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And
Division of Physiology and Neuroscience
Department of Biosciences
Faculty of Biological and Environmental Sciences
Doctoral Program Brain & Mind (B&M)
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ON THE MECHANISMS OF NEURAL DEVELOPMENT IN THE VENTRAL TELENCEPHALON

Ana Cathia Dias Magalhães

ACADEMIC DISSERTATION

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SUPERVISOR

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Neuroscience Center
University of Helsinki
Helsinki, Finland

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Marseille, France

CUSTOS

Professor Juha Voipio, PhD
Department of Biosciences
University of Helsinki
Helsinki, Finland

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LIST OF ORIGINAL PUBLICATIONS

This Thesis is based on the following original articles, which are referred to in the text by their Roman numerals:

I **Magalhães AC** and Rivera C. Superior performance of decloaking chamber-based heat-induced epitope retrieval method improves the quantification of Olig2 cells in paraffin-embedded section of embryonic mouse brain (2014) J Neurosci Methods 235: 226–233.

II **Magalhães AC** and Rivera C. *In vivo* role of NKCC1 in ventral telencephalon cell cycle reentry. Manuscript.

III Bespalov MM, Sidorova YA, Tumova S, Ahonen-Bishopp A, **Magalhães AC**, Kulesskiy E, Paveliev M, Rivera C, Rauvala H, and Saarma M. Heparan sulfate proteoglycan syndecan-3 is a novel receptor for GDNF, neurturin, and artemin (2011) J Cell Biol 192(1): 153–169.

Author's contribution to the original publications included in the Thesis:

I: Planning and performing all the experiments, analyzing all the data, and writing the manuscript

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III: Planning and performing the experiments, and analyzing the data for figure 6, and contributing to the writing of manuscript (Materials and Methods, Results, and Discussion sections)

ABSTRACT

The time of arrival of interneurons and oligodendrocytes to the neocortex is critical for proper functional brain development. Aberrances in this sequence can be detrimental, and involved in different developmental diseases. Thus, understanding the mechanisms for temporal control of the genesis and migration of neural cells is crucial. In this study we have focused on the ventral telencephalon, a major source of interneurons and oligodendrocytes. We have developed a more sensitive method for detecting and quantifying oligodendrocyte precursor cells, e.g. Olig2. Current immunohistochemistry methods for detection and quantification of this cell type are poor. Optimal immunohistochemistry often requires heat-induced epitope retrieval (HIER) method for improving the staining in paraffin-embedded sections, and therefore helping in quantitative studies. Here, we have developed an immunohistochemistry protocol using the device decloaking chamber for HIER. This method was compared to the microwave oven-based HIER method by studying the labeling of Olig2 marker in paraffin-embedded sections from embryonic mouse brain. We have demonstrated that the decloaking chamber-based HIER method is the most suitable technique for the detection of single Olig2-labeled cells in the ventral telencephalon. This qualitative result was reflected in the quantitative analyses: more Olig2-labeled cells were quantifiable with the decloaking chamber- than with the microwave oven-approach. Olig2 labeled-cells were quantified manually and automatically: both methods gave similar results, thereby confirming the efficiency of the latter method. Thus, the decloaking chamber-based HIER method constitutes a sensitive technique for the detection of oligodendrocyte precursor cells, and therefore for its quantification in the developing ventral telencephalon.

The development of telencephalon depends on fundamental processes, which include proliferation and migration of neural cells. The Na-K-Cl cotransporter isoform 1 (NKCC1) is an important protein for the process of volume regulation, and has been implicated in cell division of different cell types. Within the developing brain, the ventral telencephalon showed the highest expression of NKCC1. In this study we have characterized the expression of NKCC1 in the lateral ganglionic eminence (LGE), and identified that NKCC1-expressing cells corresponded to neural progenitor cells. Using NKCC1 knockout mice, we have studied the function of NKCC1 in cell proliferation by monitoring the cell cycle at early stage of neurogenesis. We demonstrated that NKCC1 influenced the cells to reenter the cell cycle, instead of exiting and differentiating. We, further, analyzed the expression of several markers for neural precursor cells

in the LGE. Mice lacking NKCC1 have impaired Sp8-expressing interneurons and Olig2-labeled cells at later embryonic stage. Thus, NKCC1 is crucial *in vivo* for cell cycle decision, thereby altering the production of oligodendrocyte and interneuron progenitor cells in the LGE.

