). La autorenovación y pluripotencia de las CTEs es finamente regulada por cambios epigenéticos (metilación, acetilación), microRNAs, "splicing" alternativo y modificaciones post-traduccionales (fosforilación, sumoilación, etc.) (Yeo et al., 2008)

5.2 La diferenciación in vitro de las CTEs; formación de los Cuerpos Embrioides.

La generación de los Cuerpos Embrioides (CEs) es comúnmente el paso inicial de la diferenciación *in vitro* de las CTEs. Los CEs deben su nombre a su capacidad para recapitular algunas de las etapas tempranas de la embriogénesis de mamíferos (Fig. 14) (Rathjen and Rathjen, 2001). Para generar los CEs, las CTEs se colocan en suspensión y en ausencia de LIF para promover la agregación celular y la diferenciación (Kurosawa, 2007). Después de dos días, las células de los CEs pueden adquirir dos destinos según su posición en el agregado celular. Las células de la periferia diferencian a endodermo primitivo y pierden la expresión de *Oct4* (Fig. 14) (Rathjen and Rathjen, 2001). Las células del centro se mantienen pluripotentes ($Oct4^{+}$) y además adquieren la expresión de *Fgf5*, indicando la aparición de células de ectodermo primitivo, que son equivalentes a las células del epiblasto del embrión (Fig. 14) (Rathjen et al., 2002; Rathjen and Rathjen, 2001). Entre los dos y tres días de formación del CE, el ectodermo primitivo comienza a formar un epitelio columnar a partir del cual se generarán células de las tres capas germinales del embrión, de forma similar a la etapa de gastrulación

(Fig. 14) (Keller, 2005). A los cuatro días de formación de los CEs, el endodermo primitivo ha diferenciado a endodermo parietal que expresa *Sparc* y endodermo visceral que expresa *Afp* (Fig. 14) (Rathjen et al., 2002; Rathjen and Rathjen, 2001). En esta etapa, las células del ectodermo primitivo en el CE han diferenciado a precursores ectodérmicos ($Fgf5^{+}$), mesodérmicos ($Brachyury^{+}$) y endodérmicos (Afp^{+}) (Fig. 14) (Rathjen and Rathjen, 2001). Posteriormente, estos precursores darán origen a neuronas, glia, cardiomiocitos, macrófagos y eritrocitos, entre otros tipos celulares. En etapas tardías de formación del CE, las células centrales mueren por apoptosis, formándose una cavidad central equivalente a la cavidad pro-amniótica en el embrión (Coucouvanis and Martin, 1995).

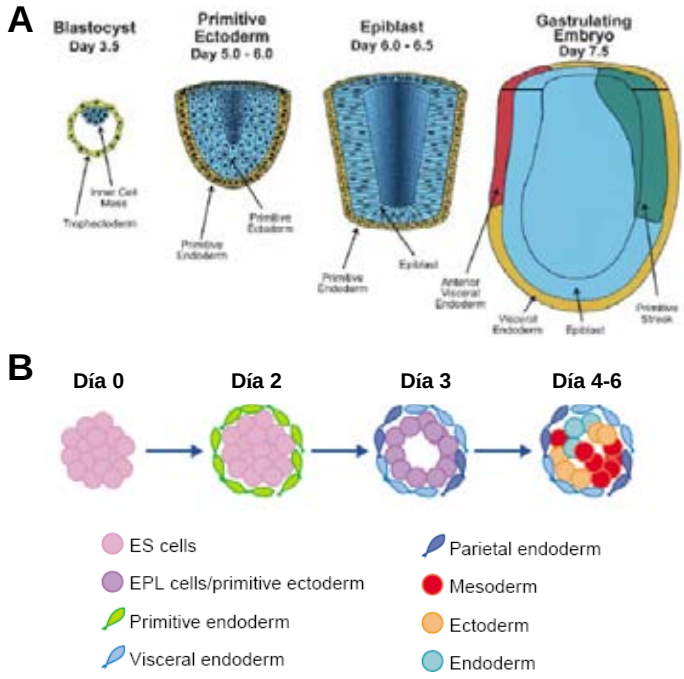

Fig. 14. La formación de Cuerpos Embrioides recapitula etapas tempranas del desarrollo. (A) Etapas iniciales del desarrollo embrionario en mamíferos desde el blastocisto hasta la gastrulación donde se generan el ectodermo, mesodermo y endodermo. **(B)** La formación de los Cuerpos Embrioides inicia al colocar en suspensión *in vitro* a las Células Troncales Embriónicas (rosa) para formar agregados (Día 0), en los cuales las células externas empiezan a diferenciar a endodermo primitivo (verde, Día 2). Al día 3, las células más internas generan un ectordermo primitivo (morado) que se organiza de forma columnar, mientras que el endodermo primitivo diferencia a endodermo visceral (azul claro externo) y parietal (azul oscuro). Del día 4 al 6, las células del ectodermo primitivo generan progenitores ectodérmicos (naranja), mesodérmicos (rojo) y endodérmicos (azul claro interno). Modificado de (Keller, 2005; Rathjen and Rathjen, 2001).

5.3 La diferenciación in vitro de las CTEs a linajes neurales.

Las CTEs murinas y humanas pueden diferenciarse *in vitro* hacia CPNs, las cuales son identificadas por la expresión de *Sox1*, *Nestina* y *Pax6*. Estas CPNs "generadas *in vitro*" pueden diferenciarse hacia neuronas, astrocitos y oligodendrocitos. En algunos casos, este proceso involucra la formación de CEs (Lee et al., 2000; Wichterle et al., 2002), mientras que en otros métodos la diferenciación se induce directamente a partir de una monocapa de CTEs (Stavridis and Smith, 2003). La neuralización de los CEs se basa comúnmente en la selección de las CPNs mediante un medio definido o en la exposición a un morfógeno como el AR (Bibel et al., 2004; Lee et al., 2000; Wichterle et al., 2002). Otras formas de neuralización incluyen el cocultivo de las CTEs con líneas celulares estromales (Barberi et al., 2003; Kawasaki et al., 2000), la formación de neuroesferas con Fgf2 y Egf (Hitoshi et al., 2004) o el uso de antagonistas de inhibidores neurales como los BMP (Sonntag et al., 2007). Estas CPNs pueden propagarse de forma pura mediante un sistema de cultivo que promueve las divisiones simétricas no diferenciativas (Conti et al., 2005). Sin embargo, la homogeneidad en este cultivo esta basada solo en la expresión de Nestina (Conti et al., 2005), por lo cual se esperaría la presencia de una población diversa de CPNs con diferentes grados de especificación, compromiso y potencial diferenciativo.

Las CPNs generadas a partir de las CTEs expresan inicialmente *Sox1*, indicando su identidad neuroepitelial (Suter et al., 2008). Estas CPNs en cultivo diferencian a GR al apagar la expresión de *Sox1* e iniciar la expresión de *Pax6* (Suter et al., 2008). La GR generada *in vitro* a partir de las CTE humanas presenta la expresión de *Rc2, Blbp, Glast* y *Gfap* (Bibel et al., 2004; Liour et al., 2006; Liour and Yu, 2003; Nat et al., 2007), de forma equivalente a la GR en el cerebro. En cultivo, la GR expresa *Pax6* y *Ngn1/2* mientras mantiene un potencial neurogénico, el cual cambia hacia potencial gliogénico al regularse negativamente la expresión de *Ngn* y aparecer Olig2, Mash1 y el receptor de EGF (Bouhon et al., 2006).

La diferenciación dirigida de las CTEs hacia diferentes subtipos neuronales ha permitido la obtención de neuronas glutamatérgicas y gabaérgicas del telencéfalo, neuronas granulares del cerebelo, neuronas motoras de la médula espinal, neuronas serotoninérgicas del metencéfalo y neuronas dopaminérgicas del cerebro anterior y medio (Barberi et al., 2003; Lee et al., 2000; Su et al., 2006; Watanabe et al., 2005; Wichterle et al., 2002; Yan et al., 2005). Esta diferenciación *in vitro* de las CTEs normalmente depende de la exposición a los morfógenos que *in vivo* controlan la especificación neuronal (Zhang, 2006). Por ejemplo, la exposición de los CEs a Shh y

AR es necesaria para producir neuronas motoras de la médula espinal (Li et al., 2005; Wichterle et al., 2002). Además, la concentración de AR determina la identidad posicional de los CEs (Okada et al., 2004). A su vez, la combinación de Shh con Fgf8 promueve la diferenciación de las CTEs a neuronas DAm (Lee et al., 2000; Yan et al., 2005), mientras que Shh y BMP7 inducen la diferenciación a neuronas dopaminérgicas hipotalámicas (Ohyama et al., 2005). Finalmente, antagonizando algunos miembros de la familia Wnt es posible dirigir la diferenciación a linajes neuronales telencefálicos (Watanabe et al., 2005).

5.3.1 La diferenciación in vitro de las CTEs a neuronas dopaminérgicas mesencefálicas.

La producción *in vitro* de neuronas dopaminérgicas a partir de las CTEs es generalmente poco eficiente en ratón y primates (máximo 24-26%), mientras que en humanos se ha logrado alcanzar hasta el 65% recientemente (Deierborg et al., 2008). La producción de neuronas DAm a partir de las CTEs murinas se reportó inicialmente utilizando un protocolo de cinco etapas: 1.- Expansión *in vitro* de las CTEs, 2.- Formación de CEs por cuatro días, 3.- Sembrar los CEs en adherencia y con medio definido para seleccionar las CPNs, 4.- Expandir las CPNs con Fgf2 y especificarlas con Fgf8 y Shh, 5.- Diferenciación de las CPNs a neuronas mDA. Mediante esta estrategia de cultivo se generan 70% de neuronas y 20% de neuronas dopaminérgicas en relación al total celular (Lee et al., 2000). Sin embargo, en un segundo reporte se logró solo 11% de neuronas TH^+ usando el mismo protocolo (Rodriguez-Gomez et al., 2007). Estas neuronas dopaminérgicas fueron caracterizadas principalmente mediante marcadores genéricos, así que aún no hay suficientes evidencias que pertenezcan al linaje mesencefálico (Lee et al., 2000; Rodriguez-Gomez et al., 2007). Otra estrategia para producir neuronas DAm *in vitro* consiste en el cocultivo de las CTEs con líneas celulares (Perrier et al., 2004). Una de las más empleadas es la línea estromal PA6, que en CTEs murinas y humanas induce la expresión de *Th* en aproximadamente 12% y 34% del total celular, respectivamente (Kawasaki et al., 2000; Vazin et al., 2008). Aún se desconoce cual molécula es responsable del efecto inductor de la línea PA6, aunque recientemente se ha sugerido que esta actividad no depende de proteínas de superficie celular (Vazin et al., 2008). La adición de Shh y Fgf8 en presencia de la línea estromal no incrementa significativamente el porcentaje de las neuronas dopaminérgicas (Kim et al., 2006a). Por otro lado, el cocultivo de las CPNs con astrocitos inmortalizados del

cerebro medio induce alrededor de 14% de neuronas TH^+ con la identidad posicional de neuronas DAm (Roy et al., 2006). El efecto de los astrocitos de cerebro medio parece ser específico porque los astrocitos corticales no incrementan a la población de las neuronas DAm (Roy et al., 2006).

Recientemente, la formación de "rosetas neurales" a partir de las CTEs se ha descrito como el método más adecuado para generar y mantener a las CPNs en un estado primitivo susceptible a ser especificado (Elkabetz et al., 2008; Pankratz et al., 2007). Estas rosetas neurales consisten en estructuras epiteliales circulares con un lúmen en el centro, emulando la organización del tubo neural (Elkabetz et al., 2008; Pankratz et al., 2007). Las CPNs en las rosetas presentan una identidad posicional similar a la del cerebro anterior, pero pueden redirigirse al linaje DAm mediante la aplicación de Shh y Fgf8 (Elkabetz et al., 2008). Hasta el momento, el método más eficiente de diferenciación dopaminérgica involucra la formación de estas rosetas neurales, donde hasta un 65% del total celular son neuronas dopaminérgicas (Cho et al., 2008). También se demostró que la mayoría de las neuronas TH^+ expresaban *En1*, confirmando la presencia de neuronas DAm (Cho et al., 2008). Sin embargo, aún se desconoce en que momento de este largo protocolo (30-40 días aproximadamente) las CTEs se especifican al linaje DAm (Cho et al., 2008). La identificación de las células precursoras competentes para la especificación dopaminérgica es esencial para el establecimiento de procedimientos más racionales de diferenciación dirigida *in vitro*.

La diferenciación dopaminérgica *in vitro* de las CTNs también se ha inducido intrínsicamente mediante la sobreexpresión de *Nurr1, Lmx1a* y *Foxa2*. En el caso de *Nurr1*, hasta el 35% del total celular expresó *Th* (Kim et al., 2002) y esta población aumento hasta 50% en presencia de Shh y Fgf8, los cuales al parecer también indujeron la expresión de marcadores del cerebro medio como *En1* y *Ptx3* (Chung et al., 2002; Kim et al., 2002). La sobreexpresión de *Nurr1* acelera la maduración de las neuronas dopaminérgicas *in vitro,* las cuales secretan mayor cantidad de dopamina al recibir un estímulo despolarizante (Chung et al., 2002). También se ha demostrado que la sobre-expresión de *Nurr1* y el cocultivo con la línea PA6 producen un incremento sinérgico de neuronas TH^+ hasta aproximadamente 54% del total celular (Kim et al., 2006a). En el caso de *Foxa2*, no se reportó el porcentaje de CTEs dirigida al linaje DAm (Kittappa et al., 2007). La sobrexpresión de *Lmx1a* bajo el control de un enhancer del promotor de *Nestina* resulta en más del 85% de neuronas, de las cuales 75-85% son TH^+ (Friling et al., 2009) .De estas neuronas, cerca del 98% presentan otros marcadores de las neuronas

DAm, tales como Foxa2, En1, Lmx1b, Nurr1 y Ptx3 (Friling et al., 2009). En las CTEs humanas, la sobreexpresión de *Lmx1a* produjo de un 50-65% de neuronas TH^+/β-III $Tubulina^+$, de las cuales hasta 85% expresó otros marcadores DAm (Friling et al., 2009). El bloqueo de la expresión basal de *Lmx1a* durante la diferenciación dopaminérgica de CTEs humanas, produce un decremento en los niveles de expresión de *Nurr1*, *Ptx3*, *Th* y *DAT* (Cai et al., 2008).

5.4 La diferenciación in vivo de los precursores derivados de las CTEs.

El potencial de diferenciación de las CTEs y sus progenitores *in vivo* se ha estudiado mediante el transplante de estas células a regiones del cerebro embrionario, postnatal y adulto. Algunas de las CTEs transplantadas al hipocampo, corteza y cerebelo del ratón adulto producen neuronas eléctricamente activas, aunque la incidencia de formación de tumores es muy alta (Harkany et al., 2004). Por otro lado, el cerebro embrionario de ratón al parecer instruye a las CTEs humanas a diferenciar de forma sitio-específica en el hipocampo y la corteza (Muotri et al., 2005).

La diferenciación específica *in vivo* de las células de los CEs también depende de señales de su entorno. Por ejemplo, las células de los CEs integradas en el embrión de pollo generaron neuronas motoras e interneuronas en la médula espinal, así como neuronas sensoriales en el ganglio de la raíz dorsal, demostrando la influencia del entorno instructivo (Plachta et al., 2004). No obstante, en ese mismo estudio las células de los CEs fallaron en generar linajes dorsales en la médula espinal (Plachta et al., 2004).

Las CPNs generadas *in vitro* a partir de las CTEs muestran capacidad para responder a nichos neurogénicos o gliogénicos en el embrión y en el cerebro adulto (Carpentino et al., 2008; Guillaume et al., 2006; Tabar et al., 2005). Algunas de las CPNs pueden migrar a través de la CMR hasta el BO donde generan nuevas neuronas (Tabar et al., 2005). Sin embargo, las evidencias indican que las CPNs derivadas de las CTEs presentan restricciones en la generación de subtipos neuronales específicos en respuesta al entorno del cerebro. Por ejemplo, la neuralización de los CEs con el AR induce la pérdida del potencial de estas células para diferenciar a neuronas sensoriales en el ganglio de la raíz dorsal (Plachta et al., 2004). En otros casos, se ha reportado que las CPNs transplantadas son capaces de generar neuronas funcionales *in vivo* y en rebanadas del hipocampo (Benninger et al., 2003; Wernig et al., 2004) pero no adquieren la identidad posicional correspondiente al sitio de integración en el cerebro

(Wernig et al., 2004). Adicionalmente, las neuronas glutamatérgicas, gabaérgicas, dopaminérgicas y serotoninérgicas fueron encontradas fuera del lugar donde se producen estas neuronas en el cerebro, descartando la diferenciación sitio-específica (Wernig et al., 2004). Por otro lado, se ha sugerido que en respuesta al entorno, las CPNs inyectadas en la RSV y el hipocampo del cerebro adulto producen neuronas calretinina^{+} y glutamato^{+}, respectivamente (Joannides et al., 2007; Tabar et al., 2005). No obstante, en estos casos no se analizaron marcadores de especificación neuronal, lo cual era necesario considerando que la calretinina y el glutamato se producen en muchos subtipos neuronales a lo largo del cerebro.

5.5 El transplante de las neuronas dopaminérgicas en modelos animales de la enfermedad de Parkinson.

La enfermedad de Parkinson se caracteriza por la degeneración de las neuronas dopaminérgicas de la SNc (Dauer and Przedborski, 2003). Estas neuronas inervan al estriado donde producen el neurotransmisor dopamina. Los pacientes con Parkinson sufren de problemas motores como temblor, dificultad para caminar y la pérdida de expresión facial. El transplante celular y la infusión de factores tróficos son algunas de las estrategias empleadas para aminorar los síntomas del Parkinson (Deierborg et al., 2008). En el primer caso, las neuronas dopaminérgicas generadas *in vitro* a partir de las CTEs parecen aliviar algunos síntomas de la enfermedad de Parkinson provocada en modelos murinos (Kim et al., 2002). Las neuronas transplantadas son colocadas en el estriado, donde funcionan como una bomba local de dopamina que puede mantenerse hasta por 32 semanas (Rodriguez-Gomez et al., 2007). Sin embargo, el problema principal en la interpretación de estos datos es determinar el grado de contribución de las neuronas dopaminérgicas a la recuperación (Ben-Hur et al., 2004). Adicionalmente, las neuronas transplantadas comúnmente muestran una viabilidad muy baja (Park et al., 2005). En una situación ideal, las neuronas dopaminérgicas tendrían que colocarse en la SNc, desde donde proyectarían axones hacia el estriado, lo que implicaría restablecer el circuito neuronal perdido durante la neurodegeneración. Pocos estudios han mostrado el transplante de las CPNs a la SNc en modelos de Parkinson y aún no se ha detectado diferenciación dopaminérgica en esa zona (Bjugstad et al., 2008). Por otro lado, un reporte indica que las neuronas dopaminérgicas transplantadas al estriado pueden desarrollar “cuerpos de Lewis”, los cuales son agregados protéicos indicativos del proceso degenerativo en el Parkinson (Kordower et al., 2008). Este dato sugiere que a

largo plazo, el cerebro de pacientes con Parkinson induce degeneración en las neuronas transplantadas. En conjunto, este panorama indica que se debe encontrar una población de CPNs susceptible a especificarse al linaje DAm para así lograr transplantes altamente enriquecidos en neuronas productoras de dopamina.

JUSTIFICACION

El potencial de diferenciación de una célula hacia un linaje específico depende de las señales que recibe de su entorno. En particular, la diferenciación dirigida de las CPNs a neuronas dopaminérgicas se ha analizado principalmente *in vitro*. Sin embargo, el nicho de diferenciación en el tubo neural es mucho más complejo y finamente regulado que el entorno de las CPNs en cultivo. Por esta razón, el potencial de diferenciación mostrado *in vitro* puede cambiar al exponer a las CPNs a un entorno natural.

Hasta el momento, solo un estudio ha reportado el transplante de CPNs al cerebro medio embrionario E10.5 *in vivo* (Olsson et al., 1997). Esto se logró mediante inyecciones guiadas por ultrasonido, lo cual resultó en muy baja viabilidad de los embriones huésped (Olsson et al., 1997). Estas dificultades técnicas hacen necesario el establecimiento de sistemas alternativos para evaluar la diferenciación de las CPNs en el nicho donde se producen las neuronas DAm. El cultivo de explantes organotípicos ha sido hasta el momento la principal estrategia utilizada para evaluar *in vitro* el potencial de las CPNs transplantadas al cerebro embrionario y adulto (Benninger et al., 2003; Carletti et al., 2002; Fishell, 1995). Sin embargo, en estos casos no se cotejó el desarrollo del tejido con la neurogénesis *in vivo,* dejando abierta la posibilidad de que el entorno del explante no sea el idóneo para analizar la diferenciación. Un sistema óptimo de cultivo de explante tiene que mostrar un desarrollo muy similar al del tubo neural durante la etapa embrionaria relevante para la neurogénesis del subtipo celular a analizar. Este tipo de sistema, permitiría probar el potencial de diferenciación de las CPNs hacia determinados linajes sin la necesidad de conocer las señales involucradas.

El ensayo de las neuroesferas fue considerado inicialmente como la mejor forma de expandir por largos periodos *in vitro* a las CPNs del cerebro embrionario y adulto. Sin embargo, al salir del entorno del tubo neural y proliferar, las CPNs alteran significativamente su información posicional (Santa-Olalla et al., 2003a). Estos cambios en la identidad de las CPNs podrían ser irreversibles, comprometiendo su destino hacia linajes específicos y evitando la capacidad de respuesta a señales de especificación (e.g. Shh, Wnt, Fgf8, etc). Al abordar este problema, algunos estudios han indicado que las

neuroesferas son susceptibles a la re-especificación al integrarse a rebanadas del cerebro embrionario (Hitoshi et al., 2002; Kim et al., 2006b). Sin embargo, estos reportes no ofrecen ninguna evidencia directa de la presencia de marcadores posicionales en las CPNs transplantadas, así como tampoco evidencias que apoyen que el entorno de las rebanadas es idóneo para analizar la especificación. Por otro lado, la diferenciación dirigida de las neuroesferas hacia linajes neuronales específicos ha sido poco eficiente. Este problema parece ser producto de dos factores: 1. El bajo potencial neuronal de las neuroesferas en general; 2. La baja capacidad de diferenciación dirigida de los pocos progenitores neurogénicas en las neuroesferas. Hasta el momento no hay una respuesta contundente sobre si las CPNs reducen su capacidad para diferenciar específicamente como resultado de la expansión como neuroesferas. Alternativamente, las CPNs en cultivo podrían no estar expuestas a un entorno adecuado para la neurogénesis. Para el caso de la diferenciación dopaminérgica en particular, la mejor forma de distinguir entre estas dos posibilidades es colocando a las CPNs en el nicho donde se producen naturalmente las neuronas DAm.

Las CTEs producen muchos tipos de neuronas *in vitro*, las cuales podrían emplearse con fines terapéuticos en pacientes con enfermedades neurodegenerativas. Durante la diferenciación neuronal *in vitro*, las CTEs generan diversas poblaciones de células precursoras, las cuales van progresivamente restringiendo su compromiso para diferenciar hacia ciertos linajes. Este proceso recapitula a nivel celular las principales etapas de la neurogénesis que ocurren en el embrión. Por ejemplo, las CTEs adquieren inicialmente una identidad ectodérmica (precursores de CEs) para después diferenciar a CPNs, las que eventualmente diferencian terminalmente a neuronas dopaminérgicas. Actualmente, se desconoce cual población celular intermedia entre las CTEs y las neuronas, tiene mayor capacidad de respuesta a señales de especificación neuronal. En principio, el entorno del cerebro embrionario en etapas tempranas de la neurogénesis proveería las señales necesarias para evidenciar a las células precursoras con mayor susceptibilidad de especificación neuronal. La identificación de esta población de células precursoras permitiría el establecimiento de estrategias de cultivo más racionales para lograr la diferenciación dirigida de las CTEs.

En resumen, el potencial de diferenciación de las CPNs a neuronas DAm debe probarse en un entorno donde las señales necesarias para la especificación de ese linaje estén presentes. Ese entorno se encuentra naturalmente en el mesencéfalo embrionario durante etapas tempranas de la neurogénesis. El enfoque que proponemos permitirá

identificar a las CPNs competentes para interpretar el nicho dopaminérgico mesencefálico. A su vez, evidenciaremos a las CPNs que presenten una reducción intrínseca en su potencial de especificación neuronal.

HIPOTESIS

Las Células Precursoras Neurales generadas y/o expandidas *in vitro* son capaces de interpretar correctamente el nicho dopaminérgico en el cerebro medio embrionario.

OBJETIVO GENERAL

Determinar el potencial de diferenciación de diversas poblaciones de Células Precursoras Neurales.

OBJETIVOS PARTICULARES

- Establecer un sistema de cultivo del mesencéfalo que recapitule los procesos de neurogénesis observados durante el desarrollo.

- Demostrar que el nicho de la línea media ventral en el explante mesencefálico es instructivo para la diferenciación de las CPNs al linaje dopaminérgico.

- Determinar la especificación neuronal y el potencial neurogénico y gliogénico de las CPNs de neuroesferas embrionarias y adultas cuando se integran en explantes de mesencéfalo embrionario.

- Evaluar en explantes de mesencéfalo embrionario la especificación neuronal y el potencial neurogénico de las poblaciones de células precursoras generadas *in vitro* a partir de las CTEs.

ESTRATEGIA EXPERIMENTAL

La estrategia experimental consiste en el cultivo ex *vivo* de regiones del cerebro, donde se transplantaran las células precursoras en entornos de diferenciación específicos. Este enfoque experimental ofrece la ventaja de ser técnicamente más accesible que los transplantes *in vivo* en cerebros de embriones tempranos.

La Fig. 1A en la publicación 1 describe la estrategia experimental. A partir de embriones E10.5 se diseca la región del mesencéfalo-metencéfalo (cortes indicados con

líneas azules discontinuas) para dejar el organizador del Istmo intacto (I). Se realiza un corte (línea azul discontinua) a través de la línea media dorsal del tejido disecado (II) para cultivarlo en forma de "libro abierto" (III). El tejido se embebe en una matriz de colágena y las células donadoras marcadas con la GFP se depositan en regiones específicas del explante (IV). La imagen inferior muestra un explante con células GFP^+ transplantadas (IV). Siete días después se analiza la diferenciación de las células donadoras integradas en el explante (V). La imagen inferior muestra un corte coronal con células GFP^+ integradas al explante (V).

RESULTADOS

1. Los explantes de mesencéfalo recapitulan la neurogénesis embrionaria.

El objetivo inicial era establecer un sistema de cultivo de explante donde existiera un entorno propicio para la diferenciación neuronal en general y dopaminérgica en particular. Elegimos el sistema de cultivo de explante en colágena, el cual se ha utilizado para estudiar las señales involucradas en la diferenciación dopaminérgica y en el crecimiento de las neuritas de estas neuronas (Hynes et al., 1995; Lin et al., 2005; Ye et al., 1998).

Inicialmente, la detección de la TH en "pieza completa" a los 13.5 días de desarrollo nos permitió ubicar con precisión el núcleo dopaminérgico en la flexura ventral mesencefálica (Fig. 15A, B). En el eje radial, las neuronas dopaminérgicas se observaron en la capa pial del tejido, lo cual fue evidenciado en cortes coronales del mesencéfalo (Fig. 15C). Con el objetivo de encontrar la etapa óptima de cultivo del explante, realizamos un seguimiento de la neurogénesis dopaminérgica en el embrión (Fig. 1A, publicación 2). En base a estas observaciones, decidimos cultivar los mesencéfalos a los 10.5 días de desarrollo, durante el inicio de la neurogénesis embrionaria (Fig. 1B, publicación 1). Después de siete días, la expresión de *β-III Tubulina* abarcó todo el eje radial del explante, indicando abundante neurogénesis durante el desarrollo del cultivo (Fig. 1B, publicación 1). La desaparición en el explante de una región ventricular constituida por células indiferenciadas fue acompañada por la aparición de GR, principalmente en la línea media ventral (Fig. 1C, publicación 2). Esta observación sugiere que las CPNs en el explante mesencefálico mantienen un desarrollo similar al observado *in vivo*. Durante el periodo de cultivo, rara vez se observó la aparición de astrocitos $GFAP^+$ u oligodendrocitos $O4^+$ (data no mostrado), demostrando

la ausencia de un entorno gliogénico en el explante. En paralelo con la abundante neurogénesis, observamos que las neuronas producidas alrededor de la línea ventral del explante adquirían el fenotipo dopaminérgico, identificado por la expresión de *Th* (Fig. 1B, publicación 1). Estas neuronas dopaminérgicas se organizaron en la SNc y el AT cuando los explantes crecieron en una matriz de colágena pero no cuando se desarrollaron en membranas para cultivo organotípico (Fig. 1B, publicación 2). En cultivos en colágena, las neuronas dopaminérgicas proyectaron sus axones hacia regiones anteriores y dorsales como ocurre *in vivo* (Fig. 1B, publicación 2).

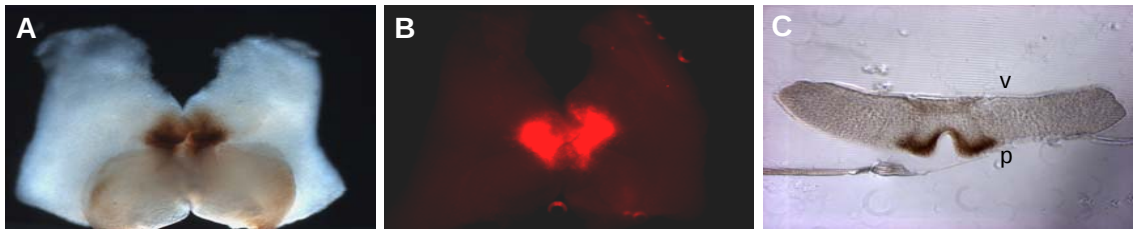

Fig. 15. Localización de las neuronas dopaminérgicas mesencefálicas. (A) Vista superior de la región pial del mesencéfalo ventral E13.5 donde se observa la región de diferenciación dopaminérgica en el centro (café oscuro) observada mediante la inmuno-detección con peroxidasa de la TH en "pieza completa". **(B)** Mesencéfalo ventral E13.5 visualizado como en "A" donde se observan las neuronas dopaminérgicas localizadas mediante la detección de TH por inmunofluorescencia (rojo intenso). El contorno del tejido se muestra como un fondo en rojo tenue. **(C)** Corte coronal en vibratomo (500 μm de espesor) del tejido mostrado en "A". Se observan las neuronas dopaminérgicas TH^+ cerca de la capa pial (p) y lejos de la superficie ventricular (v).

2. La región media ventral del mesencéfalo en cultivo promueve la diferenciación dopaminérgica.

Para analizar la capacidad del explante de dirigir la diferenciación dopaminérgica, se transplantaron las CPNs presentes en el mesencéfalo ventral E10.5 en diferentes niveles dorso-ventrales de explantes mesencefálicos en una etapa de desarrollo equivalente (Fig. 2, publicación 1). Nuestros datos indicaron que las CPNs mesencefálicas requieren del entorno de la línea media ventral en el explante para producir neuronas dopaminérgicas (Fig. 2, publicación 1).

Con la finalidad de evaluar si el explante dirigía al linaje dopaminérgico a otras poblaciones celulares del tubo neural, transplantamos a las CPNs del telencéfalo ventral en el mesencéfalo cultivado. Encontramos que a los 10.5 días de desarrollo las CPNs aisladas del telencéfalo mantienen elevado potencial neurogénico al integrarse al explante mesencefálico (Fig. 2, publicación 2). Sin embargo, estas células continuaron

produciendo el marcador telencefálico Bf1 y se mostraron comprometidas a la diferenciación gabaérgica (Fig. 3, publicación 2). En contraste, en etapas muy tempranas del desarrollo neural (E8.5) las CPNs que dan origen al telencéfalo presentaron la capacidad de intepretar correctamente el entorno mesencefálico y dar origen a las neuronas DAm en la línea media ventral (Fig. 4, publicación 2). En otras regiones del mesencéfalo, las CPNs telencefálicas E8.5 también fueron capaces de adquirir la identidad posicional correcta según el sitio de integración (Fig. 4, publicación 2).

3. Las CPNs del cerebro adulto producen neuronas al integrarse al cerebro medio embrionario.

En colaboración con el grupo del Prof. Perry Bartlett (Queensland Brain Institute, Australia) utilizamos la técnica de FACS para purificar a las CPNs de la RSV del cerebro adulto, basándonos en dos criterios: tamaño celular y afinidad por la aglutinina de cacahuate ("peanut aglutinin"; PNA). Para este estudio seleccionamos la población subventricular mayor a 12 micras con baja afinidad a la aglutinina (>12 μm/PNA^{lo}, Fig. 13). Encontramos que las células purificadas del cerebro adulto diferencian exclusivamente a neuronas en el entorno del explante mesencefálico. Estos datos se encuentran descritos en detalle en la publicación 2 (ver al final).

La técnica de FACS no permite una recuperación celular eficiente que facilite el análisis de la diferenciación en el explante. Por esta razón, utilizamos la técnica de separación magnética ("Magnetic Activated Cell Sorting"; MACS) para obtener una población enriquecida en CPNs de la RSV por selección negativa. En este caso, empleamos como criterios de selección la baja afinidad por el PNA o la ausencia de expresión del antígeno termoestable ("Heat stable antigen"; *HSA*) en las CPNs. En los experimentos de separación mediante unión a PNA o anti-HSA, se logró una reducción en la proporción de neuronas en las suspensiones celulares obtenidas de la RSV (Tabla 1). Al usar el PNA se observó un incremento de 23 veces en la frecuencia de las CPNs formadoras de neuroesferas (Tabla 1). Este dato es similar al enriquecimiento reportado usando FACS y tomando únicamente la unión a PNA como criterio de separación (Bull and Bartlett, 2005). En las condiciones usadas, la combinación de PNA y HSA no produjo una disminución aún mayor en el porcentaje de neuronas o un aumento en la frecuencia de las CPNs formadoras de neuroesferas (Tabla 1).

Tabla 1. Enriquecimiento de las CPNs del cerebro adulto por separación magnética.

	Suspensión RSV[1]	PNA[2]	anti-HSA[3]	PNA/anti-HSA[4]
Purificación 1				
vol. beads (µl)[5]	-	200	50	200/50
β-III Tubulina+[6]	62.2	16.3	30.8	31.8
neuroesferas[7]	1/2500	1/129	1/670	1/360
Purificación 2				
vol. beads (µl)	-	200	100	
β-III Tubulina +	65	17.1	18.2	n.d.
neuroesferas	1/2700	1/127	1/150	
Purificación 3				
vol. beads (µl)	-	200		200/100
β–III Tubulina +	60.1	18.5	n.d.[8]	17.5
neuroesferas	1/2850	1/124		1/153

[1]Indica los datos obtenidos de la suspensión celular de la RSV antes de la separación magnética.

[2]Indica los datos durante la separación magnética por unión a PNA.

[3]Indica los datos durante la separación magnética con el anticuerpo anti-HSA.

[4]Datos obtenidos con la combinación PNA/HSA.

[5]Volumen usado de las perlas acopladas al PNA o al anticuerpo anti-HSA.

[6]Muestra el porcentaje de células b-III Tubulina + en relación al total celular.

[7]Frecuencia de las CPNs formadoras de neuroesferas en relación al número de células sembradas inicialmente.

[8]No determinado.

Después de transplantar al mesencéfalo la población con baja afinidad al PNA, encontramos que algunas células GFP^+ presentaron típicas morfologías neuronales y expresaron *β-III tubulina*. A su vez, otro grupo de células GFP^+ mostró morfología astrocítica y expresó *Gfap*/*Nestina*, sugiriendo que estas células eran astrocitos inmaduros o células tipo B. Algunas de las células transplantadas no expresaron ninguno de los marcadores neurales analizados, mientras que aparentemente la viabilidad celular fue baja, incluso al realizar la purificación en presencia de Egf y Fgf2. Estos datos se encuentran mostrados en detalle en la publicación 2 (ver al final). Por motivos no determinados aún, experimentos posteriores de separación magnética realizados por una persona diferente, resultaron en una disminución poco significativa de la población neuronal en la suspensión de partida. Debido a esta incertidumbre, decidimos reportar estos datos como si la suspensión celular obtenida de la RSV se hubiera transplantado directamente al explante, es decir, sin un paso previo de purificación con el magneto (ver publicación 2 al final).

En conjunto, los datos obtenidos mediante las dos técnicas de purificación (FACS y MACS) sugieren que una subpoblación de CPNs en la RSV del cerebro adulto es capaz de sobrevivir, integrarse y diferenciar exclusivamente al linaje neuronal durante etapas tempranas de la neurogénesis en el mesencéfalo.

4. Las CPNs embrionarias expandidas como neuroesferas muestran un potencial neurogénico restringido en el mesencéfalo.

La formación de neuroesferas es uno de los métodos más comunes de propagación de las CPNs en cultivo. Sin embargo, el bajo potencial neurogénico de las neuroesferas *in vitro* ha abierto la posibilidad de que estas células requieran del nicho de neurogénesis en el embrión. Para investigar esta posibilidad, se generaron neuroesferas primarias GFP^+ de mesencéfalos E13.5 y posteriormente se transplantaron a explantes mesencefálicos disecados a los E10.5. En esta condición, los agregados celulares (neuroesferas sin disociar) cruzaron completamente la pared ventricular y algunas células migraron hasta alcanzar la región pial (Fig. 16A). Una cantidad elevada de las CPNs transplantadas mostraron la expresión de la *Gfap*, sugiriendo su diferenciación astrocítica (Fig. 16B). A su vez, las neuroesferas integradas indujeron la diferenciación astrocítica precoz en las células del explante (Fig. 16B).

Dado el elevado potencial neurogénico de la mayoría de las CPNs presentes en el mesencéfalo E10.5/11.5, nos preguntamos si la expansión *in vitro* de estas células en forma de neuroesferas podría afectar su diferenciación en los explantes. A los 10.5 días de desarrollo, las CPNs del tubo neural expresan *Nestina* pero son negativas para la *Gfap*, el cual es un marcador de las CPNs más tardías (Mignone et al., 2004). Después de proliferar por 7 días en presencia de Fgf2 y Egf, alrededor de un 81% de las CPNs en las neuroesferas co-expresaron *Nestina* y *Gfap*. En condiciones de diferenciación *in vitro*, esta población produjo astrocitos y neuronas, aunque estas últimas en mucho menor proporción (Fig. 4, publicación 3). Después de transplantar las neuroesferas primarias al mesencéfalo, encontramos células GFP^+/β-III $Tubulina^+$ (Fig. 16C) y GFP^+/$NeuN^+$ (Fig. 16D) en baja frecuencia, indicando pobre diferenciación neuronal. En contraste, muchas células GFP^+ mantuvieron la expresión de *Nestina* (Fig. 16E) o *Gfap* (Fig. 16F), aunque en este caso no pudimos evaluar la coexpresión de estos marcadores.

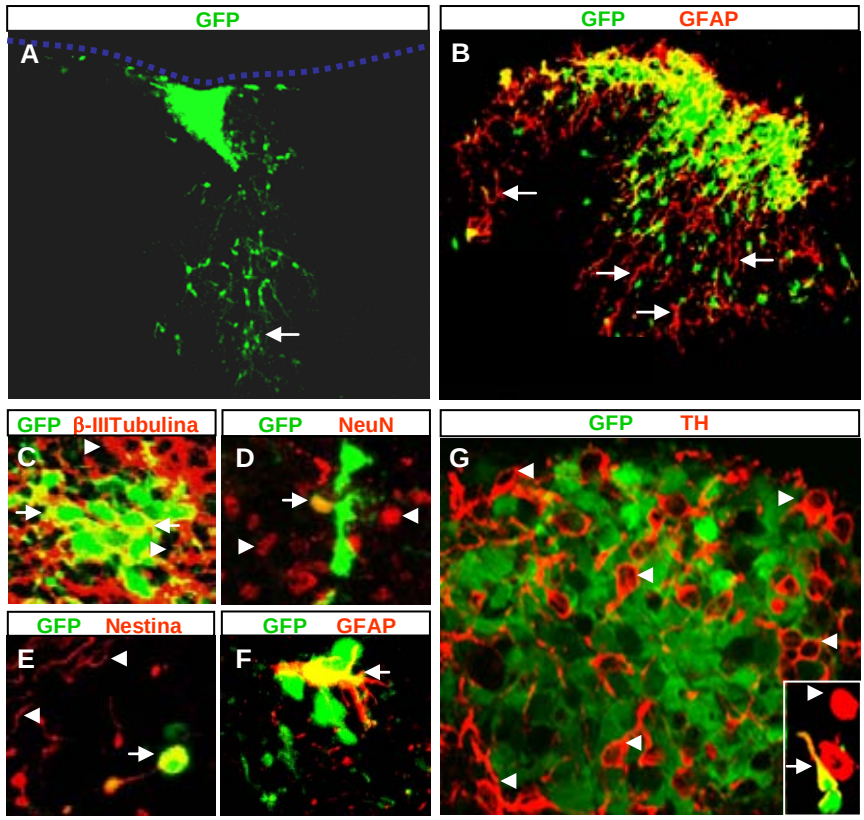

Figura 16. Diferenciación en el mesencéfalo de las neuroesferas embrionarias primarias. (A) Las neuroesferas completas (sin disociación) son capaces de atravesar completamente la pared ventricular del mesencéfalo (línea azul punteada) y algunas células logran alcanzar la región pial del tejido (flecha). **(B)** Las neuroesferas primarias expandidas de mesencéfalos E13.5 inducen la diferenciación astrocítica en las células del explante, indicado por la expresión de la *Gfap* (flechas). **(C y D)** Las neuroesferas primarias expandidas de mesencéfalos E10.5/11.5 producen pocas neuronas β-III Tubulina+ o NeuN+ (flechas) al integrarse al explante. **(E y F)** En contraste, la mayoría de las células integradas presentó la expresión de *Nestina* y/o *Gfap* (flechas). **(G)** Al integrarse en el sitio de diferenciación dopaminérgica endógena del mesencéfalo ventral, solo ocasionalmente se observaron células GFP+/TH+ (flecha en recuadro). En todas las imagenes, las cabezas de flecha señalan algunos ejemplos de la expresión del marcador indicado en el explante.

Para analizar el potencial dopaminérgico, las neuroesferas primarias de mesencéfalos ventrales E10.5/11.5 fueron transplantadas a la línea media ventral del explante obtenido de la misma etapa embrionaria. En este caso, solo ocasionalmente encontramos células GFP^{+}/TH^{+} (Fig. 16G, recuadro) en la región de inducción dopaminérgica endógena (Fig. 16G). Sin embargo, no es posible descartar que estas neuronas dopaminérgicas sean producto de la contaminación del cultivo primario con progenitores dopaminérgicos comprometidos.

5. Las CPNs embrionarias expandidas como neuroesferas secundarias muestran baja especificación neuronal en el mesencéfalo.

La formación de las neuroesferas primarias usualmente se promueve al cultivar las células del cerebro a una alta densidad inicial. Sin embargo, en esta condición de expansión, no es posible descartar la agregación de neuroblastos con las CPNs formadoras de las neuroesferas. Por lo tanto, es esperado que algunos progenitores neuronales en las neuroesferas primarias no sean producto de la expansión de las CPNs. Para reducir la frecuencia de estos neuroblastos "contaminantes" dentro de los agregados, disociamos las neuroesferas primarias, las resembramos a menor densidad y las expandimos por 7 días adicionales.

Al transplantar las CPNs de neuroesferas secundarias al mesencéfalo, observamos que algunas desarrollaban morfologías neuronales típicas (Fig. 17A), aunque la gran mayoría presentaba morfologías gliales (Fig. 17B). De acuerdo con la morfología neuronal observada, encontramos muy escasas evidencias de diferenciación neuronal en las células integradas (Fig. 17C), mientras que algunas células expresaron el marcador astrocítico *Gfap* (Fig. 17D). En un análisis preliminar de la identidad posicional de las células transplantadas en el dominio ventral Nkx6.1^{+} (Fig. 17E) y en el dorsal Pax7^{+} (Fig. 17F), no encontramos evidencias de células GFP^{+} correctamente especificadas según el sitio de integración.

6. Las neuroesferas propagadas del cerebro adulto se comportan como progenitores gliales en el cerebro embrionario.

La diferenciación neuronal de algunas CPNs purificadas del cerebro adulto nos llevó a preguntarnos si este potencial se mantenía al final de la expansión *in vitro* como neuroesferas. Antes del transplante, alrededor del 67% de las células en las neuroesferas adultas expresaron *Nestina* y *Gfap*, de forma similar a las neuroesferas embrionarias. En condiciones de diferenciación *in vitro*, las neuroesferas adultas fueron capaces de producir neuronas, astrocitos y oligodendrocitos (datos no mostrados). Sin embargo, al integrarse en los explantes de mesencéfalo, la gran mayoría de las células (95% aproximadamente) adquirió morfología de astrocitos maduros (Fig. 18A, recuadro). Esta observación fue confirmada por la expresión de la *Gfap* en estas células (Fig. 18A). Inesperadamente, no encontramos evidencias de neuronas GFP^{+}, a pesar que las células donadoras se incorporaron a un entorno exclusivamente neurogénico (Fig. 18A). Las neuroesferas sin disociar fueron capaces de integrarse completamente al tejido, pero

estas células se mantuvieron como progenitores Nestina^{+}/GFAP^{+} (Fig. 18B). En conjunto, estos datos sugieren que las CPNs de las neuroesferas adultas se comportan como glioblastos en el explante al permanecer refractarias a la influencia del entorno neurogénico.

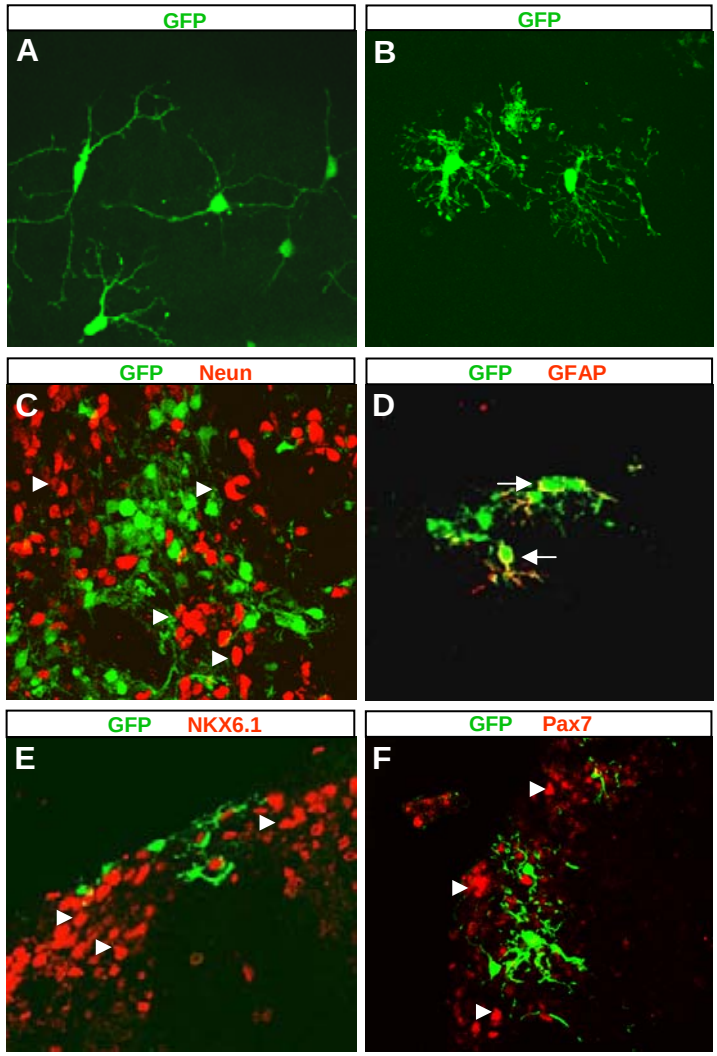

Figura 17. Potencial diferenciativo de las neuroesferas secundarias. (A) Al integrarse al mesencéfalo embrionario, pocas células GFP+ derivadas de las neuroesferas secundarias adquirieron una morfología neuronal típica al observar en "pieza completa" a las células implantadas. **(B)** La mayoría de las células integradas al explante presentaron morfología astrocítica. **(C)** Las células GFP+ no mostraron la expresión de *NeuN* un marcador de maduración neuronal. **(D)** Algunas células diferenciaron terminalmente al linaje astrocítico, evidenciado por la expresión de *Gfap* (flechas). **(E y F)** Al integrarse al sitio de expresión endógena de *Nkx6.1* y *Pax7* (cabezas de flecha) en el mesencéfalo, las células implantadas no adquirieron la expresión de estos genes de identidad posicional.

7. Efecto del ácido valproico en las CPNs propagadas como neuroesferas. El ácido valproico (AV) es una droga que modifica la información epigenética celular al inhibir

específicamente a la desacetilasa de histona I. El AV activa la expresión de algunos genes de pluripotencia en las neuroesferas (Ruau et al., 2008), lo cual podría estar vinculado a un aumento en la capacidad de estas células para reconstituir el linaje hematopoyético, aunque con baja frecuencia (Schmittwolf et al., 2005). Además, en conjunto con la sobreexpresión de *Oct4* y *Sox2*, el AV aumenta la frecuencia de reprogramación de los fibroblastos humanos a células pluripotentes (Huangfu et al., 2008). En relación a la diferenciación neuronal, el AV promueve la neurogénesis en células del hipocampo a expensas de la gliogénesis (Hsieh et al., 2004). En las neuroesferas, el AV promueve la proliferación de neuroblastos durante la etapa de diferenciación, lo cual reulta en un incremento en la proporción final de neuronas (Laeng et al., 2004). Este efecto, también se ha observado mediante el uso de otros inhibidores de desacetilasas de histonas (Siebzehnrubl et al., 2007).

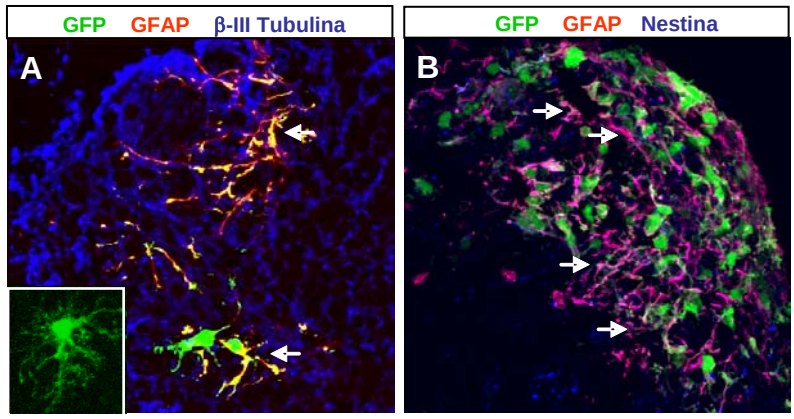

Figura 18. Las neuroesferas adultas diferencian a astrocitos en el entorno del mesencéfalo. (A) Al integrarse al explante, las Células Precursoras Neurales (CPNs) de las neuroesferas generaron astrocitos terminalmente diferenciados, evidenciado por la expresión de la *Gfap* (flechas) y la morfología observada en "pieza completa" (recuadro en A). Este compromiso al linaje glial se presentó a pesar de la abundante neurogénesis en el mesencéfalo (azul). **(B)** Al transplantar las neuroesferas completas (sin disociación celular), estas se integraron al explante pero mantuvieron la expresión de *Nestina* y *Gfap*, indicando su estado indiferenciado (magenta, flechas).

En base a estos antecedentes, decidimos probar si el AV es capaz de modificar el comportamiento en cultivo de las neuroesferas. Experimentos iniciales, han demostrado que en comparación con las neuroesferas control (Fig. 19A), el AV en concentraciones de 1-2 μM induce cambios dramáticos en la morfología de las neuroesferas embrionarias (Fig. 19B) y adultas (Fig. 19C). Estos cambios están principalmente asociados a un aumento en la adhesión celular y a la formación de células alargadas,

similares a la GR (Fig. 19B,C). Actualmente, estamos investigando la relación de estos cambios morfológicos con el potencial de diferenciación de las CPNs.

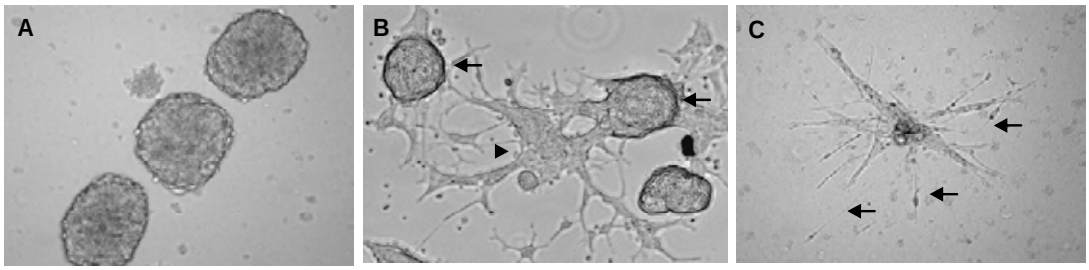

Figura 19. Efecto del ácido valproico en las Células Precursoras Neurales. (A) Neuroesferas generadas en presencia de los mitógenos FGF2 y EGF por 10 días en cultivo. **(B)** Neuroesferas derivadas de mesencéfalos ventrales E10.5 y tratadas con ácido valproico 1mM por 7 días. Algunas agregados se mantienen compactos (flechas) mientras que otros se adhieren completamente al plato de cultivo (cabeza de flecha). **(C)** Neuroesferas generadas de la región subventricular del cerebro adulto por 7 días y posteriormente tratadas por 3 días con ácido valproico. Algunas de las células presentan morfología de glia radial (flechas).

8. Potencial diferenciativo de las Células Precursoras derivadas de las CTEs.

Diversas poblaciones de células precursoras intermediarias se han identificado durante la diferenciación de las CTEs a neuronas. Sin embargo, se desconocía cual de esas poblaciones tiene mayor capacidad de especificarse eficientemente en respuesta al entorno del cerebro embrionario. Los resultados de esta parte del proyecto están presentados en la publicación 1 y en parte de la publicación 2 (ver al final).

DISCUSION

Durante el desarrollo, la diferenciación celular específica depende de interacciones complejas entre las células precursoras y su nicho. La competencia para diferenciar hacia cierto linaje es resultado de la historia de las células precursoras, durante la cual definen su capacidad de respuesta a las señales del entorno. Aunque en general se conocen algunas de las señales relevantes para la especificación de ciertos linajes celulares, aún se desconocen las combinaciones espacio-temporales precisas de los factores necesarios para la diferenciación. En este trabajo, reportamos el uso del microambiente del cerebro medio embrionario con el fin de evaluar la competencia de las CPNs para producir neuronas dopaminérgicas y otros linajes neuronales. Esta estrategia nos permitió determinar el potencial intrínseco de diversas poblaciones de

CPNs sin conocer las redes complejas de moléculas señalizadoras que inducen la diferenciación. Proponemos que nuestro enfoque experimental podría también emplearse para evaluar el potencial neurogénico de otras poblaciones de células troncales, tales como las presentes en la epidermis, la médula ósea y las células pluripotentes inducidas por reprogramación.

El nicho neurogénico en el explante mantiene su capacidad de instruir a las CPNs al linaje dopaminérgico.

Demostramos que el sistema de cultivo de explante recapitula varios aspectos del desarrollo del mesencéfalo. Sin embargo, la abundante neurogénesis observada en el explante podría ser producto del desarrollo de progenitores neuronales ya comprometidos, en cuyo caso no importaría la ausencia de señales de diferenciación en el cultivo. Como evidencia indirecta de la presencia de un entorno instructivo en el explante, demostramos que las CPNs mesencefálicas adquieren el fenotipo dopaminérgico solo al transplantarse en la región ventral. Estas CPNs fueron aisladas de la región ventral de mesencéfalo por lo cual podrían tener cierto grado de especificación hacia el linaje dopaminérgico antes del transplante. Estos datos dejaban abierta la posibilidad de que el explante solo mantuviera las señales de diferenciación terminal hacia el linaje DAm, mientras que las señales de especificación podrían estar ausentes. Sin embargo, al transplantar las células de los CEs en la línea media ventral, encontramos que recapitulan el desarrollo de las neuronas DAm (ver adelante). Como algunas de las células de los CEs a los 4 días representan una población ectodermal no neuralizada (Rathjen and Rathjen, 2001), este hallazgo sugiere la presencia de un nicho en el explante capaz de especificar completamente al linaje dopaminérgico.

Contrario a lo anteriormente reportado (Olsson et al., 1997), nuestros datos indican que las CPNs telencéfalicas incorporadas al mesencéfalo no adquieren la expresión de genes de especificación del linaje DAm, tales como *En1* y *Th*. Este hallazgo sugiere un compromiso de las células telencefálicas E10.5 al momento de ser transplantadas. En contraste las CPNs telencefálicas a los 8.5 días de desarrollo poseen la plasticidad para generar neuronas DAm. Al igual que en el caso de las CTEs (ver adelante), estos datos sugieren que la especificación neuronal debe ocurrir durante o poco después de la neuralización. Por lo tanto, las CPNs telencefálicas son susceptibles a ser re-especificadas a linajes mesencefálicos solo durante una ventana muy restringida del desarrollo temprano del cerebro (ver publicación 2 para más discusión).

Potencial diferenciativo de las CPNs mesencefálicas expandidas in vitro como neuroesferas.

Nuestros datos indican que las CPNs aisladas del mesencéfalo diferencian abundantemente a neuronas maduras al reintegrarse en los explantes mesencefálicos. Sin embargo, esta capacidad neurogénica se reduce sustancialmente cuando las CPNs proliferan *in vitro* y generan neuroesferas. Estas evidencias sugieren una reducción intrínseca en la capacidad de las CPNs de las neuroesferas para responder a señales del entorno neurogénico. De forma similar, otros estudios han demostrado que las neuroesferas embrionarias y postnatales producen principalmente células gliales al transplantarse al cerebro embrionario o postnatal (Klein et al., 2005; Winkler et al., 1998). Sin embargo, en estos casos las CPNs se integraron en cerebros de estadios avanzados de la neurogénesis (E14.5) o en etapas perinatales (P4), quedando así la posibilidad de que el entorno de diferenciación neuronal ya no fuera el idóneo (Klein et al., 2005; Winkler et al., 1998). Nuestro estudio contribuye a descartar esta posibilidad porque las CPNs fueron expuestas al entorno mesencefálico en etapas iniciales de la neurogénesis (E10.5), cuando es más probable que estén presentes las señales relevantes para la diferenciación neuronal.

Encontramos que la mayoría de las CPNs Nestina^{+} presentes en el neuroepitelio adquieren la expresión de la *Gfap* durante la formación de las neuroesferas. Estos cambios en la expresión de proteínas del citoesqueleto podrían ser reflejo de la transición de las CPNs neurogénicas a un estado con mayor potencial gliogénico en respuesta a altas concentraciones de Egf y Fgf2 (Doetsch et al., 2002). En soporte a esta hipótesis, la población de CPNs que coexpresa *Nestina* y *Gfap* aparece de forma natural durante el inicio de la gliogénesis en el cerebro embrionario (Mignone et al., 2004). Encontramos además que las neuroesferas generadas de embriones E13.5 inducían la diferenciación astrocítica precoz en las células de los explantes. Estos datos sugieren que los cambios en la identidad celular (factores intrínsecos) y la producción de señales de diferenciación gliogénicas (factores extrínsecos) restringen el potencial neurogénico de las CPNs durante la formación de las neuroesferas.

En relación a la diferenciación sitio-específica, muy pocas CPNs derivadas de las neuroesferas primarias produjeron neuronas dopaminérgicas al integrarse alrededor de la línea media ventral del mesencéfalo. Tomando en cuenta que este entorno es suficiente para instruir eficientemente a las CPNs mesencefálicas al linaje

dopaminérgico, nuestros datos indican que la expansión *in vitro* reduce la capacidad de especificación en el explante. De forma similar, estudios previos han mostrado que las neuroesferas se especifican pobremente al linaje dopaminérgico *in vitro* en respuesta a algunas de las señales que instruyen dicha diferenciación *in vivo* (Parish et al., 2008). Nuestros datos apoyan estos estudios y además sugieren que los factores extrínsecos que determinan naturalmente el linaje DAm durante el desarrollo son insuficientes para especificar a las CPNs de las neuroesferas hacia dicho linaje. Por otro lado, un estudio reciente demostró que la sobre-expresión de genes que determinan el linaje DAm (*Lmx1a, Msx1/2, Ngn2* y *Ptx3*) es insuficiente para dirigir eficientemente a las CPNs de las neuroesferas al linaje dopaminérgico (Roybon et al., 2008).

Al propagar y transplantar neuroesferas secundarias, observamos que estas CPNs no adquirieron la identidad posicional en los dominios de expresión de *Nkx6.1* y *Pax7*. Este dato es contrario a estudios previos donde se argumenta que la re-especificación de las neuroesferas es posible al exponerlas al entorno del cerebro embrionario (Hitoshi et al., 2002; Kim et al., 2006b). La debilidad de estos estudios radica en que en ningun caso se analizó directamente la presencia de los marcadores posicionales en las células transplantadas (Hitoshi et al., 2002; Kim et al., 2006b). Nuestras evidencias sugieren que durante la expansión *in vitro*, las CPNs restringen su potencial de diferenciación, lo cual no puede ser revertido por factores intrínsecos o extrínsecos. Cambios genéticos o epigenéticos más globales en las CPNs o sistemas de cultivo alternativos podrían ser necesarios para expandir poblaciones más susceptibles a ser especificadas.

Potencial diferenciativo de las CPNs aisladas de la RSV del cerebro adulto.

El enriquecimiento de las CPNs de la RSV del cerebro adulto se ha logrado mediante la selección por FACS de las células >12μm que presentan baja afinidad al PNA (Rietze et al., 2001). Los resultados en este estudio apoyan la capacidad neurogénica de esta población al integrarse al nicho embrionario. Adicionalmente, al transplantar la población de CPNs >12μm PNA^{lo}, no encontramos células $GFAP^{+}$ derivadas de las células donadoras, lo cual podría ser reflejo de una respuesta selectiva al entorno neurogénico del explante, donde la gliogénesis es escasa o nula. Una meta futura será determinar si las CPNs adultas pueden generar neuronas DAm en el mesencéfalo.

Al producir neuroesferas de la RSV del cerebro adulto, encontramos que estas CPNs diferenciaban exclusivamente a astrocitos en el explante, lo cual contrastó con el

carácter multipotente de esa población al diferenciar *in vitro*. Una observación similar se ha reportado anteriormente al inyectar Egf directamente en la RSV del cerebro adulto (Doetsch et al., 2002). En este caso, las CPNs de la RSV dejaron de migrar al BO y comenzaron a producir exclusivamente células astrocíticas *in situ* (Doetsch et al., 2002). Estas células astrocíticas mantuvieron la capacidad de generar neuroesferas que se mostraron multipotentes *in vitro* (Doetsch et al., 2002). Los datos presentados en nuestro estudio apuntan a que este mismo fenómeno sucede también al exponer ex *vivo* a las CPNs adultas a altas concentraciones de Egf. Otra posibilidad es que las CPNs adultas no son capaces de interpretar el nicho neurogénico del embrión. En contra de esta posibilidad, algunas de las CPNs purificadas del cerebro adulto produjeron neuronas en el mesencéfalo. Tomando estas evidencias en conjunto, nuestros datos sugieren que la mayor parte de las CPNs en las neuroesferas adultas restringen su potencial hacia el linaje glial.

Potencial diferenciativo de las células precursoras de los Cuerpos Embrioides al integrarse al mesencéfalo.

La diferenciación *in vitro* de las CTEs hacia diversos linajes es comúnmente iniciada mediante la formación de CEs. Por otro lado, la diferenciación neuronal de las CTEs puede inducirse *in vitro* sin la formación de los CEs (Barberi et al., 2003; Kawasaki et al., 2000; Tropepe et al., 2001; Ying et al., 2003). Nuestras evidencias indican que las CTEs deben diferenciar a células de los CEs para responder al entorno neurogénico del mesencéfalo. Esta conclusión está basada en la observación que las CTEs integradas al mesencéfalo adquieren la expresión de *Oct4* pero no de marcadores neurales. El hallazgo que las CTEs aparentemente se mantienen pluripotentes en el explante, podría deberse a la presencia de factores que activan la señalización del receptor gp130, la cual contribuye a mantener a las CTEs indiferenciadas *in vitro*. De hecho, el complejo LIF/CNTF/gp130 está presente en algunas regiones del cerebro embrionario, tales como la EGL y la médula espinal (Gregg and Weiss, 2005). La presencia de estas señales en el mesencéfalo podría definir un nicho no permisivo para la diferenciación neuronal de las CTEs. En contraste, el 70-75% de las células de los CEs diferenciaron al linaje neural en el explante. Este dato sugiere que las células de los CEs pueden interpretar el nicho de las CTNs, ya que *in vitro* los CEs no muestran una elevada tendencia a producir neuronas en ausencia de señales exógenas como el AR (Wichterle et al., 2002). El 30% restante de la población de los CEs podría estar constituída por progenitores

comprometidos a linajes endodermales y mesodermales, los cuales ya están presentes a los 4 días de formación del CE (Rathjen and Rathjen, 2001).

Los patrones de formación en el tubo neural dependen del establecimiento de gradientes de morfógenos a lo largo de los principales ejes (Ashe and Briscoe, 2006). En particular, la actividad en gradiente de Shh establece los dominios de progenitores neuronales en la region ventral del tubo neural, mientras que los BMPs organizan la region dorsal (Dessaud et al., 2008; Liu and Niswander, 2005; Machold and Fishell, 2002). En el mesencéfalo, la señalización de Shh especifica a las neuronas dopaminérgicas Lmx1a^{+} en la línea media ventral y a las motoneuronas e interneuronas inhibitorias en los dominios de expresión de *Nkx6.1* y *Nkx2.2*, respectivamente (Andersson et al., 2006b; Blaess et al., 2006). Nuestros resultados sugieren que las células de los CEs responden a la concentración local de Shh de acuerdo al sitio de integración en el mesencéfalo. Esta conclusión deriva de la observación de que las células de los CEs selectivamente producen neuronas dopaminérgicas Lmx1a^{+}, motoneuronas Nkx6.1^{+}/Islet1^{+} e interneuronas Nkx2.2^{+} en los dominios de expresión correspondientes del explante. En el mesencéfalo dorsal, las células de los CEs expresaron *Pax7*, probablemente como consecuencia de la señalización de los BMPs derivada de la placa del techo. Al igual que Shh, otros morfógenos como TGFβ, TGFα, y miembros de la familia de los Wnts y Fgfs tienen un papel esencial en diferentes estadios del desarrollo de las neuronas DAm (Abeliovich and Hammond, 2007). En este estudio, las células de los CEs no produjeron neuronas TH^{+} al encontrarse en la línea media ventral pero lejos del Istmo, sugiriendo una posible influencia de Fgf8 en la diferenciación. Cualquiera que sean las señales involucradas, la diferenciación de los CEs en el explante parece estar mediada por un mecanismo instructivo, dado que estas células no expresaban *Lmx1a*, *Nkx6.1*, *Nkx2.2* y *Pax7* antes del transplante. Futuros experimentos serán dirigidos a elucidar el papel de los morfógenos durante la neuralización, especificación y diferenciación de los CEs en el mesencéfalo.

Reportes previos han mostrado que las células de los CEs diferencian al parecer aleatoriamente a varios subtipos neuronales, incluyendo el dopaminérgico, al integrarse al estriado de murinos adultos (Bjorklund et al., 2002). En contraste, nuestros datos demuestran que el nicho del mesencéfalo dirige la diferenciación sitio-específica de los CEs a neuronas dopaminérgicas en el dominio Lmx1a^{+} y neuronas gabaérgicas en el dominio Nkx2.2^{+}. Basados en la expresión de *Lmx1a*, alrededor del 67% de las células

de los CEs se especificaron al linaje DAm, aunque solo el 38% alcanzó la diferenciación terminal y expresó *Th* al momento del análisis. Los porcentajes más bajos de especificación de los CEs en otros dominios del explante quizás reflejen la integración de las células transplantadas en zonas donde los marcadores evaluados no se expresan ubicuamente.

En conjunto, nuestros resultados indican que muchas células de los CEs tienen la capacidad de recapitular el desarrollo de las neuronas presentes en el sitio de integración en el mesencéfalo. En relación al linaje dopaminérgico, reportes recientes han demostrado que las células mesencefálicas $Shh^{+}/FP4^{+}/Foxa2^{+}$ de la placa del piso son los progenitores dopaminérgicos (Kittappa et al., 2007; Ono et al., 2007). Por lo tanto, una posibilidad interesante es que las células de los CEs diferencian inicialmente a células de la placa del piso antes de diferenciar terminalmente a neuronas DAm.

Potencial diferenciativo de las CPNs derivadas de las CTEs.

Los resultados presentados sugieren que la neuralización *in vitro* de las CTEs reduce su competencia para producir neuronas dopaminérgicas alrededor de la línea media ventral del mesencéfalo. Esta conclusión se apoya en la evidencia de que el entorno de los explantes actúa como una matriz instructiva para las CPNs del mesencéfalo y para las CPNs generadas “de novo” en el nicho del mesencéfalo a partir de los CEs. Además, las CTEs neuralizadas *in vitro* con AR también mostraron pobre especificación en los explantes. De acuerdo con estos datos, trabajos previos han demostrado que las CPNs derivadas de las CTEs generan neuronas funcionales en los cerebros de la rata E16.5 pero no muestran elevada diferenciación sitio-específica (Wernig et al., 2004). A su vez, las células de los CE neuralizadas *in vitro* con AR pierden la capacidad de generar interneuronas dorsales y neuronas sensoriales en el ganglio de la raíz dorsal (Plachta et al., 2004). Una limitante en la interpretación del estudio de Wernig et al., es que las células se transplantaron durante etapas tardías de la neurogénesis, cuando las señales de especificación neuronal podrían estar ausentes o disminuidas. En contraste, en este estudio demostramos que el nicho instructivo presente durante etapas tempranas de la neurogénesis es insuficiente para dirigir el destino de las CPNs generadas *in vitro* a partir de las CTEs. Aún no es claro, sin embargo, si las CTEs pierden su competencia poco después de ser neuralizadas o si su potencial de diferenciación se restringe progresivamente conforme avanza el cultivo. Este estudio no descarta además la posibilidad de que otras poblaciones de CPNs derivadas de CTEs, tal como las células

neurales de roseta descritas recientemente (Elkabetz et al., 2008; Li et al., 2005; Yan et al., 2005), respondan eficientemente a las señales instructivas en el explante.

La diferenciación neuronal *in vitro* de las CTEs usualmente tienen como primer paso el enriquecimiento en CPNs Nestina$^+$ o Sox1$^+$ (Lee et al., 2000; Ying et al., 2003). Este enfoque resulta en la obtención de cultivos celulares que contienen una mezcla de CPNs en diferentes estadios de desarrollo y posiblemente con un destino neuronal ya predeterminado (Pruszak et al., 2007). Nuestros datos sugieren que después de las neuralización *in vitro* de las CTEs, el entorno del mesencéfalo aún especifica a una pequeña subpoblación celular, la cual podría representar CPNs responsivas o precursores de CEs aún no neuralizados. Futuros análisis serán encaminados a probar el potencial de diferenciación de poblaciones más homogéneas de CPNs mediante las técnicas de separación celular usando antígenos de superficie o mediante condiciones selectivas de crecimiento (Pruszak et al., 2007; Roy et al., 2006).

Trabajos previos han demostrado que Fgf8 y Shh promueven la diferenciación dopaminérgica en el mesencéfalo y en los precursores derivados de las CTEs (Lee et al., 2000; Ye et al., 1998). En este estudio, el tratamiento con Shh y Fgf8 previo al transplante promovió un aumento en la proporción de las células GFP$^+$/Lmx1a$^+$ incorporadas al mesencéfalo, en comparación con las CPNs sin tratamiento. Las neuronas GFP$^+$/Lmx1a$^+$ mostraron una distribución aleatoria a lo largo del eje dorso-ventral del mesencéfalo. Es importante considerar que *Lmx1a* también es un marcador de la placa del techo (Chizhikov and Millen, 2004a; Millonig et al., 2000), aunque estas células pierden la expresión de *Lmx1a* al diferenciar (Failli et al., 2002). En contraste, la mayoría de las células Lmx1a$^+$ diferenciaron a neuronas en el explante. Esta observación sugiere que Shh y Fgf8 comprometen a una subpoblación de CPNs hacia el linaje dopaminérgico y no hacia células de la placa del techo. Esta interpretación es respaldada por el hecho que algunas células donadoras produjeron neuronas dopaminérgicas putativas independientemente del sitio de integración. En apoyo a nuestro estudio, se ha demostrado que las CPNs primitivas diferencian a CPNs más comprometidas en su potencial diferenciativo al exponerse a Fgf2, Fgf8, Shh y Egf *in vitro* (Elkabetz et al., 2008; Pankratz et al., 2007).

Diferenciación dirigida de las CTEs a linajes neuronales específicos.

En este trabajo demostramos que solo durante etapas tempranas de la diferenciación, las CTEs adquieren la competencia para responder eficientemente a señales de

especificación en el mesencéfalo. Otros reportes han sugerido que el subtipo neuronal de las CTEs debe ser especificado durante etapas tempranas de su diferenciación, incluso antes de la neuralización. Este fenómeno se debe a que solo las células ectodermales o las CPNs tempranas son responsivas a las señales que establecen los patrones de formación en el cerebro embrionario. Por ejemplo, las CTEs diferencian a neuronas dopaminérgicas *in vitro* al cultivarse sobre una línea celular estromal (Kawasaki et al., 2000). No obstante, la línea estromal solo es capaz de especificar a las CTEs al linaje dopaminérgico antes o al inicio de la expresión de *Sox1* (Parmar and Li, 2007). De forma similar, la especificación *in vitro* de las CTEs humanas a neuronas DAm, en lugar de neuronas dopaminérgicas del cerebro anterior, requiere la exposición a Fgf8 antes del inicio de la expresión de *Sox1* (Yan et al., 2005).

La especificación temprana de las células precursoras derivadas de las CTEs se ha demostrado además en linajes diferentes al dopaminérgico. Por ejemplo, la inhibición de la señalización de Wnt en los CEs, promueve eficientemente la especificación de progenitores telencefálicos $Bf1^{+}$, mientras que el mismo tratamiento es inefectivo en etapas más tardías de la diferenciación (Watanabe et al., 2005). Otro estudio indica que el neuroepitelio primitivo derivado de las CTEs mantiene una identidad similar a la del cerebro anterior y responde a señales como el AR, el cual induce la expresión de genes del cerebro posterior (Elkabetz et al., 2008; Pankratz et al., 2007). En contraste, al diferenciar el neuroepitelio primitivo a CPNs definitivas ($Sox1^{+}$) la especificación por AR disminuye significativamente (Elkabetz et al., 2008; Pankratz et al., 2007). La organización epitelial de la CPNs *in vitro* al formar las rosetas neurales, podría proveer un nicho que preserve la competencia para la diferenciación neuronal específica. Estas rosetas neurales se mantienen en cultivo mediante la señalización de Notch y Shh, mientras que Fgf2, Fgf8 y Egf promueven una pérdida de la estructura epitelial y la transición a CPNs definitivas, con la simultánea restricción en el potencial de diferenciación (Elkabetz et al., 2008).

La modificación genética es otra alternativa para controlar la diferenciación de las CTEs a linajes neuronales específicos. En particular, la diferenciación dopaminérgica dirigida se ha logrado con líneas de CTEs que sobreproducen factores transcripcionales como Lmx1a, Foxa2, Nurr1 y Ptx3 (Friling et al., 2009)(Andersson et al., 2006; Chung et al., 2002; Kittappa et al., 2007; Martinat et al., 2006). Sin embargo, aún al sobreexpresar *Lmx1a* o *Nurr1*, las CTEs necesitan de señales extrínsecas como Shh y Fgf8 para producir neuronas DAm (Andersson et al., 2006b; Friling et al., 2009;

Kim et al., 2002). Por otro lado, faltan evidencias contundentes de que la sobreexpresión de *Nurr1* y *Ptx3* genera genuinas neuronas DAm (Chung et al., 2002; Martinat et al., 2006). Nuestros datos sugieren que la combinación correcta de factores extrínsecos (señales del nicho) es suficiente para que las CPNs competentes derivadas de las CTEs, recapitulen el desarrollo dopaminérgico mesencefálico. Este proceso ocurre mediante la activación de determinantes intrínsecos tempranos (*Lmx1a*) y tardíos (*Ptx3*).

CONCLUSION

En este estudio empleamos un sistema experimental novedoso para revelar diferencias intrínsecas en el potencial diferenciativo de diversas poblaciones de CPNs expuestas al entorno del mesencéfalo. Encontramos que las células de los CEs generan una población de CPNs altamente susceptible a especificarse en repuesta a su entorno de diferenciación. En contraste, las CPNs generadas *in vitro* a partir de las CTEs o expandidas como neuroesferas, restringen progresivamente su capacidad de respuesta al nicho de diferenciación en el cerebro. La purificación y propagación de poblaciones de células precursoras que eficientemente produzcan neuronas DAm, como la identificada en este estudio, es esencial para desarrollar un tratamiento más eficiente de la enfermedad de Parkinson.

PERSPECTIVAS

- Identificar las señales del nicho del mesencéfalo involucradas en la neuralización y la especificación neuronal de los CEs. Para el caso de la neuralización, el uso de explantes obtenidos del ratón nulo para *Dll1*, proveería un entorno de diferenciación donde los niveles de señalización de Notch1 estuvieran abatidos. Este diseño experimental permitiría dilucidar si la señalización de Notch es esencial o dispensable para la neuralización de las células de los CEs. Para analizar el papel de Shh y Wnt en la especificación DAm de los CEs, el uso de inhidores de estas vías de señalización como la ciclopamina y el Dkk1, sería la ruta más directa para comenzar a identificar la etapa del proceso diferenciativo en que estas señales están involucradas.

- Determinar el potencial diferenciativo de las células de los CEs integradas en explantes del cerebro anterior y posterior. Los resultados obtenidos hasta el momento

han demostrado que las células de los CEs son capaces de neuralizarse a lo largo del eje antero-posterior del tubo neural. Al igual que en el cerebro medio, los explantes de la EGL promueven la diferenciación sitio-específica de los CEs. En la EGL, las neuronas derivadas de los CEs dan origen principalmente al linaje gabaérgico, de forma similar a las células endógenas (ver publicación 2). Futuros experimentos podrían estar orientados a comprobar la diferenciación de CEs a neuronas piramidales en la corteza o a neuronas serotoninérgicas en el metencéfalo. Este objetivo proveería un sistema *in vitro* para estudiar el proceso de diferenciación de las CPNs hacia múltiples linajes neuronales en el tubo neural.

- Analizar los mecanismos que restringen la respuesta al entorno mesencefálico de las CPNs generadas in vitro a partir de las CTEs. Esta problema es el más difícil de abordar dentro de las perspectivas y podría resolverse mediante varios enfoques. Uno de ellos sería identificar alguna propiedad que promueva la respuesta al entorno de los CEs y analizar si dicha propiedad se mantiene cuando estas células diferencian *in vitro* a CPNs. Por ejemplo, al comparar la susceptibilidad de estas células a señales como Shh, Wnt, Fgf8 es esperado que se encuentren diferencias significativas y que esto condicione la respuesta al nicho. En particular para el caso de Shh, las CPNs generadas *in vitro* podrían expresar altos niveles del receptor Ptc, el cual es un regulador negativo de la señal de Shh y disminuye considerablemente la sensibilidad de las CPNs a dicha señal (Dessaud et al., 2007).

- Utilizar a las células de los CEs como un "sensor celular" para probar el nicho de diferenciación en el cerebro medio adulto. La presencia de CPNs en el cerebro medio adulto permitiría en principio activar la diferenciación de estas células con fines terapéuticos. Sin embargo, a pesar de tener potencial neurogénico *in vitro*, las CPNs del cerebro medio adulto solo producen glia *in situ* (Lie et al., 2002). Una posible explicación de esta restricción es la ausencia de un entorno propicio para la diferenciación en el cerebro medio. Para probar esta hipótesis, los CEs se transplantaran directamente a la SNc y el AT en el adulto. La similitud entre los nichos neurogénicos en el embrión y el adulto (Alvarez-Buylla and Lim, 2004), abre la posibilidad de que las células transplantadas produzcan neuronas DAm en caso de recibir la combinatoria de señales correcta.

- Determinar si modificaciones genéticas en la cascada de señalización de Notch1, aumentan la capacidad neurogénica de las neuroesferas embrionarias y adultas. En este caso, se generarían neuroesferas nulas en *Dll1* para analizar su comportamiento en los explantes. Estas neuroesferas presentan un elevado potencial neurogénico *in vitro*, aunque subtipos neuronales específicos no fueron evaluados (Grandbarbe et al., 2003b). Este dato demuestra que la activación de la señalización de Notch1 determina la transición de las CPNs neurogénicas hacia un potencial gliogénico (Grandbarbe et al., 2003b). Esta transición podría comprometer la respuesta de las CPNs a las señales presentes durante la neurogénesis temprana recapitulada en los explantes. Por lo tanto, la alta capacidad neuronal de las neuroesferas *Dll1*-/- podría acompañarse de una mayor susceptibilidad a especificarse correctamente según su posición en el cerebro embrionario. La letalidad temprana (E11.5 aproximadamente) de la mutación nula para *Dll1*, nos permitirá evaluar únicamente a las CPNs heterócigas en el caso de la neuroesferas adultas. Aunque la ausencia de un solo alelo *Dll1* tiene un efecto menor en las CPNs (Grandbarbe et al., 2003b) la exclusiva diferenciación astrocítica de las neuroesferas adultas incorporadas al explante, nos permitirá detectar diferencias, incluso sutiles, en algún cambio de potencial gliogénico a neurogénico.

MATERIALES Y METODOS

1. Protocolo de 5 etapas para generar neuronas dopaminérgicas *in vitro* a partir de Células Troncales Embriónicas.

Soluciones

1. Dulbecco's Modified Eagle's Medium. DMEM (Gibco 12100-046).
2. Suero Fetal Bovino inactivado. SFB (Gibco 16000-044).
   - Inactivar el suero calentándolo a 56°C por 30 min.
   - Alicuotar y almacenar a -20°C. Una vez descongelada una alícuota, se puede mantener a 4°C hasta por 1 mes.
3. β-Mercaptoetanol. β-MeOH (Gibco 21985-023).
   Para 100 ml de solución 100X (10 mM):
   - Llevar 72 µl de β-MeOH 14 M a 100 ml con PBS.
   - Esterilizar por filtración a través de una membrana de 0.22 µm.
   - Almacenar a 4°C hasta por 2 meses.
4. Glutamax/Penicilina/Estreptomicina (GPS).
   Para una solución 100X:
   - Descongelar un frasco de 100 ml de Glutamax (200 mM, Gibco 35050-061) y transferir 10 ml a otro tubo.
   - Agregar 300 mg de penicilina y 500 mg de estreptomicina a los 10 ml de Glutamax. Mezclar hasta que se disuelvan los antibióticos.

- Esterilizar los 10 ml por filtración en membrana de 0.22 μm y agregarlos a los 90 ml restantes de la botella de Glutamax. Mezclar.
- Alicuotar en tubos falcon de 15 ml y guardar a –20°C. Las alícuotas descongeladas se pueden mantener a 4°C por 1 mes.

5. Piruvato de Sodio 100X (Gibco 11360-070).
6. Aminoácidos no esenciales 100X (Gibco 11140-050).
7. Solución amortiguadora de fosfatos 1X ("Phosphate Buffered saline" o PBS 1X).

Para 1 L:

NaCl	8 g
KCl	0.2 g
Na_2HPO4	1.44 g
KH_2PO4	0.24 g

- Mezclar en 900 ml de agua bidestilada.
- Ajustar pH a 7.2-7.4 con HCl.
- Aforar a 1 L en matraz de aforado.
- Esterilizar por filtración con membrana de 0.22 μm o en la autoclave.

8. Gelatina 1%.

Para 300 ml:
- Agregar 3 g de gelatina en polvo en 300 ml de H_20 grado cultivo.
- Disolver con calor en el microondas.
- Esterilizar en autoclave.
- Almacenar a temperatura ambiente hasta por 2 meses.
- Para utilizarse, preparar una solución al 0.1% en H_20 grado cultivo.

9. Verseno (EDTA 0.02%).

Mezclar 10 mg de EDTA·4Na en 50 ml de PBS. Esterilizar por filtración con membranas de 0.22 μm.

10. Tripsina 0.25% ó 0.05% (Gibco 15090-046).

Diluir el stock de tripsina al 2.5% con Verseno para obtener la concentración deseada.

11. Factor inhibidor de leucemia ("leukemia inhibitory factor" o LIF por sus siglas en inglés). (Chemicon 2010).

- Tomar 100 μl del stock y mezclarlos con 900 μl de medio M15 (ver abajo) que tenga 1 mg/ml de albúmina bovina. De la solución preparada, poner 10 μl (1000U) por ml de medio.

12. Mitomicina C (Roche 107409).

Para preparar un stock 50X:
- Con una jeringa de 1 ml tomar 0.5 ml de PBS 1X e inyectarlo en el frasco con 2 mg de mitomicina.
- Disolver completamente y tomar con la jeringa la mayor cantidad de líquido posible.
- Agregar el contenido de la jeringa a 3.5 ml de PBS 1X.
- Almacenar a 4º C en la oscuridad hasta 1 mes.

Nota: La mitomicina es un potente inhibidor de la proliferación celular. Para su manejo utilizar guantes y cubrebocas.

13. Poli-D-lisina 10 μg/ml. (Sigma P-7886).
- Del stock a 1 mg/ml tomar 100 μl y llevar a 10 ml en agua de cultivo.
- Esterilizar por filtración con membrana de 0.22 μm.

14. Medio M15.

Para 500 ml:

DMEM	405 ml
SFB inactivado	75 ml
β-MeOH 100X	5 ml
GPS 100X	5 ml
Aminoácidos no esenciales 100X	5 ml
Piruvato de sodio 100X	5 ml

Almacenar a 4°C hasta por 2 semanas.

15. Medio de cultivo para células alimentadoras STO.

Para 500 ml:

DMEM	460 ml
SFB inactivado	35 ml
GPS 100X	5 ml

Almacenar a 4°C hasta por 3 semanas.

16. Medio de Congelación 2X.

Para 25 ml:

DMEM	15 ml
DMSO (dimetil sulfóxido, grado cultivo (Sigma D2650)	5 ml
SFB inactivado	5 ml

17. Medio ITSFn para selección de Células Precursoras Neurales.

Para 50 ml:

DMEM/F12 sin HEPES (Gibco 12500-039)	48.413 ml
Insulina 4 mg/ml (Gibco 13007-018)	62.4 µl
Transferrina 4 mg/ml (Gibco 13008-016)	625 µl
Selenita de sodio 10 µM (Sigma S5261-10G)	150 µl
Fibronectina 1 mg/ml (Gibco 33016-015)	250 µl
GPS 100X	500 µl

Esterilizar por filtración con membrana de 0.22 µm. Almacenar a 4°C hasta por 1 semana.

18. Medio para expansión de Células Precursoras Neurales.

Para 50 ml:

DMEM/F12 sin HEPES	48.441 ml
N2 100X (Gibco 17502-048)	500 µl
GPS 100X	500 µl
Laminina 0.85 µg/µl (Gibco 680-3017IV)	58.82 µl

19. Factores de crecimiento.

Preparar a las siguientes concentraciones:

- Fgf2 1 ng/ml (Gibco 13256-029)
- Fgf8 5 ng/ml (Peprotech 100-25)
- Shh 25 ng/ml (Peprotech 100-45, R and D Systems 461-SH).

Las alícuotas de Fgf2, Fgf8 y Shh deben diluirse en medio DMEM/F12 sin HEPES con albúmina a 1 mg/ml. Esterilizar por filtración a través de membrana de 0.22 µm con una jeringa de insulina y un filtro de 4 mm (Millex-GV, Millipore SLGV004SL).

20. Medio para diferenciación de Células Precursoras Neurales a neuronas dopaminérgicas.

Para 50 ml:

DMEM/F12 sin HEPES	48.441 ml
N2 100X	500 µl
GPS 100X	500 µl

Laminina 0.85 µg/µl	58.82 µl
Ácido ascórbico 10 mM (Sigma A-4544)	500 µl

Esterilizar por filtración en membrana de 0.22 µm. Almacenar a 4°C hasta por 1 semana.

Nota: Todos los medios de cultivo deben calentarse alrededor de 20 min a 37º C antes de usarse.

Desarrollo del protocolo de 5 etapas para diferenciar las CTEs a neuronas dopaminérgicas.

Etapa 1: Expansión de las Células Troncales Embriónicas.

A) Cultivo de fibroblastos de la línea STO.

Las CTEs se expanden sobre una monocapa de fibroblastos alimentadores ("feeders cells" en inglés) de la línea STO.

Procedimiento para preparar la monocapa de fibroblastos alimentadores:

1. Cubrir con gelatina al 0.1% cajas de 10 cm de diámetro (Corning ¿??).
Incubar a temperatura ambiente al menos por 2 horas.
2. Aspirar la gelatina con la bomba de vacío y lavar 1 vez con PBS 1X.
3. Descongelar un vial de células STO (ver abajo).
4. Crecer las células STO a subconfluencia en cajas de 10 cm de diámetro (Corning 430167) con 10 ml de medio.
5. Agregar 200 µl de mitomicina C a la caja de 10 cm de diámetro para inactivar mitóticamente a los fibroblastos. Incubar 2 horas con la droga a 37°C.
6. Aspirar el medio y lavar 2 veces con PBS 1X para eliminar la mitomicina.
7. Aspirar el PBS y agregar 2 ml de tripsina al 0.25%; incubar brevemente a temperatura ambiente hasta que empiece a desprenderse la monocapa.
8. Inactivar la tripsina agregando 2 ml de medio M15.
9. Disociar las células mecánicamente subiendo y bajando el líquido 5 veces con una pipeta de 5 ml.
10. Centrifugar a 220 x g durante 5 min.
11. Aspirar el sobrenadante y resuspender el pellet en medio para células STO. La cantidad de medio añadida normalmente es de 5-10 ml según el tamaño del pellet.
12. Contar células en la cámara de Neubauer. Tomar 10 µl de la suspensión celular y mezclarlos con 10 µl de azul de tripano. De esta mezcla colocar 10 µl en la cámara de Neubauer y contar el número de células vivas y muertas. Las células muertas tienen alta permeabilidad para el colorante y se tiñen de azul. El conteo se realiza en los cuatro cuadrantes grandes de las esquinas, cada uno de los cuales esta dividido en 16 cuadrados pequeños. El conteo total se promedia entre cuatro. El área de cada cuadrante grande es de 1 mm^2, la cual se cubre con 0.1 µl de suspensión celular. Por lo tanto, para obtener el número de células por ml multiplicamos el promedio obtenido por $2X10^4$, ya que el factor de dilución en azul de tripano fue de 1:2.
13. Sembrar $1.0X10^6$ fibroblastos alimentadores por caja de cultivo de 6 cm de diámetro (Corning 430166) previamente tratada con gelatina.
14. Incubar toda la noche para que las células se adhieran y cambiar el medio para eliminar las células muertas. Los fibroblastos pueden mantenerse hasta por 1 mes en la incubadora pero deben inspeccionarse bajo el microscopio para verificar que la monocapa esté intacta antes de usarse.

B) Subcultivo de fibroblastos alimentadores.

En ocasiones es necesario subcultivar los fibroblastos para expandir la población y obtener un mayor número de cajas para el cultivo de CTEs.

Procedimiento:

1. Realizar los pasos 6-12 del procedimiento para cultivar fibroblastos.
2. Sembrar alrededor de 40,000 células/ml en cajas de cultivo tratadas con gelatina.

C) Cultivo de Células Troncales Embriónicas.

Procedimiento:

1. Descongelar un vial de CTEs (ver abajo)
2. Cultivar las CTEs sobre una monocapa de células STO en medio M15, remplazando el medio cada vez que se acidifique. Esto puede detectarse mediante el indicador de pH en el medio (rojo fenol usualmente), el cual cambia hacia el anaranjado-amarillo conforme disminuye el pH. El crecimiento de las colonias debe ser inspeccionado constantemente para subcultivar las CTEs antes de que lleguen a confluencia.
3. Cambiar el medio M15 de 2 a 3 h antes de subcultivar las CTEs.
4. Aspirar el medio y lavar 2 veces con PBS.
5. Añadir 1 ml de tripsina al 0.25% a la caja de 6 cm de diámetro. Incubar a 37°C por 10 min.
6. Agregar 1 ml de medio M15 para inactivar la tripsina y dispersar las células subiendo y bajando el líquido por 30 veces con una pipeta.
7. Dejar sedimentar las colonias sin disociar, transferir el sobrenadante a otro tubo y centrifugarlo a 220 x g por 5 min.
8. Resuspender las células en 5-10 ml de M15 según el tamaño del pellet. Tomar una alícuota y contar en la cámara de Neubauer como se describió anteriormente.
9. Sembrar aproximadamente $1X10^6$ células en 4 ml de M15 por cada caja de 6 cm de diámetro que tenga una monocapa de fibroblastos alimentadores.
10. Cambiar el medio diariamente para evitar que se acidifique hasta que el cultivo llegue a semiconfluencia.

D) Congelación y descongelación de células.

Procedimiento para la descongelación:

1. Sacar del tanque de nitrógeno líquido el vial de las células y transferirlo inmediatamente a un baño de agua a 37°C.
2. Una vez que el medio se descongele completamente (1 a 2 minutos), transferir la suspensión celular a un tubo de 15 ml y agregar 5 ml de medio (STO o M15, según corresponda) muy lentamente por la pared del tubo. Mezclar suavemente por inversión hasta homogeneizar la solución.
3. Centrifugar las células a 220 x g durante 5 min.
4. Aspirar el sobrenadante y resuspender el pellet en el medio apropiado. Las células alimentadoras se resuspenden en 10 ml y se siembran en caja gelatinizada de 10 cm. Las CTEs se resuspenden en 4 ml y se siembran en caja de 6 cm con fibroblastos alimentadores.

Procedimiento para la congelación:

1. Tripsinizar a las células en la forma habitual.
2. Tomar una alícuota y contar en la cámara de Neubauer.
3. Centrifugar a 220 x g por 5 min.
4. Resuspender en la mitad del volumen requerido para obtener una densidad de 1-2 $X10^6$ células/ml.
5. Etiquetar los viales de congelación con el nombre de la línea, número de pase, cantidad de células y fecha.

6. Agregar suavemente un volumen de medio para congelar 2X a la suspensión celular. Homogeneizar mezclando suavemente.
7. Hacer alícuotas rápidamente en viales de congelación (Corning 430487). Agregar 0.5 ml de la suspensión celular por vial.
8. Transferir los viales de congelación a -70°C por 24 h y posteriormente almacenar en el tanque de nitrógeno líquido.

Etapa 2: Formación de los Cuerpos Embrioides.

1. Subcultivar $3X10^6$ CTEs en una caja de 10 cm de diámetro previamente gelatinizada sin fibroblastos alimentadores.
2. Agregar medio M15 suplementado con 1000 U/ml de LIF.
3. Cultivar las células por 3-4 días cambiando el medio diariamente.
4. Aspirar el medio y lavar dos veces con PBS.
5. Agregar 2 ml de tripsina 0.25% e incubar a 37°C por 10 min.
6. Inactivar la tripsina con al menos 2 ml de medio M15 y transferir a un tubo de 15 ml.
7. Centrifugar a 220 x g por 5 min. Resuspender las células en 5 ml de medio M15.
8. Tomar una alícuota y contar en la cámara de Neubauer. Se esperan aproximadamente $15X10^6$ células por plato de 10 cm de diámetro.
9. Sembrar $2X10^6$ células por plato bacteriológico de 10 cm de diámetro en 10 ml de medio M15.
10. Cultivar las células por 4 días a 37°C, 5% CO2/95% aire atmosférico. Cambiar el medio en el día 2 de la siguiente manera:

- Transferir los CEs a un tubo Falcon de 50 ml usando una pipeta de 25 ml.
- Dejar que los CEs se asienten en el fondo del tubo (aproximadamente 5 min).
- Aspirar el medio y resuspender los CEs en 10 ml de medio M15.
- Con una pipeta de 25 ml, transferir gentilmente los CEs a la caja de 10 cm de diámetro original.

Etapa 3: Selección de Células Precursoras Neurales.

1. A los 4 días de la etapa anterior, transferir los CEs a un tubo de 15 ml con una pipeta de 25 ml.
2. Permitir que se asienten los CEs en el fondo del tubo.
3. Aspirar el medio y añadir 10 ml de medio M15.
4. Transferir los CEs a una caja de 10 cm de diámetro previamente gelatinizada. Incubar por 24 h a 37°C y 5% CO2/95% aire atmosférico. Este tiempo es suficiente para que los CEs se adhieran al plato de cultivo.
5. Cambiar el medio M15 por 10 ml de medio ITSFn.
6. Cultivar los CEs por 7 días cambiando el medio cada 2 días y al séptimo día pasar a la etapa 4.

Etapa 4: Expansión de Células Precursoras Neurales.

A) Preparación de las cajas para la expansión:

1. En una caja de 24 pozos (Corning 3524, Nunc 177437) cubrir algunos pozos con 0.5 ml de Poli-D-Lisina. Incubar toda la noche a 37°C.
2. Lavar 3 veces con PBS y dejar los pozos con 0.5 ml de PBS toda la noche a 37ºC.
3. Lavar 1 vez con PBS y cubrir los pozos con 0.5 ml de fibronectina 1μg/ml (preparada en agua grado cultivo y esterilizada por filtración a través de membrana de 0.22 μm). Incubar a 37°C al menos por 3 h.

5. Lavar los pozos 1 vez con PBS antes de usarse. Las cajas pueden mantenerse a 4°C en PBS hasta por 1 semana.

B) Expansión de las Células Precursoras Neurales.

1. A los 7 días de selección aspirar el medio ITSFn y lavar 2 veces con PBS.
2. Agregar 2 ml de tripsina 0.05%, cubriendo toda la superficie de la caja. Incubar por 7 min a 37°C.
3. Inactivar la tripsina con 2 ml de medio M15. Desprender las células del fondo de la caja de cultivo, aspirando el líquido con una pipeta y haciéndolo fluir lentamente sobre las células.
4. Transferir a un tubo y esperar 5 min para permitir que los agregados celulares se asienten en el fondo. Tomar la suspensión celular restante.
5. Disociar brevemente las células y centrifugar a 220 x g por 5 min.
6. Aspirar el medio y resuspender en medio de expansión sin factores para contar en cámara de Neubauer.
7. Sembrar 3-4X10^5 células por pozo de las cajas pre-tratadas de 24 pozos. Agregar Fgf2 (10 ng/ml), Fgf8 (100 ng/ml) y Shh (500 ng/ml). Al agregar las células y los factores ajustar para un volumen final de 500 μl por pozo. Incubar a 37°C en 5% CO2/95% aire atmosférico.
9. Cambiar el medio de expansión y los factores de crecimiento al tercer día y luego cada vez que se acidifique el medio (se torne anaranjado-amarillo) hasta el sexto día.

Etapa 5: Diferenciación de las CPNs a neuronas dopaminérgicas.

1. Cambiar el medio de expansión por medio de diferenciación e incubar las células a 37° C y 5% CO2/95% aire atmosférico por otros 6 días, cambiando el medio de diferenciación cada dos días.

2. Generación de neuroesferas.

Soluciones

1. Solución amortiguadora de fosfatos 1X (“Phosphate Buffered saline” o PBS 1X).
2. Medio Definido.

Para 25 ml:

DMEM/F12 con Hepes	23.869 ml
Glutamax 100X	0.250 ml
Insulina 25 μg/ml	0.156 ml del stock a 4 μg/ml
Transferrina 100 μg/ml	0.625 ml del stock a 4 μg/ml
Progesterona 20 nM	0.005 ml del stock a 100 μM
Putresina 60 μM	0.015 ml del stock a 0.1 M
Selenita de sodio 30 nM	0.075 ml del stock a 10 μM
Heparina 2 μg/ml	0.001 ml del stock a 100 μg/ml

El medio definido se mantiene a 4º C y puede utilizarse hasta por 15 días.

3. Medio definido con albúmina 0.1%.
4. Verseno (EDTA 0.02%).
5. Tripsina 0.1%.
7. Fgf2 a 1 ng/μl*.
8. Egf a 1 ng/μl*.

*Preparar como se explicó anteriormente pero en medio definido con 1 mg/ml de albúmina.

9. Fluído cerebro-espinal artificial (aCSF, por sus siglas en inglés) modificado.

Para 50 ml:

NaCL (124 mM)	360 mg
KCl (5 mM)	18.6 mg
MgCl2 (3.2 mM)	32.5 mg
CaCl2 (0.1 mM)	0.75 mg*
NaHCO3 (26 mM)	109 mg
D-glucose (10 mM)	90.1 mg

Aforar con agua bidestilada y filtrar por membrana de 0.22 μm.

10. Solución de digestión para región subventricular del cerebro adulto.

Para 50 ml:

Hialuronidasa	33.5 mg*
Acido kinurénico	10 mg
Tripsina	5.2 ml del stock a 2.5%

Aforar a 50 ml con aCSF modificado. Esterilizar por filtración, hacer alícuotas de 30 ml y congelarlas a -20º C.

*Pesar al final porque son altamente higroscópicos.

A) Procedimiento para generar neuroesferas embrionarias primarias.

1. Esterilizar por inmersión en etanol al 96% por 30 min todos los instrumentos de disección. Dejar secar sobre gasa estéril antes de utilizarlos.
2. Sacrificar la ratona de 11.5 días de gestación por dislocación cervical.
3. Bañar el abdomen con etanol al 96%, retirar los cuernos uterinos y colocarlos en una caja petri estéril.
4. Remover cada uno de los embriones del utero, retirar el saco amniótico y colocar los embriones en una caja petri de 35 mm con DMEM/F12.
5. Terminar de retirar los restos de saco amniótico y pasar a los embriones a otra caja petri con DMEM/F12.
6. Mediante un microscopio estereoscópico se diseca la región mesencefálica más ventral, siguiendo los siguientes pasos:

- Se realiza un corte en la parte más anterior de la vesícula mesencefálica y otro a nivel del organizador del Istmo. Estos cortes se realizan casi perpendiculares entre sí, de tal forma que convergen en un punto, lo que permite separar el mesencéfalo del resto del embrión (como referencia ver Fig.1 en publicación 1).
- Trasladar los tejidos a otra caja de 35 mm con DMEM/F12 fresco.
- Hacer un corte de la vesícula mesencefálica a lo largo de toda la línea media dorsal para abrir completamente el tubo neural.
- Viendo la zona ventricular del tejido, realizar dos cortes paralelos, uno en cada lado de la línea media ventral, para obtener un tejido enriquecido en progenitores de neuronas dopaminérgicas. Remover completamente las meninges.

Durante toda la disección, los embriones se deben mantener en hielo.

7. Colocar los tejidos en un tubo con 500 μl de tripsina al 0.1% e incubar a 37º C por 20 min.
8. Para detener la actividad de tripsina agregar 500 μl de medio definido con 10% de suero fetal bovino inactivado.
9. Disgregar mecánicamente los tejidos con una pipeta de 1 ml, subiendo y bajando alrededor de 20 veces el líquido con los tejidos, evitar en lo posible hacer burbujas.
10. Terminar de disociar el tejido con una pipeta Pasteur con la punta redondeada con fuego. Subir y bajar el líquido 20-25 veces. Dejar sedimentar 1 min los restos sin disgregar y pasar el sobrenadante a otro tubo.

11. Centrifugar la suspensión celular a 3000 r.p.m. por 5 min.
12. Con la bomba de vacío aspirar el sobrenadante y resuspender el pellet en 1 ml de medio definido a temperatura ambiente.
13. Determinar el número de células por ml (ver arriba).
14. Sembrar a una densidad de $5X10^5$ células/ml en cajas de cultivo de 12 pozos. Ajustar el volumen para que en cada pozo tenga un volumen final 960 μl de medio.
15. Agregar por pozo 20 μl de Fgf2 (1 ng/μl) y 20 μl de Egf (1 ng/μl). Incubar a 37º C en 5% CO2/95% aire atmosférico por 7 días. Reponer la mitad de ambos factores (10 ng/ml) cada 2 días.

Procedimiento para generar neuroesferas embrionarias secundarias.

1. Con una pipeta serológica recolectar las neuroesferas y colocarlas en un tubo falcon de 15 ml. Lavar los pozos para recuperar la mayor cantidad de neuroesferas posibles. Dejar que sedimenten aproximadamente por 5 min.
2. Retirar cuidadosamente la mayor cantidad de medio posible y agregar 500 μl de tripsina al 0.025%. Incubar por 10 min a 37º C.
3. Inactivar la tripsina como se describió anteriormente y dispersar mecánicamente las células con 15 pases usando una pipeta serológica de 1 ml.
4. Terminar de dispersar mecánicamente con 20 pases mediante una pipeta Pasteur con la punta roma.
5. Centrifugar a 3000 r.p.m. por 5 min y resuspender en 1 ml de medio definido.
6. Contar el número de células como se describió anteriormente y sembrar a 100,000 células por ml en cajas de 12 pozos con 20 ng/ml de Fgf2 y 20 ng/ml de Egf.
7. Incubar a 37º C en 5% CO2/95% aire atmosférico por 7 días. Agregar la mitad de cada factor (10 ng/ml) cada dos días y cambiar un cuarto del medio definido al cuarto y sexto día.

Para inducir la diferenciación *in vitro*, hacer el mismo procedimiento pero cultivando hasta por 7 días sin factores de crecimiento en medio definido con 10% de SFB en cajas recubiertas con poli-D-lisina (4 μg/ml) y laminina (5 μg/ml).

B) Procedimiento para generar neuroesferas del cerebro adulto.

1. Retirar el cerebro de la cavidad craneal y colocarlo en una caja de 35 mm con medio DMEM/F12 para remover la sangre.
2. Cambiar el cerebro a una caja de 35 mm con medio definido y bajo el microscopio de disección hacer un corte coronal a nivel del inicio anterior de los núcleos amigdalinos y un segundo corte justo delante del inicio del quiasma óptico. Mantener en hielo.
3. Separar la región subventricular de cada hemisferio y colocarlas en cajas con medio.
4. Con un bisturí hacer fragmentos de la región subventricular de aproximadamente 1 mm, evitando macerar el tejido.
5. Depositar los tejidos en 3 ml de la solución de digestión e incubar a 37º C durante una hora y media, mezclando los tejidos cada 20 minutos para evitar que se adhieran entre sí.
6. Centrifugar a 1500 r.p.m. por 5 min.
7. Descartar el sobrenadante, adicionar al menos 3 ml de inhibidor de tripsina y triturar 4 veces con pipeta de 1 ml.
8. Centrifugar a 1500 r.p.m. por 5 min., descartar el sobrenadante y resuspender en medio definido con Fgf2 y Egf (20 ng/ml cada uno).

9. Pasar la suspensión celular a través de un tamizador celular de nylon de 70 μm de apertura de poro (BD Falcon, 352350).
10. Contar células en cámara de Neubauer y sembrar 3-4X10^4 en 3 ml por pozo de 35 mm. Adicionar diariamente 20 ng de Egf y Fgf2 por cada ml de medio.

3. Cultivo de explantes en colágena.

Soluciones

1. Medio Optimix.

Para 50 ml:

Optimem con glutamax	36 ml
DMEM/F12 con HEPES	12.5 ml
Glucosa 2M en PBS	1 ml
GPS 100X	0.5 ml

2. NaCl 1.5 M *
3. $NaHCO_3$ 7.5%*

*Preparar en agua de cultivo y esterilizar por filtración con membrana de 0.22 μm.

4. Gel de colágena de rata. Hacer una mezcla con las siguientes cantidades:

Optimix	300 μl
Colágena de rata	75-100 μl
NaCl 1.5 M	10 μl
$NaHCO_3$	10 μl

Nota: La colágena fue preparada en el laboratorio del Dr. Alfredo Varela-Echavarría (Instituto de Neurobiología/UNAM). Durante la extracción de la colágena a partir de cola de rata, la concentración final puede variar de lote en lote. En general entre 75 y 100 μl de colágena resulta en un crecimiento adecuado de los explantes.

Procedimiento para cultivar explantes en colágena.

1. Disecar la región mesencéfalo-metencéfalo de embriones E10.5. Para separar la región de interés se efectúa un corte en la región más anterior del mesencéfalo y un corte posterior a la altura del "labio rómbico" (ver Fig. 1A, publicación 1). Ambos cortes tienen una orientación casi perpendicular entre sí y convergen en un punto, por lo cual no es necesario realizar más cortes para separar la región. No remover las meninges para evitar maltratar los tejidos. Durante los primeros pasos de la disección los embriones se mantienen en DMEM/F12, pero una vez separada la región de interés, los tejidos se transfieren al medio Optimix. Durante toda la disección los tejidos se mantienen en hielo.
2. Preparar el gel de colágena de rata. La mezcla para hacer el gel debe estar en hielo en todo momento para evitar la polimerización de la colágena.
3. Cortar 4 tejidos a lo largo de la línea media dorsal para cultivarlos en forma de "libro abierto" (ver Fig. 1A, publicación 1). No hacer este corte en todos los tejidos disecados porque se deforman rápidamente.
4. Con la ayuda de una punta azul con el extremo recortado, transferir los 4 tejidos abiertos a una caja petri de 35 mm, depositando cada tejido en una pequeña gota de medio.
5. Colocar la región ventricular de cada tejido hacia arriba.
6. Remover casi todo el medio, dejando solo una delgada película de líquido sobre los tejidos para evitar que se sequen completamente.
7. Agregar a cada tejido 30-40 μl de la mezcla de colágena.
8. Acomodar cada tejido para que quede completamente cubierto con la colágena.

9. Incubar 1 h a 37º C en 5% CO2/95% aire atmosférico para permitir que la colágena polimerice completamente.
10. Cubrir los explantes suavemente con 2 ml de medio Optimix e incubar por 7 días, cambiando la mitad del medio cada 2 días.

4. Transplante celular al explante.

A) Procedimiento general para transplantar células a los explantes en colágena.

1. Realizar el cultivo de explante en colágena hasta el paso 9 como se describió anteriormente.
2. Mientras la colágena polimeriza, preparar una suspensión celular como se describió para el subcultivo de las neuroesferas y de las CTEs. La suspensión celular a transplantar debe quedar muy concentrada. Como referencia se puede observar en el microscopio una gota de la suspensión en la cual idealmente no deben observarse espacios entre las células. La población de interés debe expresar constitutivamente la proteína verde fluorescente (GFP) para distinguir las células transplantadas de las del explante.
3. Con la ayuda de un mechero de alcohol, estirar a un diámetro muy fino la punta de pipetas Pasteur largas. En este paso se preparan alrededor de 10 pipetas de diferentes diámetros de grosor para utilizar la más delgada que aún permita el flujo fácil de la suspensión celular.
4. Al terminar la primera hora de polimerización de la colágena, sacar los tejidos de la incubadora y bajo el microscopio estereoscópico inyectar a través de la colágena la suspensión celular con la ayuda de las pipetas Pasteur alargadas. Las células se depositan sobre la superficie del explante sin perforarlo. Para controlar el flujo de las células, se utiliza una manguera con boquilla en el extremo de las pipetas Pasteur.
5. Incubar por 3 h a 37º C para permitir que las células se adhieran al tejido.
6. Agregar suavemente 2 ml de medio Optimix por caja de 35 mm e incubar los explantes por 7 días a 37º C en 5% CO2/95% aire atmosférico. Cambiar la mitad del medio cada dos días.

B) Procedimiento para localizar las células transplantadas específicamente en la línea media ventral del explante.

Como la línea media ventral del mesencéfalo forma un canal que dispersa fácilmente las células transplantadas, es necesaria una modificación del protocolo anterior:

1. En el paso 4 del protocolo de cultivo de explantes (ver arriba) transferir 4 tejidos sobre un inserto para cultivo organotípico (Millicell-CM, Millipore PICMORG50) colocado en una caja de cultivo de 35 mm.
2. Colocar hacia arriba la región ventricular de los tejidos y quitar la mayor cantidad de medio posible.
3. Depositar la suspensión celular sobre la línea media ventral, usando pipetas Pasteur alargadas como se describió anteriormente.
4. Transferir el inserto a una caja de 35 mm que tenga 2 ml de medio Optimix.
5. Incubar toda la noche para permitir que se adhieran las células.
6. Al siguiente día transferir suavemente los tejidos sobre el inserto a una caja limpia de 35 mm. Para desprender los tejidos de la membrana es necesario agregar un poco de medio sobre los explantes.
7. Seguir desde el paso 5 el procedimiento habitual para el cultivo en colágena (ver arriba).

8. Incubar los explantes por 7 días. Cambiar la mitad del medio cada dos días.

5. Análisis de la diferenciación de las células transplantadas.

Soluciones

1. Paraformaldehído (PFA) 4%.

Para 200 ml:

- Agregar 8 g de PFA a 90 ml de agua bidestilada.
- Calentar entre 60-70º C en campana de extracción. No sobrecalentar.
- Agregar por goteo NaOH hasta que el PFA se disuelva completamente al mezclar.
- Agregar 100 ml de PBS 2X y dejar enfriar completamente en campana de extracción.
- Ajustar a pH 7.2.
- Aforar a 200 ml con agua bidestilada.
- Filtrar para eliminar los restos de PFA sin disolver.
- Hacer alícuotas de 5 ml, las cuales se pueden guardar a -70º C indefinidamente o a 4ºC hasta 1 semana.

2. Sacarosa 30% en PBS 1X.

3. Gelatina 1% preparada en agua bidestilada.

4. Solución de bloqueo.

Para 10 ml:

PBS 1X	9,840 μl
Tritón X-100	50 μl del stock al 20% en PBS 1X
Suero Fetal Bovino	100 μl
Azida de sodio	10 μl del stock al 10% en PBS 1X

5. Dapi (Molecular probes).

6. Solución de montaje para fluorescencia: buffer 0.1 M de bicarbonato de sodio pH 9 mezclado 1:1 con glicerol.

7. Reactivos para detección de muerte celular *in situ* con la técnica de "TUNEL" ("Terminal transferase-mediated biotin dUTP nick end labeling"; Roche diagnostics), mediante la cual se detecta la fragmentación del DNA presente en las células apoptóticas.

Anticuerpos primarios monoclonales (generados en ratón).

1. Anti-Nestina (chemicon). Diluir 1:100.
2. Anti-β III Tubulina (chemicon). Diluir 1:100.
3. Anti-Oct3/4 (BD Bioscience). Diluir 1:100.
4. Anti-NeuN (Chemicon). Diluir 1:400.
5. Anti-O4 (Chemicon). Diluir 1:100.
6. Anti-Nkx6.1 (Developmental Studies Hybrydoma Bank). Diluir 1:2.
7. Anti-Nkx2.2 (Developmental Studies Hybrydoma Bank). Diluir 1:2.
8. Anti-Islet1/2 (Developmental Studies Hybrydoma Bank). Diluir 1:2.
9. Anti-Pax7 (Developmental Studies Hybrydoma Bank). Diluir 1:2.
10. Anti-En1 (Developmental Studies Hybrydoma Bank). Diluir 1:2.

Anticuerpos primarios policlonales (generados en conejo).