Once interneurons are born, they migrate to the neocortex. We have studied the implication of syndecan-3 in their tangential migration. Here, we have found that the Glial cell line-derived neurotrophic factor GDNF interacts with syndecan-3 to promote the tangential migration of calbindin-expressing interneurons within the telencephalon. Consistently, mice lacking syndecan-3 have an accumulation of migrating interneurons in the LGE.

Taken together, in this study, we have found two important mechanisms for temporal and spatial control of cortical oligodendrocytes and interneurons.

LIST OF ABBREVIATIONS

AEP	Anterior entopeduncular area
ARTN	Artemin
Ast	Astrocyte
BDNF	Brain-derived neurotrophic factor
bHLH	Basic helix-loop-helix
bRG	Basal radial glial
CCC	Cation-chloride cotransporter
Cdk	Cyclin-dependent kinase
CGE	Caudal ganglionic eminence
$[Cl^-]_i$	Intracellular concentration of Cl^- ions
CP	Cortical plate
C-R	Cajal-Retzius
CR	Calretinin
dLGE	Dorsal lateral ganglionic eminence
E	Embryonic day
ECM	Extracellular matrix
EC	Ependymal cells
ERK	Extracellular signal regulated kinase
GABA	γ-aminobutyric acid
$GABA_A$-R	$GABA_A$-receptors
GDNF	Glial cell line-derived neurotrophic factor
GFL	GDNF family ligand
GFRα	GDNF family receptor α
HB-GAM	Heparin binding growth associated molecule
HIER	Heat-induced epitope retrieval
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycan
IHC	Immunohistochemistry
IP	Intermediate progenitor
IZ	Intermediate zone
KCC	K-Cl cotransporter
KO	Knockout
L	Layer
LGE	Lateral ganglionic eminence
MAPK	Mitogen-activated protein kinase
MGE	Medial ganglionic eminence

MZ	Marginal zone
N	Neuron
NC	Neocortex
NCAM	Neural cell adhesion molecule
NCC	Na-Cl cotransporter
NE	Neuroepithelial
NKCC	Na-K-Cl cotransporter
NPY	Neuropeptide Y
NRTN	Neurturin
NT4	Neurotrophin 4
OB	Olfactory bulb
OD	Oligodendrocyte
OPC	Oligodendrocyte precursor cell
POA	Anterior entopeduncular area
PP	Preplate
PSPN	Persephin
PV	Parvalbumin
RET	Rearranged during transfection
RG	Radial glial
RTK	Receptor tyrosine kinase
SAP	Subapical progenitor
Sema	Semaphorin
SFK	Src family kinase
SNP	Short neural precursor
SP	Subplate
SST	Somatostatin
SVZ	Subventricular zone
TGFβ	Transforming growth factor β
VIP	Vaso-intestinal peptide
VZ	Ventricular zone
VZ/SVZ	Proliferative zones
WM	White matter
WT	Wild-type

INTRODUCTION

1. The development of telencephalon in mouse

The nervous system arises from the neurectoderm. The neuroectoderm is transformed into the neural plate, whose lateral margins fold inward to form the neural groove, and then close to form the neural tube at around embryonic day (E) 9 (Figure 1). The posterior part of the neural tube gives rise to the spinal cord, while the anterior part gives rise to the brain. During development, the anterior part of the neural tube becomes first subdivided into three vesicles, the prosencephalon (future forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain), and later into five vesicles, the telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon (Figure 1). The rostral prosencephalon forms the telencephalon, which is subdivided into two symmetric telencephalic vesicles (Figure 1). These vesicles include dorsal and ventral territories, termed as pallium and subpallium, respectively (Figure 2). The dorsal telencephalon becomes the cerebral cortex, and the ventral telencephalon develops into the basal ganglia. The ventral telencephalon can be subdivided into lateral (LGE), medial (MGE), and caudal (CGE) ganglionic eminences (Marín and Rubenstein, 2003).