1. Anti-β III Tubulina (Covance). Diluir 1:2000.
2. Anti-GFAP (Chemicon). Diluir 1:250.
3. Anti-TH (Chemicon). Diluir 1:500.
4. Anti-Lmx1a (donado por Michael German MD, UCSF, Estados Unidos). Diluir 1:1000.
5. Anti-Ptx3 (donado por Dr. Marten Smidt, Utrecht University, Holanda). Diluir 1:5000.

Anticuerpos secundarios.

1. Alexa Fluor 350 anti-ratón (Molecular probes). Diluir 1:50.
2. Alexa Fluor 488 anti-ratón (Molecular probes). Diluir 1:1000.
3. Alexa Fluor 488 anti-conejo (Molecular probes). Diluir 1:1000.
4. Alexa Fluor 594 anti-ratón (Molecular probes). Diluir 1:1000.
5. Alexa Fluor 594 anti-conejo (Molecular probes). Diluir 1:1000.
6. Alexa Fluor 647 anti-ratón (Molecular probes). Diluir 1:1000.
7. Alexa Fluor 647 anti-conejo (Molecular probes). Diluir 1:1000.

En cada caso el número indica aproximadamente la longitud de onda en nanómetros (nm) con la cual se obtiene la máxima excitación del fluoróforo. Alexa fluor 350 se excita con luz UV y su pico de emisión es a 442 nm en azul. Alexa fluor 488 se excita con luz azul y su pico de emisión es a 519 nm en verde. Alexa fluor 594 se excita con luz verde y su pico de emisión es a 619 nm en rojo. Alexa fluor 647 se excita con luz roja y su pico de emisión es a 666 nm en el rojo lejano.

A) Procesamiento del tejido cultivado en colágena

1. Al terminar el periodo de cultivo, los tejidos embebidos en la colágena se sumergen en tubos para hibridación con PFA 4% y se incuban a 4º C por media hora.
2. Transferir los tubos al cuarto frío e incubar 2 h con mezclado suave.
3. Hacer 3 lavados de 15 min cada uno con PBS 1X, manteniendo los tubos en mezclado a 4º C. Una vez terminado este paso los tejidos se pueden guardar alrededor de 1 mes a 4º C en PBS. Si se desea hacer los cortes del tejido, seguir al siguiente paso.
4. Crio-proteger los tejidos en sacarosa al 30%, incubando por lo menos toda la noche a 4º C con mezclado moderado. Idealmente dejar el tejido 2 días en sacarosa para obtener cortes más íntegros.
5. Congelar el tejido crio-protegido en la resina para congelar (“tissue freezing medium”; Jung) y realizar cortes de 10 μm de espesor en el criostato.
6. Recuperar los cortes en laminillas pretratadas con Gelatina 1%. En cada laminilla delimitar el área de los cortes mediante un lápiz hidrofóbico (Innogenex, Vector laboratories H-4000); esto permite utilizar menos cantidad de anticuerpos y detectar más de un marcador por laminilla.
7. Almacenar las laminillas con los cortes indefinidamente a -70º C o seguir a la siguiente etapa.

B) Inmunohistoquímica con fluorescencia

1. Dejar secar los cortes de tejido por 30 min a temperatura ambiente.
2. Fijar por 2 min con PFA 4% cubriendo completamente todos los cortes en la laminillas. A partir de este paso se debe evitar que los cortes se sequen.
3. Lavar 5 veces con PBS 1X.
4. Incubar los cortes por 10 min con solución de bloqueo. En caso de tener mucha señal inespecífica al final del procedimiento, incubar hasta 1 h.
5. Incubar los cortes toda la noche a 4º C con el anticuerpo primario diluido en solución de bloqueo.
6. Hacer 3 lavados de 5 min cada uno con PBS.
7. Incubar los cortes 1 h a temperatura ambiente con el anticuerpo secundario correspondiente diluido en solución de bloqueo.
8. Hacer 3 lavados de 5 min cada uno con PBS.
9. Incubar con Dapi 1X por 2 min y enjuagar una vez con PBS.

10. Cubrir los cortes con solución de montaje y colocar cubreobjetos. Las laminillas se pueden guardar así hasta por 5 días a 4º C.
Nota: A partir del paso 4 los cortes se mantienen en una cámara humedad para evitar que se sequen.
Las muestras se observan en el microscopio de epifluorescencia Zeiss o en el microscopio confocal LSM 510 meta.

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The embryonic midbrain directs neuronal specification of embryonic stem cells at early stages of differentiation

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ABSTRACT

Specific neuronal differentiation of Embryonic Stem Cells (ESCs) depends on their capacity to interpret environmental cues. At present, it is not clear at which stage of differentiation ESCs become competent to produce multiple neuronal lineages in response to the niche of the embryonic brain. To unfold the developmental potential of ESC-derived precursors, we transplanted these cells into the embryonic midbrain explants, where neurogenesis occurs as in normal midbrain development. Using this experimental design, we show that the transition from ESCs to Embryoid Body (EB) precursors is necessary to differentiate into Lmx1a$^+$/Ptx3$^+$/TH$^+$ dopaminergic neurons around the ventral midline of the midbrain. In addition, EB cells placed at other dorsal–ventral levels of the midbrain give rise to Nkx6.1$^+$ red nucleus (RN) neurons, Nkx2.2$^+$ ventral interneurons and Pax7$^+$ dorsal neurons at the correct positions. Notably, differentiation of ESCs into Neural Precursor Cells (NPCs) prior to transplantation markedly reduces specification at the *Lmx1a*, *Nkx6.1* and *Pax7* expression domains, without affecting neuronal differentiation. Finally, exposure to Fgf8 and Shh in vitro promotes commitment of some ESC-derived NPCs to differentiate into putative Lmx1a$^+$ dopaminergic neurons in the midbrain. Our data demonstrate intrinsic developmental potential differences among ESC-derived precursor populations.

Introduction

Embryonic Stem Cells (ESCs) are pluripotent cells capable of contributing to all somatic tissues and to the germ line upon injection into blastocyst-stage embryos. Terminal differentiation of ESCs occurs through the initial formation of tissue-specific stem and progenitor cells (Murry and Keller, 2008). In vitro, ESC differentiation is usually directed with different sets of extrinsic signalling molecules that are known to specify the desired cell type (Murry and Keller, 2008). Nevertheless, as culture conditions are not equivalent to the stem cell niche, the differentiation potential displayed in vitro by ESC-derived precursors may dramatically change in a natural environment. For adult tissues that possess limited regenerative capacity (e.g., the brain), the differentiation potential of ESC-derived precursors has to be tested during embryonic development. This can be accomplished by using embryonic tissue explants as an instructive matrix that contains all the factors that specify the lineage under study.

During early stages of midbrain development, Neural Precursor Cells (NPCs) at the ventricular zone acquire a transcriptional code that determines the neuronal identity of their derivatives upon differentiation (Nakatani et al., 2007). The ventral-most NPCs express *Lmx1a*, *Msx1/2* and *En1/2* which are required for differentiation into midbrain dopaminergic (mDA) neurons (Andersson et al., 2006; Nakatani et al., 2007; Simon et al., 2001). The expression of these genes depends on the signalling morphogens like Sonic hedgehog (Shh) and Fibroblast growth factor 8 (Fgf8), secreted by the floor plate and the isthmus organizer, respectively (Ye et al., 1998). Terminal differentiation into mDA neurons involves the expression of *Nurr1* and *Ptx3*, as well as the expression of the Tyrosine Hydroxylase gene (*Th*) for dopamine synthesis (Saucedo-Cardenas et al., 1998; Smidt et al., 2004). Adjacent to the *Lmx1a* expression domain, *Nkx6.1* is expressed in NPCs that differentiate into glutamatergic "red nucleus" (RN) neurons, that retain the expression of this gene (Nakatani et al., 2007). At the RN, some Nkx6.1$^+$ NPCs differentiate into motor neurons that express *Islet1* (Fedtsova and Turner, 2001). Between the dorsal and ventral *Nkx6.1* expression domains, *Nkx2.2* is expressed in a narrow band of NPCs that eventually differentiate into Nkx2.2$^+$ GABAergic neurons (Nakatani et al., 2007). On the other hand, the expression of *Pax7* is found in NPCs and in some post-mitotic neurons of the dorsal midbrain (Fedtsova and Turner, 2001; Thomas et al., 2006), where the superior colliculus, the primary visual centre in higher vertebrates, arises (Thompson et al., 2004). Thus, the presence of specific transcription factors, such as Lmx1a, Nkx6.1, Nkx2.2 and Pax7, identifies different midbrain precursor cells committed to specific neuronal phenotypes.

Ectodermal differentiation of ESCs through Embryoid Body (EB) formation is commonly used to derive NPCs in vitro. These NPCs show

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the ability to differentiate into a variety of neuron subtypes. For instance, specific culture conditions after EB formation promote differentiation of ESCs into mDA neurons (Kawasaki et al., 2000; Lee et al., 2000; Ying et al., 2003). However, the proportion of mDA neurons generated is generally low compared with the number of cells present at the end of the differentiation protocols (Rodriguez-Gomez et al., 2007). Progressive restrictions in the differentiation potential of ESC-derived precursors might account for their limited production of specific neuronal lineages in vitro. Alternatively, it should be considered that relevant signals present in the embryonic niche of the brain might be absent in culture.

ESC-derived NPCs differentiate into neurons and glial cells after transplantation to the embryonic and neonatal brains (Brustle et al., 1997; Zhang et al., 2001). In the embryonic mouse brain and in rat hippocampal slices, ESC-derived neurons receive excitatory and inhibitory synaptic input and display electrophysiological properties of functional neurons (Benninger et al., 2003; Wernig et al., 2004). Yet in many cases, ESC-derived neurons expressed *Th* and *En1* outside the midbrain (Wernig et al., 2004). Site-specific differentiation of ESC-derived precursors after integration into the embryonic midbrain must involve the influence of an instructive niche over a competent cell population. In the present study, we demonstrate that ESC-derived precursors neuralized within the embryonic midbrain, but not in vitro, present high developmental potential, giving rise to neurons with the specific positional identity and neurotransmitter phenotype along the midbrain dorsal–ventral axis.

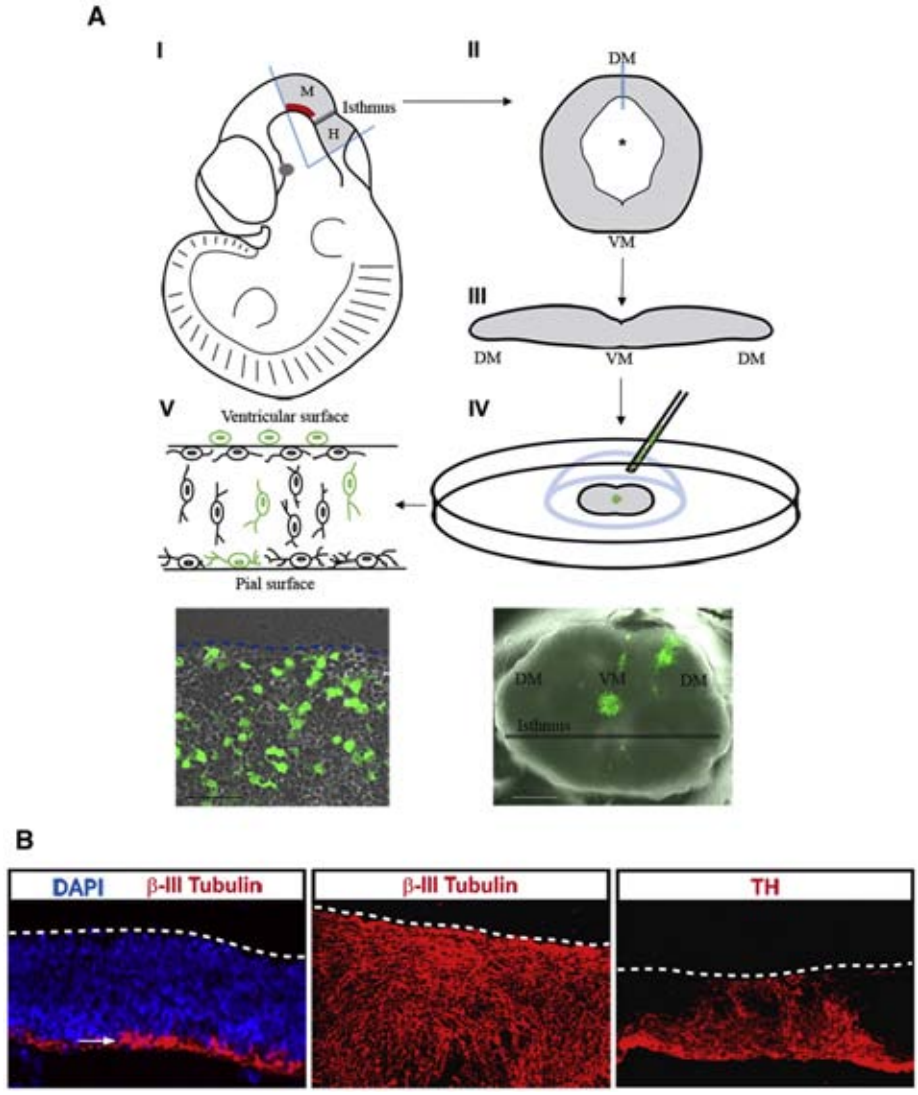

Fig. 1. "Transplant-into-explant" procedure. (A) Experimental design. (I) Lateral view representation of an E10.5 mouse embryo with the prospective midbrain–hindbrain explant highlighted in gray; blue lines indicate the antero-posterior cut sites. (II) Frontal view of the isolated midbrain–hindbrain explant; the asterisk indicates the lumen of the neural tube. For explant preparation, the ventricular zone (the stem cell niche) was exposed after cutting along the dorsal midline, indicated by the blue dashed line. (III) In the resulting "open book" explant, the ventral region is in the middle while the dorsal midbrain is split in two lateral sites. (IV) Explants with the ventricular surface facing up were embedded in a collagen matrix (outlined in blue). Using a glass capillary, donor GFP^+ cells were deposited over the ventricular surface. The image below shows a midbrain–hindbrain explant with transplanted GFP^+ cells viewed directly from above. (V) Schematic representation of a midbrain coronal section; 7 days after transplantation, GFP^+ cells had already crossed the ventricular surface and migrated to the pial surface. The image below shows GFP-labelled midbrain NPCs integrated into the explant. The midbrain tissue was visualized in bright field; the blue dashed line indicates the ventricular surface. (B) Explant development. E10.5 midbrains showed a narrow layer of β-III $Tubulin^+$ neurons (arrow) at the pial surface; while most cells within the neuroepithelium were still undifferentiated (left image). After 7 days, midbrain explants showed β-III Tubulin staining from the ventricular zone to the pial surface, demonstrating that abundant neurogenesis had occurred in vitro (middle image). Robust generation of TH^+ dopaminergic neurons around the ventral midline of the midbrain explants after 7 days in culture (right image). The white dashed lines indicate the ventricular surface in all images. M, midbrain; H, hindbrain; DM, dorsal midbrain; VM, ventral midbrain. Scale bar: 500 μm in IV; 50 μm in V.

Materials and methods

Embryonic stem cells culture and differentiation

The R1B5 ESCs line (gift from Andras Nagy), which constitutively expresses the green fluorescent protein (GFP), was used for all the studies presented here. To produce ESC-derived EB cells, NPCs and dopaminergic neurons in vitro, we used the 5-stage differentiation protocol as previously described (Lee et al., 2000). Briefly, ESCs were propagated (stage 1) on gelatin-coated culture plates with "feeders cells" or in the presence of 1000 U ml^{-1} of leukemia inhibitory factor (LIF; Chemicon). ESCs medium (M15) was composed of Dulbecco's modified eagle medium (DMEM), MEM nonessential amino acids 1X, MEM sodium pyruvate 1X, Glutamax 1X, 15% FCS (all from Gibco), 0.55 mM 2-mercaptoethanol (Sigma), plus penicillin and streptomycin (30 μg ml^{-1} and 50 μg ml^{-1}, respectively; Sigma). To form EBs (stage 2), 2.0×10^6 ESCs were seeded in non-adherent 10 cm bacterial culture dishes and cultivated 4 days in ESCs medium. NPCs were selected from EBs (stage 3) for 7 days under adherent conditions in ITSFn medium containing insulin (5 μg ml^{-1}), transferrine (50 μg ml^{-1}), fibronectin (5 μg ml^{-1}), Glutamax 1X (all from Gibco), selenium (30 nM; Sigma) and antibiotics. For in vitro expansion (stage 4), 1.5×10^5 cells cm^{-2} were seeded in 24-well culture dishes (Corning) previously treated with poly-D-lysine (10 μg ml^{-1}; Sigma) and fibronectin (1 μg ml^{-1}; Gibco). Expansion medium was composed of DMEM-F12 without HEPES buffer, N2, Glutamax 1X (all from Gibco), ascorbic acid (100 μM; Sigma) and laminin (1 μg/ml; Sigma). Expansion media contained Fgf2 (10 ng/ml), Fgf8 (100 ng/ml) and Shh (500 ng/ml) purchased from Peprotech. Cells were expanded for 6 days before promoting terminal differentiation into dopaminergic neurons (stage 5) in expansion medium without growth factors for additional 6 days.

Collagen explant culture

Midbrain–hindbrain explants were obtained from E10.5 (developmental stage: 10.5 days post-coitum) CD-1 mice embryos after cutting from the anterior limit of the midbrain to the rhombic lip (see Fig. 1). The dorsal midline of the explants was cut and the tissue was embedded in a collagen matrix with the ventricular surface facing up (Fig. 1). The collagen mixture contained: rat collagen (100 μl; gift from Dr. Alfredo Varela-Echavarría), 1.5 M NaCl (10 μl), 7.5% $NaHCO_3$ (10 μl) and explant media (300 μl). Explant media (referred in the text as OPTIMIX) was composed of Optimem with Glutamax (72% v/v), DMEM-F12 (25% v/v) from GIBCO, Glucose 2 M (2% v/v; Sigma) and GPS 1X (glutamax/penicillin/streptomycin). Collagen-embedded explants were incubated at 37 °C for 1 h to allow collagen polymerization; OPTIMIX was then added to fully cover the tissues. Explants were maintained at 37 °C in humidified 5% CO_2-95% atmospheric air incubators for 7 days.

"Transplant-into-explant" experimental procedure

Midbrain explants with the ventricular surface facing up were embedded in a collagen matrix as described above. Donor cells were dissociated with trypsin (Gibco), concentrated in 5–15 μl of OPTIMIX and aspired with the tip of a Pasteur pipette previously stretched to form a thin capillary. The tip of the Pasteur pipette was introduced into the collagen matrix and small volumes of the cell suspension were laid down over the tissue with the help of a hose with a mouthpiece. Cells were allowed to adhere to the tissue for 3 h at 37 °C before carefully adding the medium. For transplantation of ventral midbrain progenitors (see Fig. 2), a transgenic mice line expressing GFP was used (gift from Andras Nagy). The exposed ventricular surface of GFP^+ midbrains was gently aspired with a thin glass capillary and cells were dissociated with 0.025% trypsin. Using this technique, we consistently found around 50% of $Nestin^+$ progenitors in the final cell suspension.

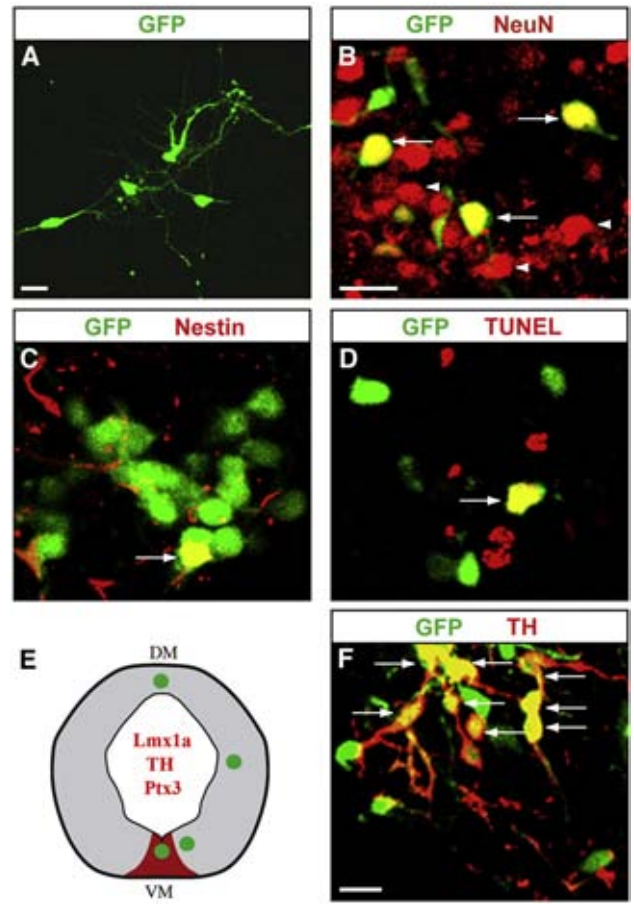

Fig. 2. Neuronal progenitors differentiate into dopaminergic neurons at the ventral midbrain. NPCs isolated from E10.5 midbrains were transplanted to age-matched midbrain explants. After 7 days, GFP visualization in whole mount explants demonstrated that most donor cells presented neuronal morphologies (A). Expression of *NeuN* in many donor cells (B, arrows) and host cells (B, arrowheads) indicate robust neuronal differentiation by the end of the explant culture. In contrast, few donor cells remained as $Nestin^+$ progenitors (C, arrows) after the peak of neurogenesis in the midbrain explants. At several stages of the explant culture, few GFP^+ cells were labelled by the TUNEL technique (D, arrow) demonstrating high survival of transplanted cells. (E) Schematic representation of a midbrain coronal section. The zone of dopaminergic neuron production around the ventral midline ($Lmx1a^+/TH^+/Ptx3^+$ domain) is highlighted in red. Green circles indicate the equivalent dorsal–ventral sites of transplantation in the midbrain explants. Donor NPCs differentiated into TH^+ putative dopaminergic neurons (F, arrows) only after integration around the ventral midline. DM, dorsal midbrain; VM, ventral midbrain. Scale bar: 20 μm; B–D same magnification.

Tissue processing and immunofluorescence

Midbrain explants were fixed with 4% PFA for 2 h at 4 °C, washed with PBS and cryo-protected overnight in 30% sucrose. Midbrain explants were then sliced in 10 μm thick cryostat sections that were fixed with 4% PFA for 2 min. Tissue sections were rinsed with PBS and covered with blocking solution (0.1% Triton X-100, 1% FCS and 0.01% sodium azide) for 10 min at room temperature. Primary antibodies were diluted in blocking solution and incubated on tissue sections overnight at 4 °C. Next day, tissue sections were washed with PBS and then incubated for 1 h at room temperature with secondary antibodies diluted in blocking solution. Finally, tissue sections were washed with PBS and mounted in 0.1 M sodium carbonate buffer (pH 9) 1:1 with glycerol. Primary antibodies were used to detect Nestin (1:100; mouse), β-III Tubulin (1:100; mouse), GFAP (1:250; rabbit), NeuN (1:100; mouse), TH (1:500; rabbit), O4 (1:100; mouse), all from Chemicon; GABA (1:4000; rabbit; Sigma), β-III Tubulin (1:2000; rabbit; Covance), Oct3/4 (1:100; mouse; BD Biosciences), Lmx1a (1:1000; rabbit; gift from M. German), Ptx3 (1:5000; rabbit; gift from

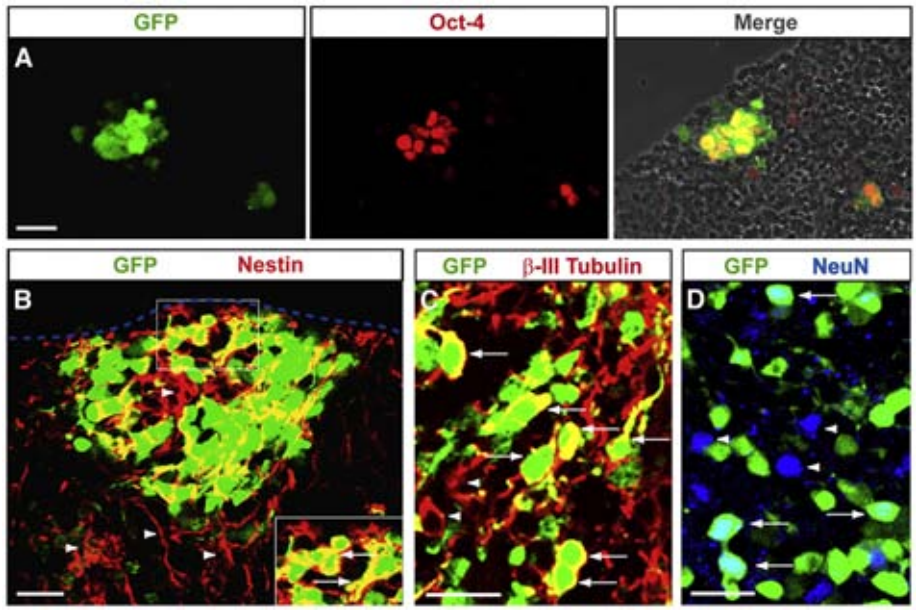

Fig. 3. Differentiation of ESCs through EB formation is necessary for neuralization within the embryonic midbrain. Undifferentiated ESCs or those differentiated in vitro by EB formation were transplanted to midbrain explants. Seven days after transplantation, ESCs integrated into the midbrain parenchyma and maintained the pluripotency marker Oct-4 (A). Transplanted EB cells migrated into deeper layers of the midbrain (B). The blue dashed line indicates the ventricular surface. Some integrated donor cells expressed *Nestin* (B inset, arrows). Most EB cells integrated within the embryonic midbrain had differentiated into β-III Tubulin$^+$ neurons 7 days after transplantation (C, arrows). Despite their immature state at the time of transplantation, some EB cells reached terminal neuronal differentiation as assessed by *NeuN* expression (D, arrows). Scale bar: 25 μm.

M. Smidt), Islet1/2, Nkx2.2, Nkx6.1, and Pax7 (supernatants 1:2; mouse; Developmental Studies Hybridoma Bank). Primary antibodies were detected with the following secondary antibodies: anti-mouse IgGs (goat) coupled to Alexa Fluor 488, 594 and 647 and anti-rabbit IgGs (goat) coupled to Alexa Fluor 488, 594 and 647, all from Molecular Probes. In situ cell death detection was based on labelling DNA breaks by the TUNEL technique (Roche diagnostics). Confocal images were obtained with a ZEISS LSM 510 Meta microscope.

Quantification and data analysis

For cell quantification, single optical planes were analyzed independently within the confocal stacks. The pinhole aperture of all channels was maintained as close as possible to obtain high confocality and only clearly double-labelled cells were counted. Every image was divided with a grid to avoid double-counting GFP$^+$ cells. For Nestin, β-III Tubulin, GFAP and TH, only donor cells that clearly displayed a GFP$^+$ nucleus surrounded by the cytoplasmic markers were considered (e.g. Fig. 3C). Donor cells from all cell populations were obtained from at least three independent cultures. After transplantation, quantification was done by averaging the percentage of positive donor cells for a given marker in at least 3 different midbrains explants. Numbers on cell counting were confirmed by a second independent investigator. Data are presented as the average ± standard deviation; "*n*", indicates the total number of GFP$^+$ cells counted.

Results

Midbrain explant development

Dopaminergic differentiation in the midbrain starts during early stages of embryonic neurogenesis (E11) and is almost complete 4 days later (Bayer et al., 1995). During this developmental period, an instructive niche within the ventral midbrain could direct transplanted NPCs to mDA neurons. However, transplantation of cells to precise locations of the early murine brain is technically difficult (Olsson et al., 1997). To overcome this problem, we designed an experimental strategy based on transplantation of donor cells to E10.5 midbrain collagen explant cultures (Fig. 1A). Collagen explant cultures of developing midbrain have been previously used to study differentiation and neurite growth of dopaminergic neurons (Hynes et al., 1995; Lin et al., 2005; Ye et al., 1998). Midbrains obtained from E10.5 mouse embryos had a thin layer of β-III Tubulin$^+$ neurons at the pial surface (Fig. 1B) indicating the beginning of neurogenesis. After 7 days in culture, β-III Tubulin was detected from the ventricular zone to the pial surface of the explant (Fig. 1B). This indicates that robust neurogenesis occurred during the culture period. Furthermore, although TH was not detected in E10.5 midbrains, after 7 days in culture, many TH$^+$ neurons appeared around the ventral midline (Fig. 1B). Noteworthy, glial cells were rarely observed, suggesting that abundant gliogenic signals are absent in our culture system. These data demonstrate that midbrain explants recapitulate the peak of embryonic neurogenesis, making this experimental system suitable for analyzing the differentiation potential of transplanted NPCs.

The ventral midbrain directs neural progenitors into the dopaminergic fate

We next evaluated whether the neurogenic environment within midbrain explants promotes neuronal differentiation of donor cells. GFP-labelled NPCs isolated from the midbrains of E10.5 embryos were transplanted to the ventricular zone of age-matched midbrain explants. Donor cells reached deep layers of midbrain parenchyma after 7 days (see Fig. 1A, part V). This process required significant migration of GFP$^+$ cells along the radial axis of explants, as transplanted cells were not injected into the tissue but rather deposited over the ventricular wall. Seven days after transplantation, most GFP$^+$ cells had neuronal morphology (Fig. 2A) and expressed *NeuN* (86.88 ± 11.05%; $n = 97$; Fig. 2B), which encodes a nuclear marker of mature neurons. Consistent with this finding, few donor cells expressed *Nestin* (10.66 ± 0.66%; $n = 131$; Fig. 2C), which encodes an intermediate filament marker that identifies NPCs. Cell death quantification by the TUNEL technique showed few apoptotic GFP$^+$ cells (6.05 ± 0.9%, $n = 963$; Fig. 2D) at various times during the entire culture period. Because the majority of donor cells survived, these

Table 1
Percentage[a] of ESC-derived precursors expressing specific neural markers after transplantation to embryonic midbrain explants

	EB	NPC	NPC Fgf8+Shh
Nestin	10.74±1.97%	4.57±2.66%	ND[d]
	n=465	*n*=392	
β-III Tubulin	66.32±5.08%	74.39±9.38%	70.61±7.1%
	n=1002	*n*=296	*n*=326
NeuN	8.09±1.22%	40.65±2.43%	ND
	n=442	*n*=359	
Lmx1a			
In-site[b]	65.53±4.86%	ND	15.21±6.02%
	n=517		*n*=86
Out-site[c]	0%	ND	13.39±7.58%
	n>100		*n*=446
TH			
In-site	38.14±5.66%	4.19±0.92%	6.38±5.03%
	n=1333	*n*=375	*n*=109
Out-site	0%	6.33±5.0%	5.83±4.73%
	n>100	*n*=868	*n*=236
Nkx6.1			
In-site	51.51±2.31%	8.35±3.23%	ND
	n=648	*n*=334	
Out-site	0%	0%	ND
	n>100	*n*>100	
Nkx2.2			
In-site	37.47±10.04%	ND	ND
	n=239		
Out-site	0%	ND	ND
	n>100		
Pax7			
In-site	43.67±3.52%	10.44±8.96%	ND
	n=244	*n*=210	
Out-site	0%	0%	ND
	n>100	*n*>100	

[a] Data are the average percentage±standard deviation of positive cells for a given marker. "*n*" indicates the total number of cells counted. More than a hundred GFP$^+$ cells were visualized (*n*>100) when positive cells for a specific marker were not observed.
[b] Indicates transplanted cells found inside the expression domain of the marker.
[c] Indicates transplanted cells found outside the expression domain of the marker.
[d] Not determined.

data suggest that most transplanted NPCs had differentiated into neurons after 7 days To test the specificity of the ventral midline niche to induce dopaminergic differentiation, donor NPCs were transplanted to different dorsal–ventral levels of E10.5 midbrain explants (Fig. 2E). Donor-derived neurons expressed *Th* around the ventral midline of the midbrain (Fig. 2F) but not elsewhere (data not shown). These results demonstrate that within the neurogenic environment of the midbrain explants, the only region that supports development of donor-derived dopaminergic neurons is around the ventral midline.

Midbrain neurogenesis promotes neuronal differentiation of EB cells

Previous studies have demonstrated that ESCs readily acquire a neural fate in vitro, even under minimal conditions (Smukler et al., 2006). Therefore, we decided to test whether the embryonic midbrain is permissive for ESCs differentiation into neural lineages. Seven days after transplantation to the midbrain, GFP-labelled ESCs were observed fully integrated into the tissue (Fig. 3A). Integrated cells, however, presented a rounded morphology characteristic of undifferentiated ESCs and expressed *Oct4* (Fig. 3A), suggesting that donor cells maintained a pluripotent state. Accordingly, donor cells did not express *Nestin* or β-*III Tubulin* (supplemental online Fig. S1A), confirming the absence of ESC-derived NPCs or neurons, respectively. These data indicate that the midbrain explant environment maintains ESCs undifferentiated.

We next asked whether ESCs differentiation to the ectodermal lineage through EB formation (Rathjen and Rathjen, 2001) may result in a cell population responsive to midbrain neurogenic signals. Aggregation of ESCs during 4 days in the absence of LIF produced EB cells that did not express neural markers, while *Oct4* was expressed at low levels in few cells at the periphery of the aggregate (data not shown). Two days after transplantation of the whole EBs (without cell dissociation), they expressed low levels of *Nestin*, even in zones with not direct contact with the tissue (supplemental online Fig S1B). As EB cells integrated completely into the tissue, some donor-derived NPCs expressed *Nestin* (10.74±1.97%; Table 1) at levels comparable to those of the endogenous NPCs (Fig. 3B). At the end of the explant culture, most integrated donor cells expressed β-*III Tubulin* (66.32±5.08%; Table 1; Fig. 3C) demonstrating robust neuronal differentiation of EB cells. In addition, some EB-derived cells produced NeuN (8.09±1.22%; Table 1; Fig. 3D) and the axonal protein NF-M (supplemental online Fig. S1D), indicating terminal neuronal differentiation. However, the expression profile of most differentiated EB-derived cells was Nestin$^-$/β-III Tubulin$^+$/NeuN$^-$ showing the presence of many immature neurons. This finding was further supported by the immature bipolar morphology of many donor cells (supplemental online Fig. S1C). Importantly, donor cells did not differentiate to GFAP$^+$ astrocytes or O4$^+$ oligodendrocytes (data not shown) indicating that the embryonic midbrain explants exclusively directed EB cells into the neuronal lineage.

EB cells efficiently differentiate into mDA neurons around the ventral midline of the midbrain

To evaluate site-specific differentiation of EB cells, single-cell suspensions were implanted at different dorsal–ventral levels of midbrain explants (see supplemental online Fig. S2A for a precise localization of transplanted cells). Clusters of GFP$^+$ cells roughly remained at their original dorsal–ventral positions after transplantation (Fig. S2A), indicating that lateral movement of EB cells within adjacent dorsal–ventral domains was less prominent than radial migration. Day-4 EB cells did not express any marker of the mDA lineage before transplantation (data not shown). After integration around the ventral midline of the midbrain, close to the isthmus organizer, a large proportion of EB cells expressed *Lmx1a* (65.53±4.86%; Table 1; Fig. 4A). Many of these Lmx1a$^+$ donor cells expressed β-*III Tubulin* (supplemental online Fig. S2B) indicating robust differentiation of donor cells into mDA neurons. The Lmx1a$^+$ donor cells that did not express β-*III Tubulin* likely represented mDA progenitors. In addition, some of those EB cells located around the ventral midline terminally differentiated into TH$^+$ dopaminergic neurons (38.14±5.66%; Table 1; Fig. 4B). Importantly, donor-derived TH$^+$ cells always expressed *Lmx1a* (Fig. 4C), confirming the midbrain identity of GFP$^+$ dopaminergic neurons. In contrast, EB cells integrated lateral to the ventral midline zone produced few, if any, dopaminergic neurons (supplemental online Fig. S2C). Similarly, EB cells did not express *Th* when placed around the ventral midline but far from the isthmus organizer or at the dorsal midbrain (data not shown). Some TH$^+$ donor cells migrated away from the transplantation site to the pial surface of the tissue (supplemental online Fig. S2D), like the endogenous dopaminergic neurons do. As expected, donor cells around the ventral midline expressed *Ptx3* (Fig. 4D), supporting the notion that some GFP$^+$ acquired the identity of mature mDA neurons. Altogether, these data indicate that EB cells have the potential to recapitulate the developmental program responsible for dopaminergic neuron production when exposed to the embryonic midbrain environment.

EB cells acquire positional identity along the midbrain dorsal–ventral axis

As EB cells readily acquired the positional identity around the midbrain ventral midline (Lmx1a$^+$/Ptx3$^+$/TH$^+$ domain), we evaluated the corresponding identity of EB cells integrated at other dorsal–

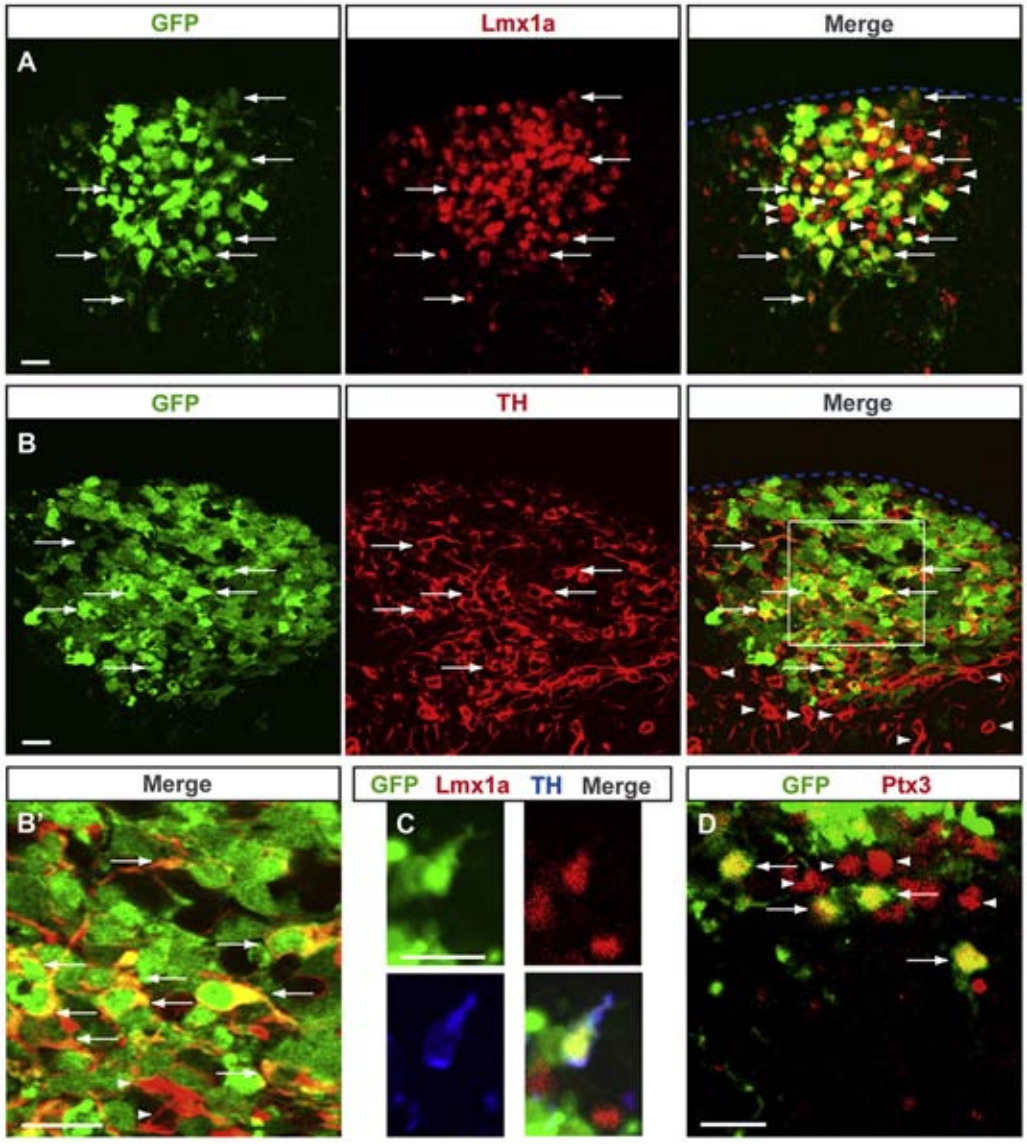

Fig. 4. EB cells efficiently produce mDA neurons around the midbrain ventral midline. Site-specific differentiation of EBs into dopaminergic neurons was evaluated 7 days after transplantation into the midbrain. Many EB cells integrated around the ventral midline expressed *Lmx1a* (A, arrows), which specifically identifies the entire mDA lineage. Terminal differentiation of some transplanted EB cells resulted in significant numbers of GFP^+/TH^+ dopaminergic neurons around the ventral midline (B, arrows). Note that donor cells are integrated at the site of robust dopaminergic differentiation in the midbrain (B, arrowheads). A higher magnification of the marked area is shown in B'. Donor-derived TH^+ cells always expressed *Lmx1a* (C) demonstrating selective generation of mDA neurons. Donor cells around the ventral midline also expressed *Ptx3* (D, arrows), another marker gene of mature mDA neurons. The blue dashed lines indicate the ventricular surface. Arrowheads indicate midbrain expression in all images. Scale bar: 20 μm.

ventral levels of the midbrain explants (Fig. 5F and see supplemental online Fig. S3A for a precise localization of transplanted cells). Before transplantation, day-4 EB cells did not express *Nkx6.1*, *Nkx2.2* or *Pax7* (data not shown). Seven days after transplantation of EB cells at the RN, many donor cells expressed *Nkx6.1* (51.51 ± 2.31%; Table 1; Fig. 5A), a gene normally expressed in neurons of that region. In contrast, a small number of EB-derived cells expressed *Nkx6.1* at places where few endogenous cells expressed this gene (supplemental online Fig. S3B). Moreover, EB cells did not express *Nkx6.1* at the dorsal midbrain (data not shown). Interestingly, we observed $GFP^+/Nkx6.1^+$ cells as soon as 3 days after transplantation (data not shown). After 7 days, most EB-derived $Nkx6.1^+$ cells had already differentiated into β-III $Tubulin^+$ RN neurons (supplemental online Fig. S3C). In addition, some donor cells differentiated into $Islet1^+$ motor neurons at the RN (Fig. 5B). Besides, donor-derived $Nkx2.2^+$ interneurons (37.47 ± 10.04%; Table 1; Fig. 5C) appeared when EB cells integrated into more lateral levels of the ventral midbrain (see Fig. 5F). At the *Nkx2.2* expression domain, GFP^+ neurons acquired the GABAergic phenotype, such as the endogenous midbrain neurons do (Fig. 5D). Finally, EB cells expressed *Pax7* at the dorsal midbrain (43.67 ± 3.52%; Table 1; Fig. 5E), while no expression of *Pax7* was detected in EB cells integrated near but outside the expression domain of this gene (supplemental online Fig. S3D). These data indicate that EB cells have the developmental plasticity to produce multiple neuronal lineages along the dorsal–ventral axis of the midbrain.

ESC-derived NPCs show high neuronal potential but poor lineage specification in the midbrain

Although different protocols have been developed to neuralize ESCs in vitro, the proportion of specific neuronal subtypes obtained is generally low (Kawasaki et al., 2000; Lee et al., 2000; Ying et al., 2003). Thus, we investigated whether in vitro neuralization of ESCs prior to transplantation affects neuronal differentiation and plasticity in the midbrain explants. We obtained cultures enriched in NPCs (55.5 ± 2.12% $Nestin^+$ cells) after keeping EB cells in adherent conditions with

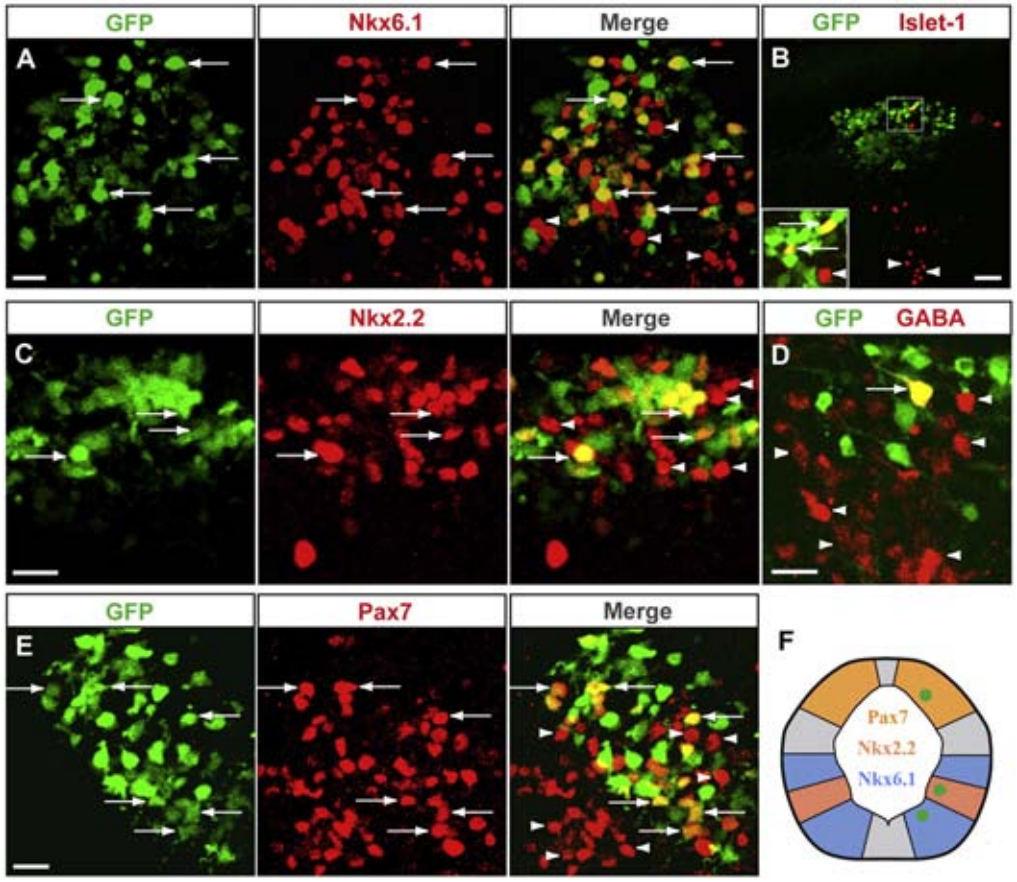

Fig. 5. Lineage specification of EB cells along the midbrain dorsal–ventral axis. EB cells were transplanted at different positions of the embryonic midbrain and positional identity markers for each location were analyzed. Donor cells expressed *Nkx6.1* (A, arrows) when placed lateral to the ventral midline. Some donor cells at the *Nkx6.1* expression domain also expressed *Islet1* (B, arrows), a gene active in motor neurons; the inset shows a magnified view of the marked area. EB cells acquired *Nkx2.2* expression (C, arrows) only after integration into the *Nkx2.2* expression domain in the midbrain, where some donor (D, arrow) and host cells (D, arrowheads) differentiated into GABAergic neurons. After integration into the dorsal midbrain, many donor cells expressed *Pax7* (E, arrows). (F) Schematic colour-coded representation of the approximate *Nkx6.1*, *Nkx2.2* and *Pax7* expression domains in a E10.5 midbrain coronal section (based on Nakatani et al., 2007). The green circles indicate the equivalent sites of donor cell transplantation at the midbrain explants. Arrowheads indicate endogenous midbrain expression in all images. Scale bar: 20 μm in A and C–E; 50 μm in B.

a defined medium for 7 days. Even though some cells had already differentiated into neurons before transplantation (16.5±2.12%), only a small number of them expressed *Th* (0.18±0.22%; $n=1646$). Seven days after transplantation, few donor cells expressed *Nestin* (4.57±2.66%; Table 1). This finding was consistent with robust neuronal differentiation of donor cells, based on cell morphology (Fig. 6A) and β-*III Tubulin* expression (74.39±9.38%; Table 1). Furthermore, the proportion of donor-derived NeuN$^+$ neurons (40.65±2.43%; Table 1; Fig. 6B) was greater than that observed when EB cells were transplanted (see above). This observation probably reflects the more mature state of ESC-derived NPCs at the time of transplantation. Despite the observed robust neuronal differentiation, donor NPCs produced few TH$^+$ putative dopaminergic neurons around the ventral midline (4.19±0.92%; Table 1). In some cases, we observed that none of the GFP$^+$ cells integrated around the ventral midline expressed *Th* (Fig. 6C). Donor-derived TH$^+$ cells were also located outside the *Th* expression domain (6.33±5.0%; Table 1), including regions of null (Fig. 6D) or scarce (supplemental online Fig. S4A) endogenous dopaminergic differentiation. These data suggest that this population of ESC-derived NPCs is not susceptible to respond to the dopaminergic niche within the explant culture.

Analysis of positional identity revealed that few integrated donor cells expressed *Lmx1a* (supplemental online Fig. S4B) around the ventral midline. Moreover, some donor cells expressed *Nkx6.1* (8.35±3.23%; Table 1; Fig. 6E) and *Pax7* (10.44±8.96; Table 1; Fig. 6F) after integration at the RN and the dorsal midbrain, respectively. Interestingly, we did not find significant numbers of GFP$^+$ cells expressing these markers outside their respective endogenous domains (Table 1), suggesting site-specific differentiation of some donor cells. Together, these data suggest that in vitro-neuralization of ESCs for 7 days results in NPCs with high neurogenic potential but low responsiveness to specification signals within the embryonic midbrain explants.

Morphogen exposure in vitro promotes mDA lineage commitment of some ESC-derived NPCs

Exposure of ESC-derived NPCs to high concentrations of Fgf8 and Shh enhances dopaminergic differentiation in vitro (Lee et al., 2000). To analyze the effect of morphogen exposure on neuronal differentiation in the midbrain, ESC-derived NPCs were treated in vitro with Fgf8 and Shh before transplantation. Morphogen exposure for 6 days in a culture dish resulted in a cell monolayer mainly composed of Nestin$^+$ NPCs with a minor proportion of β-III Tubulin$^+$ neurons (8.2±0.57%) and GFAP$^+$ astrocytes (0.77±0.1%). When these cells are allowed to differentiate in a culture dish, they show the presence of TH$^+$ neurons (in our hands up to 11%) at the end of the differentiation protocol. After transplantation into the midbrain, morphogen-treated donor cells presented neuronal morphologies (Fig. 7A), expressed *NeuN* (Fig. 7A, inset) and β-*III Tubulin* (70.61±7.1%; Table 1; Fig. 7B). In addition, a greater proportion of GFAP$^+$ donor cells (6.15±0.55%, $n=165$; Fig. 7C) was present in comparison with the initial cell culture (see above), suggesting that some donor cells differentiated into astrocytes in the

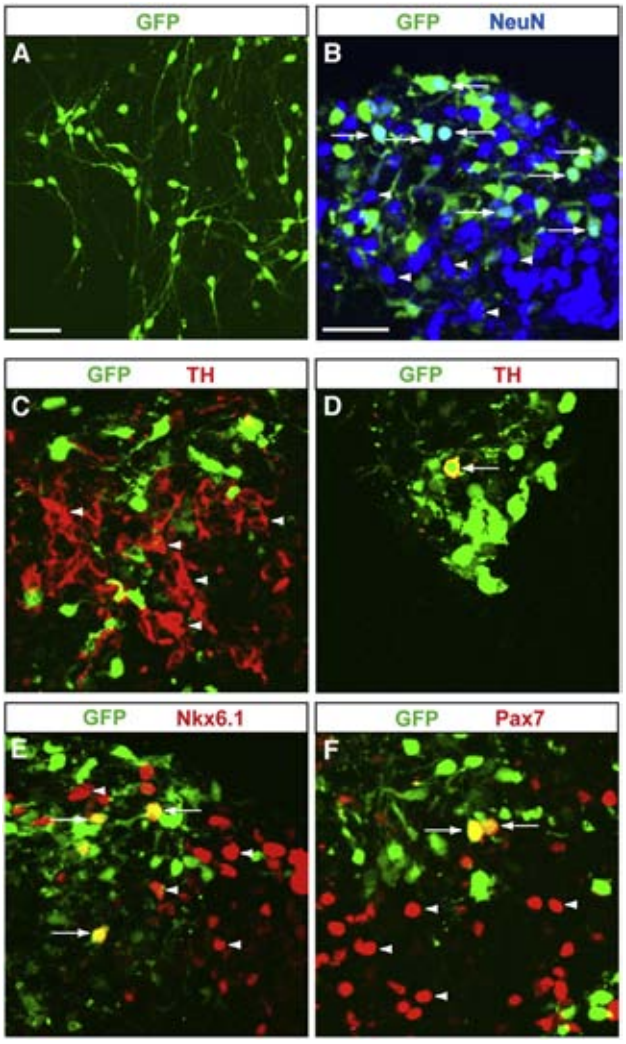

Fig. 6. Neural differentiation of ESCs in vitro reduces their response to midbrain specification signals. ESCs were differentiated in vitro into NPCs, then transplanted to midbrain explants, where specification and differentiation of donor NPCs were analyzed after 7 days. In vitro-generated NPCs integrated into the midbrain and presented neuronal morphologies (A). Many donor cells showed expression of *NeuN* (B, arrows), indicating neuronal differentiation and maturation. Although many donor cells were integrated around the ventral midline of the midbrain, in some cases we did not find evidence of donor-derived TH$^+$ neurons (C). Instead, donor-derived TH$^+$ neurons were often located far from the ventral midline (D, arrow) at positions without *Th* expression in the midbrain. Positional identity analysis showed that some donor cells expressed *Nkx6.1* (E, arrows) or *Pax7* (F, arrows) after integration in their respective expression domains. Arrowheads indicate endogenous midbrain expression in all images. Scale bar: 50 μm; B–F same magnification.

midbrain. Besides, donor cells apparently induced astrocyte differentiation at the integration site in the midbrain explant (Fig. 7C).

Some morphogen-treated NPCs showed *Lmx1a* (8.36±0.77%; n=1413) and *Th* (0.45±0.3%; n=2776) expression before transplantation. After transplantation to the midbrain, Lmx1a$^+$ donor cells were distributed with no apparent relationship to the site of integration. In fact, Lmx1a$^+$ donor cells outside the *Lmx1a* expression domain appeared with approximately the same frequency (13.39±7.58%; Table 1; Fig. 7D) as Lmx1a$^+$ donor cells at the main induction site (15.21±6.02%; Table 1; Fig. 7E). Seven days after transplantation, most Lmx1a$^+$ donor cells expressed β-*III Tubulin* (supplemental online Fig. S4C). As expected, morphogen-exposed NPCs expressed *Th* around the ventral midline (6.38±5.03%; Table 1; Fig. 7F, left image) and far from it (5.83±4.73%; Table 1; Fig. 7E, right image) at sites with no local dopaminergic neurons, thus excluding site-specific differentiation. Altogether, these data suggest that in vitro exposure to Shh and Fgf8 commits the fate of some NPCs to Lmx1a$^+$/TH$^+$ neurons without enhancing their response to inductive signals of dopaminergic differentiation within the midbrain.

Discussion

During development, specific cell differentiation depends on complex interactions between precursor cells and the niche of differentiation. Competence is gained or lost as a result of precursor cell history, which define how the cells will respond to specific factors. Although in general some factors are known to be relevant for the specification of some cell lineages, the complete spatial and temporal combinations of factors required for a differentiation pathway are largely unknown. In this work, we report the use of the natural environment in which mDA neurons arise during development to test the competence of ESC-derived precursor cells to differentiate into dopaminergic neurons. This approach allowed us to determine the intrinsic differentiation potential of precursor cells without knowing all the complex networks of signalling molecules and factors that induce differentiation.

EB cell differentiation potential in the embryonic midbrain

In vitro differentiation of ESCs within different lineages is facilitated by the formation of EBs. Nonetheless, neuronal differentiation of ESCs can be induced without forming EBs (Ying et al., 2003). Our data indicate that competence to respond to midbrain patterning signals is only acquired after ESCs differentiate into EB cells. This conclusion is based on the observation that ESCs expressed *Oct4* after integration into the embryonic midbrain explants, while neural markers were not detected. The fact that ESCs do not differentiate after transplantation could be due to factors that activate the gp130 signalling pathway, which contributes to maintain ESCs undifferentiated in vitro. Actually, the LIF/CNTF/gp130 receptor complex is present at some regions of the embryonic brain (e.g., the lateral ganglionic eminence and the spinal cord (Gregg and Weiss, 2005)). Those signals within the embryonic midbrain could define a condition that is not permissive for ESCs neuronal differentiation. In contrast, roughly 65% of EB cells differentiated into neurons, which is an indicative of their ability to interpret the neural stem cell niche after integration into the midbrain explants. The remaining EB cells that were not neuralized might represent progenitor populations committed to mesodermal or endodermal cell lineages, which are already present at day 4 of EB formation (Rathjen and Rathjen, 2001).

Pattern formation in the neural tube depends on gradients of morphogens acting along the main axes. In particular, Shh signalling establishes progenitor's domains in the ventral region, while BMP organize the dorsal region (Liu and Niswander, 2005; Lupo et al., 2006). Our results suggest that EB cells respond to the local Shh concentration at the site of integration in the midbrain, as donor cells specifically acquired the identity of surrounding cells. Accordingly, EB cells expressed *Pax7* at the dorsal midbrain, probably as a consequence of BMP signalling from the roof plate (Liu and Niswander, 2005). Whichever signals are involved, EB cell differentiation in the embryonic midbrain appears to be mediated by an instructive mechanism, as these cells had no expression of patterning genes (e.g., *Lmx1a*, *Nkx6.1*, *Nkx2.2* and *Pax7*) before transplantation. Future experiments will be directed to elucidate the role of midbrain patterning signals during neuralization and differentiation of EB cells into specific neuronal lineages.

Previous work has shown that EB cells randomly differentiate into several neuronal subtypes, including dopaminergic neurons, after transplantation into the adult murine striatum (Bjorklund et al., 2002). Our data demonstrate that the embryonic midbrain milieu at midgestation is able to direct site-specific differentiation of EB cells into mDA neurons at the *Lmx1a* expression domain and GABAergic

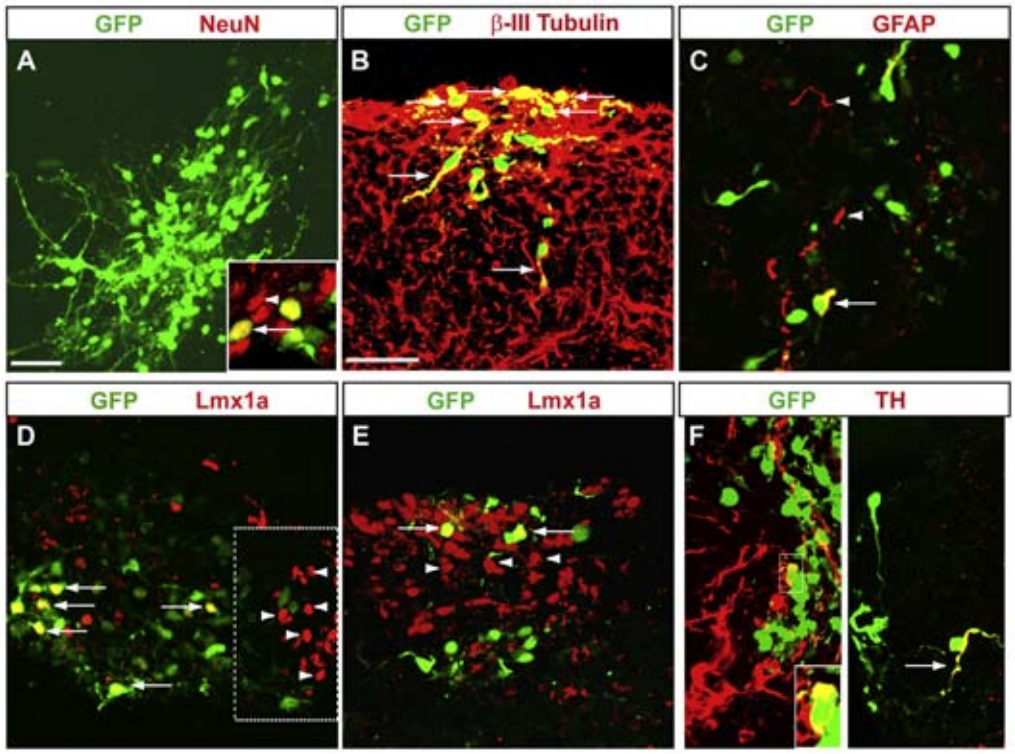

Fig. 7. Shh and Fgf8 commit the fate of some ESC-derived NPCs into putative Lmx1a$^+$ dopaminergic neurons. EB cells were neuralized in vitro, expanded with Fgf2 in the presence of Fgf8 and Shh for 6 days and then transplanted to midbrain explants. Most integrated cells had neuronal morphology (A) and expressed *NeuN* (A, inset) 7 days after transplantation. Many donor cells also expressed β-*III Tubulin* (B, arrows) in the midbrain parenchyma. A minor proportion of donor cells expressed *Gfap* (C, arrow), indicating differentiation to the astrocytic lineage. Note that some host-derived GFAP$^+$ fibres appeared at the site of integration of donor cells (C, arrowheads). Analysis of positional identity revealed that some donor cells expressed *Lmx1a* with no relationship to their integration site. *Lmx1a* expression was found in GFP$^+$ cells (D, arrows) located lateral to the ventral midline (outlined with a dashed line) or at the ventral midline (E, arrows). Few GFP$^+$/TH$^+$ putative dopaminergic neurons appeared around the ventral midline (F, left image). The inset shows a higher magnification of the double-labelled cell (F, left image). Some donor cells differentiated to dopaminergic neurons at sites of null dopaminergic differentiation in the midbrain (F, right image), suggesting that site-specific differentiation did not occur. Scale bar: 50 μm; B–F same magnification.

neurons at the *Nkx2.2* expression domain. Based on *Lmx1a* expression, roughly 67% of EB cells differentiated along the dopaminergic lineage in the midbrain, although only around 38% reached terminal differentiation and expressed *Th* at the time of analysis. Lower percentages of specification of EB cells at other sites of the midbrain (i.e. *Nkx6.1*, *Nkx2.2* and *Pax7* expression domains) correlate with the mosaic composition of endogenous specified cells. Thus, our data demonstrate that many EB cells have the developmental capacity to be specified according to the midbrain region where they integrate. Regarding the dopaminergic lineage, recent reports indicate that Shh$^+$/FP4$^+$/Foxa2$^+$ floor plate cells are mDA progenitors (Kittappa et al., 2007; Ono et al., 2007). Hence, an interesting possibility is that EB cells transit through a floor plate-like state before differentiating into mDA neurons.

ESC-derived NPCs differentiation potential in the embryonic midbrain

Our data suggest that in vitro neuralization of EB cells reduces their competence to differentiate into dopaminergic neurons at the ventral midbrain niche. This conclusion is supported by the evidence that midbrain explants act as an instructive matrix for endogenous midbrain NPCs and for EB-derived NPCs generated within the explant environment. Besides, poor lineage specification within the midbrain explants was also observed after in vitro neuralization of ESCs with retinoic acid (Baizabal JM and Covarrubias L, unpublished). In agreement with our results, previous work demonstrated that ESC-derived NPCs generated functional neurons in the embryonic E16.5 rat brain but failed to show significant site-specific differentiation (Wernig et al., 2004). In addition, mouse EB cells transplanted to the chick dorsal root ganglia acquired regional identity, but this response capacity was lost after in vitro neuralization with retinoic acid (Plachta et al., 2004). Yet, it is not clear whether ESC-derived precursor cells lose competence soon after in vitro neuralization or rather their differentiation potential is progressively restricted as the cell culture advances. In addition, our work does not rule out the possibility that other ESC-derived NPC populations, like the recently described neural rosette cells (Elkabetz et al., 2008), efficiently respond to instructive signals within the explants or in vivo.

In vitro neuronal differentiation protocols of ESCs usually enrich for the generic NPC markers Nestin and Sox-1 as a first step (Ying et al., 2003). This approach results in highly heterogeneous cell cultures that contain a mixture of NPCs at different stages of development and, possibly, with a defined neuronal fate (Pruszak et al., 2007). Our data suggest that after in vitro neuralization of ESCs, a minor subpopulation still responds to the midbrain milieu, which could represent either NPCs at an early stage of specification or undifferentiated EB precursors. Future experiments will be directed to test the differentiation potential of more homogenous precursor populations obtained by sorting for specific surface antigens or by growing in selective conditions (Pruszak et al., 2007; Roy et al., 2006).

Previous work has shown that Fgf8 and Shh induce dopaminergic differentiation of midbrain explants and ESC-derived precursors (Ye et al., 1998). In our study, treatment of ESC-derived NPCs with Fgf8 and Shh prior to transplantation apparently raised the proportion of Lmx1a$^+$ donor cells observed in the midbrain as compared with untreated NPCs. However, Lmx1a$^+$ cells derived from morphogen-treated NPCs were distributed randomly across the midbrain explant, which contrast with the few, but restricted to the ventral midline, Lmx1a$^+$ cells derived from untreated NPCs. These observations suggest that Shh and Fgf8 do not enhance the response of NPCs to

developmental cues within the midbrain, but rather commit the fate of some NPCs to the dopaminergic lineage. This interpretation is further confirmed by the fact that donor cells produced TH^+ putative dopaminergic neurons independently of their integration site. Supporting our data, other study indicates that primitive NPCs become more compromised after in vitro exposure to morphogens like Fgf2, Fgf8 and Egf (Elkabetz et al., 2008).

Directing specific neuronal differentiation of ESCs

Neuronal specification within the embryonic brain involves the induction of lineage-specific genes that actively repress alternative neuronal fates (Lee et al., 2008). Our data indicate that cell competence for neuronal specification is only present during a restricted developmental window between the ectoderm (EB cells) and the early neuroectoderm stages. Thus, it is likely that simultaneously or soon after neuralization of EB cells within the explants, these cells are directed to specific neuronal lineages by expression of fate determinants (e.g. *Lmx1a, Nkx6.1*) in response to environmental cues. In contrast, EB cell neuralization in vitro occurs in the absence of the correct developmental context, which results in NPCs not susceptible to further specification within the embryonic explants.

Other studies have also suggested that neuronal fates have to be specified during early stages of ESCs differentiation, even before neuralization, as only primitive cells are highly responsive to patterning signals. For example, ESCs growing over a stromal cell line differentiate to dopaminergic neurons in vitro, but the unknown stromal inducing activity specifies dopaminergic neurons before or at the start of *Sox1* expression (Parmar and Li, 2007). Similarly, in vitro specification of human ESCs into mDA neurons instead of forebrain dopaminergic neurons requires exposure of precursor cells to Fgf8 before the onset of *Sox1* expression (Yan et al., 2005).

Early specification of ESC-derived precursors is not restricted to the dopaminergic lineage. Inhibition of the Wnt signalling pathway in EB cells, efficiently promotes specification of $Bf1^+$ telencephalic progenitors, while the same treatment is ineffective at later stages of differentiation (Watanabe et al., 2005). In a different study, ESC-derived primitive neuroepithelium with an anterior identity efficiently responds to caudalizing signals like retinoic acid, whereas definitive NPCs lose the epithelial organization and become more restricted in their differentiation potential (Elkabetz et al., 2008; Pankratz et al., 2007). Epithelial organization as neural rosette might provide a niche that favours the maintenance of competent cells for specific neuronal differentiation (Elkabetz et al., 2008).

Intrinsic modifications are another alternative to direct ESC-derived precursors into specific lineages. Efficient dopaminergic differentiation in vitro has been achieved with engineered ESC lines that overproduce transcription factors required for mDA development, like Lmx1a, Foxa2, Nurr1 and Ptx3 (Andersson et al., 2006; Chung et al., 2002; Kittappa et al., 2007; Martinat et al., 2006). Our data suggest that the correct combination of extrinsic factors (i.e., signals from the niche) is sufficient for competent ESC-derived precursors to fully recapitulate dopaminergic development through activation of early (*Lmx1a*) and late (*Ptx3*) intrinsic mDA determinants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.09.024.

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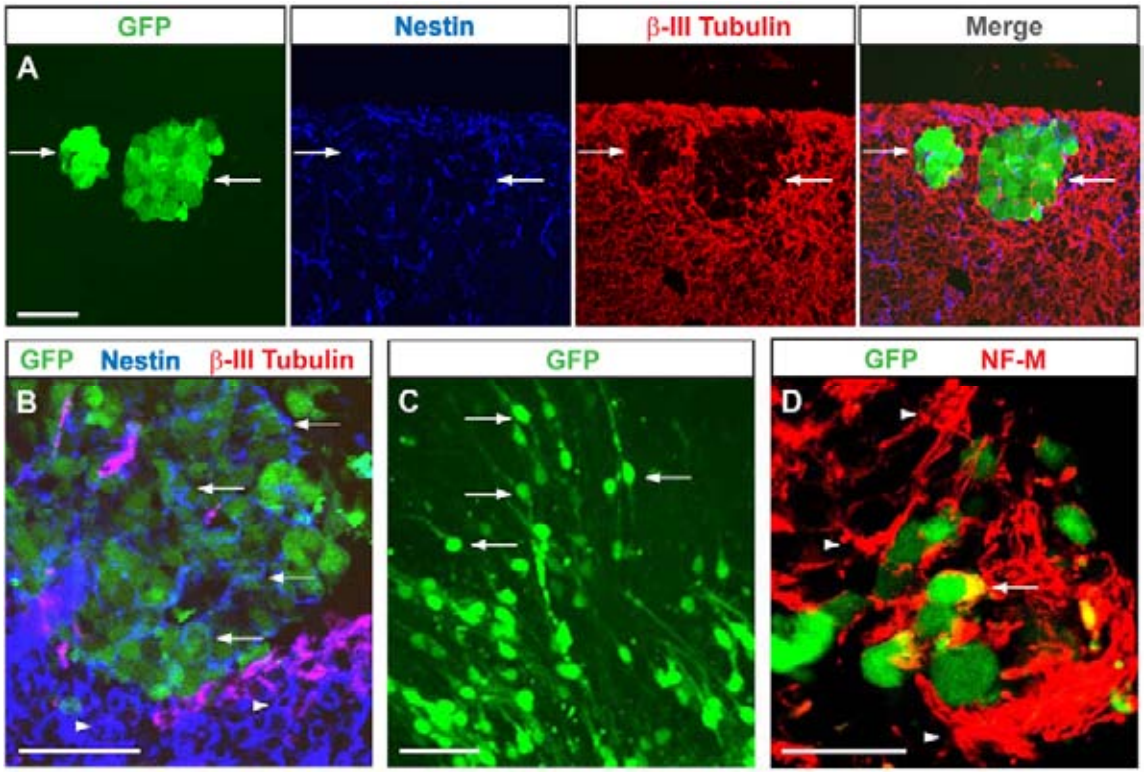

Fig. S1. Neural differentiation potential of ESCs and EB cells integrated into the embryonic midbrain. (A) GFP-labelled ESCs (arrows) fully integrated into the midbrain neuroepithelium but did not differentiate into Nestin^{+} NPCs or β-III Tubulin^{+} neurons. (B) Two days after transplantation, *Nestin* expression (arrows) was detected at low levels in partially integrated whole EBs. (C) Seven days after integration, most donor cells showed a bipolar morphology (arrows) that is often associated with immature neurons. (D) Few EB-derived cells expressed the axonal protein *NF-M* (arrow) confirming the immature state of most donor-derived neurons. Arrowheads indicate endogenous expression in all images. Scale bar: 50 μm.

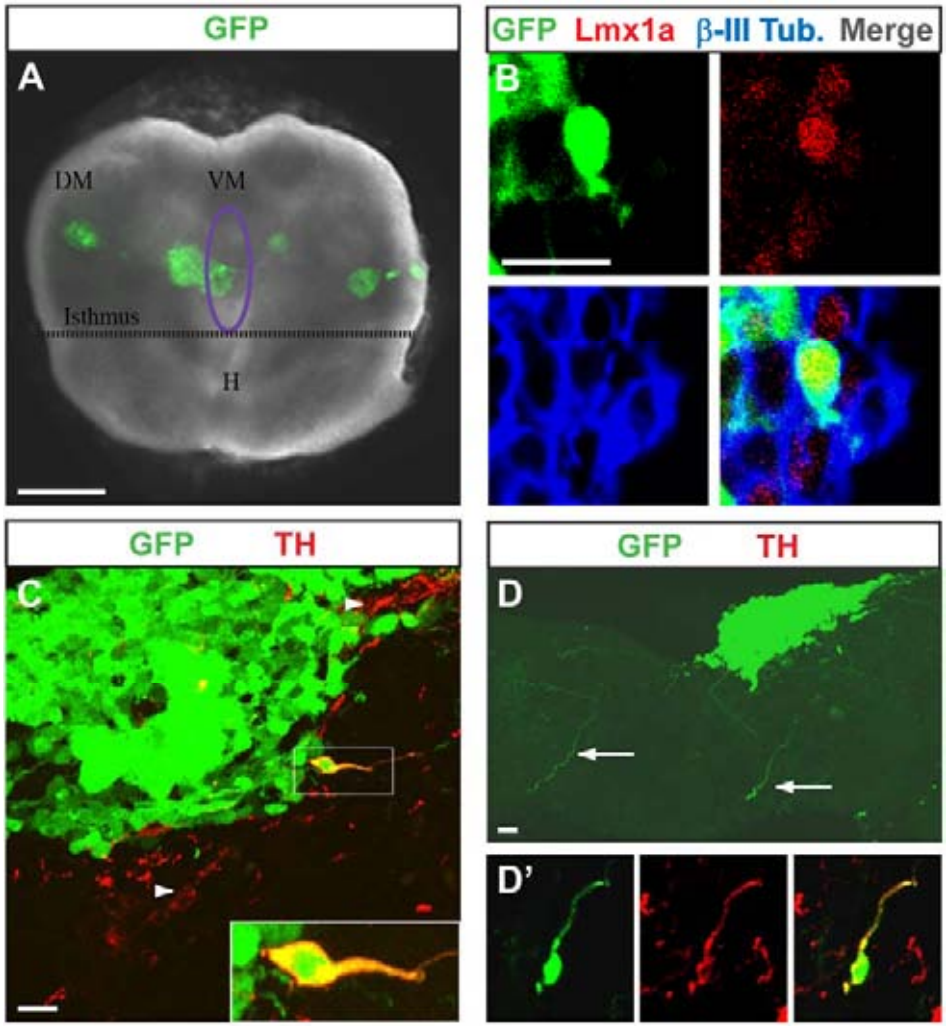

Fig. S2. EB cells recapitulate dopaminergic neurogenesis only around the ventral midline of the midbrain. (A) Midbrain-hindbrain explant with several clusters of GFP$^+$ cells viewed directly from above; the ventral midline (the dopaminergic niche) is roughly delimited with a purple ellipse, while the approximate location of the isthmus is indicated with a dashed line. Only EB cells located inside the purple ellipse differentiated into Lmx1a$^+$/ TH$^+$ mDA neurons. (B) Lmx1a$^+$ donor cells also expressed *β-III Tubulin*, demonstrating the correct positional identity of donor-derived neurons around the ventral midline. (C) Few donor cells expressed *Th* when located close but outside the purple ellipse shown in A. The inset shows a magnified view of the marked area. Note the small number of endogenous TH$^+$ cells (arrowheads). (D) Some GFP$^+$ cells (arrows) migrate away from a partially integrated EB; occasionally, migrating

GFP$^+$ cells expressed *Th* (D'). DM, dorsal midbrain; VM, ventral midbrain; H, hindbrain. Scale bar: 500 μm in A; 20 μm in B-D.

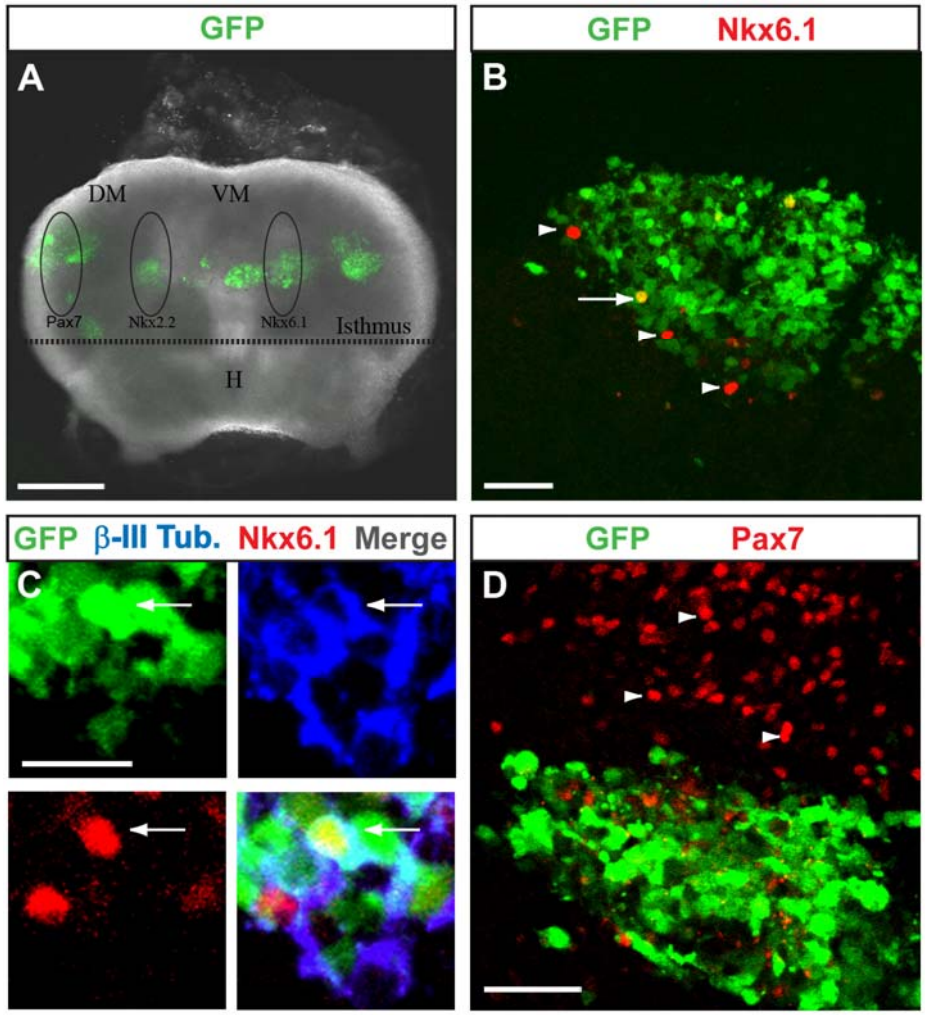

Fig. S3. Positional identity of EB cells strictly depends on location in the midbrain. (A) Midbrain-hindbrain explant with several clusters of GFP+ cells viewed directly from above. The black ellipses roughly indicate the zones that induced *Nkx6.1*, *Nkx2.2* and *Pax7* expression in transplanted EB cells. For clarity, black ellipses are indicated unilaterally, although Nkx6.1 and Nkx2.2 have a bilateral distribution around the ventral midline (see Fig. 4F). The approximate location of the isthmus is indicated with a dashed line. (B) Few EB cells acquired *Nkx6.1* expression (arrow) when the endogenous number of Nkx6.1+ cells in the midbrain was also small (arrowheads). (C) Co-expression of *Nkx6.1* and *β-III Tubulin* suggest differentiation of donor cells into

RN neurons. (D) EB cells did not express *Pax7* after integration outside the main expression domain of this gene, supporting site-specific differentiation into midbrain dorsal fates. DM, dorsal midbrain; VM, ventral midbrain; H, hindbrain. Scale bar: 500 μm in A; 50 μm in B, D; 20 μm in C.

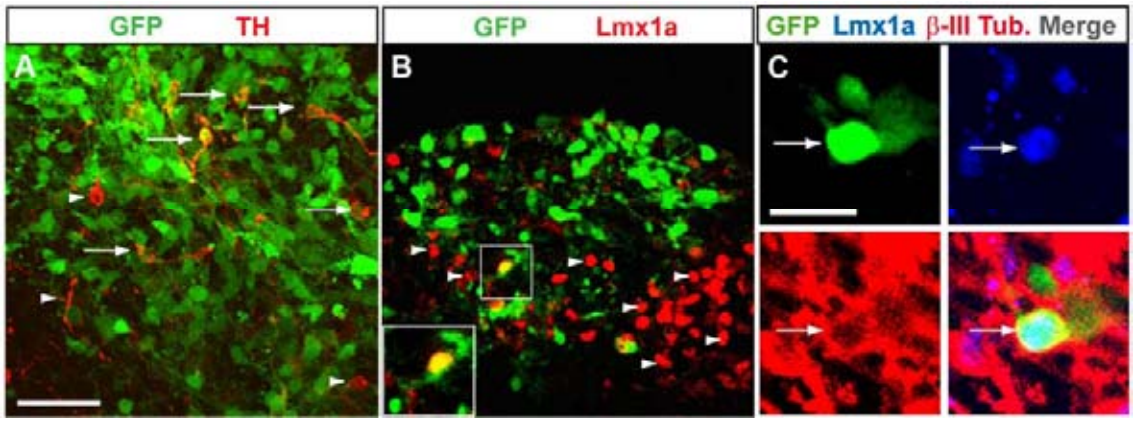

Fig. S4. In vitro neuralization of ESCs reduces their differentiation potential in the midbrain. (A) Several donor-derived TH^+ putative dopaminergic neurons (green nucleus, red cytoplasm; arrows) integrated into a site with scarce number of host TH^+ cells (arrowheads). Note the low proportion of donor-derived TH^+ neurons in relation with the total number of GFP^+ cells. (B) Few ESCs-derived NPCs acquired *Lmx1a* expression at the appropriate domain. The inset shows a magnified view of the marked area. (C) Some morphogen-expanded NPCs differentiated into β-III $Tubulin^+$ neurons that also expressed *Lmx1a*. Scale bar: 50 μm in A, B; 20 μm in C.

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Forebrain Neural Stem Cells show transient competence to readout the dopaminergic niche of the embryonic midbrain

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Running Title: Dopaminergic potential of forebrain Neural Stem Cells

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Abstract

Neural Stem Cells (NSCs) generate complex stereotypic arrays of neuronal subtypes in the brain. This process involves the integration of patterning cues that progressively restrict the fate of specific NSCs. Yet, the capacity of NSCs to interpret foreign microenvironments remains poorly defined. The aim of this work was to test the competence of forebrain NSCs to respond to the embryonic midbrain (eMB) milieu as well as to examine the inductive properties of the telencephalic niche. Our data indicate that the eMB explants foster neuronal but not glial differentiation of NSCs isolated from the telencephalon or purified from the adult subventricular zone. However, telencephalic NSCs isolated from the neural tube (E10.5) were largely unable to produce midbrain dopaminergic (mDA) neurons at the eMB explants. Instead, donor cells behaved as compromised gabaergic progenitors that expressed *Bf1*. In contrast, earlier (E8.5) telencephalic NSCs isolated from the neural plate succeeded to differentiate into $Lmx1a^{+}/TH^{+}$ mDA neurons at the eMB explants. In addition, these NSCs showed region-dependent expression of *Nkx6.1*, *Nkx2.2* and gabaergic differentiation. Paralleling the behavior of telencephalic NSCs, rapid restrictions limiting the production of mDA neurons also occurred after retinoic acid-mediated neuralization of Embryonic Stem Cells. Moreover, we show that extinction of inductive signals within the telencephalon lag behind the commitment of residing NSCs. Our data suggest that mDA neurons might be derived from alternative sources of NSCs, in view of their use for Parkinson's disease therapies.

Key words: Neural Stem Cells; Forebrain; Dopaminergic Neuron; Midbrain; Niche; Embryonic Stem Cells; Neurogenesis.

Introduction

Differentiation of Neural Stem Cells (NSCs) during embryogenesis gives rise to a wide diversity of neuronal and glial subtypes. Directed differentiation of NSCs depends on their residing niche, which provides the required signals to guide specific fates (Edlund and Jessell, 1999). In principle, lineage determination of NSCs in a culture dish would require to emulate at least some of the properties of the niche. This goal has been attained through exposure of NSCs to signaling molecules (e.g. morphogens) (Zhang, 2006) or to the "natural" environments present in explant cultures (Carletti et al., 2002; Fishell, 1995).

NSCs emerge during the induction of the neural plate (NP) around day 7 of embryonic development (E7) in mice (Wood and Episkopou, 1999). The most anterior part of the NP is then specified to become the telencephalon (i.e. the embryonic forebrain) around E8.5. This event is marked by the presence of the forkhead transcription factor Bf1 (also known as Foxg1) at the prospective telencephalic region (Shimamura and Rubenstein, 1997; Tao and Lai, 1992). Genetic studies have implicated *Bf1* as a critical determinant of the telencephalic fate, especially at the ventral region (Dou et al., 1999; Martynoga et al., 2005; Shimamura and Rubenstein, 1997). After closure of the neural tube (NT), the telencephalon becomes regionalized in dorsal and ventral domains; the former giving rise to the cerebral cortex while the latter produces the medial, lateral and caudal ganglionic eminences. Most NSCs at the dorsal telencephalon differentiate into pyramidal glutamatergic neurons, whereas ventral NSCs generate several subtypes of gabaergic interneurons (Hebert and Fishell, 2008). Cortical inhibitory interneurons are also generated within the ganglionic eminences, a process that involves migration from ventral to dorsal telencephalic regions (Nery et al., 2002; Wichterle et al., 2001). After birth, NSCs continue the generation of new neurons

exclusively in two telencephalon-derived structures: the subventricular zone (SVZ) of the anterior ventricle and the dentate gyrus of the hippocampus (Zhao et al., 2008).

The region surrounding the ventral midline of the mesencephalon (i.e. the embryonic midbrain; eMB) is the niche where NSCs become specified into the dopaminergic lineage. This event is molecularly defined as the beginning of *Lmx1a* expression (E9), which actively represses alternative ventral fates (Andersson et al., 2006). The Lmx1a^{+} dopaminergic domain forms the medial region of the floorplate (FP) (Kittappa et al., 2007; Ono et al., 2007), the organizer center that patterns the ventral NT through production of the morphogen Shh (Lupo et al., 2006). Dopaminergic neurogenesis initiates around E10.5, a process marked by an increase in cell proliferation and the initial appearance of Tyrosine Hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis (Joksimovic et al., 2009). The production of midbrain dopaminergic (mDA) neurons (i.e. TH^{+} cells) peaks by E12/13 (Bayer et al., 1995). At this point, dynamic migratory patterns gradually organize mDA neurons into the ventral tegmental area (VTA) and the substancia nigra compacta (SNc). These structures regulate motor functions, reward seeking, addiction and motivation. Degeneration of mDA neurons at the SNc leads to Parkinson's disease (Dauer and Przedborski, 2003).

Embryonic Stem Cells (ESCs) reproduce in culture some of the molecular and cellular events that lead to neurogenesis in the embryo (Keller, 2005; Murry and Keller, 2008). Initial differentiation of ESCs into ectodermal cells occurs around days 3/4 of Embryoid Body (EB) formation (Rathjen and Rathjen, 2001). Within EBs, differentiation of ectodermal cells into NSCs is often induced by Retinoic Acid (RA) (Bibel et al., 2004; Wichterle et al., 2002). ESC-derived NSCs are susceptible to differentiate in culture into a wide range of lineages, including telencephalic glutamatergic and gabaergic neurons as well as mDA neurons (Lee et al., 2000;

Watanabe et al., 2005). After transplantation to the embryonic brain, ESC-derived precursors show regional specification at the spinal cord and the midbrain (Baizabal and Covarrubias, 2009; Plachta et al., 2004). At present, no clear evidence exists of niche-dependent differentiation of ESC-derived NSC incorporated into the forebrain (Wernig et al., 2004).

In this study, we present evidence that forebrain NSCs differentiate into mDA neurons upon incorporation into the eMB explants. Ectopic differentiation of forebrain NSCs was restricted to a short developmental window at stages prior to the onset of neurogenesis in the telencephalon. Furthermore, we show that committed telencephalic NSCs reside in an inductive niche that fully instructed pre-neuralized ESC–derived precursors into gabaergic neurons.

Materials and Methods

Collagen and organotypic explant cultures

Mesencephalic and telencephalic explants were obtained from E10.5 CD-1 mice embryos and cultivated in a collagen matrix as previously described (Baizabal and Covarrubias, 2009). Explant media (Heretofore referred as Optimix) was prepared by mixing Optimem with Glutamax (72% v/v) and DMEM-F12 (25% v/v) both from Gibco, Glucose 2 M (2% v/v; Sigma) and GPS 1X (glutamax/penicillin/streptomycin). For organotypic cultures, mesencephalic explants were laid over a Millicell culture plate insert (Millipore) and transferred to 35 mm culture dishes containing 2 ml of Optimix. Every tissue was then covered with a drop of media. Explants were maintained at 37°C in humidified 5% CO_2-95% atmospheric air incubators for 7 days.

Cell transplantation

This procedure was done essentially as previously described (Baizabal and Covarrubias, 2009). Forebrain NSCs were obtained from a transgenic mice line (gift from Andras Nagy) that constitutively produces the Green Fluorescent Protein (GFP). The ventral regions of 8-12 telencephalons were dissected from E10.5 embryos and treated for 10 min. at 37°C with 0.1% trypsin (Gibco) diluted in Versene (EDTA 0.02%; Gibco). Trypsin was then inactivated with Optimix containing 10% Fetal Bovine Serum (FBS; Gibco). The tissues were mechanically dissociated and the resulting cell suspension was concentrated in 5-20 μl of Optimix. Early NSCs were isolated by directly suctioning the most anterior region of the E8/8.5 NP. Trypsin dissociation was not carry out in this case in order to increase cell recovery and survival. For transplantation, a fire-stretched Pasteur pipette coupled to a hose with a mouthpiece was used to deposit donor cells over the ventricular surface of collagen explants. Transplanted cells were allowed to attach to the tissue for three hours at 37°C before adding the media.

Purification of NSCs from the adult SVZ

Purification of adult NSCs was based on cell diameter and low affinity to the Peanut Agglutinin (PNA; (Rietze et al., 2001)). The anterior SVZ of adult GFP^+ mice was removed essentially as previously described (Lois and Alvarez-Buylla, 1993). Single cell suspensions of the SVZ were passed through a 70 μm cell-strainer (Falcon) to remove debris. At this point, donor cells were either directly transplanted or incubated for 20 min. at 4°C with Phycoeritrine-conjugated PNA to proceed with purification. Following incubation, the cell suspension was rinsed twice with DMEM/F12 (Gibco) and finally in DMEM/F12+1% Bovine Serum Albumin (BSA; Sigma) before sorting in a FACS II (Becton-Dickinson) flow cytometer. Sorting was based on forward scatter and selection of Phycoeritrine positive and negative groups, with a 5-channel gap between groups. Adult NSCs were collected directly in NS-A basal serum-free media

(Euroclone) containing 0.6% glucose, 3 mM $NaHCO_3$, 5 mM HEPES, 2 mM L-glutamine, 0.1 mg ml^{-1} apo-transferrin, 25 μg ml^{-1} insulin, 60 mM putrescine, 30 mM selenium and 20 nM progesterone (Sigma).

Embryonic Stem Cells culture and differentiation

The R1B5 line of ESCs (gift from Andras Nagy) which constitutively produces the GFP was propagated and differentiated into EB precursors as previously described (Baizabal and Covarrubias, 2009). EBs cells were neuralized following a protocol based on RA treatment (Wichterle et al., 2002). The media was supplemented with 1μM RA two days after the initial aggregation of ESCs into EBs. Cultures were maintained in the presence of RA for 2 additional days before dissociation and transplantation.

Tissue processing and immunofluorescence

Collagen explants were fixed and processed for cryo-sectioning as previously described (Baizabal and Covarrubias, 2009). Primary antibodies were used to detect Nestin (1:500; mouse), β-III Tubulin (1:100; mouse), GFAP (1:250; rabbit), NeuN (1:400; mouse), $Gad_{65/67}$ (1:500; rabbit) and TH (1:500; rabbit), all from Chemicon; β-III Tubulin (1:2000; rabbit; Covance), Oct3/4 (1:100; mouse; BD Biosciences); Lmx1a (1:1000; rabbit; gift from M. German), En1 supernatant (1:2; mouse; Developmental Studies Hybridoma Bank), Bf1 (1:100; rabbit; Abcam). Primary antibodies were detected with the following secondary antibodies: anti-mouse IgGs (1:1000; goat) coupled to Alexa-Fluor 594 and 647 and anti-rabbit IgGs (1:1000; goat) coupled to Alexa-Fluor 594 and 647, all from Molecular Probes. DAPI (1:1000; Molecular Probes) was used for nuclear staining. Fluorescence images were obtained with a Nikon Eclipse TE300 inverted microscope coupled to a CCD digital camera (Coolsnap, Photometrics).

Confocal images were collected with a ZEISS LSM 510 Meta microscope and analyzed with the LSM 5 image examiner software.

RNA purification, cDNA synthesis and Polymerase Chain Reaction (PCR)

These procedures were done as previously reported (Santa-Olalla et al., 2003). Four days after the initial aggregation of ESCs, RNA purification was carried out in RA-treated and control EBs. The oligonucleotide primers used were: *Hprt*: forward CCT GCT GGA TTA CAT TAA AGC ACT G, reverse GTC AAG GGC ATA TCC AAC AAC AAA C; *Otx2*: forward ATG ATG TCT TAT CTA AAG CAA CCG CCT TAC G, reverse TCA TTG GGT CAT CAG TAT AAA CCA; *En1*: forward TCA AGA CTG ACT CAC AGC AAC CCC, reverse CTT TGT CCT GAA CCG TGG TGG TAG; *En2*: forward AAG ACG CTA TCA CTT CAC GGT GGT, reverse AGA ATA GCG CGT GCA GTA GAC CCA; *Hoxd1*: Forward AGC AAA CTG TCC GAA TAT GGA GCC A, reverse CTC TCG TTC CCT CTT CTT CTG CTT; *Pax7*: forward CGA CGA GGA AGG AGA CAA GAA AGA A, reverse TGG TGG TGG TGG GGT AGG TAG AGT; *Pax6*: forward AGA CTC GGA TGA AGC TCA GAT GCG, reverse ACT GGT ACT GAA GCT GCT GCT GAT; *Nkx6.1*: forward GGA TGA CGG AGA GTC AGG TCA AGG T reverse GCT GCC ACC GCT CGA TTT GTG CTT T; *Nkx2.2*: forward AGG AGG ACT CGA TCC TTA CCA CGG T, reverse ATT TGC CAC CAG TTG TCA GAA CGT GTA; *Foxa2*: forward AAG CGA GCT AAA GGG AGC ACC TG reverse CCT GGA GTA CAC TCC TTG GTA GTA; *Th*: forward TAC GCC ACG CTG AAG GGC CTC TAT, reverse AGG TGA GGA GGC ATG ACG GAT GTA; *Gli1*: forward GCT GGA TAT GAT GGT TGG CAA GTG, reverse GGT CCG ATT CTG GTG CTT GGC G; *Gli3*: forward AAG ACA GGA AAG CTG GCT CGT TCT reverse TGT GTT TGT GGT CCT CCT TGC CTA. Transcripts were amplified for 25, 30, 35 and 40 cycles. cDNA from specific regions of the NT were included as positive controls. In

addition, PCR mixtures without cDNA were included as negative controls. All the PCR products were of the expected size and their identity was confirmed by digestion with several restriction endonucleases.

Data Analysis

The data presented for every NSCs population is representative of at least three independent experiments. The frequency of the reported observations is summarized in Table 1. For adult NSCs, data is presented as the average ± standard deviation; "n", indicates the number of GFP^+ cells counted. For the other populations, cell counting was not made in detail because in most cases donor cells were observed as high-density aggregates after transplantation. However, our qualitative data allowed a clear distinction between competent (e.g. E8 NSCs and EB precursors) and incompetent cells (e.g. E10.5 NSCs and ESC-derived neural precursors). In most cases, the images presented are single optical planes selected within the confocal Z-stacks. The pinhole aperture of all channels was maintained close to value 1, in order to obtain high confocality.

Results

Accurate development of the dopaminergic niche within mesencephalic explants

A suitable explant culture system must reproduce, as faithfully as possible, the developmental processes observed in the embryo**.** At present, in vitro transplantation studies of NSCs have employed organotypic cultures, which involves setting the explants over a porous membrane with media beneath them (Carletti et al., 2002; Fishell, 1995; Hitoshi et al., 2002; Kim et al., 2006). In contrast, we reported the use of eMB explants inside collagen matrices to direct the differentiation of transplanted ESC-derived precursors (Baizabal and Covarrubias, 2009). Here, we extend our analysis of mDA development in collagen cultures and compare it with the organotypic paradigm.

Initially, we examined mDA neuron differentiation at several time points of development. The first TH^+ dopaminergic neurons appeared between E10.5/11 at the pial surface of the tissue (Fig. 1A). By E13.5, mDA neurons had substantially increased in number and they were found migrating from the ventricular to the pial region as two columns running parallel to the ventral midline (Fig. 1A). Around E15/16, large numbers of TH^+ neurons had already formed the VTA at the midline and the SNc bilaterally (Fig. 1A).

After 7 days in culture, collagen explants of the eMB showed a similar number and array of mDA neurons in comparison to the E15/16 embryo (Fig. 1A, B). The collagen matrix promoted the growth of the tissue along the radial axis, where TH^+ neurons emerged from the ventricular to the pial region as observed in vivo (Fig. 1A, B). Furthermore, some TH^+ cells migrated laterally and away from the ventral midline, resembling the SNc (Fig. 1A, B). In contrast, organotypic explant cultures showed few TH^+ neurons only at the pial region, without any clear distinction between the VTA and the SNc (Fig. 1B). Therefore, collagen explants present a more appropriate pattern of development. Further supporting this notion, dopaminergic axons in collagen explants grew toward anterior and dorsal regions, as in vivo (Fig. 1C). Besides, radial glial-like cells, which represent dopaminergic progenitors (Bonilla et al., 2008), appeared close to the ventral midline (Fig. 1C).

Neural Stem Cells from the embryonic and adult forebrain strictly produce neurons in the embryonic midbrain

The robust neurogenesis within the eMB could direct or allow neuronal differentiation of foreign NSCs isolated from other regions of the NT or the adult brain. We tested whether E10.5 NSCs from the ventral telencephalon (VT) initiated neurogenesis after integration into the eMB parenchyma. Two days after transplantation, some donor

NSCs still expressed the progenitor marker *Nestin* (Sup. Fig. 1A). At this point, many endogenous cells were also undifferentiated, judged by the expression of the same gene (Sup. Fig. 1A). After seven days, donor cells were completely integrated into the eMB explants and adopted neuronal morphologies (Fig. 2A). These data were further confirmed by the presence of many forebrain-derived β-III Tubulin^{+} and NeuN^{+} neurons (Fig. 2B, C). Thus, neuronal differentiation is not affected when telencephalic NSCs develop in an ectopic niche.

Lineage tracing analysis has demonstrated that telencephalic NSCs eventually generate the adult forebrain NSCs of the SVZ (Young et al., 2007). Therefore, we asked if adult forebrain NSCs posses neurogenic potential in an ectopic embryonic environment. Dissociated cell suspensions of the adult SVZ went through the ventricular surface of the eMB explants and developed neuronal and glial morphologies at deep layers of the tissue (Fig. 2D). This observation was confirmed by detection of β-III Tubulin or Gfap in forebrain cells (Fig. 2E, F). Interestingly, Gfap^{+} donor cells always expressed *Nestin* (Fig. 2E), suggesting the survival of adult astrocyte-like NSCs (Doetsch et al., 1999; Mignone et al., 2004). Some transplanted cells neither acquired neural morphology nor expressed any of the evaluated cell markers (data not shown). These cells could represent ependymal or endothelial populations.

To confirm ectopic neurogenesis of forebrain cells, FACS-purified NSCs from the adult SVZ were directly transplanted into the eMB explants. Purification was based on sorting cells with a diameter bigger than 12 μm and low affinity to the peanut agglutinin (>12 μm PNA^{lo}), which results in a significant enrichment of adult NSCs (Rietze et al., 2001). After purification, approximately 1 in 7 cells show the capacity to generate neurospheres (i.e. proliferative cell aggregates) in culture (Rietze et al., 2001). Importantly, the purified population is devoid of neuroblasts and mature neurons

(Rietze R. and Barlett P. personal communication). Data obtained from three independent purification/transplantation experiments demonstrated that a high proportion of purified cells (50.19 ± 3.64%, n = 57; three independent experiments) were β-III Tubulin$^+$ (Fig. 2G) and presented neuronal morphology (Sup. Fig. 1B) after incorporation into the eMB explants. We did not detect Gfap$^+$ donor cells (data not shown), while some GFP$^+$ cells resembled migratory progenitors (Sup. Fig. 1C). This analysis indicates that adult NSCs within the forebrain SVZ exclusively differentiate into neurons during early stages of embryonic neurogenesis.

Telencephalic Neural Stem Cells show commitment to the forebrain lineage

Currently, it is unknown whether forebrain NSCs have the potential to produce mDA neurons in response to the influence of the midbrain niche. To address this question, E10.5 NSCs isolated from the VT were placed at the ventral midline of age-matched eMB explants. Transplanted NSCs did not produce Lmx1a and En1 at the dopaminergic domain (Fig. 3A) ruling out specification into the mDA lineage. Furthermore, with very rare exceptions (Sup. Fig. 2A), forebrain-derived neurons did not produce TH (Fig. 3A) indicating the absence of dopaminergic features.

The lack of response to inductive signals within the eMB suggested that telencephalic NSCs were already compromised to forebrain lineages. As NSCs at the VT exclusively give rise to gabaergic interneurons (Hebert and Fishell, 2008), we looked for the presence of the glutamic acid decarboxylases 65 and 67 ($GAD_{65/67}$, encoded by *Gad2* and *Gad1*, respectively) in donor cells. Many gabaergic neurons derived from telencephalic cells emerged after incorporation into the eMB (Fig. 3B). Besides, although gabaergic neurons arise at most dorso-ventral levels of the eMB (Nakatani et al., 2007), telencephalic-derived $GAD_{65/67}^+$ neurons were also observed at the region of dopaminergic neurogenesis (Fig. Sup. 2B). This finding suggest that

telencephalic NSCs retain intrinsic information from their region of origin. Confirming this assumption, Bf1^{+} telencephalic cells were observed at the eMB explants (Fig. 3C).

Neural Stem Cells of the prospective telencephalon differentiate into mesencephalic dopaminergic neurons

The possibility remained that forebrain NSCs had broader plasticity at developmental stages preceding E10.5. Hence, NSCs isolated around E8/8.5 from the most anterior NP (i.e. the prospective telencephalic region) were directly transplanted into the E10.5 eMB explants. Interestingly, after integration into the eMB, some telencephalic cells formed "rosette"-like structures, characterized by a tight arrangement of large columnar cells into a round shape while leaving a lumen at the core; thus resembling the NT (Fig. 4A). Forebrain-derived Lmx1a^{+} cells appeared within neural rosettes and outside these structures when donor cells were incorporated at the ventral midline (Fig. 4A, B). At the same region, some GFP^{+} cells produced TH after seven days (Fig. 4C), in agreement with the specification and differentiation of early telencephalic NSCs into mDA neurons. Absence of forebrain-derived dopaminergic neurons outside the ventral midline (data not shown), ratified niche-dependent differentiation of donor cells.

To gain more insights about the plasticity of early NSCs, site-specific differentiation at other domains of the eMB was examined. Donor cells produced many GAD$_{65/67}^{+}$ neurons at the ventro-lateral area, together with the acquisition of *Nkx2.2* expression at the same region (Fig. 4D, E). Although *Nkx2.2* and *Gad1/2* are also expressed at the VT (Hebert and Fishell, 2008; Qiu et al., 1998), donor cells did not expressed these genes at the dorsal midbrain or the ventral midline, respectively (data not shown). Forebrain NSCs at the ventral midbrain also expressed *Nkx6.1* (Fig. 4F) a gene whose expression is exclude from the VT (Qiu et al., 1998). Together, these data

suggest an influence of the surrounding environment on early telencephalic NSCs, whom apparently were able to integrate many inputs from the niche.

Rapid restrictions on developmental plasticity are also imposed on Neural Stem Cells in culture

Our results indicate that between E8.5 and E10.5, competent NSCs restrict their fate and response to patterning cues. To investigate whether swift restrictions on NSC competence also occur in vitro, we used ESCs as a model for neural specification. ESC-derived precursors within EBs were selected as the starting population because these cells are not neuralized and respond to environmental signals at the eMB explants (Baizabal and Covarrubias, 2009). Initially, EB precursors expressed weak levels of the pluripotency marker *Oct4*, especially at the periphery of the aggregate (Fig. 5A). After a 48 hr exposure to 1 μM RA, most EB cells had differentiated into Nestin$^+$ neural precursors (Fig. 5B). The transcriptional profile of these populations revealed that naïve EB cells had an "anterior" identity, indicated by the expression of *Otx2* and the absence of *Hoxd1* (Fig. 5C). Exposure to RA promoted a shift to a "posterior" identity (i.e. *Otx2* down, *Hoxd1* up) during EB neuralization (Fig. 5C). Some genes differentially expressed along the dorso-ventral axis of the NT were also affected by RA (Fig. 5C). Thus, ventrally-restricted genes like *Nkx6.1*, *Nkx2.2*, *Foxa2*, *Th* and *Gli1* were up regulated. Accordingly, the dorsal gene *Pax7* was slightly down regulated with RA treatment, while *Pax6* expression did not change.

After transplantation, ESC-derived neural precursors produced NeuN$^+$ mature neurons (Fig. 5D). However, few donor cells produced Lmx1a and Th at the ventral midline of the eMB (Fig. 5E, G). Those GFP$^+$ dopaminergic neurons were unlikely the product of regional specification as donor cells also produced Lmx1a and Th outside the dopaminergic niche (Fig. 5F, H). Therefore, restrictions on cell competence are likely

established early (i.e. within the 48 hrs of RA treatment) during in vitro neuralization of competent EB cells.

An inductive niche within the telencephalon instructs gabaergic neurogenesis

Commitment of telencephalic NSCs might entail the extinction of inductive signals from their niche. Yet, in order to identify an instructive niche for neurogenesis, transplantation of pre-neuralized precursors is necessary, as NSCs could be biased to neuronal differentiation. Thus, we employed naïve EB precursors (Fig. 5A) as a "sensor" of environmental cues in the telencephalon.

After incorporation into the E10.5 telencephalon, transplanted EB cells acquired *Nestin* (Fig. 6A) and *β-III Tubulin* (Fig. 6B) expression. Donor-derived neurons produced $GAD_{65/67}$ only after integration into the ganglionic eminences at the VT (Fig. 6C). Besides, donor $GAD_{65/67}^{+}$ cells were negative for Nestin (Sup. Fig. 3), confirming their exit from the cell cycle and initiation of neuronal differentiation. Production of TH was never observed in donor cells (data not shown), suggesting the exclusive generation of gabaergic neurons, just like endogenous NSCs. Hence, although telencephalic NSCs are committed to forebrain lineages around E10.5, the inductive capacity of their niche still remains at that stage of development.

Discussion

Directed in vitro differentiation of NSCs into specific neuronal lineages has proven difficult in many cases [see as examples (Daadi and Weiss, 1999; Parish et al., 2008)]. Two major issues are at the bottom of this problem: (1) the precise combinatorial influence of morphogens through space and time is not easily reproducible in culture; (2) The level of commitment of NSCs is usually unknown, leading to the application of inductive signals to refractory cells. Here, we overcame the first issue by transplanting NSCs to explant cultures that faithfully reproduce embryonic neurogenesis. Using this

approach, we then addressed the second issue and demonstrated that early telencephalic NSCs are susceptible of robust neuronal specification in response to the host environment. Nevertheless, cell competence swiftly decays as development advances. Supporting these data, a rapid lost in cell competence was also observed during in vitro neuralization of ESCs.

Our study indicates that the embryo and adult forebrain NSCs maintain their neurogenic potential at the eMB. In particular for SVZ cells, a previous work demonstrated that they express neuronal markers after transplantation to the embryonic brain (Lim et al., 1997). However, it remained unclear whether donor-derived neurons resulted from differentiation of NSCs. Here, through a purification protocol based on the selection of >12 μm PNA^{lo} cells, we provide evidence that NSCs from the adult SVZ differentiate into neurons in the embryonic brain. Ectopic neuronal differentiation in the eMB could result from interactions of forebrain NSCs with a permissive niche (i.e. that allows differentiation) or an instructive niche (i.e. that directs differentiation). Notably, NSCs and even committed neuronal progenitors from the SVZ mostly generate glial cells after ectopic incorporation into non neurogenic regions (Herrera et al., 1999; Seidenfaden et al., 2006). Hence, although we can not discern between an instructive or permissive mechanism, selective neuronal differentiation of forebrain NSCs suggest an influence of the eMB explants to promote neurogenesis.

Telencephalic E10.5 NSCs do not adopt dopaminergic features after integration around the ventral midline of the eMB. This observation likely reflects the compromised state of telencephalic NSCs at that stage of development. In disagreement with this assumption, a previous work reported that some E13.5 telencephalic NSCs express *En1* after incorporation into the eMB in vivo (Olsson et al., 1997). Certainly, we can not completely rule out that this discrepancy comes up from a limited capacity

of the explants to direct differentiation of late (i.e. E10.5 onward) forebrain NSCs. Yet, the ability of the explants to efficiently conduct mDA specification of E8.5 NSCs (this study) and naïve EB precursors (Baizabal and Covarrubias, 2009) argues against that possibility. Furthermore, unlike the study from Olsson et al., we used confocal analysis of single optical planes to achieve a greater resolution of labeled cells. In support of early restrictions on cell fate, E12 telencephalic and cerebellar NSCs do not adopt region-specific identities after heterotopic transplantation (Carletti et al., 2002; Carletti et al., 2004). In fact, ectopically located telencephalic cells autonomously maintain *Bf1* expression and do not produce host diencephalic markers like Nkx2.1, oxytocin and vasopressin (Na et al., 1998).

In this study, it was not feasible to evaluate mDA differentiation of adult NSCs, as this population only represents around 1.7% of SVZ cells (Rietze et al., 2001), thus making difficult a substantial cell recovery for transplantation after FACS. Alternative purification methods, like magnetic cell sorting, might provide an easier mean to evaluate neuronal specification of adult NSCs. This is an important issue because recent reports indicate that NSCs residing at the SVZ seem to be compromised to their normal interneuron fates (Merkle et al., 2007; Milosevic et al., 2008). The possibility remains, however, that the immature environment within the embryonic brain has the capacity to confer a broader plasticity on adult NSCs.

This work shows that E8.5 NSCs, isolated from the prospective telencephalic region of the NP, produce $Lmx1a^{+}/Th^{+}$ neurons after ectopic integration in the ventral midbrain. To our knowledge, this is the first evidence of niche-dependent differentiation of forebrain NSCs into mDA neurons. This observation is unlikely due to the present of "contaminating" midbrain NSCs during transplantation as only the most anterior region of the prospective telencephalic vesicle was grafted. Importantly, although telencephalic

dopaminergic neurons are also gabaergic (Daadi and Weiss, 1999) early NSCs did not express *Gad1/2* at the ventral midline of the eMB. Besides, *Lmx1a* expression is restricted to dopaminergic neurons of the midbrain (Andersson et al., 2006). Thus, our data support a re-specification event of forebrain NSCs into a midbrain fate. The broad plasticity of E8.5 NSCs is not restricted to the dopaminergic lineage, as those cells acquired region-specific expression of *Nkx2.2*, *Nkx6.1* and *Gad1/2*. Hence, early NSCs of the anterior NP are capable of integrating ectopic environmental signals.

Taken together, our analysis of forebrain NSCs suggests that the high competence and plasticity of NP cells are rather ephemeral features. In this regard, classical studies of Shh pattern formation at the ventral spinal cord have reached a similar conclusion. In particular, Shh is necessary to repress the expression of the dorsal gene *Pax7* in neural plate explants but is not required in neural tube explants, presumably because Pax7$^-$ NSCs are already committed to ventral fates (Ericson et al., 1996). In addition, within a time lapse of just 12 hrs, neural plate explants lose their competence to produce floor plate cells and motoneurons in response to Shh (Ericson et al., 1996).

In vivo, the transition from primitive to definitive NSCs initiates around E8.5 in mice (Hitoshi et al., 2004). One distinctive feature of this event is the down regulation of the pluripotency marker *Oct4*, which might confer an increased plasticity to naïve neural cells (Akamatsu et al., 2009). Thus, it is tempting to speculate that the E8.5 NP cells isolated in this work represent the population of primitive NSCs. To advance in our understanding of cell competence, an important issue for the future will be to define the molecular profile of primitive NSCs or their equivalent produced in vitro from ESCs. Interestingly, some transplanted E8.5 NSCs formed an arrange similar to the previously described neural rosettes (Li et al., 2005). In contrast, neural rosettes were

never observed after transplantation of undissociated E10.5 telencephalic tissue (data not shown). In culture, the tendency of NSCs to form rosettes is fairly common at the NP stage (E8.25) but rapidly declines after NT formation (E9.5 onward; Elkabetz et al., 2008). Arguably, ESC-derived neural rosettes have a primitive character as they generate a broad repertoire of neuronal subtypes in response to developmental signals in culture (Elkabetz et al., 2008). In the same study, a transcriptional analysis revealed the expression of 298 rosette-specific genes, many of these codifying for transcriptions factors (Elkabetz et al., 2008). Thus, an appealing possibility is that early E8.5 NSCs have a direct lineage relationship with the rosette-forming neural precursors.

Similar to the embryonic NSCs, competent EB cells rapidly lose their responsiveness to environmental cues at the ventral midbrain. This occurred after a short neuralizing pulse (48 hrs) with 1 μM RA in culture. In contrast, a previous work showed that RA-induced neural precursors acquire ventral fates at the correct positions in the chick spinal cord (Plachta et al., 2004). The dose-dependent specification of EB-derived NSCs by RA might explain this difference, as high concentrations of RA (i.e. 0.1-2 μM) repress anterior genes like *Otx2* and activate caudal genes like *Gbx2* as well as members of the *Hox* family (this study and Okada et al., 2004). High doses of RA also promote the activation of ventral genes (this study and Li et al., 2005; Okada et al., 2004). Therefore, the ventro-caudal identity imposed by RA might bias the fate of neural precursors to hindbrain or spinal cord lineages. The irreversible RA-mediated down-regulation of essential genes for the development of mDA neurons, like *Otx2* (Omodei et al., 2008) could avoid the correct interpretation of the dopaminergic niche by NSCs.

Commitment of NSCs involves a transition from dependence on external information to dependence on intrinsic transcriptional programs (Edlund and Jessell,

1999). Thus, signals within a niche could change or even disappear when residing NSCs become intrinsically specified to a neuronal lineage. Yet, our data indicate that the inductive properties of the VT are completely retained even though the local NSCs are already specified/compromised to the gabaergic lineages. We reached this conclusion because naïve EB cells are instructed to gabaergic neurogenesis specifically at the ventral region of the E10.5 telencephalic explants. The generalized neuronal potential of EB cells across the embryonic brain should facilitate the study of the niche in a variety of null mutations backgrounds.

It is know that the spatiotemporal interpretation of signals elicited by morphogens, leads to the fine-tuned segregation of neuronal fates in vivo (Ashe and Briscoe, 2006). Altogether, our data suggest that the interpretation of complex morphogens networks at the NT is only possible during very restricted developmental windows. The identification of competent NSCs to produce the neurons affected in neurodegenerative diseases will offer a more rational approach for future cell therapies.