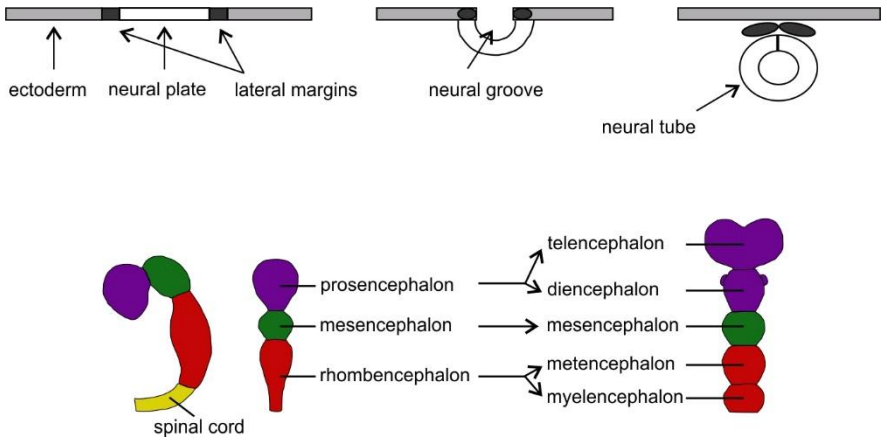

Figure 1. Development of nervous system. The lateral margins of the neural plate fold inward to form the neural groove, and then close to form the neural tube. The neural tube gives rise to the spinal cord (posterior) and brain (anterior). The neural tube is subdivided into three vesicles: the prosencephalon, mesencephalon, and rhombencephalon. Then, the neural tube is subdivided into five vesicles: the telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon.

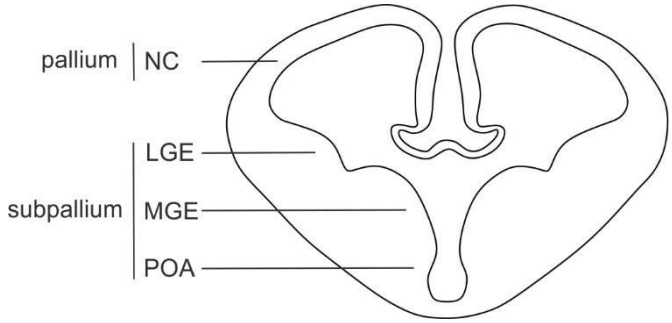

Figure 2. Schematic illustration of the telencephalon of an embryonic day 12.5 mouse. The telencephalon is subdivided into pallium (dorsal telencephalon) and subpallium (ventral telencephalon). NC, neocortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; POA, preoptic area.

1.1. Neural development within the telencephalon

The telencephalon contains the ventricular (VZ) and subventricular (SVZ) zones, which correspond to the proliferative zones (VZ/SVZ). VZ/SVZ are composed of radial glial (RG) cells that are neural progenitor cells: these cells give rise to neurons and macroglial cells. Progenitor cells located in the VZ/SVZ of the dorsal telencephalon generate excitatory glutamatergic projection neurons, which represent around 80% of cortical neurons. In contrast, progenitor cells located in the VZ/SVZ of the ventral telencephalon generate inhibitory γ-aminobutyric acid (GABA)ergic local circuit interneurons, which represent around 20% of cortical neurons. Because of their different locations of birth, the newborn neurons exhibit two distinct modes of cell migration: projection neurons migrate radially, while interneurons migrate tangentially to their final destinations within the neocortex (Figure 3; Marín and Rubenstein, 2003; Batista-Brito and Fishell, 2009).

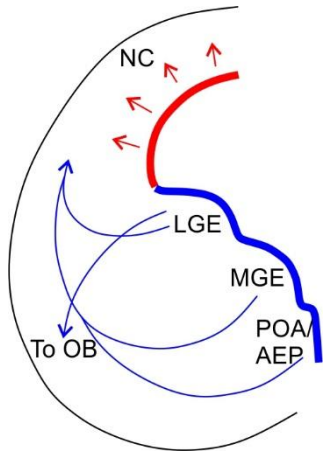

Figure 3. Schematic illustration of progenitor cell pools and neuronal migration in the telencephalon. Projection neurons that are born in the ventricular zone of pallium (in red) migrate radially within the NC (arrows in red). Interneurons that are born in the ventricular zone of subpallium (in blue) migrate either tangentially towards the NC or rostrally to the OB (arrows in blue). OB, olfactory bulb; NC, neocortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; POA/AEP, preoptic area/anterior entopeduncular area.

1.1.1. Neurogenesis and cell migration within the dorsal telencephalon

1.1.1.1. The formation of projection neurons

Before neurogenesis, the neural tube is composed of a single layer of neuroepithelial (NE) cells, termed as neuroepithelium. This layer is pseudostratified because of interkinetic nuclear migration: the nuclei of NE cells move along the apical-basal axis during the cell cycle. S-phase cells have their nuclei at the pial surface; which move to the ventricular surface for mitosis. NE cells have a short and a long process that contact the ventricular (apical) and the pial (basal side) surfaces, respectively (Figure 4). These cells are considered as stem cells, because they have the capacity to self-renew and to be multipotent. During development, NE cells undergo symmetric proliferative divisions, thereby producing NE cells, leading to an expansion of the stem cell pool (Figure 4; Götz and Huttner, 2005).