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Figure Legends

Fig. 1 Development of the dopaminergic lineage in culture. (A) Coronal sections of the ventral midbrain showing the production of TH during development. (B) TH detection in representative coronal sections of organotypic and collagen explants after 5 days in culture. Dopaminergic neurons close to the ventricular zone (A, B; arrows) or the pial region (A, B; arrowheads) are indicated; asterisks indicate the bilateral domains of the SNc. (C) Whole-mount detections of TH in collagen explants visualized from a top view. Note the presence of the dopaminergic cell bodies at the ventricular zone (left image) and the antero-dorsal projection of dopaminergic axons at the pial region

(arrows, middle image) in the same tissue. Collagen explants maintained the organization of Nestin^{+} radial glial-like progenitors at the ventral midline (arrows, right image). The dashed lines indicate the approximate level of the Isthmus organizer. A, anterior; P, posterior; D, dorsal. All images in A and B are at the same magnification. Scale bar: 20 μm. Left and middle images in C are at the same magnification; scale bar: 100 μm. Right image in C; scale bar: 20 μm.

Fig. 2 Neurogenic potential of forebrain NSCs. E10.5 telencephalic NSCs showed neuronal morphologies (A, arrows) and produced β-III Tubulin (B, arrows) and NeuN (C, arrows) seven days after transplantation to the eMB explants. Arrowheads in C indicate midbrain NeuN^{+} neurons. (D) Whole mount visualization of adult cells from the SVZ after incorporation into the eMB explants. Donor cells with glial morphology (D, arrowhead) produced Gfap and Nestin (E, arrow). Donor cells with neuronal morphology (D, arrows), produced β-III Tubulin (F, arrow). (E) Purified NSCs from the adult SVZ (>12 μm PNA^{lo}; see text for details) differentiated into β-III Tubulin^{+} neurons in the eMB explants. Scale bars: 20 μm.

Fig. 3 Neuronal commitment of telencephalic NSCs. (A) E10.5 NSCs isolated from the VT did not produce Lmx1a, En1 and Th after integration around the ventral midline of the eMB explants. Arrowheads indicate endogenous mDA neurons. (B) Many donor telencephalic NSCs differentiated into GAD$_{65/67}^{+}$ gabaergic neurons (arrows). Arrowheads indicate endogenous midbrain gabaergic neurons. (C) Donor cells maintained the production of the telencephalic transcription factor Bf1 (arrows) seven days after incorporation into the eMB explants. Note that some apparently single-labeled Bf1^{+} cells indeed had weak GFP signals (arrowheads). Scale bars: 20 μm.

Fig. 4 Niche-specific differentiation of early forebrain NSCs. (A) Rosette-like structure formed by donor cells integrated into the eMB explants. Early forebrain NSCs within

neural rosettes (A) and outside these structures (B) produced Lmx1a around the ventral midline (arrows). (C) At the same region, donor-derived TH^+ neurons (arrows) were observed. (D) In contrast, donor NSCs produced $GAD_{65/67}^+$ gabaergic neurons (arrows) at ventro-lateral levels of the eMB explants. (E, F) Within the gabaergic domain, some donor NSCs were positive for Nkx2.2 and Nkx6.1 (arrows). Arrowheads indicate endogenous midbrain cells. Scale bars: 20 μm.

Fig. 5 Developmental restrictions on neural precursors generated in vitro. (A) ESCs-derived precursors within EBs produced Oct4, especially at the periphery of the cell aggregate (arrows). (B) Two days after exposure to RA, many $Nestin^+$ precursors appeared within EBs. (C) Expression of positional identity genes in control and RA-treated EBs. The analyzed genes are classified according to their differential expression along the antero-posterior and dorso-ventral axes of the NT. Negative controls (-) consisted in the PCR mixtures without including the cDNA. The corresponding gene expression at the NT was included as a positive control (+). Amplification was carried out for 25, 30, 35 and 40 cycles in order to have a semi-quantitative estimation of mRNA levels. (D) Neural precursors within RA-treated EBs differentiated into $NeuN^+$ neurons (arrows) in the eMB explants. Yet, few donor derived-neurons produced TH close to the ventral midline (E, arrow) and far from it (F, arrow). The mDA marker Lmx1a was also detected in few donor cells inside (G, arrow) and outside (H, arrow) the dopaminergic domain. E-H are the same magnification. Scale bars: 20 μm.

Fig. 6 Inductive properties of the telencephalic niche. (A) Naïve EB cells differentiated into $Nestin^+$ neural precursors (arrows) after incorporation into the telencephalic explants. (B) Some donor-derived neural precursors differentiated into β-III $Tubulin^+$ neurons (arrows) in the telencephalon. Note the presence of undifferentiated GFP^+ aggregates within the tissue (A and B, arrowheads). (C) EB-derived $Gad_{65/67}^+$ neurons

(arrows) were strictly found at the ventral region of the telencephalon. Scale bars: 20 μm.

Sup. Fig. 1 Incorporation of forebrain NSCs into the eMB explants. (A) Some Nestin^{+} telencephalic cells (arrow) were observed two days after transplantation. Note the presence of many endogenous Nestin^{+} cells within the eMB explants. (B) Purified NSCs from the adult SVZ showed typical neuronal morphology after integration into the explants. (C) Some purified cells from the SVZ did not differentiated into β-III Tubulin^{+} neurons and presented a migratory morphology within the eMB explants.

Sup. Fig. 2 NSCs isolated from the E10.5 telencephalon do not respond to the dopaminergic niche of the eMB explants. (A) Only rarely, donor-derived neurons produced TH (arrow) around the ventral midline. (B) E10.5 telencephalic NSC differentiated into Gad$_{65/67}^{+}$ neurons in close proximity to En1^{+} dopaminergic neurons. Note, that although some noise is observed in the red channel, three clearly GFP^{+} gabaergic neurons are distinguished (arrows). Scale bar: 20 μm.

Sup. Fig. 3 Gabaergic differentiation of EB cells at the VT. Donor-derived Gad$_{65/67}^{+}$ neurons (arrowheads) were negative for Nestin. Some donor-derived Nestin^{+} cells are also indicated (arrows). Scale bar: 20 μm.

Fig. 1

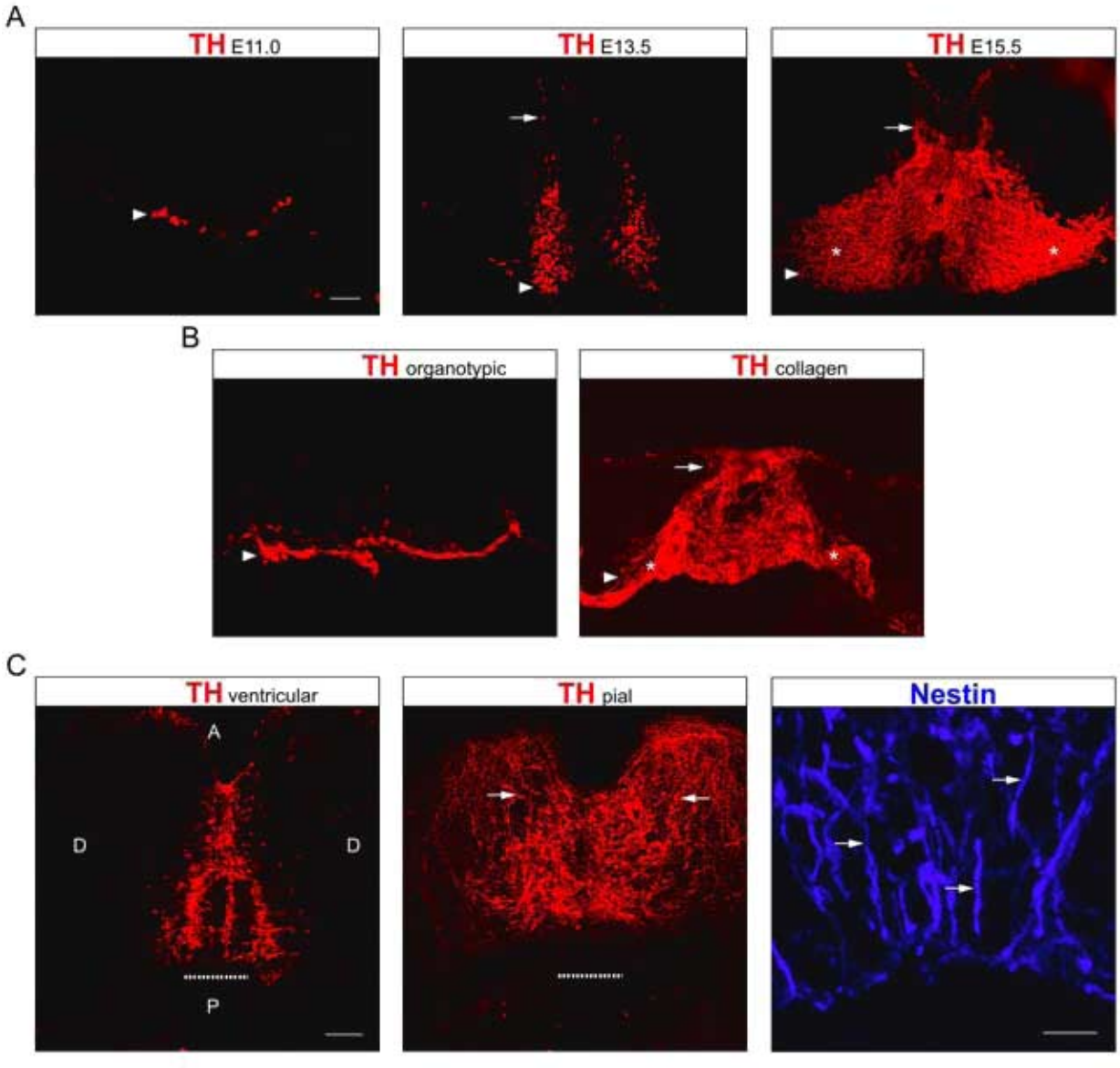

A
TH E11.0
TH E13.5
TH E15.5
B
TH organotypic
TH collagen
C
TH ventricular
A
D
D
P
TH pial
Nestin

Fig. 2

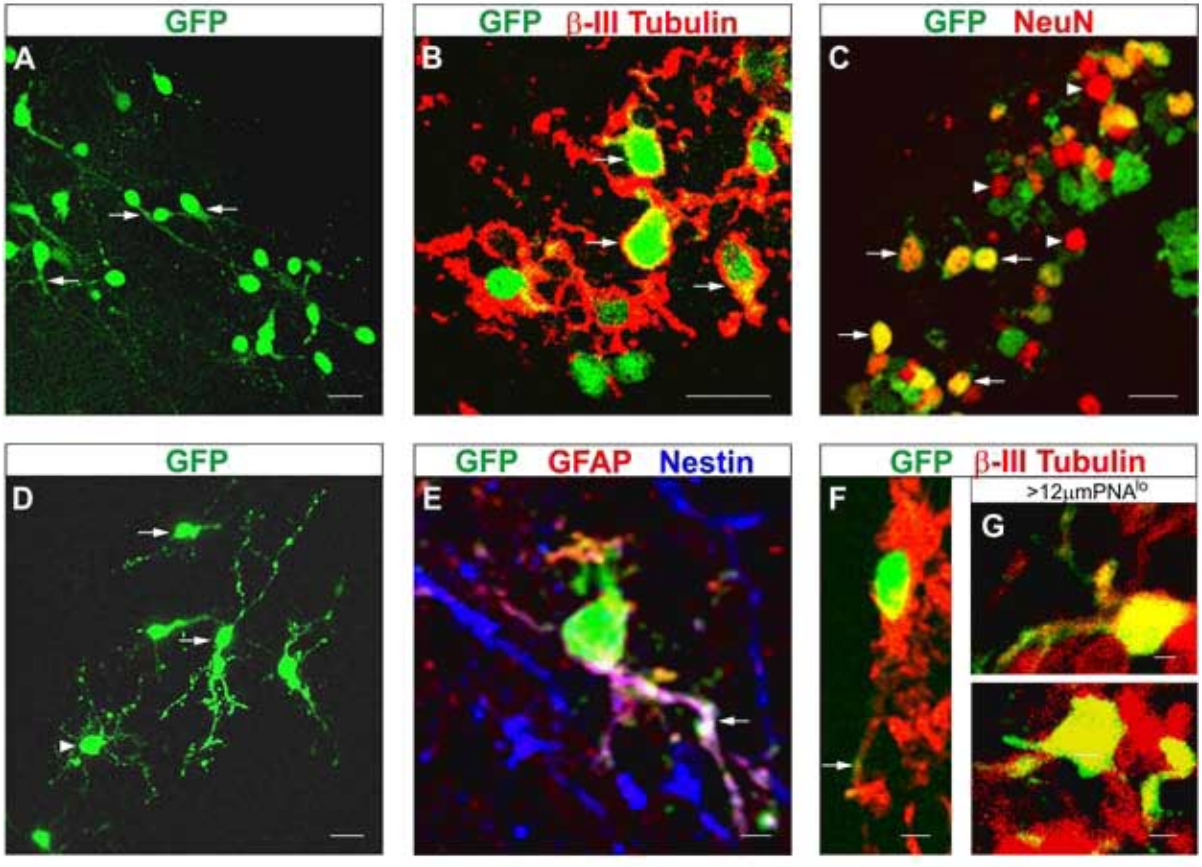

GFP
A
GFP β-III Tubulin
B
GFP NeuN
C
GFP
D
GFP GFAP Nestin
E
GFP β-III Tubulin
F
>12μmPNA^lo
G

Fig. 3

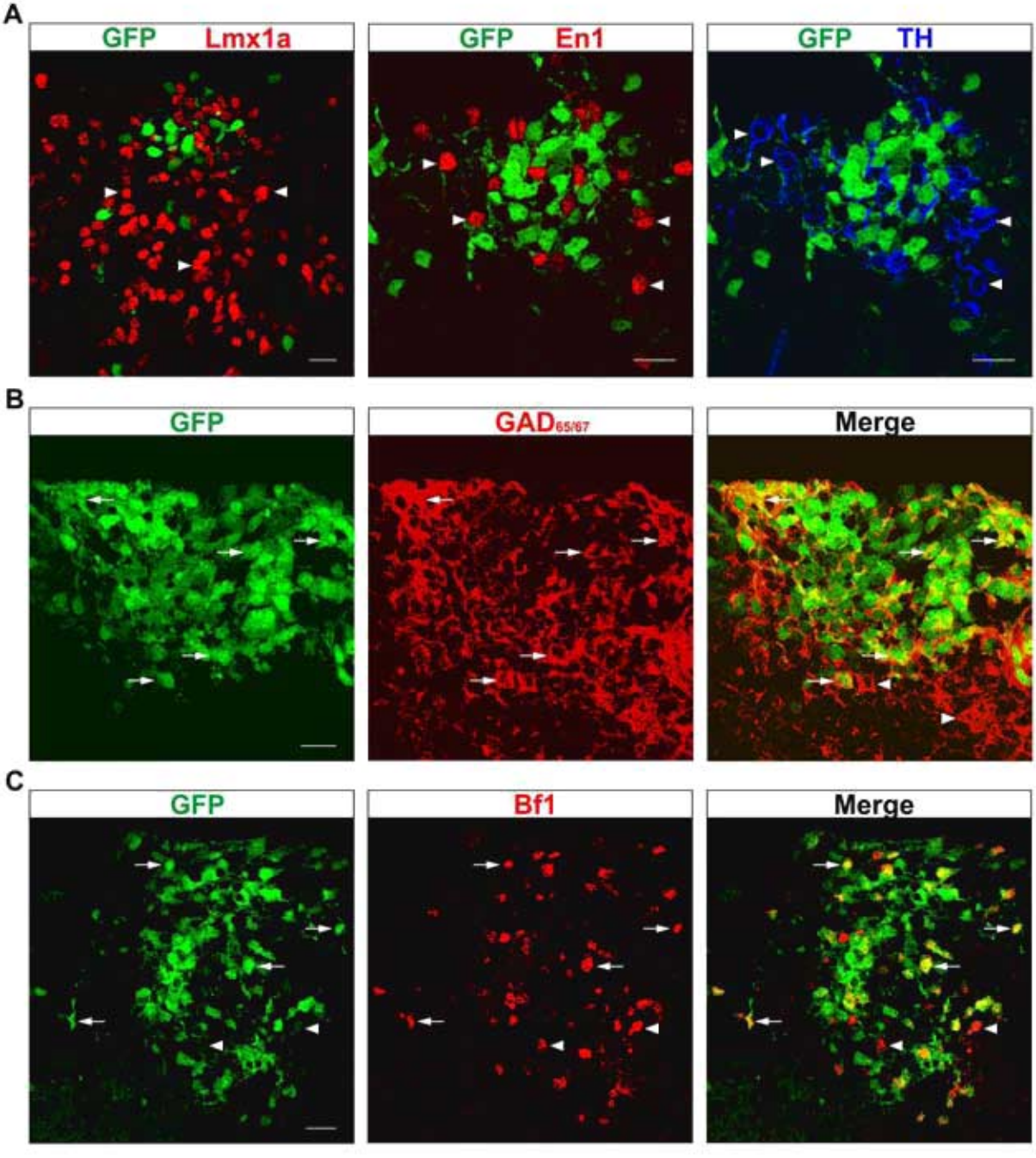


Fig. 4

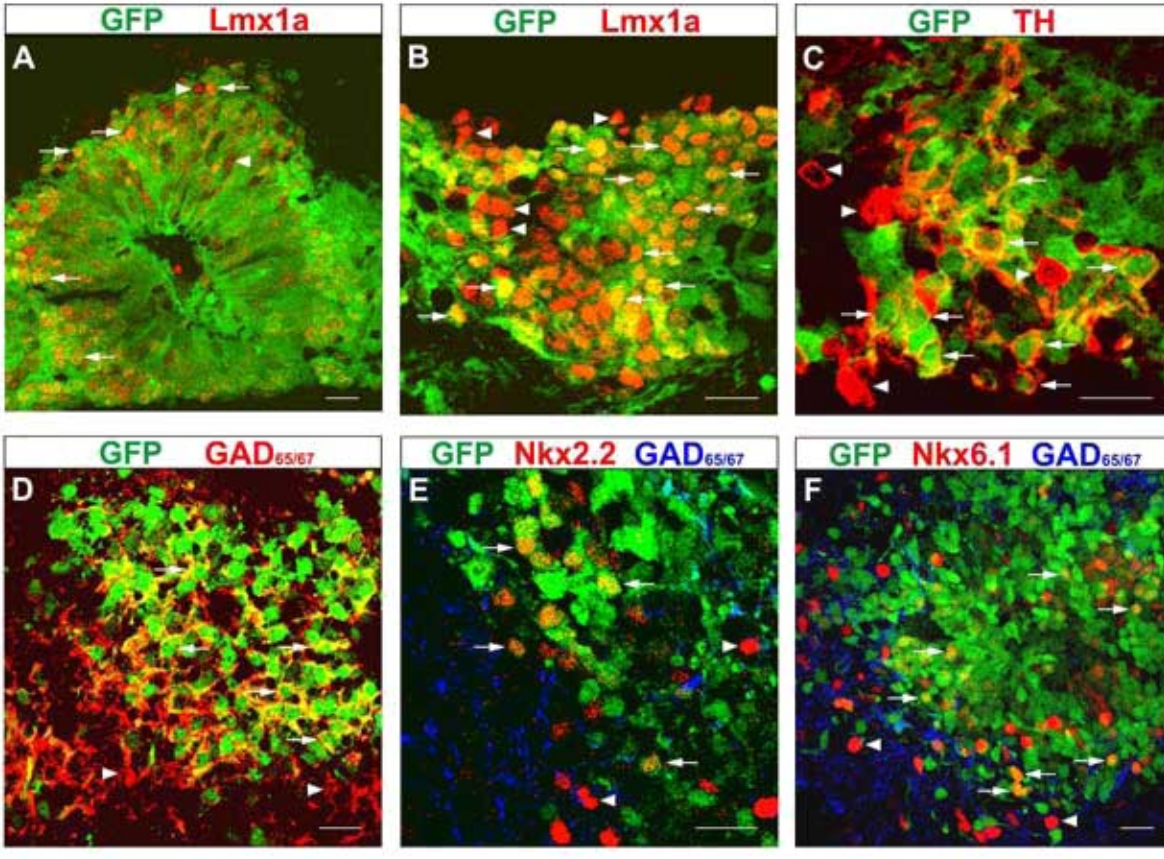

GFP Lmx1a
A
GFP Lmx1a
B
GFP TH
C
GFP GAD65/67
D
GFP Nkx2.2 GAD65/67
E
GFP Nkx6.1 GAD65/67
F

Fig. 5

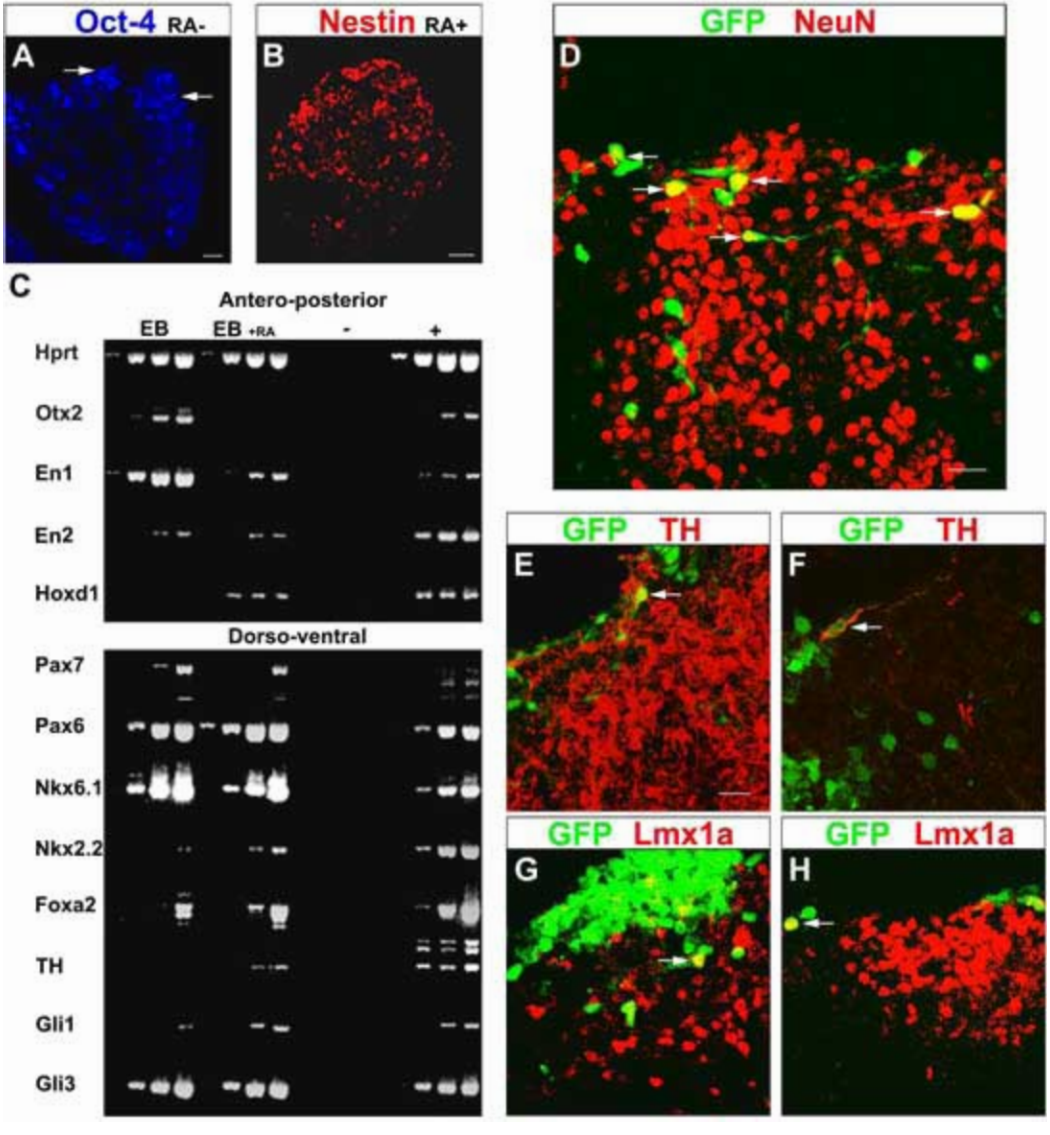
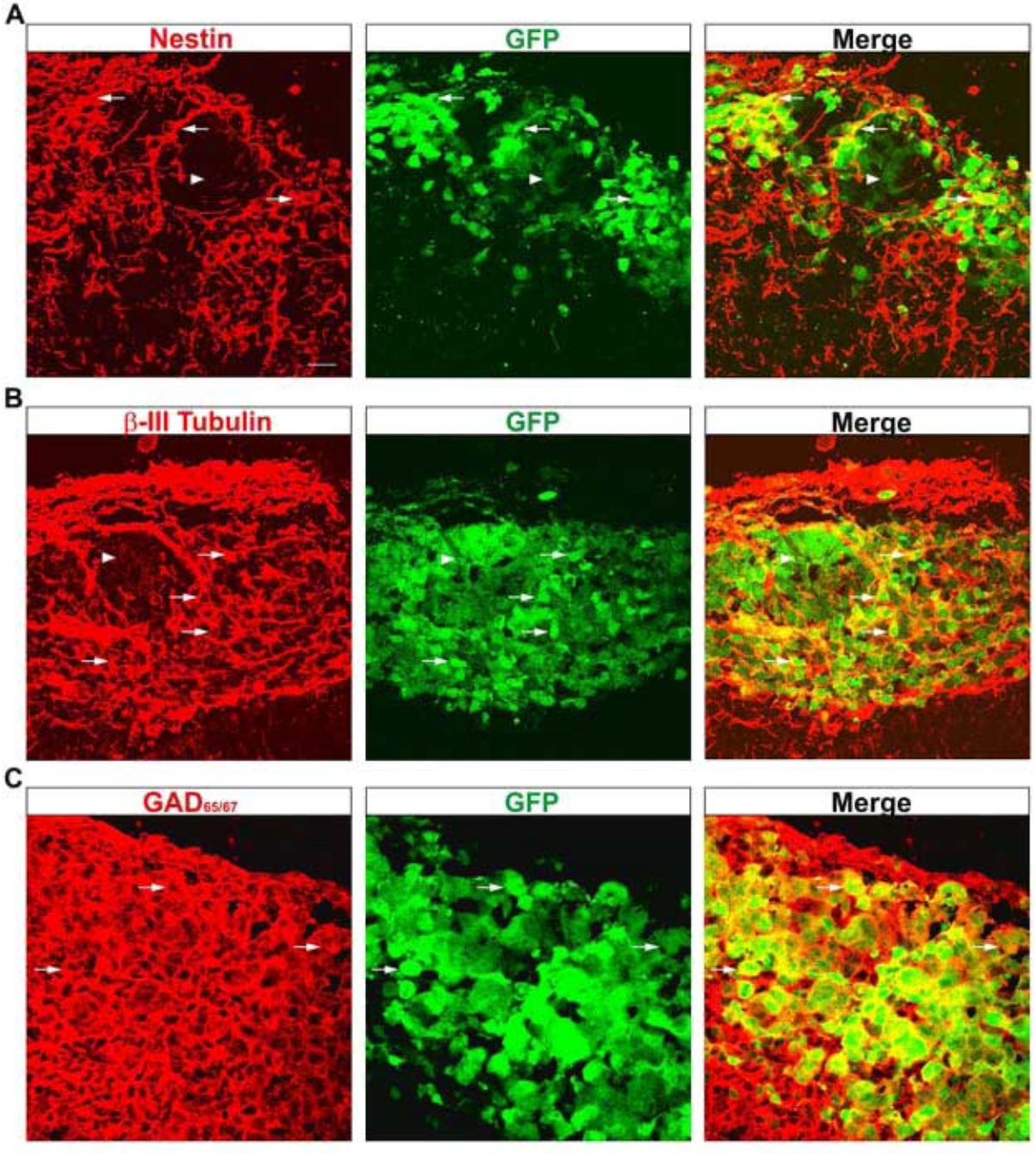

Oct-4 RA-
A
Nestin RA+
B
GFP NeuN
D
C
Antero-posterior
EB
EB +RA
-
+
Hprt
Otx2
En1
En2
Hoxd1
Dorso-ventral
Pax7
Pax6
Nkx6.1
Nkx2.2
Foxa2
TH
Gli1
Gli3
GFP TH
E
GFP TH
F
GFP Lmx1a
G
GFP Lmx1a
H

Fig. 6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Nestin
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B
β-III Tubulin
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C
GAD65/67
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Fig. Sup. 1

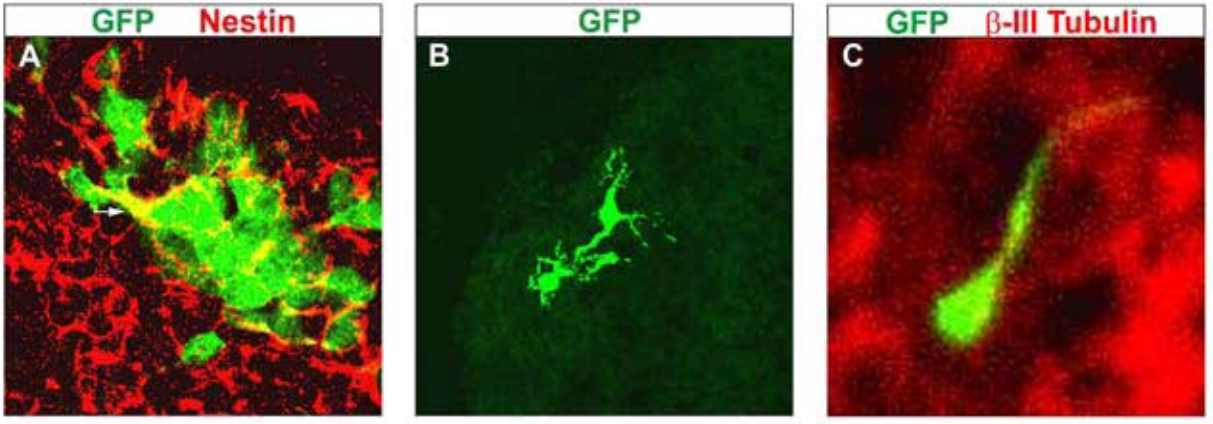

FIG. SUP. 2

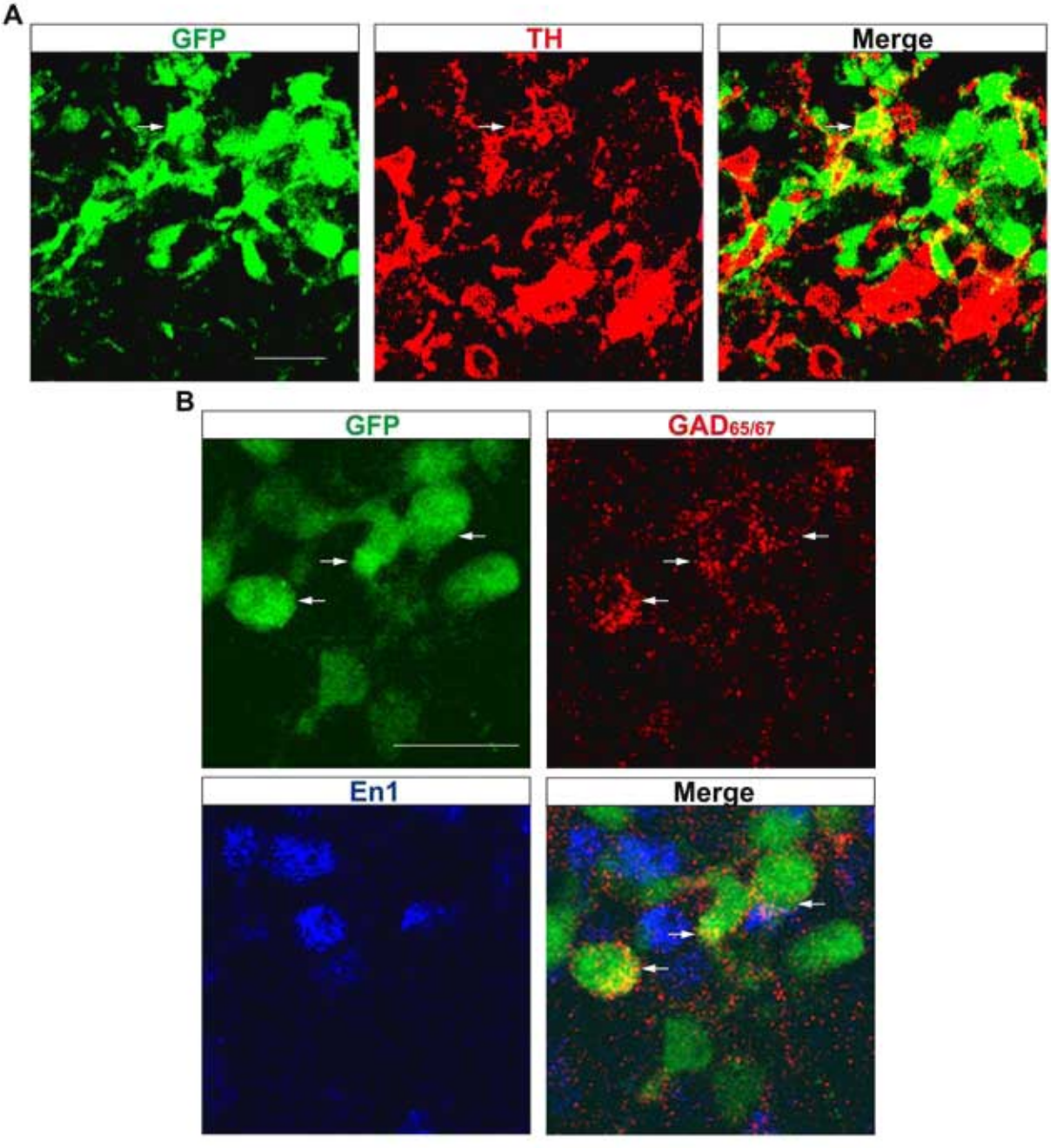

Fig. Sup. 3

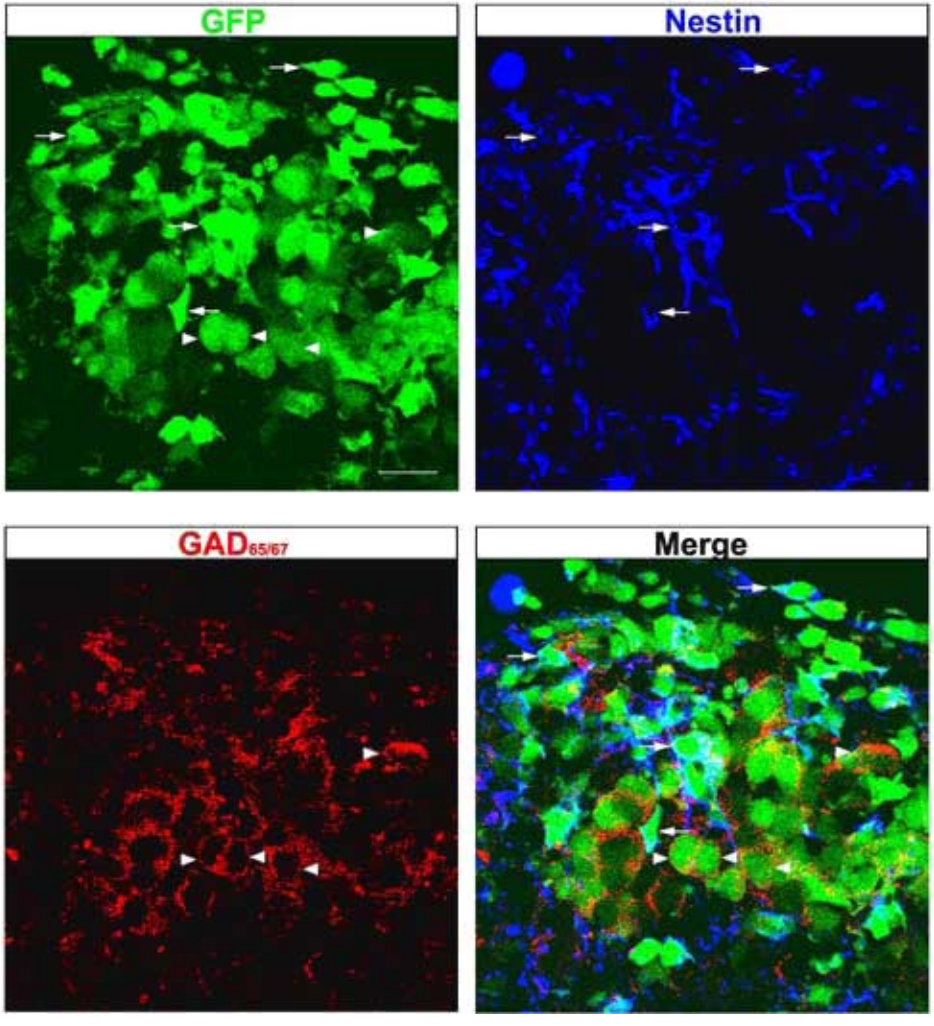

Table 1

Frequency[a] of response to their host environment of distinct stem cells populations

	Niche-dependent neuronal specification	Niche-independent neuronal specification
E10.5 NSCs[b] (ventral telencephalon)	**-** n (0/3)	**+** n (3/3)
E8.5 NSCs[c] (anterior NP)	**+** n (4/4)	**-** n (0/4)
EB-derived neural precursors[b] (RA-induced)	**-** n (0/3)	**+** n (3/3)
EB cells[c] (Day 4 of formation)	**+** n (4/4)	**-** n (0/4)

[a] "n" indicates the number of experiments where niche-dependent or independent responses were observed divided over the total number of independent transplantation experiments. At least 6 explants were analyzed for every NSCs population. Data is based on the observation of >100 transplanted cells for every independent experiment.

[b] Negligible numbers of dopaminergic neurons were observed at the ventral midline of the EM.

[c] Approximate cell countings indicated at least 15% of donor-derived mDA or gabaergic neurons over the total GFP^+ population integrated into the EM or the VT, respectively.

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REVIEW ARTICLE

Neural Stem Cells in Development and Regenerative Medicine

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In the last 10 years, enormous interest in neural stem cells has arisen from both basic and medical points of view. The discovery of neurogenesis in the adult brain has opened our imagination to consider novel strategies for the treatment of neurodegenerative diseases. Characterization of neurogenesis during development plays a fundamental role for the rational design of therapeutic procedures. In the present review, we describe recent progress in the characterization of embryo and adult neural stem cells (NSCs). We emphasize studies directed to determine the *in vivo* and *in vitro* differentiation potential of different NSC populations and the influence of the surrounding environment on NSC-specific differentiation. From a different perspective, the fact that NSCs and progenitors continuously proliferate and differentiate in some areas of the adult brain force us to ask how this process can be affected in neurodegenerative diseases. We propose that both abnormal cell death activation and decreased natural neuronal regeneration can contribute to the neuronal loss associated with aging, and perhaps even with that occurring in some neurodegenerative diseases. Furthermore, although NSC activation can be useful to treat neurodegenerative diseases, uncontrolled NSC proliferation, survival, and/or differentiation could cause tumorigenesis in the brain. NSC-mediated therapeutic procedures must take into account this latter possibility.

Key Words: Neurogenesis, Neural precursor cells, Differentiation, Neurosphere, Transplantation.

Introduction

Development begins with a totipotent cell: the fertilized egg. In mammals, self-renewal-like divisions may occur during morula stage until outer cells deviate to the trophectodermal lineage. Inner cells (founders of the inner cell mass) remain pluripotent with the ability to differentiate to all cell types. The epiblast derives from these inner cells, whose potential restricts as development proceeds through gastrulation. In all animals, ectodermal, mesodermal, and endodermal precursors segregate early in development, although they may not be definitively compromised to cell types of these major lineages. Fate of cells can be tracked very early in development and reliable fate maps can be obtained. However, specification and determination maps strongly depend on the precise environment in which the differentiation potential of a cell population is tested. Therefore, it is difficult to deduce definitively the time at which cells of a particular embryonic tissue compromise within a lineage, primarily when tissue fragments, possibly carrying specific microenvironments, are used. Definitive specification and determination maps will require testing the differentiation potential of isolated cells in neutral and specific environments.

Depending on cell characteristics such as proliferation and differentiation competence, different names have been adopted to call the precursor cells of a particular cell lineage. Stem cell is the name given to cells with broad differentiation potential that retain the capacity for self-renewal indefinitely. A totipotent stem cell has the ability to give rise to all cell types of an organism, whereas a pluripotent or multipotent stem cell produces many but not all cell types. Pluripotent stem cell is a more proper denomination for stem cells with the ability to give rise to cell types within different lineages, and multipotent stem cell should refer to stem cells with the ability to give rise to cell types belonging to a common specific lineage (e.g., hematopoietic, neural, muscle). Cells

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with broad (multipotentiality) or restricted (e.g., unipotentiality) differentiation potential and with limited proliferation capacity should be called progenitor cells. Typical progenitor cells are those denominated transit-amplifying progenitors; these rapidly proliferate for a limited number of rounds. In cell lineages with several sublineages, it is convenient to use terms confined to the lineage, so that a totipotent-specific stem cell should be that producing all cell types within that lineage.

An intrinsic characteristic of the nervous system is its enormous cellular diversity. In the adult brain, specific neurons produce specific neurotransmitters and receptors and make specific contacts with other cells. Neural stem cells (NSCs) are the source of all neuron types. They appear during neural plate formation and possibly constitute the major cell type of early neuroectoderm (1). As development proceeds, neural tube forms and NSCs become progressively less abundant and more restricted progenitor cells emerge. Afterward, when most nervous system structures are defined, complex migration and differentiation patterns initiate followed by guided axonal growth that finally allows specific cell targeting, thus defining the stereotypical neuronal wiring of the nervous system.

An old dogma establishes that generation of each neuron present in the adult occurs only once during a lifetime. However, recent evidence has supported previous data that suggest the emergence of new neurons during adult life. As expected, the source of these neurons appears to be NSCs, which apparently reside at restricted niches (see below). The functional relevance of this *de novo* generation of neurons in the adult has not yet been defined, but a role in learning and olfactory function has been suggested. Could they participate in neuron renewal or regeneration upon accidental or pathologic damage of the nervous system? We do not know the answer to this question yet; however, the well-documented presence of adult NSCs prompts researchers to consider the possibility of activating endogenous or exogenous (i.e., transplanted) NSCs to recover specific neuronal loss. In this context, it is essential to determine the differentiation potential of embryo and adult NSCs, as well as to determine whether a neurogenic environment exists in the adult brain.

Neural Stem Cells in the Developing Embryo

Origin and Identification. Neural stem cells are functionally defined as long-term, self-renewing cells with the ability to generate the three major lineages of the nervous system (neurons, astrocytes, and oligodendrocytes). Multipotent NSCs are responsible for generation of all neurons in the central and peripheral nervous systems. Central and peripheral nervous system neurons could originate from a common pluripotent NSC population during early stages of neural tube development (2). Under the influence of neighboring epidermal cells, pluripotent NSCs restrict their potential and produce neural crest stem cells (NCSCs). Interestingly, these NSCs produce not only the sympathetic and parasympathetic neurons and Schwann cells of the peripheral nervous system, but also some non-neural derivatives such as melanocytes, craniofacial mesenchyme, and smooth muscle of the aortic outflow tract (3).

The origin of all different neurons should not be attributed to a single NSC type but to a set of NSCs originated during neural plate induction and neural tube extension. It is known that during gastrulation, different embryo organizers confer positional information to NSCs, which may be crucial to define their definitive fate. The positional information allows NSCs to differentially proliferate, migrate, and differentiate to cover the needs along the anteroposterior (AP) and dorsoventral (DV) axes of the neural tube. In the absence of neural plate organizers, NSCs could remain in a primitive neural cell state, perhaps with broader developmental potential.

Apparently, central nervous system NSCs and NCSCs have a broad differentiation potential early in development and become progressively restricted as development advances. For instance, early neural tube progenitors (possibly NSCs) differentiate in response to ectopic inductive signals to adopt multiple neuronal phenotypes present along the DV axis of the neural tube (4,5). On the other hand, late neural tube progenitors lose the ability to adopt ectopic neuronal phenotypes (6), albeit these experiments do not completely discard the presence of a sub-represented NSC population with high plasticity at late developmental stages. The same differentiation potential restriction, dependent on developmental age, is observed in NCSCs. In this case, early NCSCs differentiate to noradrenergic and cholinergic neurons in sympathetic and parasympathetic ganglia, respectively, whereas late NCSCs exhibit little or no noradrenergic differentiation in sympathetic ganglia (7). This phenomenon is attributed to lower sensitivity to differentiation signals in late NCSC. This restriction mechanism could also regulate the differentiation potential of central nervous system stem cells.

The identity of embryo NSCs remains controversial. Lack of very specific NSC markers makes it difficult to demonstrate long-term self-renewal and multipotentiality *in vivo*. Furthermore, the identity of NSCs could change spatially (according to neural tube position) and temporally (according to developmental age). Given the difficulty to identify NSCs, the term neural precursor cell (NPC) is more convenient to refer to all undifferentiated neural cells.

In vivo, several transcription factors are expressed during early neural cell determination. *Sox* genes, such as *Sox1* and *Sox2*, are among the earliest genes ubiquitously expressed in the early neural plate and appear to have a conserved function in neural cell determination in all metazoans (8). Other genes coding for transcription factors expressed in the newly formed neural plate are *Otx2*, *Pax3*, *Pax7*, *Msx1*, and *Msx2* (9–11); thus, these markers could be present in the more

primitive NSCs during neural plate induction. Late NPCs markers such as the intermediate filament protein Nestin and the RNA-binding protein Musashi (encoded by the *Msi1* and *Msi2* genes) appear after Sox proteins and persist during embryo development and in some areas of the adult brain (see below). *Msi1* and *Msi2* appear to be redundant genes required to maintain the NSC population (12). Sox1, Sox2, Nestin, and Musashi can be used as NSC markers, although some are also expressed in sublineage progenitors (e.g., neuronal or glial restricted progenitors) (13–15). Some studies have characterized different types of NPCs in the neural tube by evaluating their antigenic profile (16) (see below); however, no exclusive antigens have been identified for NSCs. Therefore, as with other aspects of neural cell development (17) combinations of positive and negative markers are required to better identify NSCs.

At the cellular level, NSCs have not been clearly identified *in vivo*. Radial glial cells have characteristics that suggest they represent an important NPC population within the developing nervous system (18). Radial glial cells appear early in neural tube development (ca. embryonic day 12 in telencephalon) before any sign of neurogenesis is detected and probably constitute the direct progeny of neuroepithelial NPCs present in the neural plate and the early neural tube (19,20) (Figure 1). Radial glial cells show a bipolar morphology extending across the entire radial axis of the neural tube, playing in this way an important role in neuron migration (19,21). In addition to the structural function of radial glia, this cell type produces new neurons and glial cells *in vitro* and *in vivo* (18,22–24). Different subsets of radial glial cells have been described according to detection of three markers: RC2 (an antigen found in radial cells); GLAST (the astrocyte-specific glutamate transporter), and BLBP (brain-lipid-binding protein). RC2+/GLAST+, RC2+/BLBP+, and RC2+/GLAST+/BLBP+ radial glial cells appear during the neurogenic period but only the RC2+/GLAST+/BLBP+ population is maintained until the gliogenic period, suggesting that co-expression of the three markers could be a hallmark of gliogenic radial glia (16). Although there is no direct evidence that radial glial cells represent an NSC, this progenitor population participates in nearly all neurogenesis occurring in some neural tube regions (e.g., the cerebral cortex) (22). Interestingly, radial glial cells produce astrocytes late in forebrain development and this latter cell type has been identified as a prospective NSC of the adult brain (see below) (25,26).

Embryo NSCs in Culture. Multipotent NPCs from embryonic neural tissue were initially identified *in vitro* by assessing the capacity of neural cells to proliferate in the presence of mitogens and terminally differentiate to neurons, astrocytes, and oligodendrocytes (27–29). Multipotent NPCs appear to divide *in vitro* first symmetrically and then asymmetrically to self-renew and produce neuronal-restricted progenitors; then, after several rounds of cell division, multipotent NPCs apparently undergo a cell fate switch to begin production of glial-restricted progenitors (30).

In vitro, different NPCs populations differentially respond to growth factors. Multipotent NPCs proliferate in the presence of epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), and transforming growth factor-α (TGF-α) (31,32). At present, formation of cell aggregates called neurospheres is the most common *in vitro* method to propagate NPCs. In this procedure, neural tissue is dissociated to a single cell suspension and plated in culture plates with mitogens such as EGF, FGF2, or both. Some cells within the culture proliferate and generate neurospheres, which consist mainly of undifferentiated cells (32). The cells from the neurospheres readily differentiate to neurons, astrocytes, and oligodendrocytes upon mitogen withdrawal and serum exposure on adhesion conditions (32). Similar neurospheres can be generated in the presence of an adherent such as poly-D-lysine; however, under this condition, FGF2 generally does not produce neurospheres and flat colonies are obtained instead (31) (Figure 2).

Two distinct NPC populations appear sequentially during neural tube development: “early” NPCs that proliferate only in the presence of FGF2 but that do not produce neurospheres under adherent conditions (e.g., in poly-D-lysine coated plates), and “late” NPCs that proliferate and form neurospheres with FGF2 or EGF (33–36) (Figures 1 and 2). Recently, early NSCs and “late” NSCs have been found along the entire A–P and D–V axis of the neural tube (37). The current model suggests that FGF2-responsive “early” NSCs differentiate in the presence of FGF2 to become FGF2/EGF-responsive “late” NSCs by a mechanism involving up-regulation of epidermal growth factor receptor (EGFR) mRNA (34) and protein (35).

Clonal analyses have demonstrated that neurosphere formation begins with the proliferation of a single NPC. During neurosphere growth, symmetrical and asymmetrical divisions could occur to self-renew NSC and to produce transit-amplifying progenitors, which are mitotically very active cells. It is very likely that the majority of cells in the neurosphere are this type of progenitors, whereas NPCs with the ability to regenerate a neurosphere (neurosphere founder cells) (32) are only a very small proportion (up to 10%). Upon several passages of neurospheres *in vitro*, it is expected that the transit-amplifying progenitors are lost and only NPCs that retain long-term, self-renewal capacity produce new neurospheres (32).

The precise identity of neurosphere founder cells remains unknown. Interestingly, recent unpublished findings from Gotz et al. postulate radial glial cells as a neurosphere-forming NPC population (19) (Figure 1). Nonetheless, these data do not discard the idea that possibly other NPC populations could also originate neurospheres. An additional concern is whether the cells that constitute the neurosphere retain properties of *in vivo* NPCs. This issue has been difficult to address, as neurospheres constitute a rather heterogeneous population of neural progenitors. However, recent studies have compared the expression of positional identity genes in

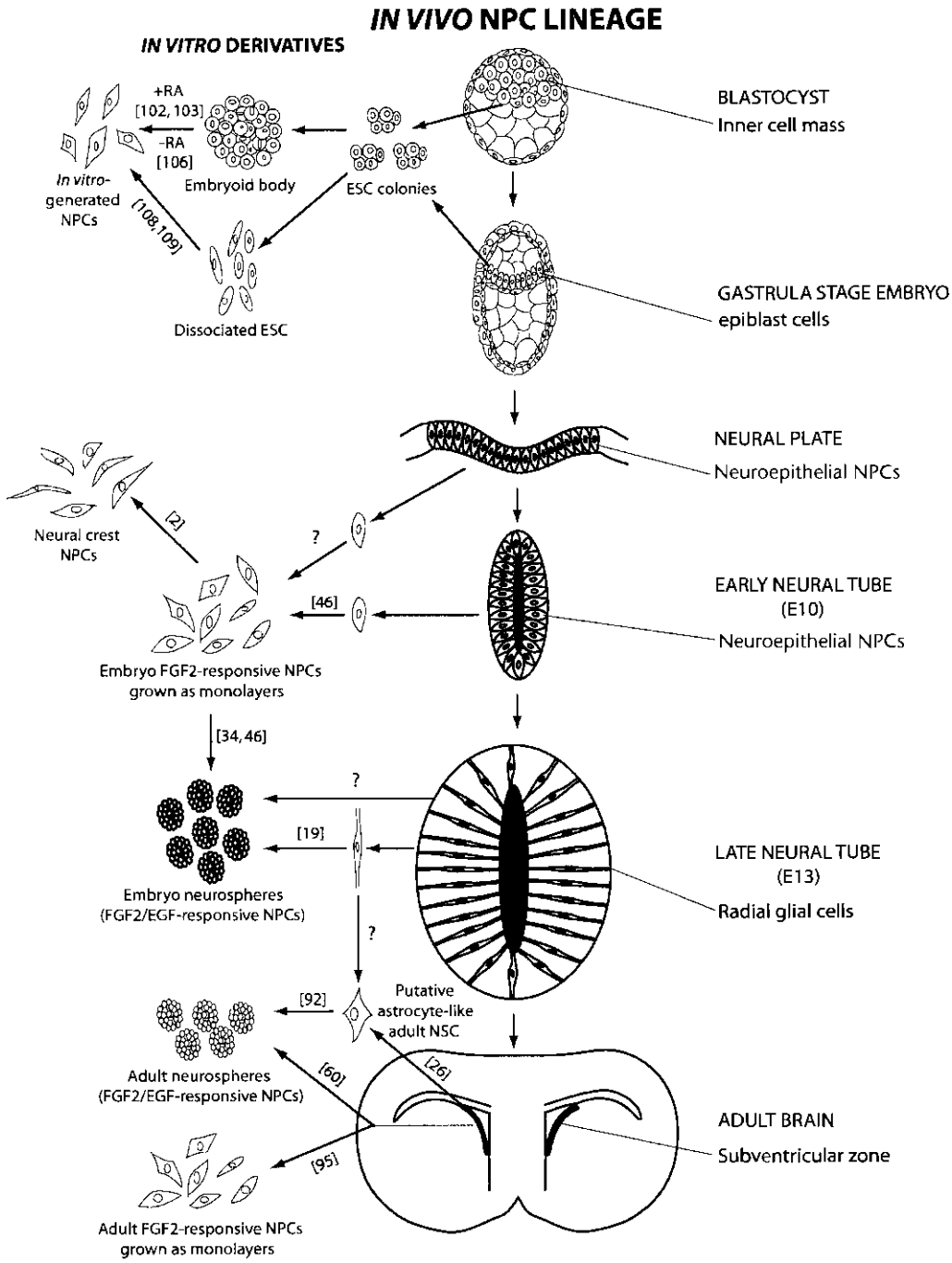


Figure 1. The origin of NPCs and *in vitro* derivatives. Different populations of NPCs appear during embryo development and in the adult brain (center of scheme). During neural plate induction and soon after neural tube closure, the majority of cells are neuroepithelial NPCs. As neural tube development proceeds, radial glial cells appear and become an important NPC population. Eventually, radial glial cells could generate the putative astrocyte-like NSC present in the adult subventricular zone. Different culture techniques allow *in vitro* expansion of NPCs (left of scheme). Multipotent FGF2-responsive NPCs can be expanded *in vitro* under adherent conditions from neural tubes at very early developmental stages. Embryo FGF2-responsive NPCs generate *in vitro* the FGF2/EGF-responsive, neurosphere-forming NPCs. Radial glial cells and probably other precursors naturally become FGF2/EGF-responsive, neurosphere-forming NPCs during neural tube development. NPCs from the adult brain can be expanded as FGF2-dependent monolayers or with the neurosphere assay. The lineage relationship between embryo and adult NPCs and these relationships with their *in vitro* derivatives remains uncertain ("?" in the scheme). Finally, NPCs can be completely generated *in vitro* from ESCs, pluripotent stem cells derived from the blastocyst inner cell mass (top of the scheme). ES cells differentiate into NPCs *in vitro* through formation of embryoid bodies or as adherent monolayers from dissociated cells. Selected references describing NPC populations represented are indicated in the scheme (see text for details).

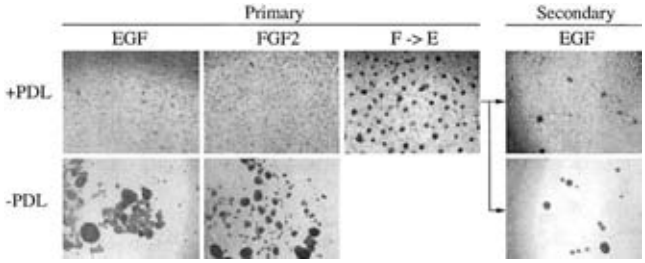


Figure 2. Neurosphere generation in the presence or absence of an adherent. Primary cell cultures from E11.5 telencephalon were incubated for 7 days in the presence of EGF, FGF2, or a sequential treatment (1 day with FGF2 and then 6 days with EGF [F→E]). Cultures were carried out in the presence (+) or absence (−) of poly-D-lysine (PDL). Bright-field pictures show the typical neurosphere morphology in cultures with EGF or FGF2 in the absence of PDL and, at the developmental stage used here, only with the sequential treatment in the presence of PDL. Some primary neurosphere cells have self-renewal capacity indicated by the ability to generate secondary neurospheres.