After E9, NE cells undergo asymmetric neurogenic divisions to self-renew and produce either neurons or neural progenitor cells, including RG cells and intermediate progenitor (IP) cells (Götz and Huttner, 2005). With the switch to neurogenesis, the layer lining the ventricle is referred to as the VZ. In the VZ, NE cells become RG cells. Similarly, RG cells have a bipolar morphology and show interkinetic nuclear migration (Figures 4 and 5). RG cells act as neural stem cells

(Malatesta et al., 2000; Hartfuss et al., 2001; Miyata et al., 2001; Noctor et al., 2001; Noctor et al., 2002; Götz and Huttner, 2005). After E13, RG cells produce neurons either directly via neurogenic divisions in the VZ, or indirectly via the production of short neural precursor (SNP) cells in the VZ, IP cells in the SVZ, and basal radial glial (bRG) cells in the superficial SVZ (Figure 4; Noctor et al., 2001; Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Götz and Huttner, 2005; Gal et al., 2006; Stancik et al., 2010; Shitamukai et al., 2011; Tyler and Haydar, 2013; Florio and Huttner, 2014).

SNP cells that are born at the apical side of the VZ have an apical process contacting the ventricular surface. In the VZ, SNP cells undergo symmetric neurogenic divisions to produce neurons (Gal et al., 2006; Stancik et al., 2010; Tyler and Haydar, 2013; Florio and Huttner, 2014).

IP cells that are born at the apical side of the VZ migrate to the basal side, thereby retracting their extensions. These cells form the SVZ. In the SVZ, IP cells undergo symmetric neurogenic divisions to produce neurons (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Götz and Huttner, 2005; Stancik et al., 2010; Shitamukai et al., 2011; Florio and Huttner, 2014).

bRG cells that are born at the apical side of the VZ have a basal process contacting the pial surface: these cells undergo mitotic somal translocation (section 1.1.1.2.) to migrate to the superficial SVZ (Miyata et al., 2004; Shitamukai et al., 2011; Wang et al., 2011). In the superficial SVZ, bRG cells undergo asymmetric divisions to self-renew and produce neurons (Shitamukai et al., 2011; Wang et al., 2011; Florio and Huttner, 2014).

After neurogenic stages, RG cells produce ependymal cells in the embryonic and early postnatal brains (Spassky et al., 2005), and become astrocytes (Figure 4; Malatesta et al., 2000; Noctor et al., 2004).

The production of neurons increases progressively, while cell proliferation decreases in the developing neocortex. During development, cell cycle progression depends on key regulatory molecules, which include cyclins and cyclin-dependent kinases (cdks). Cyclin D1 and cdk4 promote the progression of cells from the G1- to S-phase, which is also mediated by the MAPK/ERK (mitogen-activated protein kinase/extracellular signal regulated kinase) pathway (Wei and Liu, 2002; Hardwick et al., 2014).

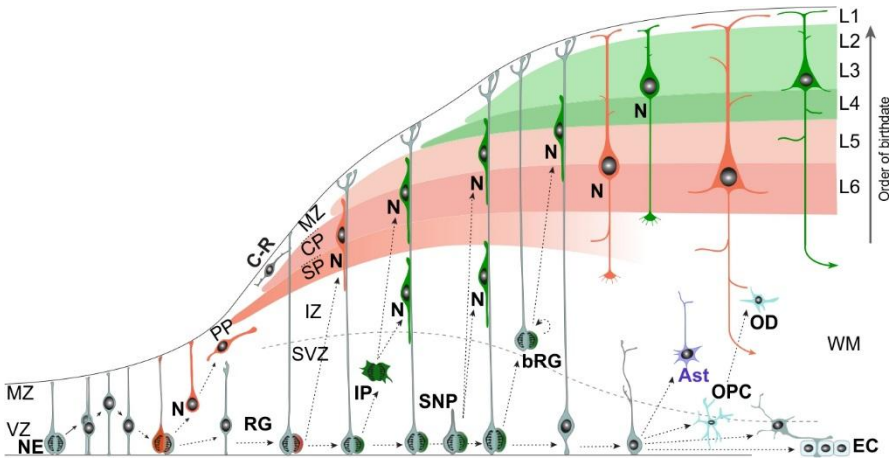

Figure 4. Neurogenesis in the mouse neocortex. NE cells divide symmetrically to expand their pool, and progressively become RG cells. RG cells produce N either directly or indirectly via either IP, SNP or bRG cells. IP cells form the SVZ and divide symmetrically to produce N. SNP cells divide symmetrically to produce N in the VZ. In the superficial SVZ, bRG cells divide asymmetrically to produce N. N migrate radially to form the PP, and then the MZ, SP, and CP. The MZ contains C-R cells. Later, RG cells produce Ast, EC, and OPC that give rise to OD. Ast, astrocyte; bRG, basal radial glia; CP, cortical plate; C-R, Cajal-Retzius; EC, ependymal cells; IP, intermediate progenitor; IZ, intermediate zone; L, layer; MZ, marginal zone; N, neurons; NE, neuroepithelial; OD, oligodendrocyte; OPC; oligodendrocyte precursor cell; PP, preplate; RG, radial glia; SNP, short neural precursor; SP, subplate; SVZ, subventricular zone; VZ, ventricular zone; WM, white matter (Adapted from Kwan et al., 2012 and Shibata et al., 2015).

1.1.1.2. Radial migration

Newborn neurons migrate radially throughout the neocortex using two different modes of cell migration to reach their final laminar positions: somal translocation and locomotion (Figures 3-5; Nadarajah et al., 2001, 2003; Nadarajah, 2003; Marín and Rubenstein, 2003; Noctor et al., 2004).

At the early stages of cortical development, around E11, newborn neurons migrate radially by somal translocation. Neurons have their somata in the VZ and two processes: a long leading process contacting the pial surface, and a short trailing process directed toward the ventricular surface. As their leading processes shorten, neurons translocate rapidly and continuously toward the pial surface (Figures 4 and 5; Nadarajah et al., 2001).

As the neocortex thickens during development, after E14, newborn neurons migrate radially by locomotion. Neurons have a short leading process directed toward the pial surface to migrate along the processes of RG cells (Figures 4 and 5; Noctor et al., 2001; Nadarajah et al., 2001). Locomoting neurons move slowly and discontinuously: they undergo four phases of migration, characterized by a

saltatory pattern (Nadarajah et al., 2001). First, neurons have a bipolar shape and move rapidly from the VZ to the SVZ; second, they adopt a multipolar shape and remained in the SVZ for 24 h or longer; third, they develop a leading process to contact the ventricular surface and move back to the VZ; and fourth, they migrate to the CP along the processes of RG cells (Noctor et al., 2004).

During the final stages of cell migration, many locomoting neurons migrate via somal translocation: their leading processes contact the MZ, and shorten as their somata move within the CP (Nadarajah et al., 2001).

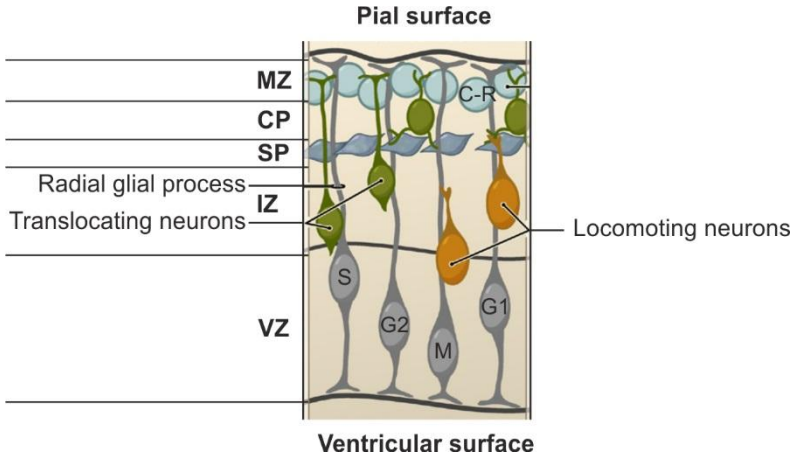

Figure 5. Modes of radial migration in the neocortex. Radial glial cells that are attached to the pial and ventricular surfaces undergo interkinetic nuclear migration: S-phase cells are at the basal side, and M-phase cells at the apical side of the VZ. From the VZ, neurons are either translocating by retracting their leading processes or locomoting with their leading processes along the radial glial processes to reach their laminar positions within the neocortex. CP, cortical plate; C-R, Cajal-Retzius cells; IZ, intermediate zone; MZ, marginal zone; SP, subplate; VZ, ventricular zone (Adapted from Ayala et al., 2007).