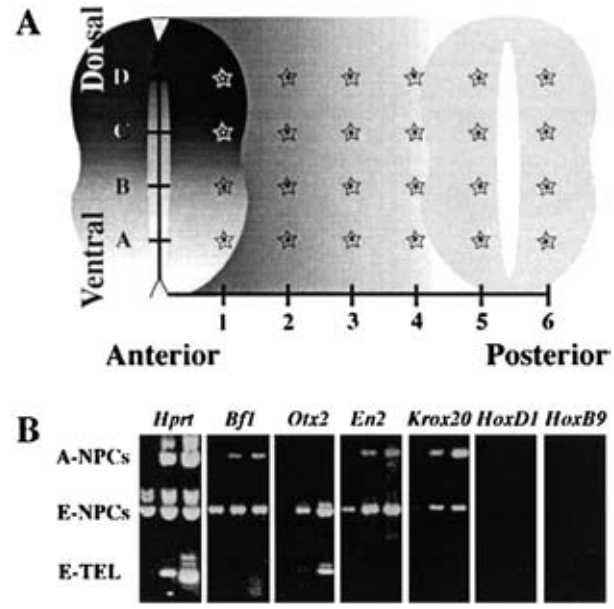
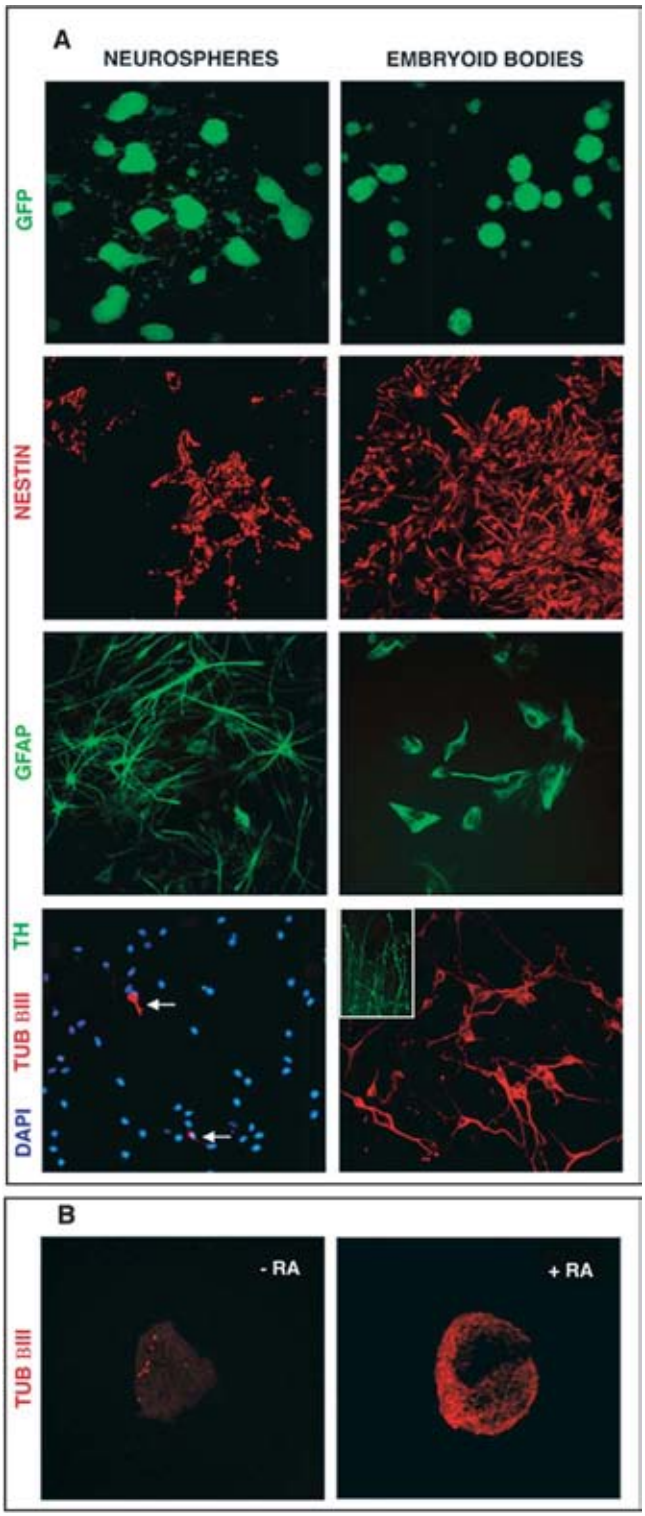


Figure 3. Identity of NSCs depending on their neural tube position. During development, anteroposterior (AP) and dorsoventral (DV) organizers instruct NSCs so that they acquire information according to their position within the neural tube. It is postulated that positional information is coded through the combined expression of specific genes. (A) Specific neuronal identity is represented by Cartesian coordinates defined as intersections according to AP and DV position. (B) Expression pattern of AP gene markers in neurospheres derived from mouse NSCs of adult SVZ (A-NPCs) and of embryonary telencephalon (E-NPCs) is shown in comparison with the natural pattern observed in the embryonary telencephalon (E-TEL). Gene expression was determined by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) protocol as reported by Santa-Olalla et al. (37). Although some genes were expressed in neurosphere cells according to the position of the founder NSC (*Bf1* in A-NSC and E-NSC, and *Otx2* in E-NSC), *En2* and *Krox20*, mesencephalon and rhomencephalon markers, respectively, were unexpectedly expressed in adult SVZ and embryo telencephalic neurospheres. These data suggest that the original gene expression code is lost in cultured NPCs, which could affect their differentiation potential.

neurosphere cells with that in neural tube regions where they were derived from (37–39). In this case, positional identity is defined as the gene code that a cell normally would express when developing in a specific neural tube region (Figure 3A). Studies from Hitoshi et al. (38) and Parmar et al. (39) pointed out that neurosphere cells preserve positional identity of origin in culture; however, they were not irreversibly specified and could, at least partially, change their positional identity when exposed to different environments (38). A more detailed study provided by Santa-Olalla et al. (37) demonstrated that the specific positional identity is indeed partially lost upon neurosphere formation. For instance, *En2* and *Krox20* are transcription factors expressed in the mesencephalic-metencephalic (mes-met) border (40) and the rhomboencephalon (41), respectively. If neurosphere cells preserve positional identity, then *En2* and *Krox20* should be exclusively found in neurospheres expanded from the mes-met border and the rhomboencephalon, respectively. However, this is not the case, as neurospheres cells expanded from all regions of the developing neural tube acquire *En2* and *Krox20* expression (37) (Figure 3B). Furthermore, neurosphere cells expanded from the adult forebrain also acquire *En2* and *Krox20* expression (Figure 3B). Additional experiments should be directed to demonstrate whether this change in positional information alters the differentiation outcome of neurosphere cells. Despite the data described above, cells with self-renewal capacity within the neurospheres solely represent a small proportion of total cells; therefore, observations may be restricted to progenitors derived from the founder cell.

Do neurosphere founder cells change their differentiation potential upon *in vitro* expansion? As mentioned before, neurosphere cells differentiate to neurons, astrocytes, and oligodendrocyes *in vitro*. However, irrespective of the growth factor regimen used to grow neurospheres, the majority of cells terminally differentiate into astrocytes (reaching >80%) and only a minor proportion differentiate into neurons (ca. 5%) and oligodendrocytes (ca. 2%) (32,42,43) (Figure 4). Indeed, many cells within neurospheres co-express RC2, GLAST, and BLBP, a feature of gliogenic radial glia (16). In addition, directed differentiation of neurosphere-derived neurons has been difficult to achieve because these cells do not easily respond to the environmental signals that naturally induce specific differentiation *in vivo* (our own observations) (44). For instance, dopaminergic neurons are strongly induced *in vivo* with growth factors such as Sonic hedgehog (Shh) and FGF8 (5), but neurosphere cells under differentiation conditions do not generate dopaminergic neurons when exposed to Shh and several other factors involved in dopaminergic neuron development (our own observations) (44). Interestingly, neurosphere cells become non-responsive to environmental signals from the early proliferation stage, as the positional identity of neurospheres does not change when expanded in the presence of morphogens such as Shh,



FGF8, and retinoic acid (37). This phenomenon is not observed in NPCs populations generated *in vitro* from more primitive ESCs (see later). Thus, it is possible that neurosphere culture conditions promote expansion of NPCs with very restricted differentiation potential, possibly by inhibiting the response of these cells to environmental signals. However, to test the real differentiation potential of neurosphere cells, they must be exposed to natural environments *in vivo* (transplantation approach) or *in vitro* (explant culture approach) (see below).

NSCs from telencephalon (45) and spinal cord (46) have also been expanded *in vitro* as adherent monolayers without neurosphere growth. In these cases, telencephalic and spinal cord NSCs proliferate in response to FGF2 and show little or no proliferation in response to EGF (45,46), whereas neurosphere cells usually proliferate with both growth factors (47,48). This differential response to growth factors suggests that monolayer FGF2-expanded NSCs could represent a different population from FGF2/EGF-responsive neurosphere cells (Figure 1). Additionally, although both populations behave as multipotent stem cells *in vitro*, their differentiation potential is dramatically different. On the one hand, FGF2-expanded NSCs from the spinal cord differentiate *in vitro* into many neurotransmitter phenotypes (2,49) (Figure 1). This NSC population in conditioned media can also be directed to differentiate into neural crest derivatives, probably through the generation of the intermediary NCSC fate (2) (Figure 1). On the other hand, the *in vitro* differentiation of neurosphere cells shows a more astrocyte-biased potential along with less efficiency to produce specific neurotransmitter phenotypes (44) (Figure 4). Probably, FGF2-expanded NSCs grown as monolayer represent a multipotent NSC population that under certain *in vitro* conditions could produce a more restricted FGF2/EGF-responsive, neurosphere-forming cell (Figure 1).

It is important to consider that growth factors acting on a NSC could not only promote its proliferation (i.e., self renewal) but also instruct or select cells within specific restricted lineages. For instance, platelet-derived growth factor (PDGF) appears to promote proliferation and/or survival of neuronal-restricted precursor (50), whereas EGF and FGF2 stimulate generation of glia-restricted precursors that terminally differentiate to astrocytes in the presence of cilliary neurotrophic factor (CNTF) or oligodendrocytes when exposed to triiodothyronine (T3) (50,51).

Prospective Purification of NPCs. Commonly, NPCs are identified retrospectively by their *in vitro* behavior (see

Figure 4. Differentiation potential of *in vitro*-expanded NPCs vs. *in vitro*-generated NPCs. (A) The left column shows fluorescent neurospheres expanded from a GFP+ transgenic mouse line. The majority of neurosphere cells expressed the neural precursor marker Nestin, but under differentiation conditions many GFAP-positive astrocytes and few tubulin-β III-positive neurons are generated. The right column shows fluorescent embryoid bodies. Nestin-positive NPCs were derived from embryoid bodies adhered to culture dishes. These *in vitro*-generated NPCs differentiated into astrocytes and also into many neurons. Inset shows tyrosine hydroxylase-positive fibers of putative dopaminergic neurons; (B) Embryoid bodies treated with retinoic acid (+RA) efficiently differentiated into neurons after 6 days in culture compared with control embryoid bodies (–RA).

previously mentioned observations). However, prospective purification of embryo and adult NPCs was recently accomplished in murines (45,51). These procedures were based on negative-selection protocols, which means that NPCs were purified considering markers they do not express. Although these procedures are useful to obtain very enriched NPCs populations, lack of positive markers for these cells does not allow following their development *in vivo*. Using a positive-selection protocol, NPCs were purified from human fetal brain and spinal cord (53), but *in situ* identification of these cells remains to be carried out.

Neural Stem Cells in the Adult Brain

Neurogenesis and Location of NSCs in the Adult Brain. First evidences that neurogenesis occurs in adult mammals were found by Altman about 40 years ago (54). However, little attention was given to those studies, perhaps because they were considered of no functional relevance. Later, studies in birds correlated neurogenesis with song learning, suggesting that generation of new neurons was a necessary process to establish a specific behavior (55,56). These discoveries, together with *in vivo* (57,58) and *in vitro* (59,60) identification of neural precursor cells capable of giving rise to neurons and glial cells late in development, prompted several groups in the last decade to study neurogenesis in adult mammals.

Neurogenesis in the adult brain has been demonstrated *in vivo* with several experimental strategies. The simplest approach to detect cells born from precursor cells is by incorporation of nucleotide analogs such as [^{3}H]-thymidine or bromodeoxyuridine into dividing cells (i.e., precursor cells). Normally, labeled cells detected a short period (i.e., 2 h) after a single application of the nucleotide analog should be considered as rapidly dividing progenitor cells, whereas cells retaining the label for a long period after application of the nucleotide analog are either slowly dividing or nondividing differentiated cells. When this type of labeling is used to trace new neurons, few injections are given (depending on the expected cell division rate of the precursor) followed by analysis of labeled cells several weeks or months after label application. An alternative approach to determine the fate of dividing cells is by infecting precursors with retrovirus carrying a reporter gene whose provirus can only integrate into replicating DNA.

The largest region in which neurogenesis occurs throughout life is found surrounding the lateral ventricles of the forebrain (61). Putative NSCs with astrocyte characteristics have been identified in the subventricular zone (SVZ) near the ependyma (the cell layer lining the ventricles) (26) (Figure 1). At present, it is unknown whether a specific astrocyte type functions as NSC, or whether this behavior is promoted by the specific SVZ niche. Ependymal cells themselves have also been proposed as a source of NSCs (62). NPCs from the SVZ generate transit-amplifying progenitor cells, which in turn differentiate into neuroblasts (i.e., neuron progenitors) that migrate to the olfactory bulb. These neuroblasts terminally differentiate into interneurons in the olfactory bulb. Continuous neurogenesis also occurs in the dentate gyrus of the hippocampus of several mammalian species (63–66). In this case, NPCs reside in the subgranular zone of the dentate gyrus (67). These cells proliferate and migrate into the granule cell layer, where they differentiate into neurons and glial cells. Newly generated neurons project axons into the CA3 region of the hippocampus. It is important to mention that NPCs detected in the tissues described above have not been definitively identified as multipotent NPCs *in vivo*, because they could represent a mixture of more restricted progenitor cells (e.g., unipotent progenitors) (see below).

Neurogenesis has been observed at other sites of the adult brain; however, the difficulties to detect this might be due to either slow neuron renewal kinetics or that this occurs only under specific conditions. In fact, newborn dopaminergic neurons were recently detected in the adult substantia nigra, the number of which was increased when dopaminergic neurons were experimentally killed (68). In another instance, proliferation of progenitor cells in the spinal cord has been associated with gliogenesis; however, these cells are able to generate neurons *in vitro* (69,70) or under an *in vivo* neurogenic environment (71). Similarly, NPCs produce mostly glial cells in the cortex, but when a subset of pyramidal neurons is destroyed without affecting the surrounding cortical tissue, new neurons appear, constituting up to 2% of cells produced (72). These data suggest that although continuous neurogenesis is principally located in the olfactory bulb and the hippocampus, many regions within the adult brain could possess a slow latent neurogenic program that could be induced or enhanced by damage or other stimuli.

To date, there is no clear indication under which natural condition neurogenesis is stimulated in the adult olfactory bulb. However, specific growth factors have been identified that affect NPC proliferation, differentiation, or both. For instance, infusion of TGFα, EGF, and/or FGF2 into the lateral ventricules of the forebrain induces proliferation of NPCs lying in the SVZ (73,74). Interestingly, the fate of NPCs also appears to be differentially affected with these growth factors, because FGF2 infusion induces neurogenesis and gliogenesis and EGF reduce neurogenesis and increase gliogenesis (74). Aside from these studies, Noggin, through their inhibitory activity on BMPs, have been shown to reduce gliogenic and promote neurogenic activity (75). This evidence suggests that Noggin controls NPC differentiation in the SVZ niche, a process that eventually leads to neuron production in the olfactory bulb.

In the adult hippocampus, several environmental or physiologic conditions have been found to regulate neurogenesis. In murines, neurogenesis is augmented during pregnancy (76), in enriched interactive environments (77), and when

free exercise is allowed (78). In addition, hormones, estrogens, and prolactin are capable of stimulating NPC proliferation and neurogenesis (74,76,79). Interestingly, ischemic (80) or convulsive stimuli (81,82) in the hippocampus, two insults that can cause neuronal death, also stimulate neurogenesis. On the other hand, stress, aging, and glucocorticoids decrease the rate of neurogenesis (83,84).

Many of the new generated neurons die within a few weeks after they are born, questioning the relevance of adult neurogenesis. However, functional synaptic connections have been detected among the surviving neurons. Electrophysiologic responses have been observed in neurons derived from GFP retrovirally labeled adult hippocampal NSCs, which receive synaptic inputs *in vitro* and *in vivo*, similar to those found in mature dentate granule cells (85). In addition, adult-born neurons in the substantia nigra can be retrogradedly labeled with an axonal tracer injected into the striatum (68). Supporting the functional role of neuron production in the adult brain, learning and memory performance significantly decrease when neurogenesis is blocked with cytostatic drugs (86). In contrast, neurogenesis induced by convulsive stimuli is also accompanied by neuronal connections but many are aberrant, which may be irrelevant or worsen the pathologic condition (82). In summary, neurogenic environments appear to be present in the adult brain, at least in the SVZ, hippocampus, and substantia nigra, and they can drive NSC differentiation to neuronal phenotypes according to these regions.

Some molecular markers of embryo NPCs such as nestin and musashi 1/2 are preserved in adult NPCs (60,63,87). In contrast, GFAP is expressed in NPCs of the adult SVZ (26) but is not expressed in murine radial glial cells, an embryo NPC population (88). Microarray technology could help to identify the set of markers that define an adult NSC. However, confidence in identified markers by this method requires a quasi-pure NSC population. Purification criteria must meet a more detailed definition of different NPC populations based on molecular markers and/or cellular behavior. Analysis done with cultured NPCs (i.e., neurospheres) is limited by heterogeneity and the uncertainty whether or not they are an actual expansion of *in vivo* NPCs. Furthermore, genes detected as active may or may not have functional relevance. Nonetheless, these considerations, a comparative analysis of different stem cell populations, have identified genes that could be representative of or have functional relevance for the "stemness" property of precursor cells (89,90).

Adult NSCs In Vitro. Strong support for adult neurogenesis is the ability to grow and expand NPCs from adult neural tissue. In general, two protocols have been used to grow adult NPCs. The first was initially developed by Weiss' group. In this protocol, a cell suspension from adult striatum is placed in a tissue culture plate at relatively low cell density without an adherent and cultured in defined medium with EGF (60). This protocol generates the now well-known neurospheres referred to previously. At present, it is known that neurospheres from the striatal tissue actually derive from a cell population residing in the SVZ of the forebrain (Figure 1). Neurospheres have been derived from adult striatum, hippocampus (see below for discussion on possible SVZ contamination below), mesencephalon, and spinal cord in the presence of EGF, FGF2, or both (60,68,69,91). Recently, Doetsch et al. (92) showed evidence indicating that a multipotent progenitor rather than the putative astrocyte-like NSC is the major cell initially responding to EGF *in vitro* to form neurospheres. Furthermore, EGF-expanded NPCs remain multipotent *in vitro* but behave as an invasive gliogenic precursor *in vivo* (92). This conclusion is in agreement with the common observation that murine neurosphere cells have the propensity to differentiate into astrocytes *in vivo* and *in vitro* though maintaining some multipotentiality *in vitro* (see below). Neurosphere formation does not appear to be a unique property of multipotent NPCs. Sphere-like aggregates can be obtained from oligodendrocytic- (93), astrocytic-, and neuronal-restricted progenitors (94). However, as expected primary neurospheres generated from compromised progenitors have a limited proliferative capacity; frequently they are small and do not generate secondary neurospheres.

The second protocol to grow adult NPCs is that developed by Gage's group. In this case, the cell suspension is cultured in the presence of FGF2 initially with serum and then with serum-free media under adherent conditions (95) (Figure 1). These cells can be maintained in culture only at high density due to the recently identified requirement of cystatin, a secreted factor produced by the cultured cells (96). Using this protocol, NPCs can be cultured from different adult tissues such as hippocampus (95) and spinal cord (70).

Is the NPC population cultured with the protocols described above the same? Current evidence suggests that self-renewing NPCs in adherent and non-adherent cultures derive from a common NPC population present in the SVZ (94). These NPCs with long-term, self-renewal ability are present in the hippocampus at early postnatal ages but practically disappear in the adult rodent (94). However, in short-term cultures astrocytic and neuronal progenitors have been identified in the adult dentate gyrus. These neuronal progenitors could be those responsible for most neurogenesis occurring in the adult hippocampus, and their limited proliferation capacity might be the cause of neurogenesis decline with age (67). This is in contrast with the putative NSCs present in the SVZ, the number of which does not decline with age (97).

Embryonic Stem Cells

Origin and Differentiation Potential. Thirty years ago, it was found that cell lines derived from human and mouse embrionary carcinomas (EC) could differentiate into neurons, particularly in the presence of retinoic acid (RA). These

cell lines have an enormous differentiation potential but show many chromosomal abnormalities. Later, karyotypically normal cell lines (embryonic stem cells, ESC) were derived from pre-implantation mouse embryos, which somehow appear related to EC cell lines (reviewed in Reference 98) (Figure 1). Although it is not certain whether ESC lines are the actual expansion of epiblast cells, they behave *in vivo* as epiblast cells and express characteristic markers of this tissue. Mouse ES cells *in vivo* can contribute to all tissues including the germ line. *In vitro*, ES cells proliferate and remain undifferentiated (i.e., self-renewal) in the presence of the leukemia inhibitory factor (LIF) and/or a fibroblast feeder layer; however, in their absence they differentiate, apparently stochastically, within different lineages. Some of the most relevant features of ES cells include clonability, stable diploid karyotype, ability to be part of all adult tissues upon reintroduction into the blastocyst, and undefined wide differentiation potential *in vitro*. The capacity of ES cells to derive all cell types present in the developing embryo has made them a powerful tool for gene manipulation and for the study of specific gene function (99). However, ES cells can produce teratomes when ectopically transplanted at different places in adult animals (100,101), which is considered a disadvantage for their therapeutic use.

A common procedure to promote ESC differentiation involves the formation of embryoid bodies (EBs) (Figure 1). EB formation occurs totally *in vitro* and involves cell aggregation and cavitation. The formation of this structure reflects the early developmental stages that lead to development of epiblast and primitive endoderm, immediately previous to gastrulation. Subsequently, different culture conditions can promote differentiation into cell types within ectodermal, mesodermal, and endodermal major lineages (reviewed in Reference 98).

NSCs from ESCs. When EBs are formed in the presence of retinoic acid, neurons appear after dissociation and plating under adherent conditions (102,103) (Figure 1); nonetheless, a large amount of cell death is observed (104). Improved procedures apply retinoic acid only at late stages of EB formation, which allows neuralization within it (105) (Figure 4). Under these circumstances, retinoic acid has a double function: it provides posteriorizing positional information and promotes neuralization. Addition of Shh, a ventralizing morphogen of neural tube, further contributes to specification of neural cells so that motoneurons are obtained. Neuralization with this latter procedure shows, first, emergence of putative NSCs (i.e., expression of Sox2) and then of neurons, suggesting that a comparable-to-normal differentiation program is switched on.

Because, as mentioned previously, retinoic acid is able to confer posteriorizing positional information, neuralization with this compound may restrict differentiation into posterior neuron types. Protocols that do not involve retinoic acid to promote neural differentiation of EB cells have been developed (106) (Figures 1 and 4). In these cases, NSC selection in a serum-free neural medium and expansion with FGF2 is performed prior to induce neuronal differentiation. These protocols allow obtaining >70% of cells positive for the NSC marker nestin. FGF2 removal from the culture medium produces neurons and astrocytes. Oligodendrocyte can be selectively obtained from EBs using a modification of the protocol described above (107).

Despite the fact that EBs have been used commonly to obtain differentiated cells, the inherent heterogeneity of the system hampers identification of factors involved in specific NSC differentiation. Retinoic acid can promote neural differentiation directly from dissociated ESCs, although at very low efficiency (104). More recent procedures involve first the selection and expansion of NSC, which can be attained by seeding cells at relatively low density in serum-free media defined and in the presence of FGF2 (108,109) (Figure 1), or by co-culturing ESCs with bone marrow-derived stromal cells (specifically from PA-6 cell line) (110). Using these procedures, a large enrichment in NSCs is obtained.

Studies, particularly in *Xenopus leavis*, have established that neural lineage is the default fate of animal pole cells (corresponding to epiblast cells in mammals) (111). Neural differentiation appears to be repressed by BMP4 and released from this repression by sequesters of BMP molecules such as Noggin. In agreement with this view, neural differentiation also appears to be sensitive to BMP4 repression in EBs and in dissociated ESCs (109,112); therefore, it is likely that ESC are prompted to neural differentiation in the absence of BMPs. NSCs grown in the presence of FGF2 derived from ESCs resemble those derived from mouse neural tube in their differentiation potential (113), but culture requirements are different (109).

After NSCs are derived from ESCs, these precursor cells could hypothetically differentiate into any neuron type of the nervous system. However, this could only be true if during ESC differentiation, NSCs do not acquire positional information or another type of instruction that might compromise subsequent NSC differentiation. Furthermore, differentiation to a specific neuron type might not only be restricted by intrinsic properties of NSCs generated *in vitro*, but also by lack of extrinsic requirements at defined times during culture. Under uninduced conditions, the favored neuron type (>50%) derived from ESCs appears to be the GABAergic (106,108,114). Efforts to attain specific neuron-type differentiation by adding specific inducers have only given partial results. As mentioned previously, motoneurons can be obtained from EBs treated with retinoic acid and Shh. In another example, an average of 20% of total neurons produced are dopaminergic when NSCs from ESCs are cultured with FGF8 and Shh (115), natural inducers of the dopaminergic neuron phenotype (5). A similar percentage of dopaminergic neurons can be obtained when NSCs are cultured on PA-6 cells (110), although in this case the inducer molecule

has not been identified. In addition to indicators of neurotransmitter synthesis and release, in all cases neuron functionality has been determined, but only in a few cases has specific axon projection been tested.

Toward Regenerative Medicine for the Central Nervous System

Differentiation Potential of Uncultured NSCs in Ectopic Sites. Cell cultures are a useful tool to selectively enrich and maintain relatively homogeneous NPCs populations for long periods *in vitro*, making genetic manipulations more feasible. *In vitro* differentiation assays also have provided valuable insights regarding the differentiation potential of NPCs. Nevertheless, NPCs *in vitro* can disclose either a narrower or a broader differentiation potential than observed *in vivo* and under no circumstance reflects the actual differentiation pattern observed *in vivo*. It is expected that differentiation in culture lacks the correct combination of activators and inhibitors required for the site-specific differentiation that give rise to a particular neuronal population. Furthermore, specific axonal projections and their role in definitive phenotype acquisition are not easy to evaluate in culture. In fact, several studies have demonstrated that *in vitro* differentiation analysis of NPCs does not predict the *in vivo* differentiation potential of NPCs. For example, O2A progenitors are capable of generating astrocytes and oligodendrocytes *in vitro* but only generate oligodendrocytes *in vivo* (116), and late NCSCs only differentiate to cholinergic neurons *in vivo* but retain the plasticity to differentiate to the noradrenergic lineage *in vitro* upon exposure to high levels of BMP2 (7). Moreover, EGF-neurosphere cells are multipotent *in vitro* but exclusively differentiate into glial cells *in vivo* (117) (see below).

Many transplantation experiments have challenged the differentiation potential of NPCs by exposure to different natural environments in the embryo and adult brain. Initial transplantation experiments using entire pieces of neural tube regions allowed to reach several conclusions: (i) at early stages of development, neural progenitors could change their identity in response to inductive cues secreted by ectopic organizers; for instance, diencephalic cells from prosomere 1 acquire a caudal midbrain fate when exposed to signals emitted by the isthmus organizer (118); (ii) NPCs plasticity is progressively restricted as development advances; for example, anterior rhombomere NPCs appear to be re-specified to a posterior rhombomere fate when transplanted to posterior rhomboencephalon, but this property is eventually lost late in development (119), and (iii) at least within some regions of the neural tube, AP specification of NPCs precedes DV specification. Classic examples here come from rhombomere transposition experiments in which NPCs in the ectopic location are re-specified in the DV axis but no longer respond to AP cues (120).

Although the transplantation experiments mentioned previously provide relevant information regarding neural tube development, transplanted NPCs within the graft remain under the influence of the original microenvironment from the donor tissue, making it difficult to reach a conclusion with regard to NPC plasticity at the single cell level. For this reason, several studies have analyzed the differentiation potential of dissociated NPCs heterotopically transplanted into the embryo brain. Intraventricular transplantation experiments demonstrated that E13.5–E15 mouse striatal precursors widely incorporate homotopically to the rat lateral ganglionic eminence (LGE) and heterotopically to the cortex, medial ganglionic eminence (MGE), and midbrain (121,122). The incorporation pattern of striatal precursors depends on the integrity of cell surface molecules or interaction with other cells, as heterotopic integration only occurs when striatal precursors are trypsinized prior to transplantation (123). Moreover, mouse striatal precursors developing in the ventral region of telencephalon differentiate into cortical neurons after transplantation into rat dorsal telencephalon (122). Despite having DV plasticity within the telencephalon, E12–E14 mouse striatal precursors already appeared restricted to the telencephalic fate, because these cells were unable to acquire host-specific markers when transplanted into rat diencephalon and mesencephalon (124). Nonetheless, this apparent restriction could be attributed to the intraventricular injection technique employed because intraparenchymally injected striatal precursors acquire a midbrain marker upon implantation into the mesencephalon (6). Conversely, E10.5 mouse midbrain progenitors acquire an MGE marker upon intraparenchymal transplantation into the basal forebrain (6).

Freshly dissociated NPCs from the adult SVZ have also been transplanted into the neonatal and adult brain. After transplantation into the adult striatum and cortex, NPCs from SVZ show limited survival and mainly differentiate into astrocytes, whereas neuronal differentiation is only observed after transplantation into the olfactory bulb (125). On the other hand, NPCs from the perinatal SVZ, although they extensively migrate and differentiate within multiples regions of the E15 mouse brain, only generate interneurons and not the long projecting neurons characteristic of the developing neural tube (126). At present, it is unknown whether perinatal and adult SVZ NPCs are able to differentiate into neuron subtypes generated during embryo development.

To date, no transplantation experiment using freshly dissociated NPCs has evaluated the acquisition of specific neuronal phenotypes in NPCs located ectopically. This is a very important issue, in that trans-specification events based entirely on cell morphology studies could be deceiving. Moreover, expression of positional identity markers in transplanted NPCs does not necessarily mean that NPCs will terminally differentiate in a site-specific fashion. Considering the study by Olsson et al. (6), some striatal NPCs express

En1 when located in the midbrain, but it is unknown whether acquisition of this midbrain marker provides implanted precursors with the ability to differentiate toward mesencephalic phenotypes (i.e., substantia nigra dopaminergic neurons). To test site-specific differentiation, NPCs must be exposed to all signals inducing a specific neuronal phenotype during development. This evaluation would require implantation of NPCs in a very specific neural tube region at the beginning of neurogenesis. At present, this goal would be difficult to achieve with conventional *in vivo* transplantation techniques. However, the explant culture approach could be a valuable tool to evaluate site-specific neuronal differentiation of implanted NPCs. In this case, NPCs are implanted in a dissected neural tube region, which is explanted in a tissue culture dish and cultivated for several days (122,127).

Transplantation experiments carried out with fresh dissociated NPCs reveal the *in vivo* behavior of very heterogeneous populations that could contain multipotent NPCs but also more restricted precursors and differentiated cells, limiting conclusions at the population level. Purification of NPCs offers a valuable opportunity to test *in vivo* the differentiation potential of relatively homogeneous NPCs populations. Using a transgenic mouse expressing the green fluorescent protein (GFP) under control of the nestin promoter, it was possible to isolate nestin-positive NPCs from the ventral mesencephalon (128). This population differentiated into dopaminergic neurons upon transplantation into the adult striatum (128). Furthermore, purified adult SVZ NPCs transplanted into the adult brain generated neurons, astrocytes, and oligodendrocytes, although in this case quantification and site-specific differentiation were not reported (52). Comparing the *in vivo* behavior of isolated NPCs with the *in vivo* behavior of the NPCs expanded *in vitro* from the sorted populations will provide the basis to study changes occurring in NPC plasticity during cell culture.

In Vivo *Differentiation Potential of NSCs Generated* In Vitro. *In vivo* differentiation potential has also been tested for NPCs expanded *in vitro*. Several groups have transplanted neurosphere cells into the embryo, postnatal, and adult brain. Some studies have revealed that murine neurosphere cells behave as glial progenitors *in vivo* (117,129–131). Particularly interesting is the work from Winckler et al. (117), which showed that EGF-generated neurosphere cells from E14 mouse telencephalon and mesencephalon differentiated exclusively into glial cells after widespread incorporation into the E15 rat forebrain. It will be very important to test whether E14 EGF-generated neurosphere cells remain glial-restricted when transplanted at the beginning of neurogenesis (i.e., E10 neural tubes). As neurons appear before glia during development, it is possible that early neurosphere cells possess neurogenic potential *in vivo*; thus, it is relevant to analyze the behavior of neurosphere cells obtained from earlier stages of neural tube development and transplanted into early embryos. These experiments should evidence, if it exists, the neurogenic potential of murine neurospheres.

Some other reports have attributed neurogenic potential to murine neurosphere cells (132–135). However, none of these studies evaluated the proportion of contaminating differentiated cells in the precursor pool used for transplantation. This is very important because some cells within neurospheres express astrocytic and neuronal differentiation markers (i.e., GFAP and tubulin-β-III, respectively) (39). Moreover, these studies remain controversial because in most cases cells were not transplanted into neurogenic environments. This limitation makes difficult to discern whether donor precursors differentiated to neurons by a default or by an instructive mechanism. For instance, Ogawa et al. (134) transplanted neurosphere precursors into the non-neurogenic environment of the injured spinal cord. After several weeks, donor-derived neurons, astrocytes, and oligodendrocytes were found in a very similar proportion to the *in vitro* differentiation outcome (ca. 6% neurons, 30% astrocytes, and 4% oligodendrocytes). On the other hand, postnatal neurospheres generated from PSA-NCAM+ cells apparently differentiated into neurons in the neurogenic environment of the adult rostral migratory stream (132). However, in this case the transplanted cells were labeled with Hoescht, and this molecule easily diffuses from donor to host cells *in vivo* (136); therefore, the experiments from Vitry et al. (132) await further confirmation. Finally, SVZ neonatal mouse neurosphere cells could differentiate into neural crest derivatives upon transplantation into the chick embryo (133). Unfortunately, the reproducibility of the differentiation event and the percentage of neurosphere cells that acquire a neural crest phenotype were not reported (133).

Human neurosphere cells have shown more reliable evidence of neuron differentiation because considerable numbers of neurons are often found to differentiate in a site-specific manner after transplantation into the embryo, postnatal, and adult brain (137–141). Human neurosphere cells transplanted into the neonatal rat brain acquired neuronal morphologies according to site of integration (hippocampus, striatum, and neocortex) and showed Tau expression, an axonal marker (141). Furthermore, human neurosphere cells specifically produce neurochemical phenotypes corresponding to site of integration (137). Site-specific differentiation is also observed after transplantation of human neurospheres into the developing primate neocortex (141). As murine and human neurosphere cells are generated under equal culture conditions, the different *in vivo* behaviors of these populations after transplantation could reflect intrinsic inter-species differences in differentiation potential.

In addition to neurosphere cells, embryo and adult NPCs expanded *in vitro* as monolayers (see above) have been transplanted to different environments *in vivo*. Neuronal-restricted NPCs derived from spinal cord multipotent NPCs (13) extensively migrate to the cortex and olfactory bulb and differentiate into multiple neuronal phenotypes upon

transplantation into the neonatal forebrain (142). On the other hand, adult hippocampal NPCs expanded with FGF2 as monolayers (see above) have shown remarkable plasticity because they generated region-appropriate neurons in hippocampus, retina, cerebellum, and olfactory bulb after transplantation into these regions of the adult brain (143–145). These data, together with *in vitro* studies (see above), suggest that FGF-2-responsive NPCs could possess more plasticity than murine FGF2/EGF-responsive, neurosphere-forming NPCs (see above). Interestingly, because FGF-2-responsive NPCs generate FGF2/EGF-responsive NPCs *in vitro* and *in vivo* (33,34) (Figure 1), this process could reflect a transition from a more primitive and multipotent NPCs population (FGF2-responsive) to a more restricted NPC population (FGF2/EGF-responsive).

NPCs generated *in vitro* from more undifferentiated ESCs have proven to be a valuable source for transplantation because these cells are capable of integration and differentiation in multiple regions of the developing neural tube (146). ESC-derived NPCs can differentiate *in vivo* in a temporally accurate manner, in that neurons appear first, astrocytes arise in the perinatal period, and oligodendrocytes are found only in postnatal mouse brains (146). Transplanted cells in the cortex acquired morphologic features characteristic of cortical projection neurons, suggesting site-specific differentiation, whereas ES-derived glial cells apparently acquired functional properties, because some astrocytes were found in proximity to blood vessels, possibly contributing to blood-brain barrier formation, and oligodendrocytes were exclusively located along the fiber tracts (146). Further experiments are needed to fully evaluate the neurogenic potential of NPCs in response to environmental signals present in the developing embryo brain.

Functional Recovery After NPC Transplantation in Animal Models of Human Neurodegenerative Diseases. Transplantation experiments intended to describe the functional properties of NPC-derived neurons should evaluate features such as axon projection and targeting along with synaptogenesis with host neurons (147). However, behavioral recovery of transplanted animals could be considered the ultimate functional test for transplanted NPCs. Murine models for Parkinson's disease and myelinization disorders such as multiple sclerosis are both common models to study behavioral recovery. Rats affected by Parkinson's disease have been alleviated after transplantation of sorted/expanded precursors from the embryonic ventral mesencephalon (128,148) and with ES-derived dopaminergic neurons (101,149). Recently, adult neurosphere cells were observed to differentiate *in vivo* into oligodendrocytes that eventually remyelinated axonal tracts of the spinal cord, improving clinical features in a chronic model of multiple sclerosis (135). Unexpectedly, NPCs also promote behavioral recovery by interacting with host cells to enhance regeneration after injury (150,151). For instance, transplantation of NPCs into midbrains with impaired but nonapoptotic dopaminergic neurons induced regeneration and survival of these neurons, leading to restoration of innervation in the mesostriatal system along with behavioral recovery (150). Because in this study few donor cells differentiated into dopaminergic neurons that contributed to the mesostriatal system, this finding could be attributed to neuro-protective factors secreted by transplanted NPCs (150).

NSCs in the Origin and Treatment of Diseases Affecting the Nervous System

Distinct diseases associated with reduction in specific neuronal populations affect humans (152). Dopaminergic neurons of substantia nigra, GABAergic neurons of striatum, motor neurons of spinal cord, and cholinergic neurons of cortex degenerate into Parkinson's, Huntington's, amyotrophic lateral sclerosis, and Alzheimer diseases, respectively. Moreover, unspecific neuron degeneration occurs during aging and neuron death can occur as consequence of a trauma affecting the nervous system. In all these cases, recovery requires either prevention of degeneration or replacement of lost neurons. In animal models of Parkinson's disease, the most recurrent model to develop therapeutic transplantation procedures, rather than replacing lost neurons with functional ones, usually transplanted cells produce dopamine locally in an attempt to cover the functional deficit (153). Although this strategy promotes some improvement, it is expected that specific neuronal replacement will result in a much better and long-term recovery. More recent procedures propose to transplant cells that provide specific survival factors for the degenerating neuron population (154).

Generally, abnormal activation of cell death is considered the cause of neuronal loss in neurodegenerative diseases and in aged animals (152). A recent proposal also considered axon degeneration as a possible cause of neuronal loss (cell body degeneration would follow axon degeneration) (reviewed in Reference 155). Here we propose that affections in neuron regeneration could also contribute to neuronal loss during adult life (Figure 5). It is now clear that during adult life there is continuous *de novo* generation of neurons, evident in some areas and difficult to detect in others. In the olfactory bulb, hippocampus, and substantia nigra, up to 0.1% of total neurons may be generated daily (68,156). This probably is a low estimate because the labeling method could limit the detection sensitivity, particularly when the generation process is slow. In any case, in general, an accumulation of neurons in regions where neurogenesis occurs in the adult has not been observed, indicating that generation of new neurons is accompanied by a constant neurodegeneration (see, for instance, Reference 68). Under this perspective, neuronal loss associated with aging or even with certain diseases could result from an increase in neuron death, a decrease in neuron generation, or both (Figure 5).

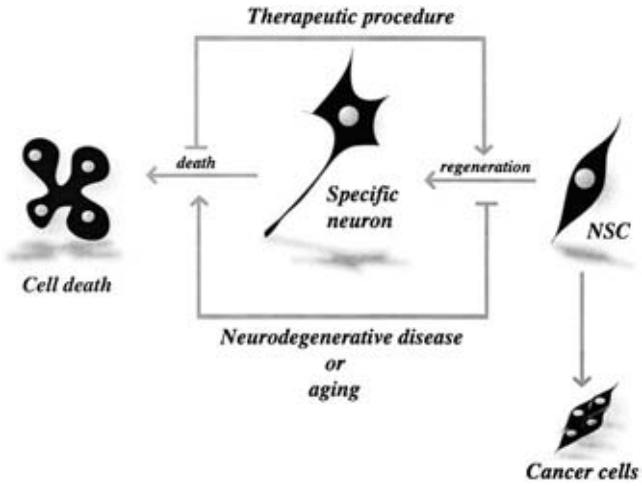


Figure 5. Causes of neuronal loss and therapeutic strategies to prevent them. During adult life some neurons die naturally. To compensate for this neuronal loss, neurons are continuously born from NSCs. Every neuronal population may have its own replacement kinetics, but this can be altered by hormonal or environmental cues (see text). Specific neuronal death can considerably increase (vertical arrow) or generation of new neurons can abnormally decrease (vertical blunted line) as a consequence of aging or disease. Increase in neuronal loss would result by either means. Depending on the original cause, therapeutic procedures must decrease (vertical blunted line) the rate of neuronal death or increase (vertical arrow) the rate of specific neuronal regeneration to restore the balance, preventing malfunction of specific neuronal circuitries and thus the cause of the characteristic disease-associated symptoms. NSCs could also derive into cancer cells.

Two general strategies could be envisaged to repopulate an affected area with specific neurons. One considers the activation and specific differentiation of endogenous NSC (Figure 5). At present, we do not know whether endogenous NSC are compromised to differentiate only into certain neuron types (see above), and perhaps limited to some brain areas. Therefore, it is fundamental to determine the full differentiation potential of these NSCs. Furthermore, it is also essential to define where specific neurogenic environments exist in the adult brain. Alternatively, if the required neurogenic environment is not found in the adult it will be important to determine which components conform a neurogenic environment, so that this could be recreated. Recreating a neurogenic environment may provide the conditions for patient self-repair. Additional research will help to define the therapeutic limitations of artificially induced specific neurogenesis.

The other general strategy to treat neurodegenerative diseases is to provide new neurons through transplantation of NSCs or their differentiated derivatives (Figure 5). At the present time, there are different possible sources of NPC that could be used for neuron replacement (reviewed in previous sections). Nonetheless, the actual differentiation potential of all these NSC types is largely unknown. Use of NSCs derived from ESC is particularly interesting because they appear to have the widest differentiation potential and are able to respond to site-specific differentiation signals (see above). However, practical use of ESC in regenerative medicine may require use of therapeutic nuclear transfer technology (commonly known as therapeutic cloning) (157). In this case, a nucleus from the patient is transferred into an enucleated oocyte. The resulting embryo is then allowed to develop up to the blastocyst stage *in vitro*, at which point an ESC line is derived from it. In addition, recent reports suggest that some non-neural adult stem cells could also serve as a source of NSCs (see, for instance, Reference 158). More research is needed to define the best source of NSCs for therapeutic purposes.

Therapeutic procedures to be used in humans must consider the undesirable consequences of either neuron replacement strategy referred to above. Recent evidence has revived an old hypothesis proposing that cancer cells derive from a small pool of undifferentiated cells living in the adult organism (159). Presently, these undifferentiated cells can be the stem cells existing in different adult tissues including neural tissues (Figure 5). The inherent self-renewal property of stem cells makes them the best candidate to accumulate mutations that promote the advantageous capacity to grow rapidly and invade different tissues, characteristics of cancer cells. In agreement with this hypothesis, stem cells are able to respond to several factors with well-known oncogenic effect (159). Therefore, neuron replacement therapeutic procedures using endogenous or exogenous NSCs must evaluate the oncogenic potential of the reactivated NSCs to be safe for humans.

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The *in vivo* positional identity gene expression code is not preserved in neural stem cells grown in culture

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Abstract

Neural stem cell specification depends on antero-posterior (AP) and dorso-ventral (DV) information provided during development. In the present study we identified similar neural stem cell (NSC) populations along the AP axis of the mouse central nervous system: the 'early' NSCs responsive to fibroblast growth factor-2 and the 'late' NSCs responsive to epidermal growth factor (EGF). Gene expression analysis shows that AP and DV transcription factor code is not preserved in NSCs in culture. Neurospheres generated with EGF from different regions showed *Emx2*, *En2* and *Krox20* expression beyond their corresponding AP restricted areas (telencephalon, mesencephalon and rhomboencephalon, respectively). *Hox* genes were rarely expressed. DV markers such as *Pax7* and *Dbx1* were not expressed in neurosphere cells, whereas *Pax6* and *Nkx2.1* were highly expressed independently of the NSC source region. In general, this pattern was found under different culture conditions. We propose that signals surrounding NSCs determine their positional identity gene expression code, which may be relevant to establish their definitive fate.

Introduction

The characterization of neural stem cells (NSCs) is the basis for understanding how cell diversity is generated during central nervous system (CNS) development. NSCs originate after neural induction and constitute the essential component of the neural plate. The whole diversity of neural phenotypes present in the adult CNS derive from these NSCs (Altmann & Brivanlou, 2001). According to the embryo developmental plan, NSCs respond directly or indirectly to signals that determine the antero-posterior (AP) and dorso-ventral (DV) axis organization (Rubenstein & Beachy, 1998; McMahon, 2000; Altmann & Brivanlou, 2001). These signals define the fate of NSCs *in vivo*; however, up to which point AP and DV organizers restrict the potential of the just formed NSCs is yet to be determined.

Early during CNS development [8.5 days post coitum (d.pc) in the mouse] morphologically distinctive structures appear along the AP axis: the prosencephalon, the mesencephalon, the rhomboencephalon and the spinal cord. Each region expresses specific transcription factors that are thought to be relevant for their formation (Altmann & Brivanlou, 2001). A combination of these transcription factors could molecularly define the boundaries between each region (Pfaff & Kintner, 1998; Rubenstein *et al*., 1998). In this sense, the *Hox* genes are particularly important in delimiting different territories within the rhomboencephalon and the spinal cord (Krumlauf *et al*., 1993). Rostrally, *Otx2*, *En1*, *En2* and *Gbx2* organize the mesencephalon and metencephalon (Wassef & Joyner, 1997), whereas *Bf1*, *Dlx1*, *Dlx2*, *Emx1* and *Emx2* support the neuromeric organization (i.e. prosomeres) of telencephalon and diencephalon (Rubenstein & Beachy, 1998). Null mutations in some of those genes support their essential role in CNS development. For instance, *Bf1* null mutations cause abnormal telencephalon development (Xuan *et al*., 1995), whereas *En1* or *En2* null mutations specifically affect the mesencephalic–metencephalic region (Joyner *et al*., 1991; Wurst *et al*., 1994). Furthermore, mutations in some *Hox* genes produce homeotic transformations accompanied by changes in the CNS developmental pattern (Mark *et al*., 1993). In some cases, NSC populations in the mutant embryos appear to be affected (Wurst *et al*., 1994; Xuan *et al*., 1995).

In the DV axis of the neural tube, a combination of transcription factors defines a code that specifies the neuronal differentiation pattern of NSCs. In the spinal cord, *Dbx1*, *Dbx2*, *Pax6* and *Pax7* express dorsally (Lee & Jessell, 1999), whereas *Nkx2.1*, *Nkx2.2* and *Nkx6.1* express ventrally (McMahon, 2000). Interestingly, some of these genes are mutually repressed, thus refining the limits between different domains (Ericson *et al*., 1997). However, others co-express within a domain, which is a requirement for differentiation of some specific neuronal phenotypes (Briscoe *et al*., 2000). A transcription factor code also appears to work in the telencephalon DV axis. However, complex migration patterns occurring in this region make it difficult to relate cell fate to a particular code (Parnavelas, 2000).

Presently, it is widely known the existence of multipotent NSCs during CNS development (Gage, 2000). There is also evidence indicating that NSCs progressively restrict their fate during development (Olsson *et al*., 1997; Mujtaba *et al*., 1999). The ability to grow NSCs in culture using epidermal growth factor (EGF) or basic fibroblast growth factor (FGF2) has been extremely useful (Gage *et al*., 1995; Weiss & van der Kooy, 1998). EGF- and FGF2-responsive cells are present in several CNS regions and at different developmental stages (Murphy *et al*., 1990; Kalyani *et al*., 1997; Santa-Olalla & Covarrubias, 1999; Tropepe *et al*., 1999), including the adult brain (Palmer *et al*., 1995; Reynolds & Weiss, 1996). These NSCs in culture appear to produce a heterogeneous population of neural precursor cells (NPCs) that have

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been used to characterize several responses to extracellular stimuli (Gage, 2000; Panchision & McKay, 2002). Nonetheless, there is little information about the positional identity of *in vitro* grown NSCs (Zappone *et al*., 2000; Hitoshi *et al*., 2002), which could be relevant in estimating their differentiation potential. Here we show that, on the basis of their responsiveness to EGF and FGF2, similar NSCs exist along the AP and DV axis. In addition, we provide data indicating that NSCs grown as neurospheres do not express the characteristic *in vivo* AP and DV positional identity gene expression code.

Materials and methods

Primary cell cultures

The care and use of animals was carried out in accordance with NIH guidelines. The procedure was carried out as previously reported (Santa-Olalla & Covarrubias, 1995). Briefly, pregnant CD-1 mice were killed by cervical dislocation, embryos at several developmental stages (11.5–15.5 d.pc) were removed from the uterine horns and collected into ice-cold PBS-glucose (5.4 mM potassium chloride, 138 mM sodium chloride, 22 mM glucose, 2 mM sodium–potassium phosphate, pH 7.2; all from Sigma, St Louis, MO, USA). Brains from embryos were dissected in the same solution. Telencephalon, diencephalon and mesencephalon were separated using as reference the secondary vesicle limits. For metencephalon and myelencephalon, the caudal limit was the pontine flexure and the cervical flexure, respectively. The spinal cord fragment used corresponds only to the cervical region with the rostral limit at the cervical flexure. The meninges associated with all regions were discarded. Telencephalon was divided into dorsal and ventral regions, the temporal cortex and the ganglionic eminence. Ventral and dorsal mesencephalon regions were separated taking as reference the presumed sulcus limitan. The tissues to be cultured were exposed to 0.1% trypsin in Versene (Invitrogen, Carlsbad, CA, USA) for 15 min, inactivated by adding fresh medium with 10% serum (1 : 1 v/v) and mechanically dissociated with a 5-mL pipette and a fire-polished Pasteur pipette. Cells were collected by centrifugation at 800 ***g*** for 5 min and resuspended in serum-free medium composed of: high-glucose Dulbecco's Modified Eagle Medium (D-MEM) and Ham's F12 1 : 1 v/v supplemented with glutamine (2.92 mg/mL), insulin (25 μg/mL), transferrin (100 μg/mL), putrescine (60 mM), progesterone (20 nM), selenium (30 nM), all reactives from Invitrogen, EGF (100 ng/mL; Peprotech, Jalapa, Ver., México), or FGF2 (50 ng/mL; Peprotech), hereafter referred to as the growth medium. Cells were counted in a haemocytometer and adjusted to 1×10^6 cells/mL, and trypan blue exclusion was carried out to verify cell viability. Aliquots of this suspension (500 000 cells) were seeded in one 16-mm-diameter well precoated with poly-D-lysine (10 μg/mL; Sigma) for 2 h. Non-adherent conditions mean that plates were not coated with poly-D-lysine. Cultures were maintained at 37 °C in a humidified 5% CO_2−95% atmospheric air. Growth factors were added 1 h after seeding. Half of the medium was replaced every 3 days with fresh growth medium containing the same concentration of growth factors.

NSCs subcultures

After 7 days of culture, generated primary neuropheres were harvested one by one from cultures in adherent condition, or in bulk from cultures in non-adherent conditions. Neurospheres were incubated in 0.025% trypsin in Versene (Invitrogen) for 10 min, inactivated by adding 1 : 1 (v/v) 1 mg/mL trypsin inhibitor (Sigma) and mechanically dissociated with a 200-μL micropipette. Cells were collected by centrifugation at 400 ***g*** for 5 min and resuspended in growth medium. Cells were counted in a haemocytometer and adjusted to 2×10^4 cells/mL; trypan blue exclusion was carried out to verify cell viability. Aliquots of this suspension (10 000 or 1000 cells) were plated in 16-mm-diameter wells, under the same conditions used for the primary cell cultures (with or without poly-D-lysine) and in the presence of EGF (100 ng/mL), FGF2 (50 ng/mL) or both. Cultures were maintained at 37 °C in humidified 5% CO_2−95% atmospheric air for 7 days. Half of the medium was replaced every 4 days with fresh growth medium containing the same concentration of growth factors. FGF1 (100 ng/mL), FGF2 (1–100 ng/mL), FGF4 (100 ng/mL), FGF8 (100 ng/mL), FGF10 (100 ng/mL), SHH (100 ng/mL) and retinoic acid (0.01–5 μM) were added at day 1 or at day 6 of culture. For clonal analysis, primary neurospheres were harvested, pooled and dissociated as described above. Cells were plated by limiting dilution at an average of 1 cell/well in 200 μL of growth medium in 96-well plates (Falcon, San Francisco, CA, USA) containing EGF (100 ng/mL) and FGF2 (50 ng/mL). Every 4 days, 50 μL of fresh growth medium containing the same concentration of growth factors as in primary culture was added. Neurospheres produced were harvested after 15 days of culture.

RNA purification, cDNA synthesis and polymerase chain reaction (PCR) procedures

RNA was purified from cultures using the protocol previously reported (Santa-Olalla & Covarrubias, 1999). Ten microlitres of RNA solution was used for cDNA synthesis using M-MLV Reverse Transcriptase (Invitrogen) in 30 μL final reaction volume according to the manufacturer's recommendations. Routinely, 3 μL of cDNA reaction was used in a PCR reaction containing 2.5 U *Taq* DNA polymerase (Biotecnologias Universitarias, México City, México), 200 mM deoxyribonucleotide-5′-triphosphates (BioLabs, Beverly, MA, USA), 1.86 mM $MgCl_2$, in the buffer provided, and 30 pmol of each forward and reverse primers. All reactions were carried out in a programmable heating block (Hybaid, Middlesex, UK). The PCR protocols were: for hypoxanthine phosphoribosyl transferase gene (*Hprt*; coding for a house keeping enzyme) and *Bf1*: cycles 92 °C 1 min, 58 °C 1 min, 72 °C 1 min. For *Otx2*, *En1*, *En2*, *Krox20*, *HoxB9*, *Dbx1*, *Dbx2*, *Pax6*, *Pax7*, *Gbx2* and *Fgf8*: cycles 92 °C 1 min, 60 °C 1 min, 72 °C 1 min. For *HoxD1*: cycles 92 °C 1 min, 62 °C 1 min, 72 °C 1 min. For *Nkx6.1* and *Nkx2.2*: cycles 92 °C 1 min, 64 °C 1 min, 72 °C 1 min. In all cases, PCR was ended with 10 min at 72 °C. Oligonucleotide primers were: *Hprt*: forward CCT GCT GGA TTA CAT TAA AGC ACT G, reverse GTC AAG GGC ATA TCC AAC AAC AAA C; *Bf1*: forward CCC TAC AGC TCC GTG TTG ACT CAA, reverse CGT GCT GGT CTG CGA AGT CAT TGA; *Otx2*: forward ATG ATG TCT TAT CTA AAG CAA CCG CCT TAC G, reverse TCA TTG GGT CAT CAG TAT AAA CCA; *En1*: forward TCA AGA CTG ACT CAC AGC AAC CCC, reverse CTT TGT CCT GAA CCG TGG TGG TAG; *En2*: forward AAG ACG CTA TCA CTT CAC GGT GGT, reverse AGA ATA GCG CGT GCA GTA GAC CCA; *Krox20*: forward AGT GGC GGG AGA TGG CAT GAT CAA, reverse AGT TCA GGC TGA GTC TGG GAC ATG; *HoxD1*: forward AGC AAA CTG TCC GAA TAT GGA GCC A, reverse CTC TCG TTC CCT CTT CTT CTG CTT; *HoxB9*: forward TTG CGA AGG AAG CGA GGA CAA AGA G, reverse TTG CCT GCT CCG TTA TTC ATC TTC T; *Pax7*: forward CGA CGA GGA AGG AGA CAA GAA AGA A, reverse TGG TGG TGG TGG GGT AGG TAG AGT; *Dbx1*: forward TCC TCC TAT TTC CCA GCT TCC TCC, reverse CGC CCT CCT CAT CCT CAT CAG AAT; *Dbx2*: forward TTG CTG ACC CAG GAC TGA AAT TCC AA, reverse CCT CGG AGT GAA TCT GCT TCC AGG A; *Pax6*: forward AGA CTC GGA TGA AGC TCA GAT GCG, reverse ACT GGT ACT GAA GCT GCT GCT GAT; *Nkx6.1*: forward GGA TGA CGG AGA GTC AGG TCA AGG T, reverse GCT GCC ACC GCT CGA TTT GTG CTT T; *Nkx2.2*: forward AGG AGG ACT CGA TCC TTA CCA CGG T,

reverse ATT TGC CAC CAG TTG TCA GAA CGT GTA; *Gbx2*: forward GAG GGC TCG CTG CTC GCT TTC TCT, reverse GGA TGG GGA CGA CAA TCT TGG GGT T; *Fgf8*: forward GCA TGT GAG GGA GCA GAG CCT, reverse GTA GTT GTT CTC CAG CAC GAT C. All PCR products were of the expected size, and their identity was confirmed by digestion with several restriction endonucleases. In order to have the best estimate of the relative amount of each specific mRNA, we took an aliquot of each PCR reaction performed in the present work every five cycles for the next 15–20 cycles, starting with the 20th, 25th or 30th cycle depending on the starting material (e.g. 20th for tissue fragments, 30th for individual neuropheres).

Results

Similar 'early' and 'late' NSCs are present along the AP axis

We have identified two classes of NSCs in the embryonic mesencephalon: the FGF-responsive cells ('early' NSCs) and the EGF-responsive cells ('late' NSCs). We have proposed that both populations are lineage-related (Santa-Olalla & Covarrubias, 1999). In this model, the 'early' NSCs proliferate and generate the 'late' NSCs upon FGF2 exposure; the 'late' NSCs proliferate and form neurospheres *in vitro* in the presence of EGF. Using a different experimental strategy, a similar conclusion was reached for telencephalic NSC populations (Tropepe *et al*., 1999; Ciccolini, 2001). It is important to mention that neurospheres must grow on an adherent surface (i.e. poly-D-lysine) in order to detect the 'early' and 'late' NSCs, otherwise the EGF-responsive population appears spontaneously (without FGF2 pretreatment; Santa-Olalla & Covarrubias, 1999).

We examined the presence of 'early' and 'late' NSCs along the whole neural tube AP axis. As shown in Fig. 1, EGF alone allowed the formation of very few and small aggregates when it was applied to cells from any of the regions tested of embryos at early stages (11.5 d.pc). As development proceeded (12.5–15.5 d.pc), an increasing number of larger neurospheres formed in response to EGF alone (Figs 1 and 2A). The production of neurospheres with EGF alone was particularly inefficient for the metencephalon and spinal cord. In our culture conditions, FGF2 alone commonly promotes limited cell proliferation, resulting in 'flat' neurospheres (Santa-Olalla & Covarrubias, 1995); however, we obtained neurosphere-like aggregates using this growth factor alone with cells from embryos at some stages (e.g. 11.5 d.pc) and/or from some regions (e.g. telencephalon) (Fig. 1). By contrast with the response to the growth factors alone, when we applied FGF2 for 1 day and then EGF for 6 days to cultures, neurospheres formed with cells from any of the regions and from the earliest stages tested (Fig. 1, F–E). This treatment identifies the 'early' NSC population. To estimate the number of 'early' NSCs, the number of neurospheres formed with EGF is subtracted from the number obtained with the sequential treatment (Fig. 2B). As can be seen, the 'early' NSC population appears at earlier developmental stages in all regions, and as development advances its number decreases and becomes negligible in the most anterior regions (telencephalon through mesencephalon). By contrast, 'early' NSCs are always present in posterior regions, representing most of the NSC population in the spinal cord (note that small aggregates obtained with EGF at 12.5 d.pc from this region might not be actual neurospheres).

In summary, these results suggest the existence of similar NSC populations along the whole CNS. 'Early' NSCs require transitory action of FGF2 to proliferate in response to EGF, whereas 'late' NSCs do not. Furthermore, these studies also show that the population responsive to EGF increases naturally in anterior regions of the developing CNS, suggesting that this NSC type has a role late in development or conceivably in the adult.

NPCs in culture lose their original AP positional identity gene expression code with prominent expression of En2 *and* Krox20

During development, NSCs restrict their differentiation potential. The neuromeric organization has been suggested to support the compromise between specific phenotypes in particular areas of the CNS (Krumlauf *et al*., 1993; Shimamura *et al*., 1997). In order to determine whether NSCs grown *in vitro* retain their original transcription factor code, we examined AP region-specific gene expression in neurospheres. In the following description, NSCs are used to designate the cells that give rise to neurospheres and NPCs the cells forming the neurospheres (a popoulation that it is thought contains NSCs). Initially, the genes tested were: *Bf1*, *En2*, *Krox20*, *HoxD1* and *HoxB9*. Primary neurospheres were collected individually such that most primary cells unable to produce neurospheres were left in the plate. These enriched NPCs were subcultured at low density (at least a 100–500 dilution factor with regard to the total cells of the primary culture) in medium containing EGF to produce secondary neurospheres. In this condition, secondary neurospheres grow isolated with a surrounding monolayer. We estimated that following this protocol very few cells from the primary culture contaminate the subculture and therefore analysis was restricted to the NPCs directly derived from the original NSCs growing in response to the growth factor. Owing to the very low cell number available, gene expression was determined by semiquantitative RT-PCR. As a reference, we also analysed gene expression in embryo regions and in primary neurospheres from which subcultures were derived. Results from two different developmental stages are presented here. As shown in Fig. 3, subcultured NPCs appeared to lose their AP positional identity gene code. Of particular note from the subculture experiments are: (1) *Bf1* expression in NPCs from more posterior regions (NSCs from 11.5 d.pc diencephalon and mesencephalon, and NSCs from 15.5 d.pc diencephalon); (2) extended AP *En2* and *Krox20* expression; (3) *HoxD1* and *HoxB9* lack of expression. These alterations started in primary neurospheres that expressed ectopically *Krox20*, and did not express *Hox* genes in NPCs from myelencephalon and 15.5 d.pc spinal cord (Table 1).

NPCs in culture lose their original DV positional identity gene expression code characterized by low expression of Pax7 *and* Dbx1

Gene expression in the DV axis has been studied in more detail in the spinal cord. Thus the genes studied here were selected according to the expression patterns in the spinal cord. We followed the same protocol as described in the previous section but analysed the following genes: *Pax7*, *Dbx1*, *Dbx2*, *Pax6*, *Nkx6.1* and *Nkx2.2* (Fig. 3). Note, we divided only two regions from 15.5 d.pc embryos in their dorsal and ventral parts: the telencephalon, in cortex and ganglionic eminence; and the mesencephalon, in dorsal and ventral mesencephalon. However, genes such as *Pax7* and *Dbx1* that are expressed along the whole AP axis were not expressed or expressed at very low levels in NPCs in culture. In addition, *Nkx6.1* was also expressed at low levels in NPCs from anterior regions. *Pax6* and *Nkx2.2* were expressed at very high levels and did not show a restriction in NPCs from either dorsal or ventral compartments.

Fgf8 is expressed in NPCs in culture but does not determine their AP expression pattern

It has been reported that *Bf1*, *En2* and *Krox20* are genes regulated by FGF8 (Lee *et al*., 1997; Marin & Charnay, 2000; Kobayashi *et al*., 2002). Therefore, we decided to study the expression of *Fgf8* as a candidate gene that could be defining the expression pattern described above. Furthermore, we also determined the expression of other genes up-regulated (*En1* and *Gbx2*) or down-regulated (*Otx2*) by FGF8

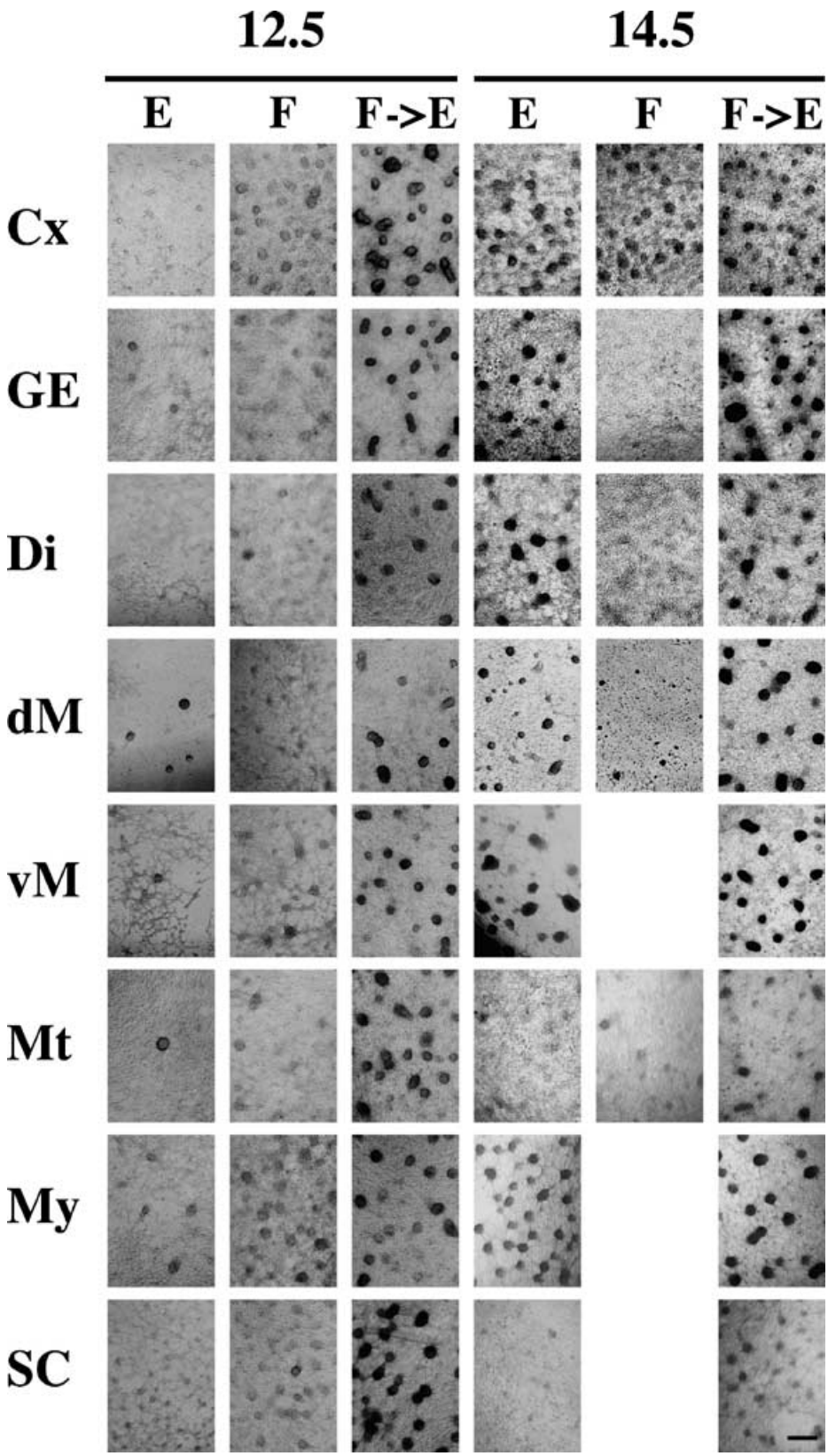

FIG. 1. Neurosphere formation in response to EGF, FGF2 or sequential treatment in primary cell cultures. Primary cells from cortex (Cx), ganglionic eminence (GE), diencephalon (D), dorsal mesencephalon (dM), ventral mesencephalon (vM), metencephalon (Mt), myelencephalon (My) and spinal cord (SC) were cultured in the continued presence of EGF (E) or FGF2 (F) for 7 days, or a sequential treatment, 1 day with FGF2 and then 6 days with EGF (F→E). Tissues were dissected from embryos at 11.5, 12.5, 13.5, 14.5 and 15.5 d.pc, but only representative pictures of the two stages indicated are shown (see Fig. 2). Bright-field microphotographs show the typical morphology of neurospheres generated with each treatment. Scale bar, 250 μm.

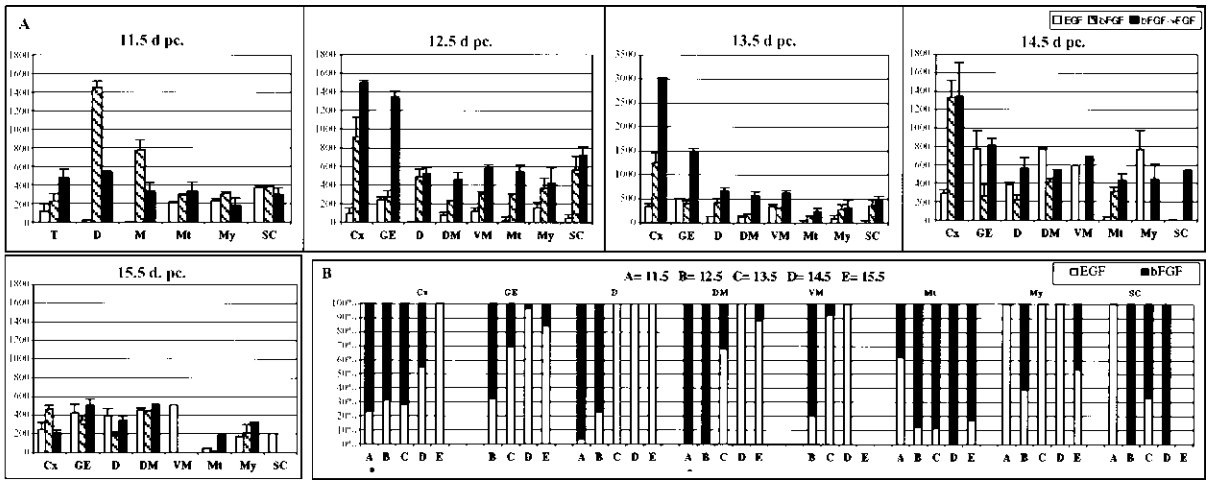

FIG. 2. Estimation of the number of 'early' and 'late' NSCs at different stages of CNS development. Neurospheres generated in the conditions described in Fig. 1 were counted (A). The developmental stage used is indicated in each panel. The values represent the mean ± SEM of generated neurospheres in individual 16-mm wells from a representative experiment ($n = 3$). The contribution of the FGF2-responsive NSC population was estimated by subtracting the number of neurospheres generated with EGF alone from the number of neurospheres generated with the sequential treatment. The percentage contribution of the EGF- and the FGF2-responsive cells to the total NSC population in different regions and at different developmental stages is shown B. Cx, cortex; GE, ganglionic eminence; dM, dorsal mesencephalon; vM, ventral mesencephalon; asterisk (*) indicates that the whole telencephalon or mesencephalon was used at 11.5 d.pc.

activity (Liu & Joyner, 2001). In this case, we used subcultures from 13.5 d.pc embryo regions (Fig. 4). In agreement with the previous data, *Bf*1, *En2* and *Krox20* showed an extended expression pattern in NPCs grown in culture. *Fgf8* as well as *En1* and *Gbx2* also showed expression in NPCs from all regions tested. Nonetheless, *Otx2* showed a similar pattern in subcultures of NPCs to those genes up-regulated by this growth factor.

Attempts to demonstrate a role for FGF8 in the observed gene expression patterns have not been successful. Addition of FGF8 or inhibition of FGF8 activity have shown no perceptible changes in gene expression (data not shown). We also evaluated the effects of several FGFs, retinoic acid (RA) and sonic hedgehog (SHH) on the genes studied above. FGFs and RA are thought to be relevant signals for the establishment of the AP gene expression pattern (Durston *et al*., 1989; Kengaku & Okamoto, 1995) whereas, SHH is a key ventralizing signal that helps to define the DV gene expression pattern (Ericson *et al*., 1997). Growth factors were added during neurosphere growth (Fig. 5A) or for the last 24 h after neurosphere growth (i.e. after 6 days) (Fig. 5B). Surprisingly, the genes studied were refractory to the effects by these growth factors, despite the fact that genes coding for their receptors are expressed in neurosphere cells (Sakurada *et al*., 1999; Tropepe *et al*., 1999; our unpublished data). Figure 5B also shows that after eight passages of 4×10^3 neurophere cells (i.e. a minimum of 32 divisions *in vitro*), the gene expression pattern described above is not altered.

Clonal neurospheres coexpresses several positional markers

In the previous experiments, gene expression was determined in pools of neurospheres generated in subcultures from primary neurospheres. Hence, it may be possible that the expression patterns determined are not attributable to what is going on in each neurosphere, but rather the average of different individual patterns in subcultured neurospheres. Therefore, we generated clonal neurospheres from the ganglionic eminence and the ventral mesencephalon, and determined the expression patterns for some of the genes analysed above (Table 2). Most of the AP markers were not expressed in each neurosphere from the same NPC pool, but we did observe coexpression of some of the genes studied. As expected, *En2* and *Gbx2* was expressed in most neurospeheres from mesencephalon (53% and 75%, respectively) but only in a fraction of those from the ganglionic eminence (10% and 10%, respectively). By contrast, *Emx2*, a telencephalon marker gene, was expressed in most neurospheres from ganglionic eminence (88%) and only in a small fraction of those derived from the ventral mesencephalon (35%). In the two regions tested, *Krox20* was expressed in a high proportion of clonal neurospehres (61% in ganglionic eminence and 88% in ventral mesencephalon). *Bf1* was not detected in clonal neuroshperes, whereas *Otx2* was detected at a low frequency (5% in ganglionic eminence and 17% in ventral mesencephalon). By contrast, DV markers in individual neurospheres followed the same pattern observed in neurosphere pools with the exception of *Nkx-6.1* which was barely detected. Despite the variety of patterns observed for each gene, coexpression of telencephalic and mesencephalic markers was observed in some clonal neurospheres. Surprisingly, genes that are known to be mutually repressed such as *Otx2*/*Gbx2* and *Nkx2.2*/*Pax6* were also coexpressed, with the latter being more prominent.

NPCs grown under different culture conditions also show extended Krox20 *and* En2 *expression pattern*

Several procedures have been used to grow NSCs in the presence of EGF, FGF2 or both. In order to determine whether the gene expression pattern observed results from the specific culture condition used in previous experiments, we analysed the positional information of NSCs grown in different conditions. Of particular interest were the conditions under which NSCs are grown non-adherently (without poly-D-lysine – PDL) and in the presence of EGF or FGF2, because these are the conditions most frequently used (Reynolds & Weiss, 1992; Gritti *et al*., 1996; Ciccolini & Svendsen, 1998; Tropepe *et al*., 1999). As before, we studied NSCs in subcultures in order to reduce the contamination from primary cells. For these experiments, the NSCs were from different regions of 13.5 d.pc embryos. As seen in Fig. 6, independently of the culture conditions or the growth factor used, *En2* and *Krox20* expression was detected in NPCs from regions where normally these genes are not expressed. Interestingly, low-density cultures with EGF + FGF-2 (EF/EF-PDL protocol) also showed extended *En2* and *Krox20* expression, in contrast to results from a previous report (Hitoshi *et al*., 2002). The pattern of other genes analysed was similar

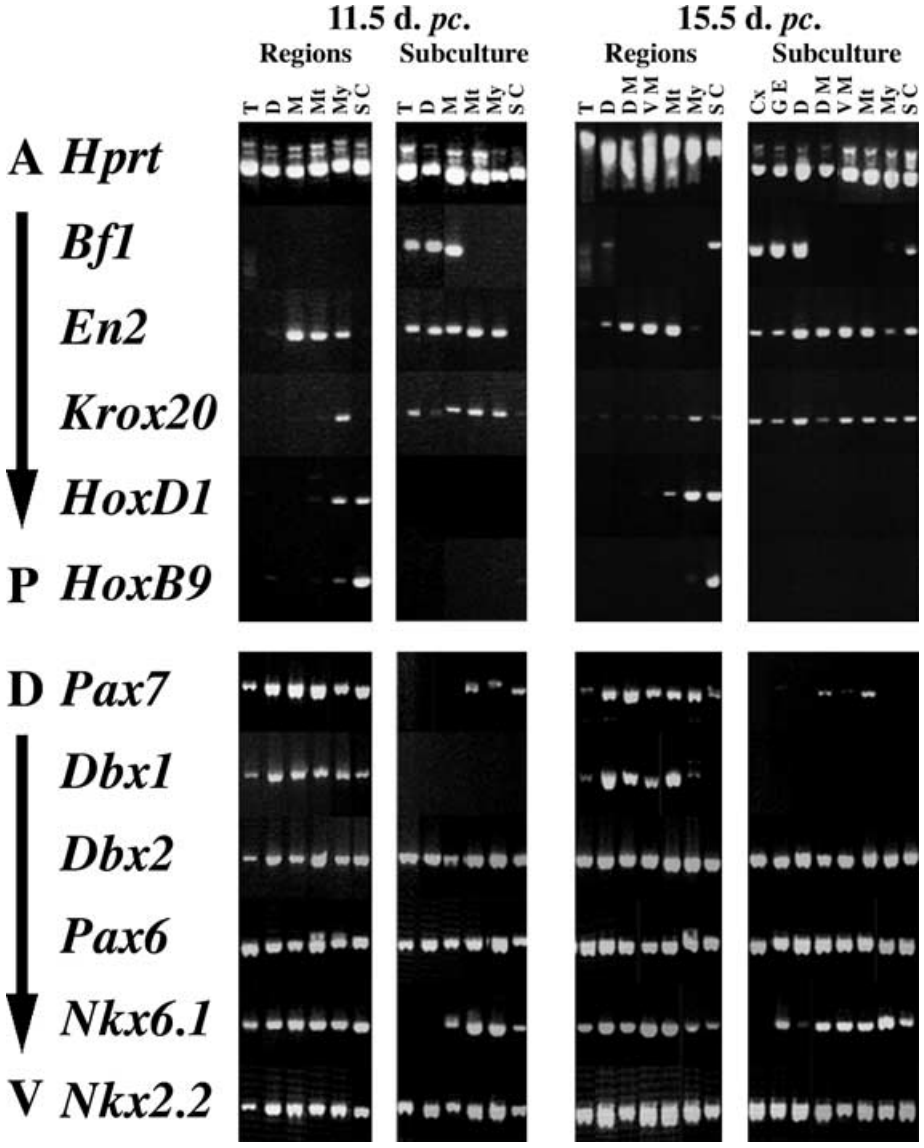

FIG. 3. Expression of AP and DV positional marker genes in NSCs. Individual primary neurospheres generated from the regions indicated were collected, pooled and dissociated. 1×10^3 cells per 16-mm tissue culture well were subcultured in the presence of EGF for 7 days. Cells were harvested at the end of culture. A semiquantitative RT-PCR procedure for specific positional marker genes was performed as described in Materials and methods with RNA extracted from cells in each individual well. As reference, the RT-PCR procedure was carried out on equivalent tissue fragments as those used to grow the NSCs. The results from two different developmental stages are shown. The lane presented for each PCR reaction corresponds to the aliquot taken after 30 cycles for tissue fragments, and 35 cycles for subcultured NSCs (see Materials and methods). These experiments were repeated three times with similar outcome.

to those described above, with the exception of *Otx2*, which was down-regulated when FGF2 was present (F/F-PDL protocol). Therefore, the positional identity gene expression code is not preserved in NSCs in culture, with little influence of the condition used.

Discussion

The present work focused on the characterization of two populations of NSCs rising along the whole developing CNS. These two populations, one responsive to FGF2 and the other responsive to EGF, can easily be distinguished when they are grown in adherent conditions. Under these conditions, a differential growth response to FGF2 or EGF is evident, such that neurospheres are mainly formed in response to EGF. As expected, 'early' NSCs were more abundant at earlier developmental stages in all regions and, as development proceeded, their number decreased with a concomitant increase in 'late' NSCs. Nonetheless, this correlation is more clearly observed in NSC populations from anterior regions (telencephalon through mesencenphalon) and is not evident at posterior regions (e.g. spinal cord; see below). We also evaluated the gene expression code that is thought to specify NSCs according to their region of origin. We found that, independent of the culture condition used, the specific combination of expressed genes that establish the AP or DV cell position identity is not preserved in NSCs grown in culture.

The EGF-responsive cell population may be a cell type with a function late in development and in the adult

Several groups have considered that generation of neurospheres results from expansion of an NSC population (Svendsen *et al*., 1995; Reynolds & Weiss, 1996). In the developing telencephalon and mesencephalon, this NSC population arose from NSCs responsive to an FGF family member (Santa-Olalla & Covarrubias, 1999; Tropepe *et al*.,

1999); the action of FGF is necessary to produce the NSC population responsive to EGF. The present study shows that FGF-responsive NSCs exist along the whole developing CNS, including the spinal cord, whose NSCs have been difficult to amplify (Kalyani *et al*., 1997).

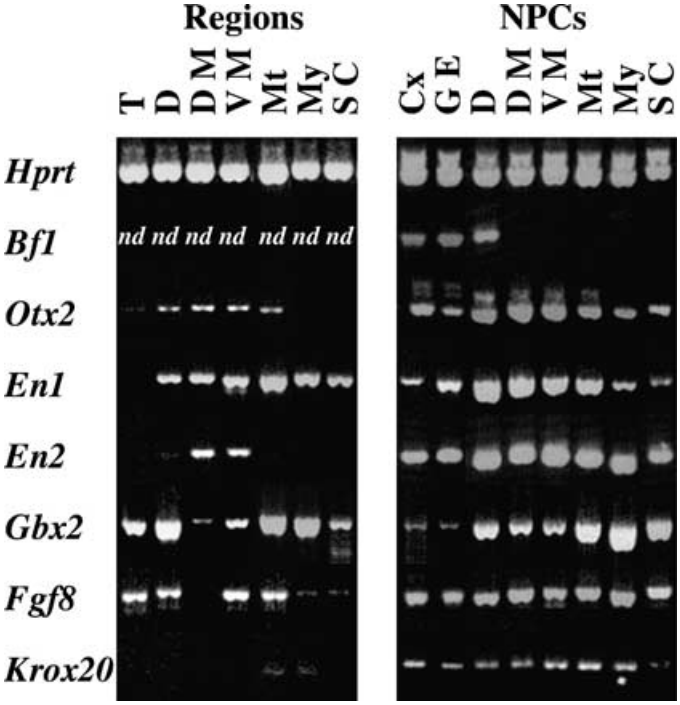

FIG. 4. Expression pattern of genes regulated by FGF8. NSCs from the regions indicated of 13.5 d.pc embryos were subcultured in the presence of EGF and harvested after 7 days. RT-PCR procedures specific for the positional markers indicated were performed. The lane presented for each PCR reaction corresponds to the aliquot taken after 40 cycles (see Materials and methods).

As expected, this population appears in all regions at earlier stages than the one responsive to EGF. These data suggest that a common NSC lineage emerges during development of most CNS regions.

The FGF-responsive cell population could contain the immediate derivatives of the primogenic NSCs. As shown here and in a previous report, these cells cannot be expanded with FGF because they readily differentiate to more mature EGF-responsive cells. However, it has recently been shown that an NSC population derived from embryonic stem cells can be expanded with FGF when leukaemia inhibitory factor (LIF) is present (Tropepe *et al*., 2001). Furthermore, a recent report (Maric *et al*., 2003) presents a negative selection protocol that appears to allow the purification of this cell population from the embryonic telencephalon. These purified cells proliferate in response to FGF2 and very few of them express the EGF receptor. This study confirms that more compromised cell populations express the EGF receptor, which are probably those capable of generating neurospheres. If this interpretation is correct, the early differentiation effect of FGF2 we reported earlier (Santa-Olalla & Covarrubias, 1999), which promotes the generation of NSCs responsive to EGF, may result from the response to this growth factor in combination with additional factors present in the primary culture.

It is unlikely that the cells responsive to EGF represent the *in vivo* founder NSCs, because they arise, at least in the diencephalon, mesencephalon and ganglionic eminence, after neurogenesis has already begun (Angevine, 1970; Bayer *et al*., 1995; Olsson *et al*., 1998). During stem cell differentiation, it is thought that the primogenitor stem cell differentiates through stages in which they divide rapidly before compromising to specific cell types. These transitory multipotent progenitors are generally named transit amplifying precursors and may serve to procure the cellular demand required at specific times or in particular regions. The EGF-responsive cells could belong to this type of neural precursor. In agreement with this suggestion, more EGF responsive cells are found in the largest regions of the CNS, the telencephalon and diencephalon. By contrast, the

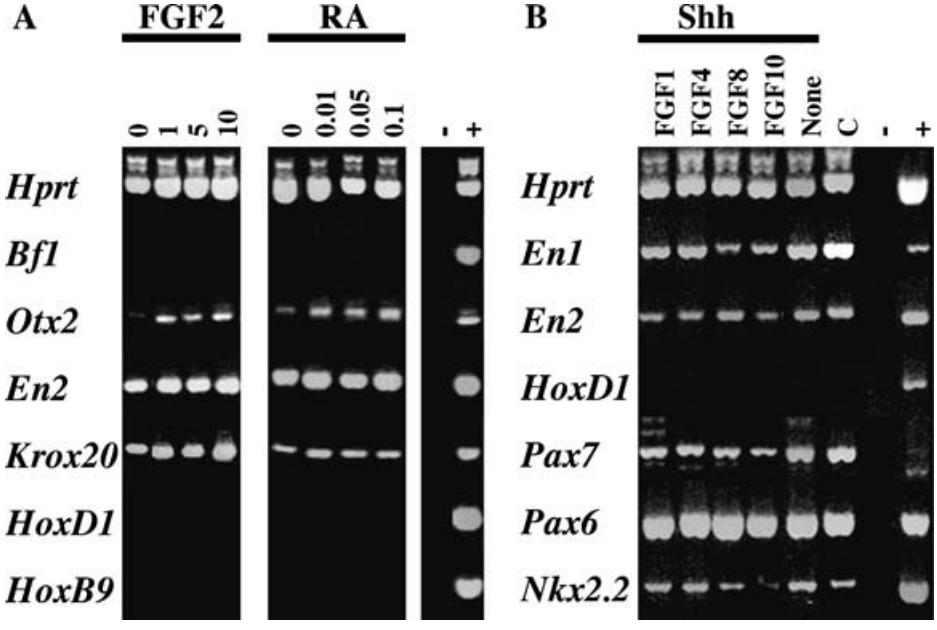

FIG. 5. Effect of several morphogenes on NPCs. (A) Individual primary neurospheres generated from dorsal mesencephlon were collected, pooled and dissociated. 4×10^3 cells per 16-mm tissue culture well were subcultured for 7 days in the presence of EGF and either FGF2 (1–10 ng/mL) or retinoic acid (RA; 0.01–0.1 μM). Cells were harvested at the end of culture. RT-PCR procedure for specific positional marker genes was performed as described in Fig. 3. The lane presented for each PCR reaction corresponds to the aliquot taken after 40 cycles (see Materials and methods). (B) 4×10^3 neurophere cells were passaged in 16-mm tissue culture wells every 7 days. At the eighth passage, all NPCs grown after 6 days in culture were exposed to the growth factors indicated and harvested after 24 h in culture. Samples were processed as described for panel A. (–) and (+) are the negative and positive controls of the PCR. These experiments were repeated twice with similar outcome.

TABLE 1. Expression of AP positional marker genes in primary neurospheres

	Colonies 11.5 d.pc.						Colonies 15.5 d.pc.							
	T	D	M	Mt	My	SC	Cx	EG	D	dM	vM	Mt	My	SC
Bf1	+	+	+	−	−	−	+	nd	+	−	−	−	−	−
Otx2	+	+	+	+	−	−	−	nd	+	+	+	−	−	−
En2	−	+	+	+	−	−	−	nd	+	+	+	+	−	−
Krox20	+	+	+	−	+	+	+	nd	+	+	+	+	+	+
HoxD1	−	−	−	−	−	−	−	nd	−	−	−	−	−	−
HoxB9	−	−	−	−	−	+	−	nd	−	−	−	−	−	−

A pool of individual primary neurospheres derived from the region indicated (abbreviations are as denoted in Figs 1 and 2) were harvested after 7 days of culture. For 11.5 d.pc embryos, FGF2 was used for 1 day followed by EGF for 6 days whereas, for 15.5 d.pc embryos, EGF was used for 7 days. mRNA detection (+) or non-detection (−) by RT-PCR of the genes indicated is shown.

TABLE 2. Expression of antero-posterior (AP) and dorso-ventral (DV) positional marker genes in clonal neurospheres

	Ganglionic eminence																				Ventral mesencephalon																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	+/*n*	(%)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	+/*n*	(%)
AP																																							
Bf1	−	−	−	−	−	−	−	−	−	x	x	x	x	x	x	x	x	x	0/9	(0)	−	−	−	−	−	−	−	−	x	x	x	x	x	x	x	x	x	0/8	(0)
Emx2	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	16/18	(88)	−	+	−	−	−	−	−	−	+	−	+	+	−	−	−	+	+	6/17	(35)
Dlx2	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	+	+	6/18	(30)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0/17	(0)
Otx2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	1/18	(5)	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	3/17	(17)
En2	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	2/18	(10)	+	+	−	+	+	+	+	+	−	−	−	−	+	+	−	−	−	9/17	(52)
Gbx2	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	3/18	(15)	+	+	+	+	−	+	+	−	+	+	+	+	+	+	+	+	+	15/17	(88)
Krox20	+	+	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	11/18	(61)	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	15/17	(88)
HoxD1	x	x	x	x	x	x	x	x	x	−	−	−	−	−	−	−	−	−	0/9	(0)	x	x	x	x	x	x	x	x	−	−	−	−	−	−	−	−	−	0/9	(0)
DV																																							
Dbx1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0/18	(0)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0/17	(0)
Dbx2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	18/18	(100)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	17/17	(100)
Pax6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	18/18	(100)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	17/17	(100)
Nkx6.1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0/18	(0)	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	1/17	(6)
Nkx2.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	18/18	(100)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	17/17	(100)

Individual primary neurospheres derived from the regions indicated were picked after 7 days of culture in the presence of EGF. Neurospheres were collected, dissociated and subcultured at a very low cell density such that a single cell was expected to be present in every well of a 96-well tissue plate. Incubation proceeded for over 15 days in a medium containing EGF and FGF2. mRNA detection (+) or non-detection (−) by RT-PCR of the genes indicated in clonal neuropheres is shown (x, not done).

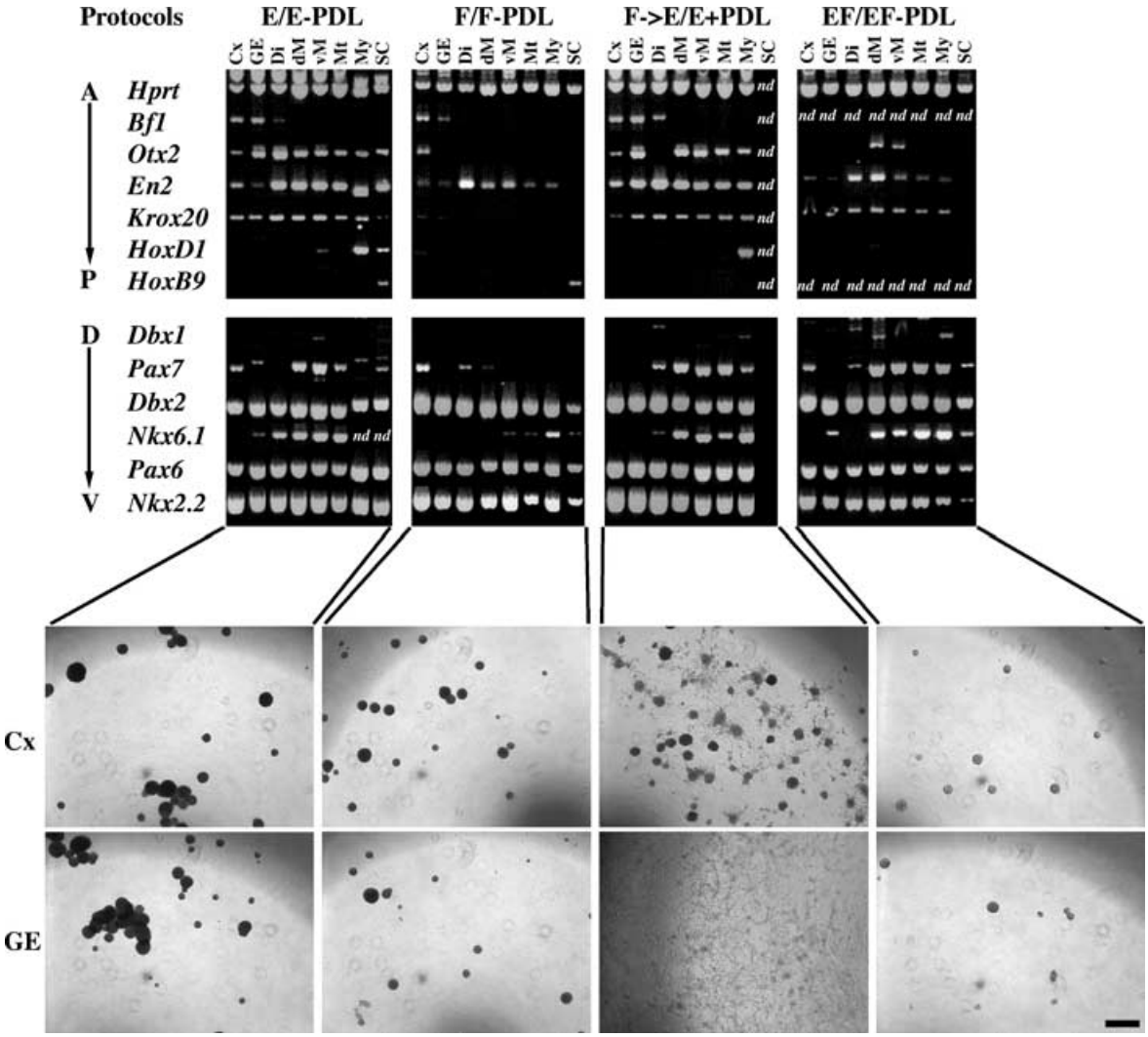

FIG. 6. Expression of AP and DV positional marker genes in neurospheres generated under different culture conditions. Primary tissue of the regions indicated was obtained from 13.5 d.pc embryos. The same pool of cells was used for the different culture conditions described below. 5×10^5 cells from each region were cultured in 16-mm wells without adherent in the presence of EGF (protocol E/E-PDL) or FGF-2 (protocol F/F-PDL), or 5×10^4 cells in the presence of both growth factors (protocol EF/EF-PDL). As a reference, we also cultured 5×10^5 cells on a poly-D-lysine-coated tissue plate with the sequential treatment (protocol F→E/E + PDL), the condition used in the experiments described previously in this work. After 7 days of culture, the colonies generated were collected, mechanically dissociated and 1×10^4 cells subcultured in a 16-mm tissue culture well without adherent in the presence of EGF (protocol E/E-PDL) or FGF2 (protocol F/F-PDL), or 1×10^3 cells in 16-mm tissue cultures in the presence of both growth factors (protocol EF/EF-PDL) for 7 days. Also, 1×10^4 cells from neurospheres grown on poly-D-lysine were grown again on a plate with poly-D-lysine for 7 days (protocol F→E/E + PDL). Cells were harvested at the end of culture. RT-PCR procedures for AP and DV marker specific genes were performed with RNA extracted from cells in individual tissue plate wells. The lane presented for each PCR reaction corresponds to the aliquot taken after 40 cycles (see Materials and methods). Photomicrographs of subcultures following the protocol indicated from the cortex (Cx) and ganglionic eminence (GE) are also shown (scale bar = 250 μm).

spinal cord, with expected low cellular demand during development, produces very few cells responding to EGF. In a similar view, different NSC populations could differentiate at different times according to the cellular demand. For instance, neurogenesis in the lamina V of the cortex occurs at 15.5 d.pc (Contamina & Boada, 1992), and precursor cells giving rise to this neuronal population can be identified at 14–16 d.pc (Malatesta *et al*., 2000). This could be the population responding to EGF, as we detected it in the cortex at around 14.5 d.pc.

In the adult, the same lineage relationship between cells responding to FGF2 and EGF could also exist. Preliminary data show that neurospheres from the adult ventricular zone can be generated in adherent conditions with EGF alone and with the sequential treatment FGF2→EGF, and more neurospheres appear to grow in the latter. Furthermore, cells proliferating in response to FGF2 and EGF have been detected *in vivo* in the adult mouse (Craig *et al*., 1996; Martens *et al*., 2002). The population responsive to EGF could represent an early astrocyte precursor, which still retains some neurogenic potential. In agreement with this possibility, cells responsive to EGF differentiate more readily to astrocytes than to neurons *in vitro* (Kilpatrick & Bartlett, 1995). Furthermore, a null mutation in the gene coding for the EGF receptor produces mice with defective gliogenesis, which may be the cause of neurodegeneration (Sibilia *et al*., 1998). A tempting possibility is that the EGF responsive cell is a radial glia precursor that shows NSC characteristics during development and in the adult. Interestingly the radial glia can give rise to neurospheres, whose cells develop into cell types of the major neural lineages (Gotz *et al*., 2002). More recently, and in agreement with the proposal described above for the embryo, transit amplifying progenitors were identified as a major source of neurospheres in the adult mouse subventricular zone (Doetsch *et al*., 2002). These authors

suggested that this cell population becomes reprogrammed to a more glial cell type in response to high doses of EGF, though retaining some multipotential characteristics.

The identity gene expression code characteristic of specific CNS regions is not preserved in NSCs grown in culture

It has been proposed that neural cell fate is determined by the expression of a set of specific genes that turn on progressively during development. Within the CNS, the gene expression code is restricted to distinct areas from which specific neurons originate. This gene expression code should be established within the NSCs at early stages of their differentiation. In our study, we chose genes known to confer identity to defined CNS regions and expressed early in development (i.e. before neural differentiation). The results presented suggest that NSCs in culture do not retain the original gene expression code that defines the identity of specific CNS regions. This conclusion is supported by detection, in NSC subcultures, of markers not appropriate to the region of origin of the neurosphere NSC founder. Nonetheless, expression of some AP-specific genes is retained in cultured NPCs according to the region of origin, such as *Bf1* (NSCs from telencephalon), *En2* (NSCs from mesencephalon), *Krox20* (NSCs from myelencephalon), and in some culture conditions the *Hox* genes. AP positional identity genes expressed broadly are *Otx2*, *En2*, *Krox20* and *Gbx2*, and the genes rarely expressed are *Hox* genes such as *HoxD1* and *HoxB9*. This is an unexpected finding because transplantation experiments have suggested that AP information is acquired by the embryo early in development (Simon *et al*., 1995).

Restrictions of DV axis markers in cultured NSCs were only studied in the telencephalon and mesencephalon. However, neurospheres from either ventral or dorsal telencephalon and from mesencephalon did not display major differences in gene expression pattern and, in fact, the pattern was similar to that for neurospheres from other regions. Neurospheres from all regions highly expressed *Pax6*, *Dbx2* and *Nkx2*, but not *Dbx1*; *Pax7* and *Nkx6.1* were expressed at low levels and were frequently not detected in rostral regions. In summary, NSCs cultured as neurospheres expressed *Otx2*, *En2*, *Gbx2*, *Krox20*, *Pax6*, *Dbx2* and *Nkx2*, and did not express *HoxD1*, *HoxB9* and *Dbx1* independently of the region of origin. Rostral cultured NPCs expressed *Bf1*, whereas caudal cultured NPCs favour the expression of *Pax7* and *Nkx6.1*. This general expression pattern could be modified by the conditions used to culture NSCs. For instance, *HoxB9* was detected in neurospheres derived from spinal cord NSCs grown in suspension independently of the growth factor used, and neurospheres show a clear decrease in expression of *Krox20*, *Otx2*, *Pax7* and *Nkx6.1* when FGF was used alone. Nonetheless, we did not find the normal gene expression code with any of the conditions used. The many common genes that are expressed in cultured NSCs from different regions may be an indication that the pattern observed is a cell-autonomous property of NSCs in a neutral environment that can only be modified by external cues. Alternatively, the culture conditions could allow the expression of some positional identity genes. However, it is worth noting that we have been unable to modify significantly the characteristic gene expression described by exogenous growth factors (e.g. EGF, FGFs, RA, SHH) that, *in vivo*, can regulate the expression of AP or DV positional identity genes. Furthermore, the gene expression pattern determined is stable for at least 32 divisions *in vitro* (Fig. 5B).

The gene expression code observed in NSCs in culture could be similar in every neurosphere or could be the result of a combination of different codes in each neurosphere. Clonal analysis shows that none of the generated neurospheres displays the code of the region from where they originate, despite the fact that the gene expression pattern observed in each neurosphere was not identical. Clonal neurospheres from ganglionic eminence more frequently expressed *Emx2* and *Dlx2*, whereas those from mesencephalon more frequently expressed *Otx2*, *En2* and *Gbx2*. However, NPCs from both regions expressed *Krox20*, *Dbx2*, *Pax6* and *Nkx2.2*, and did not express *HoxD1*, *Dbx1* and *Nkx6.1*. The pattern observed in neurospheres grown in isolation does not appear to be an exact reflection of the pattern determined in neurospheres in subcultures. Neurosphere development in isolation may vary from that occurring in subcultures (e.g. due to slower proliferation rate) and, consequently, a different expression pattern can be established.

Our results contrast with reports suggesting that NSCs are regionally specified. For telencephalic progenitors, region-specific markers have been found in primary cell cultures (Nakagawa *et al*., 1996) or in progenitors grafted into a heterotypic region (Na *et al*., 1998). These data suggest that the molecular identity becomes an intrinsic property of NSCs during early development. However, it is possible that the NSC population is under-represented in those experiments and that the observations are due to more compromised progenitor cells. A similar conclusion was reached for NSCs grown as neurospheres. In one of these studies (Zappone *et al*., 2000), only anterior marker genes were studied, finding *Bf1*, *Otx1* and *Tbr2* expression in neurospheres from anterior regions but not in those from posterior regions; however, *Emx2* expression (an anterior marker) was detected in neurospheres from both anterior and posterior regions. In another study, Hitoshi *et al*. (2002) reported the expression of *Emx1*, *Dlx2*, *En1* and *HoxB1* in neurospheres according to the region of origin, though this is more evident in primary neurospheres (which may be contaminated with primary cells) and becomes less stringent in subculture or in clonal neurospheres. The previous report also found that *Emx2* expression is not restricted to telencephalic neurospheres but is present in primary neuropheres from all regions tested. The restriction of *Emx1* expression is similar to that we observed for *Bf1*, with little spread to neighbouring regions. In our study, we confirmed *Bf1* expression in neurospheres from anterior regions, *Dlx2*-specific expression in neurospheres from the ganglionic eminence and the broad expression of *Emx2* (Table 2 and data not shown). However, we could not confirm the restricted expression of *Otx1* (data not shown) and *En1* determined in the studies of Zappone *et al*. (2000) and Hitoshi *et al*. (2002), and rather a broad expression was found in neurospheres from any of the regions tested. When neuropheres grew in suspension, we frequently observed restricted *Hox* gene expression, though in these conditions contamination by primary cells cannot be discarded. Despite the differences between Hitoshi's and Zappone's study and ours, the three studies observed broad expression of *Emx2*, which is an indication that the original identity code has been lost in expanded NSCs. The broad expression of *Krox20*, *En1*, *En2*, *Gbx2*, *Otx2* and *Fgf8* determined in our study, even in conditions where restricted expression was determined for some genes, supports this conclusion. A recent report also shows a partial retention of specific markers in neurospheres from telencephalic subregions (Parmar *et al*., 2002), but ectopic expression or lack of expression of some specific genes indicates that the original specification code has been altered in culture.

Does the gene expression pattern observed in neurospheres resemble the one present in early NSCs *in vivo*? *Pax-6* expression is an early marker of the neural plate (Walther & Gruss, 1991) and its presence has been linked with NSC pluripotentiality (Marquardt *et al*., 2001) and proliferation (Yamamoto *et al*., 2001). By contrast, *Otx2* and *Krox20* are expressed after ectopic induction of neural tissue (Alvarez *et al*., 1998). Furthermore, *Fgf8* expression occurs in the chick caudal neural plate; FGF signalling attenuation in the prospective spinal cord, including repression of *Fgf8* transcription, appears to be a prerequisite for the onset of neuronal differentiation (Diez del Corral

et al., 2002). Interestingly, *En1*, *En2* and *Otx1* are genes expressed when neural differentiation of embryonic stem cells is induced (Okabe *et al*., 1996; Rathjen *et al*., 2002). Therefore, at least *Pax6*, *Otx1*, *Otx2*, *Krox20*, *Fgf8*, *En1* and *En2* could be early NSC markers preserved in culture.

It is known that the surrounding tissue has a marked influence on the expression of AP and DV determinant genes in neural cells. Although some signals have been identified, the mechanisms that define their expression pattern are still poorly understood. NSCs grown in culture could be an interesting system to study those mechanisms. However, although it is predicted that the fate of NSC alters as a consequence of an improper positional gene expression code, no definitive conclusion has been reached. It has been found that cells from different regions and even with an apparent specific positional identity gene expression code are capable of integrating into different regions of the neural tube, though the specific code in the grafted cells has not been characterized (Brustle *et al*., 1995; Campbell *et al*., 1995; Fishell, 1995; Olsson *et al*., 1997). Therefore, the effect of this gene expression code on NSC specification still needs to be defined.

Acknowledgements

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Abbreviations

AP, antero-posterior; CNS, central nervous system; Cx, cortex; D, diencephalon; (d), dorsal; dM, dorsal mesencephalon; DV, dorso-ventral; EGF, epidermal growth factor; FGF2, fibroblast growth factor-2; GE, ganglionic eminence; M, mesencephaon; Mt, metencephalon; My, myelencephalon; NSC, neural stem cell; NPC, neural precursor cell; PDL, poly-D-lysine; SC, spinal cord; T, telencephalon; (v), ventral; vM, ventral mesencephalon.

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Growth Factor Deprivation Induces an Alternative Nonapoptotic Death Mechanism That Is Inhibited by Bcl-2 in Cells Derived from Neural Precursor Cells

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ABSTRACT

Although apoptosis has been considered the typical mechanism for physiological cell death, presently alternative mechanisms need to be considered. We previously showed that fibroblast growth factor-2 (FGF2) could act as a survival factor for neural precursor cells (NPCs). To study the death mechanism activated by the absence of this growth factor, we followed the changes in cell morphology and determined cell viability by staining with several dyes after FGF2 removal from mesencephalic NPC cultures. The changes observed did not correspond to those associated with apoptosis. After 48 h in the absence of FGF2, cells began to develop vacuoles in their cytoplasm, a phenotype that became very obvious 3–5 days later. Double-membrane vacuoles containing cell debris were observed. Vacuolated cells did not stain with either ethidium bromide or Trypan Blue, and did not show chromatin condensations. Nonetheless, during the course of culture, vacuolated cells formed aggregates with highly condensed chromatin and detached from the plate. NPCs grown in the presence of FGF2 did not display any of those characteristics. Vacuolated phenotype could be reversed by the addition of FGF2. Typical autophagy inhibitors such as 3-MA and LY294002 inhibited vacuole development, whereas a broad-spectrum caspase inhibitor did not. Interestingly, *Bcl-2* overexpression retarded vacuole development. In conclusion, we identified a death autophagy-like mechanism activated by the lack of a specific survival factor that can be inhibited by Bcl-2. We propose that antipoptotic Bcl2 family members are key molecules controlling death activation independently of the cell degeneration mechanism used.

INTRODUCTION

CELL DEATH plays an important role during normal development and in degenerative and neoplastic diseases. Presently, apoptosis has been considered the typical mechanism for physiological cell death (1,2). Apoptosis is characterized by cell shrinkage, changes in plasma membrane permeability, chromatin condensation, nucleosomal DNA fragmentation, and, at late stages, by nuclear and cell fragmentation (3,4). At the molecular level, caspases are central for triggering apoptosis (5). Different pathways can activate caspases. Some involve mitochondria and are generally associated with the release of cytochrome *c* followed by activation of caspase 9 and caspase 3 (6), whereas others are initiated by death domain-containing receptors resulting in activation of caspase 8 and caspase 3 (7). Additionally, initiation of apoptosis could be regulated by members of the Bcl-2/

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ced9 family, including proapoptotic (e.g., Bax, Bak) and antiapoptotic (e.g., Bcl-2, Bclx-L) proteins (8), and by direct caspase inhibitors such as IAPs (9).

Morphological description of dying cells during development suggests that, apart from apoptosis, other physiological mechanisms of cell death exist (10–12). Morphological characteristics can contrast, from those that define typical apoptosis, to many characteristics associated with necrotic cells, such as lack of chromatin condensation at early stages, cell swelling, and presence of vacuoles or large lysosomes in the cytosol (13,14). Nonapoptotic death can be observed in developing cells and cells affected by some diseases (11,15–17). From the mechanistic point of view, cell death is not always caspase-dependent (10) and Bcl-2 is able to block necrotic-like cell death (13,18,19). Furthermore, active capases are not essential for the appearance of some apoptotic characteristics (20). Hence, morphological features are not definitive parameters for identifying specific cell degeneration mechanisms.

Among the nonapoptotic mechanisms of cell death, autophagy is a well-characterized process by which cells degenerate (11,21). Autophagy consists of the random sequestration of cytoplasm through the formation of double-membrane vesicles (i.e., autophagosomes or autophagic vacuoles). Usually autophagosomes are small (0.5–1.5 μm), but in some particular cells autophagosomes are large enough to be seen by light microscopy (22). Autophagosomes could become intermediate autophagic vacuoles by fusion with endosomes or other autophagosomes (23,24). These vacuoles acquire hydrolases for degradation of the cytoplasmic contents by fusion with lysosomes (25). The resulting vacuole is named autolysosome or degradative autophagic vacuole (26).

Many conditions trigger cell death in culture. In general, factors regulating cell survival can be divided in activators and inhibitors of cell death. Typical activators of cell death are molecules such as tumor necrosis factor-α (TNF-α) and Fas ligand (FasL), which act on specific death domain-containing receptors (7). On the other hand, it is considered that most cells require survival factors to grow in culture; therefore, the basic components of the cell death execution machinery must be present in all cells. Among the growth factors with survival activity are glial-derived neurotrophic factor (GDNF) (27,28), brain-derived neutrophic factor (BDNF) (29,30), epidermal growth factor (EGF) (31), and several members of the fibroblast growth factor (FGF) family (32).

Several years ago we proposed that FGF2 could have survival activity on neural precursor cells (NPCs; (33)). In subcultures, FGF2 was able to support the survival of EGF-responsive NPCs from both ventral and dorsal mesencephalon. In the present study, we characterized the death mechanism of neurosphere-derived cells deprived of growth factors. Cells grown in the presence of FGF2 retained their precursor characteristics, and, as density increased, astrocyte markers were detected. When neurosphere cells were grown in the absence of FGF2, cells also displayed astrocyte markers and progressively developed vacuoles in their cytoplasm and eventually died. The data presented indicate that cell degeneration occurred by an autophagy-like process but, interestingly, over-expression of *Bcl2*, an antiapototic gene, retarded vacuole development.

MATERIALS AND METHODS

Primary cell cultures

The procedure to grow NPCs was carried out as previously described (33). NPCs from dorsal mesencephalon were used for most of the studies presented, in which cases, EGF (100 ng/ml) and basic FGF2 (50 ng/ml) were added together to the culture medium [Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (1:1 vol/vol) supplemented with: glutamine (292 μg/ml), penicillin (0.5 U/ml and 0.5 mg/ml respectively), insulin (25 μg/ml), transferrin (100 μg/ml), putrescine (60 μM), progesterone (20 nM), and selenium (30 nM)]. Cultures were maintained at 37°C in a humidified 5% CO_2 incubator. After 7 days of culture, individual primary neurospheres were picked, pooled, and dissociated by exposing to 0.025% trypsin for 5 min and inactivated with 1 mg/ml trypsin inhibitor (Invitrogen, Carlsbad, CA). For subcultures (i.e., passages), 5–10 $\times$ 10^4 cells from previous suspension were seeded in an uncoated 100 mm diameter plate and cultured for 7 days in the same medium but containing only 100 ng/ml EGF. The subculture protocol was successively repeated up to 10 times for each pool of primary NPCs. NPCs from passages 4–6 were used for the experiments described here. To induce vacuolization, we subcultured 1 $\times$ 10^5 cells in 16-mm-diameter wells precoated with poly-D-lysine and fed with 100 ng/ml FGF2 for 24 h. After this time, cells were washed twice and cultured in the absence of growth factors for several days. When required, enzymatic inhibitors were included in the culture medium at the concentration indicated and incubation done for no longer than 24 h; culture continued in absence of inhibitor. Inhibitors used were: z-Val-Ala-Asp(Ome)-fluoromethylketone [zVAD; 20 mM stock in dimethylsulfoxide (DMSO); Biomol, Plymouth Meeting, PA], cycloheximide (CHX; 100 μM stock in ethanol; Sigma, St. Louis, MO), 3-methyl adenine (3-MA; 500 mM in water; ICN,

Costa Mesa CA), LY294002 (20 mM stock in DMSO; Calbiochem, San Diego CA).

Viability assays and cell death detection

Cell viability was determined by Trypan Blue (Invitrogen, Carlsbad, CA) exclusion and with the live/dead Viability/Cytotoxicity kit (Molecular Probes, Eugene, OR) that contains the mixture of two fluorogenic dyes: calcein AM and the homodimer 1 of ethidium bromide (EthD-1). Chromatin condensation, an indicator of apoptoic death, was determined by staining with 4′,6′-diamidino-2-phenylindole dihydrchloride (DAPI; Molecular Probes, Eugene, OR). Finally, cells with degraded DNA were detected in the plate by the TUNEL (TdT-mediated dUTP nick end labeling) technique (Roche, Indianapolis, IN). Total and stained cells were visualized directly in the culture plate or counted in a hemocytometer using epifluorescence microscopy (Eclipse TE300; Nikon, Japan).

Retroviral infection

The pCL-GFPN vector used in the present study was derived from the pCLNRX retroviral vector (34) (IMGENEX, San Diego, CA); it retains the vector backbone except for the fragment from the *Eco* RI site to the *Cla* I site, containing the neomycin cassette, the RSV promoter, and the multiple cloning sites region. We substituted this fragment with a fragment synthesized by PCR, using as template the plasmid pQBIpolII (Qbiogene, Inc., Carlsbad, CA), containing some multiple cloning sites of pQBIpolII and the GFP*neo*^{r} gene under the control of the constitutive RNA polymerase II promoter. The GFP*neo*^{r} gene codes for a protein resulting from the fusion between the red-shifted green fluorescent protein (GFP) and the product of the neomicin resistance gene (*neo*^{r}). Primers used for this reaction also provided an extra cloning restriction site (*Eco* RI). The restriction map of pCL-GFPN is shown in Fig. 5B (below). Human Bcl-2 (hBcl-2) cDNA obtained from vector pBABE-hBcl-2 (a gift from Gerald I. Evan laboratory) was inserted in the *Eco* RI site of pCL-GFPN vector (pCL-GFPN-hBcl2). The unique property of the retroviral vectors constructed is the ability to select and identify in vivo the infected cells in a heterogeneous cell population. The retroviruses were produced following the protocols and using the ecotropic packaging vectors described by Naviaux et al. (34). Retroviruses were filtered through a 0.45-μm filter and stored at −70°C for later use. Viral titers were determined by infecting STO fibroblast cells and counting either fluorescent cells 48 h after infection or colonies after selection of infected cells in G418 (400 μg/ml). Titers obtained were generally around 1–2.5 $\times$ 10^6 colony-forming units (CFU)/ml.

Infection protocol for primary NPCs

Dorsal or ventral mesencephalon cell suspensions from 13.5-days post coitum (dpc) embryos were prepared and seeded at densities of 5 $\times$ 10^5 cells on a 16-mm dish previously coated with poly-D-lysine. NPCs were cultured as described above in the presence of either 100 ng/ml EGF (for ventral mesencephalon NPCs) or 100 ng/ml FGF2 plus 100 ng/ml EGF (for dorsal mesencephalon NPCs). Forty-eight hours later, 100 ml of conditioned media containing 1 $\times$ 10^6 rvGFPN or rvGFPN-Bcl-2 CFU/ml and 2 μg/ml Polybrene, were added and incubation continued for 12 h. At this time, the medium was changed for the regular DMEM-F12 medium supplemented only with EGF. After 1 week of culture, 27–88% of generated neurospheres were fluorescent. To increase the proportion of infected cells, G418 selection was performed as follows. Forty-eight hours post-infection, cells were harvested and split at 1:3 dilution (seeded on a poly-D-lysine coated 16-mm dish). Twelve hours later, G418 was added (at 400 μg/ml final concentration), and cells were cultured in this condition for 7 days in the presence of EGF. After this selection procedure, 90–100% of neuropheres were fluorescent. Cell death of NPCs infected with rvGFPN or rvGFPN-Bcl-2 was induced as described above.

RNA purification, cDNA synthesis, and polymerase chain reaction

RNA purification was performed using the Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RT-PCR was performed with RNA obtained from NPCs cultured in the presence or absence of FGF2 for 5 days as previously described (33). Sequence of oligonucleotides used for PCR and product size are: *Hprt*, forward CCTGCTGGATTACATTAAAGCACTG, reverse GTCAAGGGCATATCCA ACAACAAAC, 353 bp; *Bax*, forward GAACAGATC ATGAAGACAGG, reverse GCAAAGTAGAAGAGGGCAAC, 290 bp; *Bcl-xL*, forward GACTTTCTCTCCTACA AGC, reverse CGAAAGAGTTCATTCACTAC, 300 bp; *Bcl2*, forward CATCTTCTCC TTCCAGCCTG, reverse TGTGTGTGGAGAGCGTCAAC, 320 bp; *Beclin1*, forward GATCGGGGAGGCATCTGATGG, reverse CACATCTGGCACAGCGGACAG, 461 bp. PCR was performed for up to 35 cycles with the following protocols for each cycle. For *Hprt* and *Bcl-2*: 1 min at 92°C, 1 min at 57°C, and 1 min at 72°C; for *BclxL*: 1 min at 92°C, 1 min at 51°C, and 1 min at 72°C; for *Bax*: 1 min at 92°C, 1 min at 54°C, and 1 min at 72°C; and for *Beclin1*: 1 min at 92°C, 1 min at 60°C, and 1 min at 72°C. All protocols had an extra extension for 7 min at 72°C. In all cases, we obtained the right fragment when the RT-PCR procedure was performed with RNA from 10.5-dpc embryos. A PCR reac-

tion performed with RNA previous to the reverse transcriptase reaction did not generate a specific product.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature, followed by three washes in PBS. After the PBS rinse, cells were incubated for 30 min in blocking solution containing PBS, 10% sheep serum, and 0.1% Triton. Primary antibodies were diluted in blocking solution and added to the cells for 2 h at room temperature. After primary antibody incubation, cells were washed three times in PBS and incubated for 30 min with the appropriate secondary antibody diluted in PBS, containing 10% sheep serum and 1% Triton. Three additional washes with PBS were done after secondary antibody incubation. Finally, cells were mounted with a solution of 50% glycerol in 0.1 M sodium carbonate buffer, pH 9.0, containing 0.4 mg/ml of *p*-phenilendiamine (Sigma, St Louis, MO) to avoid fluorescent quenching. Antibodies and dilution were as follows: anti-Nestin monoclonal antibody 1:100 (Chemicon, Temecula, CA), anti-GFAP polyclonal antibody 1:250 (Chemicon, Temecula, CA), anti-β-tubulin III monoclonal antibody 1:100 (Chemicon, Temecula, CA), or anti-β-tubulin III polyclonal antibody 1:2000 (Covance, Berkeley, CA), Alexa Fluor 594 goat anti-mouse IgG 1:1000 (Molecular Probes, Eugene, OR), Alexa Fluor 488 goat anti-rabbit IgG 1:1000 (Molecular Probes, Eugene, OR), and Alexa Fluor 594 goat anti-rabbit IgG 1:1000 (Molecular Probes, Eugene, OR). Cells were examined with a BIO-RAD MRC-600 confocal microscope. Stacked optical sections were merged with the COMOS 7.0a software (BIO-RAD Microscience Ltd) and Metavue 4.5 software (Universal Imaging Corporation).

Electron microscopy

After culture, cells were pelleted and fixed with cold 3% glutaraldehyde in 0.1 M PBS, pH 7.2, for 2 h at 4°C. After rinsing with cold 0.1 M PBS pH 7.2, cells were postfixed with 1% osmium tetroxide in the same PBS solution at 4°C. The pellet was then treated with uranyl acetate, dehydrated with alcohol and propylene oxide, and embedded in Epon resin. Ninety-nanometer sections were collected and stained with uranyl acetate and lead citrate. The sections were examined and photographed under a Jeol 1200EXII electron microscope operated at 90 kV.

RESULTS

Vacuole formation in cells derived from neural precursor cells in the absence of growth factors

Previously, we presented data suggesting that FGF2 has survival activity on NPCs from mesencephalon (33). However, due to the very small number of cells seeded in those experiments (250 cells/cm^2), evaluation of cell survival was only indirect, estimated by the ability of NPCs to generate neurospheres in response to EGF. To observe the death process directly, NPCs from neurospheres were seeded at high density (5×10^4 cells/cm^2) and compared the morphological changes occurring in cultures with or without FGF2 (+FGF2 or −FGF2, respectively). To avoid artifacts due to plating efficiency, cells were first seeded in the presence of FGF2 for 12–24 h. After this period of time, most cells were attached to the plate. Plates were washed to remove the growth factor and dead cells emerged during neurosphere growth. Thereafter, medium was changed for the corresponding culture condition. At this time (0 d), fibroblast-like morphology was generally observed (Fig. 1, 0d) and almost all cells were positive for the NPC marker nestin (Fig. 2A, 0d). In the presence of FGF2, NPCs proliferated very rapidly in the next few days, and by 5 days in culture most of them expressed the astrocyte marker GFAP and only a minor proportion expressed the neuronal marker b-tubulin III (data not shown). Differentiation into astrocytes could result in this case from the high cell density (35) (see discussion). However, in absence of FGF2 proliferation did not occur, and, after 1 day in this condition, most cells started to develop small vacuoles in their cytoplasm (Fig. 1, 1d). The number and size of vacuoles increased in the following days. After 5 days in the absence of FGF2, most cells contained many vacuoles filling the whole cytoplasm (Fig. 1A, 5d). The majority of vacuolated cells expressed the GFAP marker, whereas vacuole formation was not observed in neurons (Fig. 2A, 5d –FGF2). Approximately from this stage on, vacuolated cells degenerated, slowly and asynchronously, forming very condensed aggregates, in some cases involving more than one cell (Fig. 1B); these condensed aggregates detached from the plate at different stages of culture. During this process, a large vacuole frequently emerged that usually was attached to the nucleus (Fig. 1B,C).

F1 F2 AU4

The vacuolated cell phenotype was reversed by readdition of FGF2 to culture medium. Vacuolated cells generated after culture for 7 days without FGF2 reduced markedly the number and size of vacuoles, after 5 days in culture with FGF2 (Fig. 1C). The increase in cell density at this stage suggests that proliferation had reinitiated, and confluence was reached few days later. Interestingly, many recovered cells coexpressed nestin and GFAP (Fig. 2A, 7d −FGF2 → 5d +FGF2), although in high cell density areas, cells expressing only GFAP predominated (data not shown). These results suggest that vacuole development is not associated to an irreversible change in cell viability and that some vacuolated cells are not terminally differentiated.

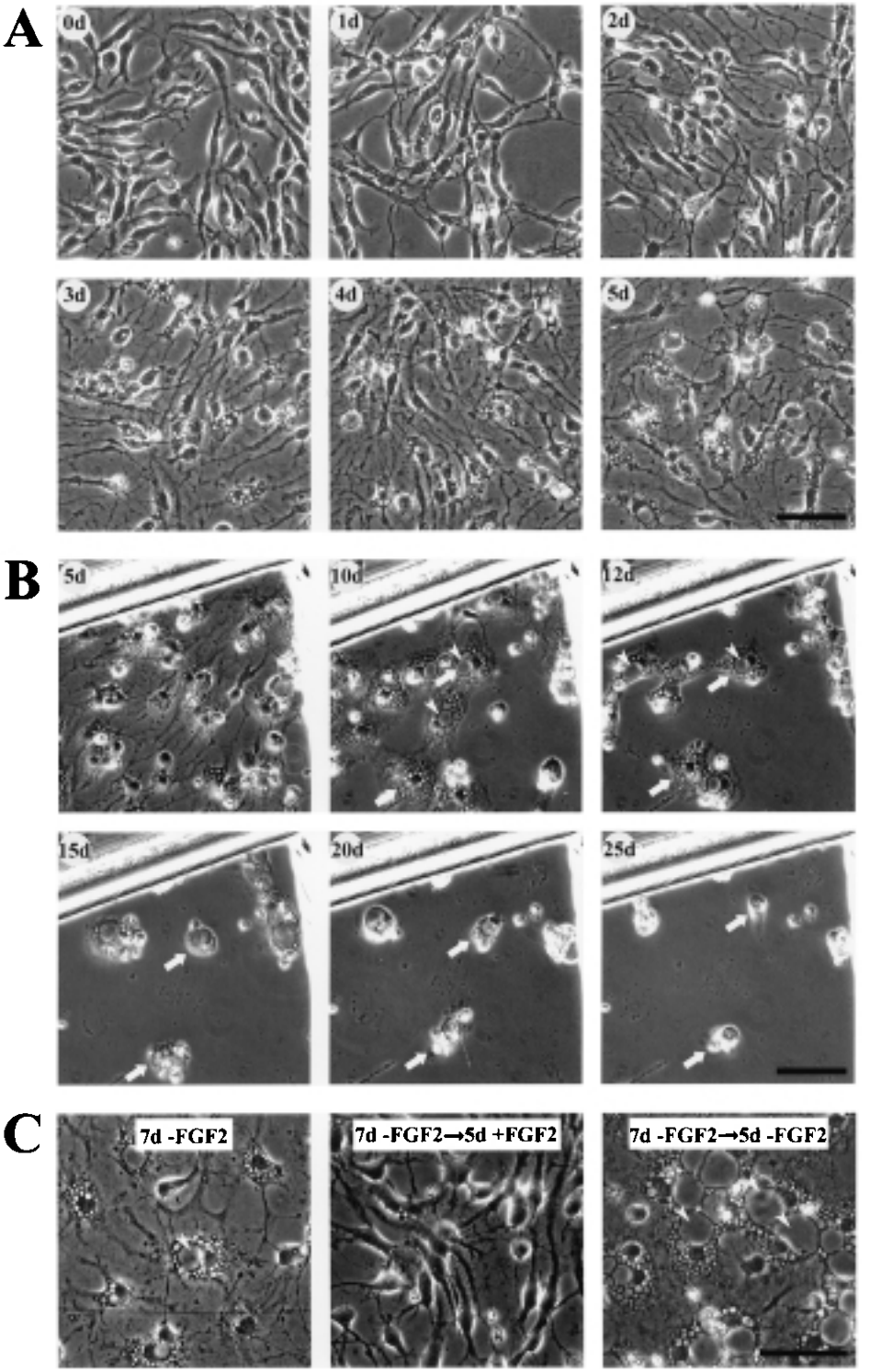

FIG. 1. Vacuole development in the absence of FGF2 is reversible but eventually leads cells to death. (**A**) Vacuole development after FGF2 withdrawal. NPC-derived cells one day (1d) after FGF2 withdrawal from the culture medium showed small vacuoles in the cytoplasm. Vacuole number and size progressively increased with culture time (2d–10d). It is interesting to note that after 5 days in culture in the absence of FGF2 some cells showed a large vacuole attached to the nucleus, and 2 days later (7d) almost all vacuolated cells displayed this large vacuole (see **B** and **C**). (**B**) Fate of individual vacuolated cells. Individual vacuolated cells were selected at day 5 of culture in the absence of FGF2 (white arrows) and a scratch nearby (seen in right upper corner of pictures) was made on plate to allow their continuous observation along the culture length. Vacuolated cells appeared to degenerate by progressive cytoplasm and nuclear condensation. Degeneration of vacuolated cells occurred asynchronously; therefore, the cells shown in photomicrographs do not necessarily represent the degeneration pattern for every cell. (**C**) Recovery of vacuolated cells after FGF2 refeeding. After 7 days of culture in the absence of FGF2, culture continued in the absence or presence of FGF2 for additional 5 days. At this time cell morphology was analyzed. Observe that vacuolated cells refed with FGF2 recovered a healthy appearance. Scale bar, 50 μm.

Characteristics of vacuolated cells

We initially determined the staining properties of vacuolated cells with dyes typically used to detect cell death (Fig. 2B). In agreement with the preservation of viability, vacuolated cells stained with calcein AM, a permeable dye that is retained by live cells, but not with EthD-1, an impermeable dye for live cells. Vacuolated cells also did not stain with Trypan Blue, a characteristic of viable cells. DAPI staining indicates that chromatin was not highly condensed in vacuolated cells, in contrast with the expected highly condensed chromatin in apoptotic cells. Furthermore, TUNEL reaction indicates that DNA was not degraded in vacuolated cells (data not shown). It should be noted that degenerated vacuolated cells observed as 'condensed bodies' (Fig. 1B) did strongly stain with EthD-1, Trypan Blue, and DAPI, and were positive for the TUNEL reaction (data not shown), indicating that degeneration is accompanied by changes in plasma membrane permeability, chromatin condensation, and DNA degradation.

To characterize vacuolated cells further, we examined their ultrastructure by electron microscopy. At day 5 in the absence of FGF2, cytoplasm of most cells was filled with single-membrane vacuoles that generally appeared empty. There was no evidence of apoptotic death at this stage because, for instance, chromatin condensation and plasma membrane blebbing were not observed (data not shown). To determine the origin and characteristics of vacuoles, cells at early stages after growth factor removal were analyzed (2 days in medium without FGF2; Fig. 3). We identified large vesicles that were classified as degradative vacuoles or autolysosomes (Fig. 3). Occasionally, we detected small vacuoles with several vesicles inside, possibly representing early autolysosomes (Fig. 3A). Many vacuoles had engulfed intracellular organelles, particularly mitochondria (see, for instance, Fig. 3D). Although the outer membrane of most vacuoles was a single membrane, it was interesting to observe that some had a double membrane (Fig. 3D). Furthermore, we frequently found double-membrane small 'vacuoles' inside a larger vacuole (Fig. 3C). These latter structures could originate from vacuoles engulfing other vacuoles, an event detected in several instances (Fig. 3A). We consider that the presence of vacuoles with cellular debris including organelles (i.e., autolysosomes) is consistent with the idea that an autophagy-like process was occurring in NPCs after FGF2 withdrawal. An involuting nucleus was also frequently observed in vacuolated cells (Fig. 3A,B), an additional autophagy characteristic. However, it is important to remark that the characteristics described above do not correspond precisely to a typical autophagy; for instance, we were unable to identify typical autophagosomes (i.e., double membrane vesicles with cytoplasmic electrodensity).

F3

Inhibition of vacuole development

The data described above suggest that NPCs in the absence of FGF2 degenerate by autophagy. To support this hypothesis, we incubated growth factor-deprived cells with inhibitors that can block autophagy in well-characterized model systems. Treatments were commonly done for the initial 12–24 h after growth factor removal, since the inhibitors used were toxic for NPCs when treatments were extended for longer periods. 3-MA and LY294002, two phophatidyl inositol-3 kinase (PI3K) inhibitors (21,36,37), prevented vacuole development (Fig. 4 and Table 1). We also found that CHX blocked vacuole formation ((21,38–40); Fig. 4 and Table 1). These data imply that a protein synthesis-dependent autophagy process is initiated after FGF2 withdrawal. In agreement with the data described above indicating that an apoptotic program was not activated, the broad-spectrum caspase inhibitor zVAD-fmk did not affect vacuole formation even when used at relatively high dose (100 μM; Fig. 4 and Table 1). Therefore, although a genetic program appears to be involved in this vacuole-associated cell degeneration process, this is not controlled by caspases, a key component of the apoptotic execution machinery.

F4
T1

Vacuole development in cells over-expressing Bcl-2

NPCs expressed several proapototic (*Bax*) and anti-apoptotic (*Bcl-2*, *Bcl-xL*) genes as well as the autophagy-associated gene, *Beclin1* (41); however, their expression was not markedly modified by FGF2 withdrawal (Fig. 5A). *Beclin1* is a gene expressed ubiquitously (41); therefore, the autophagy-promoting activity of its product must be controlled by protein modifications or by interaction with other protein products. Beclin1 was originally described as a Bcl-2 interacting protein (42). Therefore, if Beclin is involved, we considered possible that Bcl-2 overexpression could prevent autophagy in NPCs initiated by FGF2 withdrawal. To determine whether Bcl-2 has the ability to interfere with the formation of vacuoles, we infected NPCs with either a novel retroviral vector carrying the GfpNeo^r coding sequences (rvGFPN), or the same vector with the human Bcl-2 coding sequences inserted (rvGFPN-hBcl-2).

F5

NPCs infected with rvGFPN or rvGFPN-hBcl-2 retroviruses were generated as described in Material and Methods, seeded at high density, and cultured in the absence or presence of FGF2. Cells infected with the rvGFPN retrovirus developed vacuoles with a pattern similar to that observed in wild-type cells (Fig. 5). On the other hand, NPCs infected with the rvGFPN-hBcl-2 retrovirus showed a significant reduction in vacuolated cells (Fig. 5 and Table 1) accompanied by a reduction in the number of cells staining with EthD-1 (data not

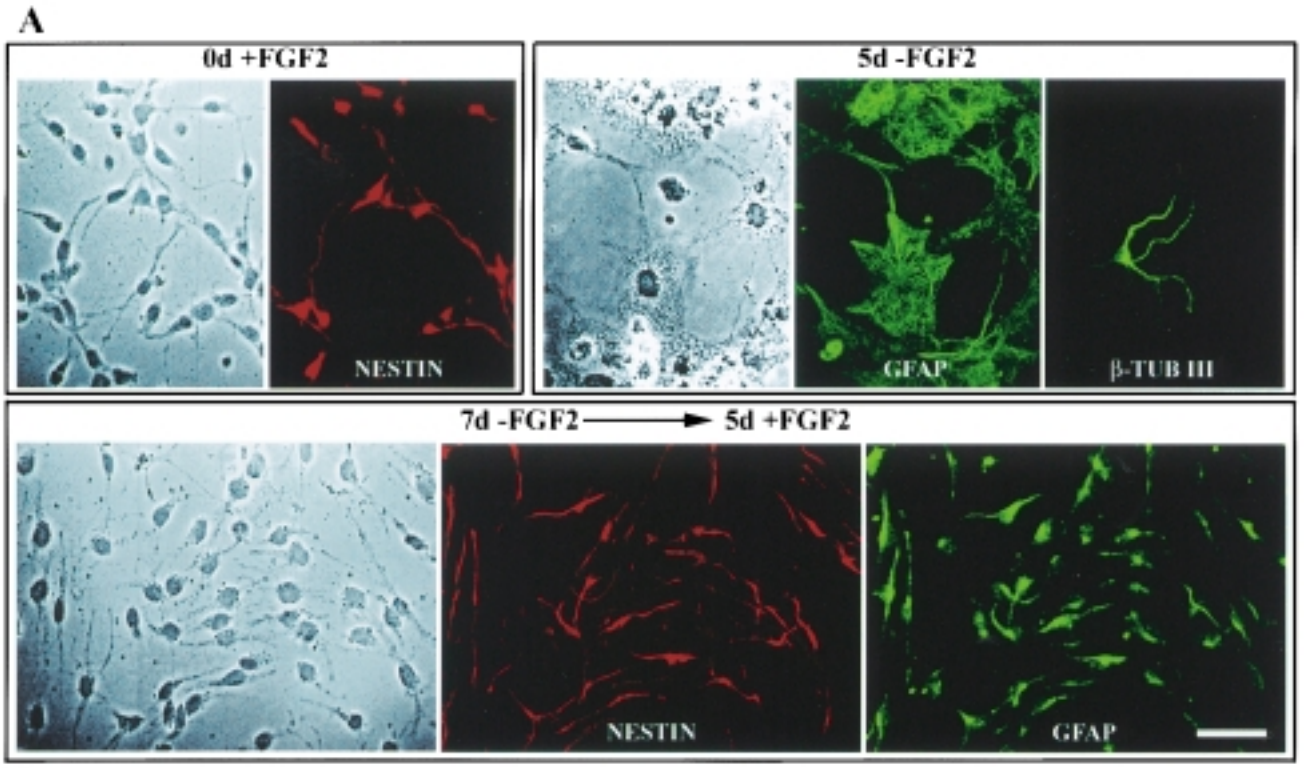

FIG. 2. Phenotype and viability of vacuolated cells. (**A**) Most of the NPC-derived cells expressed Nestin after 12–24 h in the presence of FGF2 (0d +FGF2). After 5 days of FGF2 withdrawal, the majority of cells expressed GFAP and only few cells expressed β-tubulin III. Only GFAP-positive cells developed a vacuolated cytoplasm (5d −FGF2; left and middle pictures show the same field). Readdition of FGF2 to the culture medium significantly reduced the number of vacuolated cells as described in Fig. 1C, and the resulting cells co-expressed Nestin and GFAP within low cell density areas of culture plate (7d −FGF2 → 5d +FGF2; the three pictures show the same field). Scale bar, 50 μm. (**B**) Vacuolated cells stained with Calcein AM and were impermeable to the EthD-1 (left). Additionally, vacuolated cells did not show an intense DAPI signal (middle, white arrows) and excluded Trypan Blue (right, white arrows). The upper row shows the phase-contrast photomicrographs for every staining procedure. Scale bar, 50 μm.

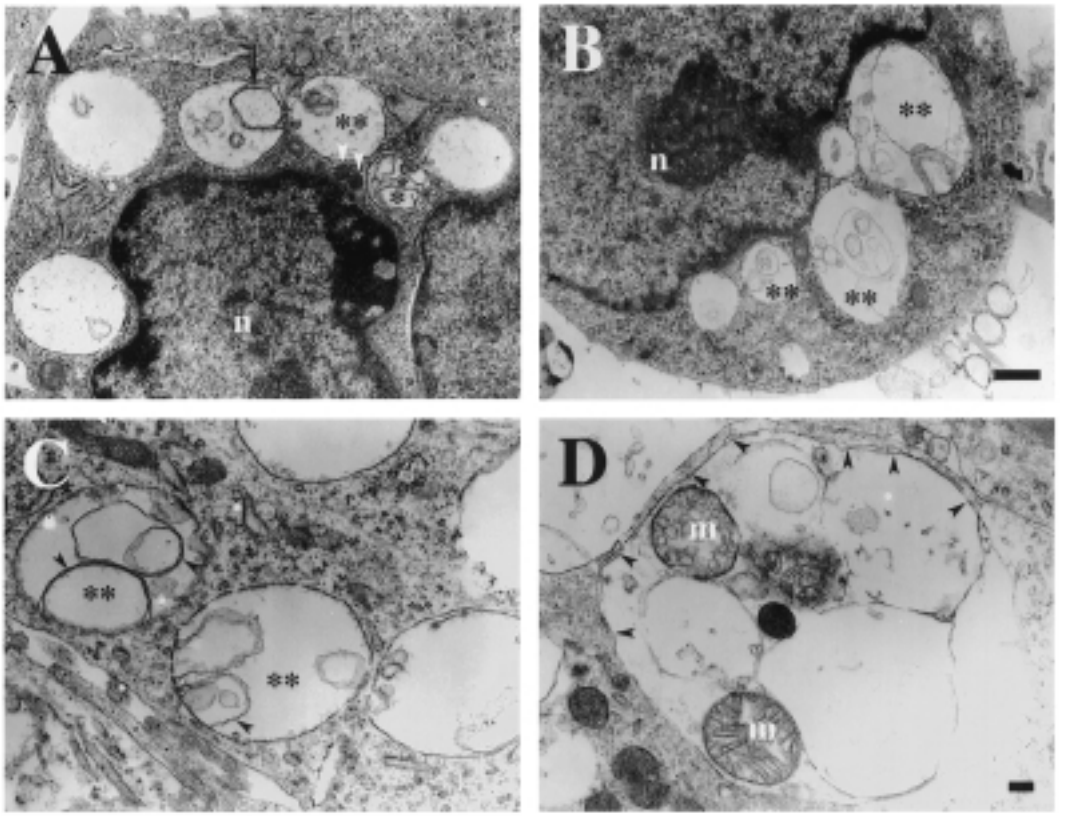

FIG. 3. Vacuolated cells show autophagic characteristics. Electron microscopy studies were carried out to characterize the vacuoles developed in our cultures. **A** and **B** (scale bar, 500 nm) are low-magnification electron micrographs showing many vacuoles containing cytoplasmic debris (some marked by a double asterisk). These single membrane vacuoles share some characteristics with the degradative vacuoles or autolysosomes observed during autophagy. Some single-membrane vesicles inside these vacuoles may represent the autophagic bodies generated after autophagosome fusion with a lysosome or a vacuole. The single asterisk marks what appears to be an autolysosome at an early stage. The black arrow in **A** points to a vacuole apparently engulfing another vacuole, and the white arrowheads point to lysosomes. Observe also the involuting nucleus (n) in **A** and **B**, a characteristic of autophagic cells. **C** and **D** (scale bar, 200 nm) show a closer view of some vacuoles. It is interesting to observe that some vacuoles contained double-membrane vesicles (black arrowheads point to the double membrane in these vesicles), which could arise from the ability of some vacuoles to engulf other vacuoles. The large vacuole appeared to have a double membrane in some areas (black arrowheads). This vacuole contained several vacuoles carrying cellular debris and several mitochondria (m) inside.

shown). These data suggest that Beclin1 activation is involved in the autophagy process initiated in NPCs by FGF2 withdrawal.

DISCUSSION

Morphological and biochemical evidence suggests that autophagy-like processes are among the physiological pathways for active self-destruction (11,21). Cells derived from NPCs displayed autophagic-like characteristics after FGF2 withdrawal. We observed double-membrane vacuoles containing cytoplasmic debris including mitochondria. However, we were unable to identify nascent or just-formed autophagosomes, which appeared to be due to the rapid vacuole development after FGF2 withdrawal. In agreement with this possibility, autophagosomes have been detected 15 min after autophagy activation (22). Vacuoles inside other vacuoles were observed frequently. It has been proposed that fusion between vacuoles and endosomes, and finally with lysosomes, occurs during autophagy (26,43). Surprisingly, we detected what appeared to be vacuoles engulfing other vacuoles. This unprecedent observation accounts for the appearance of double-membrane vacuoles inside larger vacuoles. Either vacuole fusion or vacuole engulfing may explain the rapid development of large vacuoles in some cells. An additional interesting observation is the appearance of single large vacuole in every degenerating cell that might represent the ‘central vacuole’ observed in some cell types and under certain conditions (44,45). In yeast, the central vacuole appears to be associated with

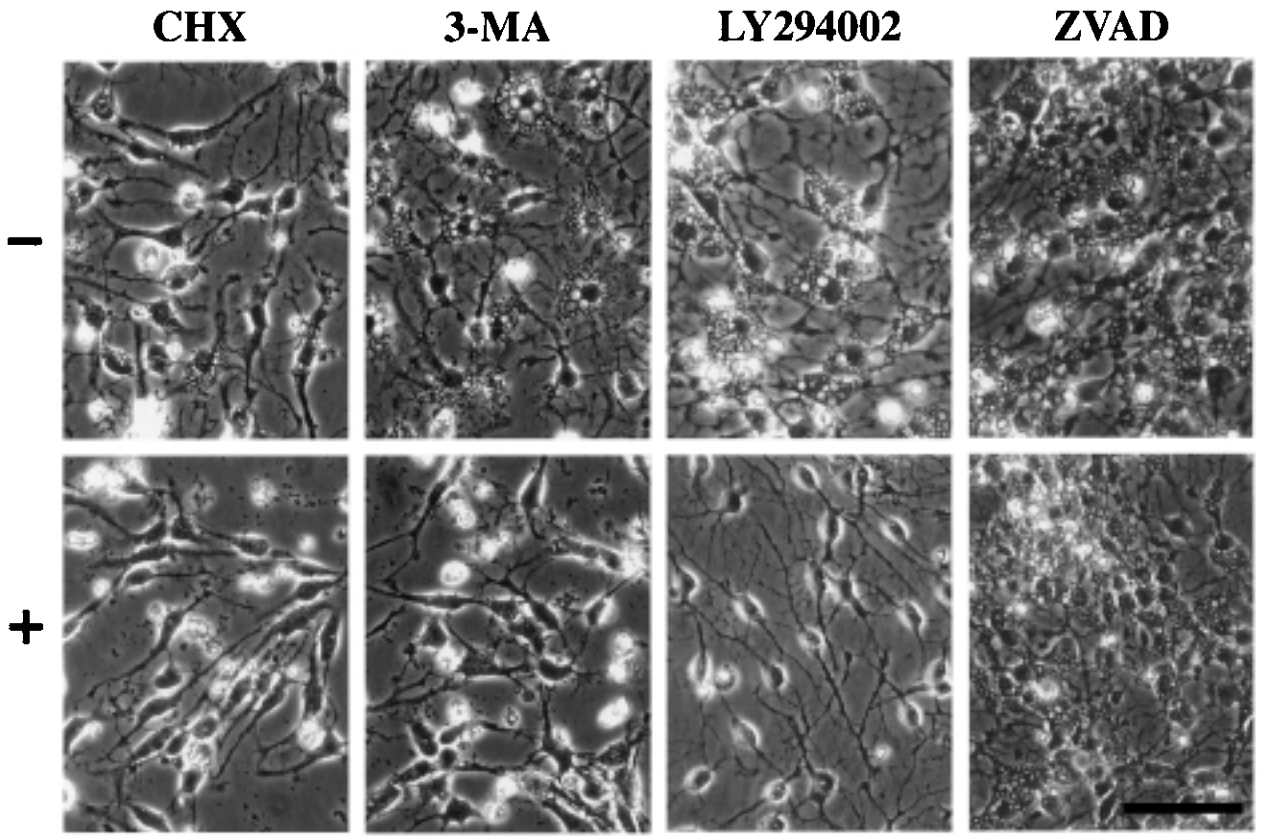

FIG. 4. Typical autophagy inhibitors prevent vacuole development in NPC-derived cells cultured in the absence of FGF2. NPCs were cultured in the presence of FGF2 for 24 h. At the time of FGF2 withdrawal, cells were cultured in the presence of cycloheximide (CHX) for 12 h, and 3-MA or LY294002 for 24 h. After washing, culture continued for 3 days in the absence of FGF2 to promote vacuole development. The caspase inhibitor zVAD was added after FGF2 withdrawal and maintained for the whole culture length (3 days). Cultures were also performed with the inhibitor vehicle alone (data not shown; see Table 1). At the end of culture, cell morphology was analyzed by phase-contrast microscopy. As shown in these photographs, CHX, 3-MA, and LY294002 inhibited vacuole development but not zVAD. At least three independent experiments gave a similar result.

autophagy (46), representing the mechanism for final degradation of sequestered cytoplasm. To our knowledge, our data show the first reproducible generation in mammalian cells of such 'central vacuole' during autophagy.

Although autophagy was originally studied as a conserved inducible pathway for general turnover of cytoplasmic components (47), it is now considered a relevant pathway for cellular degeneration (11,21). Autophagy has been observed during development of different organisms, particularly in regions where abundant cell death occurs (48–52), thus suggesting that it contributes to programmed cell death (PCD). Our data show that autophagic cells derived from NPCs, at the stage where most cells contained the central vacuole referred above, could reverse all autophagic morphological characteristics to normal when cells were cultured again in the presence of FGF2. However, autophagic cells clearly degenerated, which was estimated by the evident loss of cells from the plate and by following individual autophagic cells (Fig. 1). Because death of autophagic cells appeared to occur asynchronously in our cultures, it was difficult to determine when they were irreversibly compromised to die. Future work should be directed to identify this critical stage.

Cell degeneration by autophagy does not use the same execution machinery used by apoptotic cells. Caspases, enzymes considered essential for the execution of apoptosis, are active in autophagic cells (52). However, as demonstrated in our study, autophagy is not prevented by caspase inhibitors (see also Refs. 18,53). Conversely, in the developmental process in which PCD appears to be mainly executed by apoptosis, it has been observed that reduction in caspase activity, by enzyme inhibition (54) or by null mutations in genes coding triggering caspases (e.g., caspase 9, caspase 3) (55), does not prevent cell degeneration but, instead, PCD is now executed by autophagy. These observations indicate that PCD regulators can activate different death mechanisms, either of

TABLE 1. QUANTITATIVE ANALYSIS OF AUTOPHAGY INHIBITORS OR *BCL-2* OVER-EXPRESSION ON VACUOLE FORMATION IN NPCS CULTURED IN THE ABSENCE OF FGF2

Treatments	*% of cells without vacuoles (± SD)*	*% of cells with vacuoles (± SD)*
+ FGF2 3d	95.1 ± 0.7	4.9 ± 0.7
− FGF2 3d	16.2 ± 8.4	83.8 ± 8.4
+ FGF2 3d + *hBcl2*	86.1 ± 5.7	13.9 ± 5.7
− FGF2 3d + *hBcl2*	88.5 ± 4.4	11.5 ± 4.4
+ DMSO (0.25%)	9.0 ± 2.5	91.0 ± 2.5
+ zVAD (50 μM)	5.9 ± 3.0	94.1 ± 3.0
+ zVAD (100 μM)	6.1 ± 1.6	93.9 ± 1.6
+ LY294002 (20 μM 24 h)	52.3 ± 9.0	47.5 ± 8.8
+ FGF2 5d	99.7 ± 0.4	0.4 ± 0.4
− FGF2 5d	5.2 ± 3.6	94.8 ± 3.6
− FGF2 5d + 3-MA (20 mM 24 h)	94.6 ± 2.7	5.4 ± 2.7
+ ETOH (0.5% 12 h)	34.4 ± 12.0	65.6 ± 12.0
+ CHX (0.5 μg/ml 12 h)	88.9 ± 2.9	11.1 ± 2.9
+ ETOH (0.5% 20 h)	28.4 ± 12.8	74.2 ± 10.1
+ CHX (0.5 μg/ml 20 h)	83.0 ± 11.3	17.0 ± 11.3

Treatments were done as described in Fig. 4. The data show the average percent of vacuolated or nonvacuolated NPC-derived cells from a minimum of five experiments at each condition. Dimethylsulfoxide (DMSO) was the vehicle for zVAD and LY294002 and ethanol (ETOH) was the vehicle for cycloheximide (CHX). The final inhibitor or vehicle concentration in the medium is indicated. Three-methyl-adenine (3-MA) was dissolved in water. SD, standard deviation.

which can take over cell degeneration (see below). Under certain circumstances, apoptosis dominates in autophagy, perhaps because it is the fastest death execution mechanism; but weight balance between these two death mechanisms can change, for instance, when caspases are inactivated (54,55).

Bcl-2 is a typical antiapoptotic molecule whose overexpression, rather than blocking apoptosis, normally slows down the appearance of apoptosis indicators (56, and references therein). Interestingly, in contrast with the results obtained with the caspase inhibitor, *Bcl-2* overexpression was able to retard vacuole development after FGF2 withdrawal from NSC cultures. The anti-autophagic effect of Bcl-2 is supported by a recent report showing that *Bcl-2* down-regulation causes caspase-independent autophagy in human leukemia HL60 cells (18). Under the view referred to above, *Bcl-2* could be considered an upstream signal regulating a key step toward death activation by different mechanisms (e.g., apoptosis or autophagy; see below). Recently, on the basis of the lymphocyte numbers obtained in mice lacking a complete apoptosome and those over-expressing Bcl-2, it was concluded that Bcl-2 regulates a death program independently of the apoptosome (57). In this latter study, occurrence of autophagy was not determined, but caspases were still considered to be involved. We favor the idea that, during development, different death mechanisms cooperate for the secure and efficient removal of cells in specific processes.

How does Bcl-2 regulate autophagy? Beclin1 was originally identified as a Bcl-2-interacting protein (42), whose corresponding gene is homologous to Apg6/Vps30, a recognized yeast autophagy gene. *Beclin1* promoted autophagy when over-expressed in yeast and in MCF-7 breast carcinoma cells (41). *Beclin1* is ubiquitously expressed in adult tissues (42) and in embryonic tissues including cells within the neural lineage (Fig. 5 and data not shown). Therefore, Beclin1 autophagic activity could be regulated by direct protein modifications or by interaction with different proteins. Apg6/Beclin1 is a subunit of the autophagic specific class III PI3K complex constituted by Apg14, Vps34, and Vps15 with a role in autophagosome formation (58). Phosphatidyl inositol 3-phophate has been shown to be essential for autophagy in yeast and mammalian cells (59). Furthermore, a common autophagy characteristic is the ability to prevent it by PI3K inhibitors, such as the ones used in the present work (3-MA and LY294002) (21,36,37). The fact that these inhibitors, as well as cycloheximide, were only required during the first 24 h after FGF2 withdrawal to prevent vacuole appearance, is an indication that they are acting at the triggering stage of autophagy. Apg6/Beclin1 could be essential for PI3K complex activity. Therefore, retarded autophagy in cells over-expressing Bcl-2 could

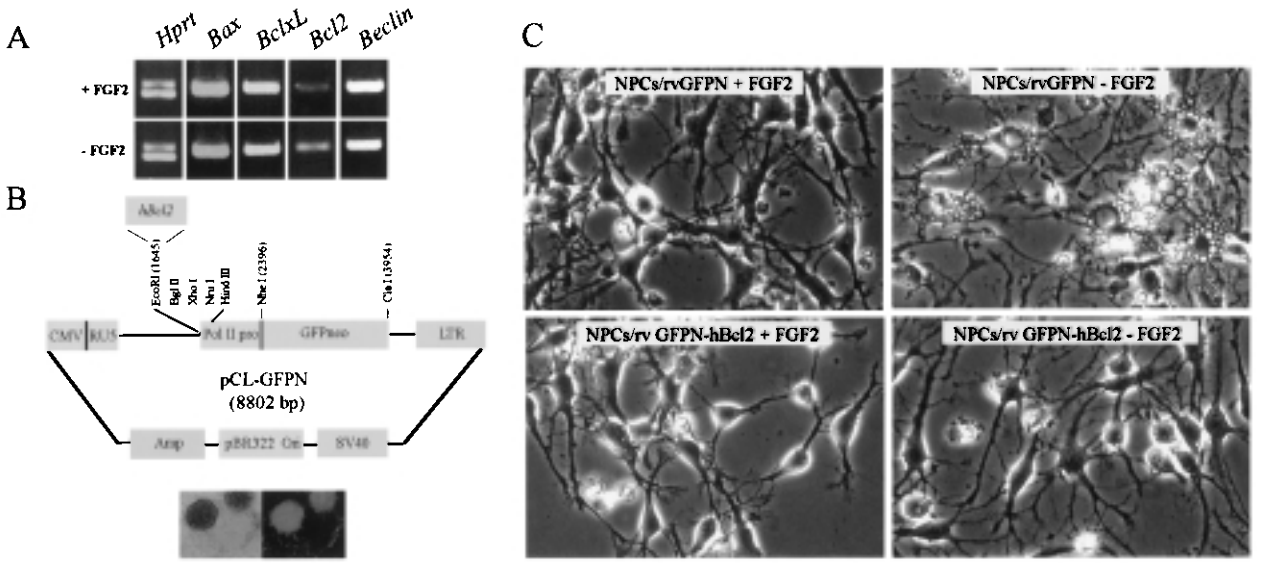

FIG. 5. Bcl-2 over-expression prevent vacuole development. (**A**) *Bax, Bcl-xL Bcl-2*, and *Beclin* expression in NPC-derived cells cultured in the presence or absence of FGF2 for 5 days. Gene expression was determined by RT-PCR; *Hprt* expression was used to confirm that similar amount of RNA from cells in each condition was present in RT-PCR procedures. FGF2 withdrawal did not promote significant changes in the expression of genes tested. (**B**) Endonuclease restriction map of pCL-GFPN retroviral vector showing the site where the *hBcl-2* coding sequence was inserted. Photographs below the plasmid map show typical infected neuropheres obtained with the infection protocol described in Materials and Methods (left, phase contrast; right, epifluorescence). (**C**) Morphology of cells derived from NPCs infected with either rvGFPN (*top panels*), or rvGFPN-hBcl2 retrovirus (*bottom panels*), cultured in the presence (+) or absence (−) of FGF2 for 5 days. Observe that cells infected with the retrovirus carrying hBcl-2 did not develop vacuoles in the absence of FGF2.

be due to Beclin1 sequestration by Bcl-2 causing a reduction in the PI3K activity required at very early autophagy stages. Recently, another Bcl-2-interacting protein with the capacity to induce autophagy was described (53).

In parallel with the regulation of the proapoptotic activity of Bax, Bcl-2 could regulate Beclin1 autophagy-promoting activity controlling, like a rheostat, the death cell fate. We propose that Bcl-2 and presumably other Bcl-2 family members with antiapoptotic activity represent key molecules where different signals converge to control cell survival. However, the mechanism that the cell uses to kill itself depends on additional factors acting downstream within the specific pathway that regulates apoptosis or autophagy. This hypothesis is supported by the fact that opening of the mitochondrial permeability transition pore occurs in autophagic cells, an event that has been associated with apoptosis (60,61). Similarly, lysosomal enzymes appear to be implicated early in apoptosis (62). Due to the rapid caspase-mediated cell death execution, it would be expected that in the presence of a functional apoptosis pathway evident autophagy characteristics would not be detected. However, in the presence of a 'weak' functional apoptosis pathway or when apoptosis is experimentally inhibited, autophagy characteristics would be disclosed (54,55).

In general, autophagy is experimentally induced by gross changes in culture conditions such as starvation, serum deprivation, and several types of cellular stress (21,47). In the present work, autophagy was triggered by the removal of a single growth factor, FGF2, condition that is only similar to the activation of autophagy by TNF-α (63). Autophagy activation by the presence or absence of a single growth factor opens the question of how a specific receptor signaling cascade targets the autophagy machinery. On the basis of the above discussion, we consider that Bcl-2 antiapoptotic family members are possible targets of growth factor death regulation. It has been reported that *Bcl-2* expression can be up-regulated by an ERK-dependent pathway activated by FGF (32), but in our case, changes in Bcl-2 mRNA levels were not detected upon FGF2 withdrawal from the culture medium, although regulation at the protein level still needs to be determined. Alternatively, growth factors could directly regulate autophagy by modulating the activity of the class III PI3K complex. Although not classified as classical autophagy, two recent reports describe the formation of vacuoles in dying cells by activation of two different specific signaling pathways (12,64). The inability of antipototic Bcl-2 family members to inhibit vacuole formation in these latter two studies suggests that these pathways are ei-

ther activating autophagy downstream Bcl-2 or activating a distinct nonautophagy mechanism.

We observed that, concurrent with autophagy activation, NPC underwent differentiation into astrocytes. Even in the presence of FGF2, astrocytes were the major cell type detected in our conditions after 5 days in culture (data not shown). This pattern also occurred in the absence of FGF2, suggesting that differentiation was not affected by autophagy activation and is not determined by FGF2 in these cells. However, when autophagy was reversed by readdition of FGF2, astrocytes were detected in areas of local high cell density, whereas cells expressing both nestin and GFAP were almost exclusively detected in areas of local low density. This latter cell population may encompass cells that did not terminally differentiate into astrocytes after FGF2 withdrawal. It has been determined that NPCs at high density are prone to differentiate into glial cells (35). In addition, Rajan et al. (65) recently reported that glial differentiation under this latter condition also involves bone morphogenetic protein-4 (BMP4). Glial differentiation by BMP4 is mediated by a novel pathway involving FRAP (FKBP12/rapamycin-associated protein), a protein identical to the mTOR (mammalian target-of-rapamycin). On the other hand, rapamycin, through its inhibitory activity on mTOR, is a very well-known generic autophagy inducer; thus, mTOR act as an autophagy suppressor (66). It is thought that the class III PI3K complex negatively regulates mTOR, releasing suppression of autophagy. Interestingly, rapamycin blocks glial differentiation by BMP4 (65). It has been proposed that the serine-threonin kinase mTOR inhibits autophagy by inhibiting the activity of a phosphatase (67), whereas mTOR promotes glial differentiation by phosphorylating Stat3 (65). Therefore, we conclude that autophagy and glial differentiation are two independent process occurring simultaneously in NPCs grown in the absence of FGF2; however, glial differentiation could be interrupted due to mTOR inhibition in autophagic cells.

Autophagy is being recognized as relevant in developmental processes as well as in diseases (26). Like genes regulating apoptosis, some genes that regulate autophagy have been identified as tumor suppressors in humans (41,68). In addition, neuron degeneration in several neurodegenerative syndromes resembles autophagy rather than apoptosis (15,16). The identification in the present study of Bcl-2 as an upstream regulator of autophagy could be of importance in the design of therapeutic procedures based in the activation or inhibition of cell death.

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Competence and plasticity of neural stem cells: insights from pattern formation in the nervous system

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Introduction

In developmental biology, cell competence is defined as the capacity to respond to an inductive signal. Competence depends on the expression of specific transcriptional factors that indirectly sensitize the cell to a particular stimulus (Edlund and Jessell, 1999). These inductive signals are usually secretable molecules that activate transductions cascades inside the cell through an autocrine or paracrine mechanism. An external inducer is referred as “instructive” when changes gene expression, whereas “permissive” signals maintain gene expression in already specified cells in order to assure proper differentiation and maturation. Secretable molecules are called “morphogens” when induce different changes in gene expression according to their local concentration in the embryo (Ashe and Briscoe, 2006).

The integration of multiple instructive signals over a cell eventually determinates its final identity (Anderson, 2001). Hence, cell competence might also be defined as the capacity to acquire a specific fate. In general, competence to generate a cell lineage is an ephemeral property that appears within undifferentiated cells, although some differentiated cells are able to regain competence through genetic manipulation and eventually trans-differentiate (Odelberg et al., 2000; Zhou et al., 2008).

During development, competence to produce the extremely vast and diverse array of neural subtypes within the mammalian nervous system (NS) might initiates around the time of the neural plate (NP) induction. At this point, most stem cells at the medial ectoderm have differentiated into neural stem/progenitor cells (NPCs). Differential gene expression along the rostrocaudal and mediolateral axes of the NP initiates regionalization of NPCs, a phenomenon that leads to neuronal subtype production in stereotyped positions (Jessell, 2000; Lumsden and Krumlauf, 1996; Muhr et al., 1999).

In recent years, much hype has been raised for the use of NPCs as a source of new transplantable neurons for the treatment of neurodegenerative diseases (Murry and Keller, 2008). NPCs have been isolated from the embryonic and adult NS and propagated in culture (Baizabal et al., 2003). Furthermore, NPCs as well as their neuronal and glial derivatives arise completely in vitro after differentiation of Embryonic Stem Cells (ESCs)(Cai and Grabel, 2007; Zhang, 2006). Substantial evidence suggests that along the differentiation of ESCs into the neural lineage, competence to produce a neuronal subtype is initially gained and then progressively lost (Baizabal and Covarrubias, 2009; Bouhon et al., 2006; Li et al., 2005; Parmar and Li, 2007). Yet, the populations of NPCs susceptibles to respond to inductive signals are only starting to be defined. Besides, another equally important issue is the molecular mechanisms that confer competence. In this matter, studies on pattern formation in the NS are continually providing important knowledge that opens the possibility of translating this information to a culture dish in order to control NSPC differentiation with a higher efficiency.

Early restrictions on NPCs competence and plasticity

The neural plate is formed of long undifferentiated neuroepithelial (NE) cells that contact the basal membrane (BM) and the ventricular surface (Merkle and Alvarez-Buylla, 2006). Around embryonic day (E) 9/E10, just before the beginning of neurogenesis in the neural tube (NT), NE cells differentiate into radial glial (RG) progenitors (Pinto and Gotz, 2007). Similar to NE cells, the RG population maintains contact with the apical (i.e. ventricular) and basal (i.e. pial) surfaces and produce most neurons in all regions of the early NT (Anthony et al., 2004). Later on development, RG cells switch their fate to produce glial (i.e. astrocyte or oligodendrocyte) lineages. To some extend, NPCs autonomously maintain this neuronal to glial differentiation bias as

divide in culture (Qian et al., 2000; Ravin et al., 2008). In the embryonic brain, extracellular factors like Fgf2 and CNTF modulate the transition of multipotent NPCs from neuronal to glial competence (Morrow et al., 2001).

Developmental programs that orchestrate the sequential fate restrictions of NPCs operate at two main levels. At one level, NPCs must "decide" whether to become a neuronal or glial cell, whereas the other level of "decision" operates within lineages and involves the acquisition of subtype-specific features (Fig. 1). Although these two levels of fate choices might work in parallel in some cases, they do not necessarily dependent on each other. Thus, NPCs referred as multipotent for their ability to produce neurons, astrocytes and oligodendrocytes are usually able to differentiate into a very limited number of neuronal subtypes (Fig. 1). As one example, multipotent NPCs propagated in vitro as neurospheres (i.e. proliferative cell aggregates) appear restricted to produce small interneurons when derived from the forebrain and projection neurons when isolated from the midbrain (Ostenfeld et al., 2002). In addition, forebrain and cerebellar multipotent neurospheres are competent to produce several neuronal subtypes from their respective regions of origin but not from other regions of the NT (Klein et al., 2005). Not surprisingly, NPCs isolated from the adult subventricular zone (aSVZ) behave as multipotent cells in vivo and in culture (Rietze et al., 2001); yet, within the neuronal lineage, their plasticity appears to be restricted to olfactory bulb (OB) interneuron fates (Merkle et al., 2007; Milosevic et al., 2008).

The evidence described above suggests an early restriction in neuronal competence of multipotent NPCs during mammalian NS development. However, it is not clear yet how early these neuronal restrictions are imposed and how broad is the developmental potential of NPCs before they circumscribe their fates to a limited number of neuronal subtypes. Transplantations studies have provided some insights about this matter

through the analysis of NPCs differentiation at ectopic locations (Baizabal et al., 2003; Gaiano and Fishell, 1998). As culture of NPCs might change their molecular/cellular identity and differentiation potential (Kalyani et al., 1998; Mignone et al., 2004; Santa-Olalla et al., 2003; Winkler et al., 1998; Yang et al., 2000), it is preferable to transplant uncultured cells when analyzing the timing of developmental restrictions occurring in the NS. Using this approach, some reports indicate that around midgestation in mice, most NPCs are restricted to region-specific fates (Carletti et al., 2002; Carletti et al., 2004; Na et al., 1998; Olsson et al., 1998); whereas other studies support a higher plasticity of donor cells (Olsson et al., 1997; Vicario-Abejón et al., 1995). Comparison of this data is particularly difficult as donor cells from different origins were grafted into diverse niches at several developmental stages However, it is relatively clear that restrictions are continually imposed on NPCs as development advances (Grimaldi et al., 2005). A good example about this comes from cerebral cortical development, where the sequential set up of cell layers allowed to determinate that NPCs produce laminar fates of older, but not younger, environments (Desai and McConnell, 2000). Besides, embryonic cerebellar NPCs generate “early” and “late” born cerebellar neurons, while postnatal cerebellar NPCs exclusively acquire the latter identities (Carletti et al., 2002).

Restrictions on NPCs at midgestation indicate that commitment to specific fates might arise even before the onset of neurogenesis. On this respect, telencephalic NPCs incorporated into the mesencephalon behave as compromised progenitors when isolated from the NT around E10.5 (Baizabal, et. al. 2009). Instead, donor cells at the same niche differentiate into midbrain dopaminergic (mDA) neurons when isolated from NP stages (i.e. around E8.0) (Baizabal, et. al. 2009). Hence, it is possible that high neuronal plasticity is a property present only within very early neuroepithelial cells. As no other transplantation study compares the differentiation potential of early NPCs (i.e. isolated

between E8/E10), it is necessary to look toward pattern formation in the early NS to provide further support to this idea. To set an example, it is known that only before the formation of the NT (stage 12 in chicks) ventral cells in the NP are competent to respond to Shh and to differentiate into floor plate (FP) cells and motoneurons in the spinal cord (Ericson et al., 1996). Along more rostral levels of the NP, ectopic serotonergic neurons appear in the prospective midbrain region when treated with Fgf4 before the 10 somites stage in rat embryos (Ye et al., 1998). Furthermore, ectopic dopaminergic neurons develop after Shh and Fgf8 treatment at the E9 NP, whereas Fgf8 alone is able to induce *Bf1* expression in the anterior NP up to the 6 somites stage in rat embryos (Ye et al., 1998).

If NPCs with a high developmental plasticity emerge at early stages of NP development, then it might be suggested that competence to interpret a wide range of signals arises around the time of neuralization of ectodermal cells. Important lessons about this issue have recently originated from ESCs differentiation into diverse neural subtypes in culture (Zhang, 2006). This process involves intermediate steps of ESCs differentiation into ectodermal and neural precursors (e.g. RG and NE-like cells) that eventually leads to terminal neuronal differentiation (Cai and Grabel, 2007; Desbaillets et al., 2000; Gotz and Barde, 2005; Lee et al., 2000; Rathjen et al., 2002; Rathjen and Rathjen, 2001). Substantial evidence indicates that competent cells appear early as ESCs enter into the neural lineage. Thus, dopaminergic neurons, telencephalic neurons and motoneurons are more efficiently generated from ESCs in culture when patterning signals like Shh, Fgf8, Wnt and RA are added to ESC-derived ectodermal or primitive neuroectodermal cells (Li et al., 2005; Pankratz et al., 2007; Watanabe et al., 2005; Yan et al., 2005). In contrast, when primitive neuroectodermal cells advance into definitive neuroepithelial cells, a concomitant decrease in competence to produce specific

neuronal lineages is observed (McCarthy et al., 2001; Watanabe et al., 2005). The restriction of ESCs to respond to patterning signals or the inductive activity of stromal cell lines, initiates close to the appearance of *Sox1* expression (Pankratz et al., 2007; Parmar and Li, 2007; Yan et al., 2005) a marker gene of NSCs that is expressed from E8 onward in the NP (Wood and Episkopou, 1999). A progressive limitation in the developmental competence of ESC-derived precursors is also observed when these cells are exposed to the niche of the embryonic brain. In this case, primitive ESC-derived Embrioid Body (EB) cells produce NSCs within the embryonic midbrain milieu; a niche that eventually directs specification of donor cells into several lineages, including mDA neurons, at stereotypic positions (Baizabal and Covarrubias, 2009). EB cells also produce region-specific neurons at the ventral spinal cord and dorsal root ganglia in the chick embryo (Plachta et al., 2004). Yet, prolonged in vitro neuralization of EB cells (with and without RA) prior to transplantation significantly reduced their competence to interpreted the niche of embryonic neurogenesis (Baizabal and Covarrubias, 2009; Plachta et al., 2004). Hence, only ESC-derived NPCs at early stages of differentiation posses high competence and plasticity; resembling somehow those precursors at the early NP.

In summary, the current perspective suggests that within the neural lineage, early NP precursors are highly malleable and even capable of interpreting foreign developmental contexts that are not part of their normal ontogeny. Later on development, restrictions on competence and plasticity rapidly emerge, probably even before the onset of neurogenesis. By midgestation, some NPCs still generate multiples fates but these are confined to neuronal subtypes present within ontogeny-related regions. Reaching postnatal ages and adulthood, NPCs narrow down their choices a bit further and mostly generate “late-born” neurons and region-restricted neurons, respectively.

- Molecular mechanisms of NPCs competence

Although the molecular mechanisms that confer competence on NPCs are in general unknown, here we review some findings that together could provide a theoretical and experimental framework in the near future. The information gathered in this topic has been obtained from diverse NPCs populations and therefore might seem fragmentary at first. Yet, it is expected that competence mechanisms share some general principles across cell lineages.

During multiple cell divisions, NPCs undergo dynamic changes in their sensibility to environmental signals. Some NPCs maintain competence to respond to specific signals as development advances and even in the adult. Yet, based on studies of cell plasticity (see above) the capacity of NPCs to interpret those cues within diverse environmental contexts rapidly declines during development (Fig.?). This phenomenon implies that a particular signal could elicit radically different effects depending on the cell it reaches. Taking Shh signaling as an example, this morphogen promotes cell differentiation, proliferation, migration and death. This repertoire of interpretations to the same molecule depends on the intrinsic status of the affected cells, determined by its own combination of transcription factors, signal transducers and receptors.

The broad developmental plasticity of early NPCs at the NP (see above) likely reflects a more permissive state to change the balance of intrinsic determinants under the influence of external signals. A nice example about this comes from studies on NPCs positional identity at the ventral NT. In the ventral midline of the SC, NPCs progressively change their gene expression and identity. The sequence observed is: dorsal progenitors (Pax7+), motoneurons progenitors (Olig2+), V3 interneurons progenitors (Nkx2.2+) and finally FP progenitors (Foxa2+) (Dessaud et al., 2008). This fluctuation in gene expression at the ventral SC is the product of the temporal and

spatial integration of the Shh morphogen gradient by FP progenitors (Dessaud et al., 2007).

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