1.1.1.3. Cortical laminae formation

Several waves of newborn neurons leave the VZ/SVZ toward the pial surface (Figures 3 and 4; Angevine and Sidman, 1961; Marín and Rubenstein, 2003).

- At around E11, the first wave of migrating neurons forms the preplate, just below the pial surface.
- At around E13, the second wave of migrating neurons separates the preplate (PP) into the marginal zone (MZ) above and the subplate (SP) below, in between which the cortical plate (CP) forms. The MZ becomes the layer (L) 1, and the CP is the precursor of cortical L2-6.

- After E14, further successive waves of neuronal migration form the different layers in the CP.

The formation of cortex occurs in an inside-out manner, because each layer of cells appears at a different time point: early-born neurons form the deep layers, whereas late-born neurons occupy the more superficial layers of the cortex (Figures 4 and 6; Angevine and Sidman, 1961).

The formation of the six-layered cortex depends on the migration of different types of neurons within each layer. The MZ consists of Cajal-Retzius cells. Diverse subtypes of projection neurons occupy the different cortical layers. The callosal projection neurons are located in L2/3, L5, and L6: they send axons to the contralateral cortex. The subcerebral projection neurons are located in L5: they send axons to the optic tectum, brainstem, or spinal cord. The corticothalamic projection neurons are located in L6: they send axons to the thalamus (Molyneaux et al., 2007). The cortical layers are also composed of different types of interneurons (section 1.1.2.3.).

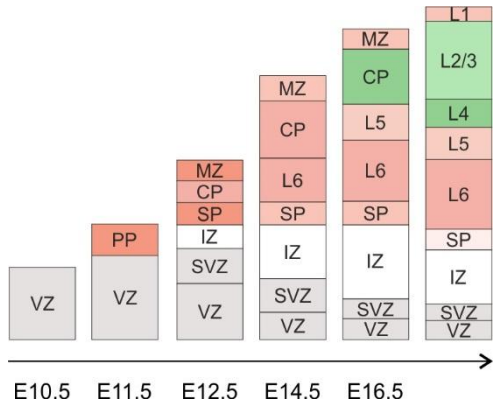

Figure 6. Schematic organization of the developing neocortex. Early-born neurons forms the PP. The PP is later separated into the MZ and SP. In between these two layers is formed the CP. The MZ becomes L1. The CP produces L2-6 of the cortex. CP, cortical plate; E, embryonic day; IZ, intermediate zone; L, layer; MZ, marginal zone; PP, preplate; SP, subplate; SVZ, subventricular zone; VZ, ventricular zone (Adapted from Molyneaux et al., 2007).

1.1.2. Neurogenesis and cell migration within the ventral telencephalon

1.1.2.1. The formation of interneurons

The production of interneurons in the ventral telencephalon is similar to the production of projection neurons in the dorsal telencephalon. In the ventral telencephalon, cells lining the ventricle are RG cells. These cells undergo

interkinetic nuclear migration within the VZ, and divide at the ventricular surface via asymmetric neurogenic divisions to self-renewal and produce either IP cells or interneurons. Newborn interneurons migrate radially along RG cells, and then tangentially to the neocortex. IP cells migrate from the VZ to the SVZ. In the SVZ, these cells undergo symmetric neurogenic divisions to produce interneurons (Brown et al., 2011; Ciceri et al., 2013). Furthermore, RG cells undergo asymmetric divisions to self-renew and produce either subapical progenitor (SAP) or SNP cells. SNP cells either self-renew in the VZ or generate SAP cells. SAP cells divide at subapical positions within the VZ: these cells either self-renew or generate either IP or bRG cells (Pilz et al., 2013).

Interneurons arise from the ventral telencephalon, including the GEs and preoptic area (POA). These ventral territories generate different subgroups of interneurons based on their timing of production, their pathways of migration, and their phenotypes that vary in morphology, physiology, and expression of neurochemical markers. Here, I will focus on the formation of neurochemical-expressing interneurons within the ventral telencephalon (Wonders and Anderson, 2005, 2006; Marín, 2013).

Medial ganglionic eminence

The MGE is the primary source of cortical interneurons, producing around 60% of cortical interneurons. Most of the MGE-derived interneurons are born at around E13.5. MGE-derived interneurons migrate tangentially to the neocortex through the MZ, lower IZ (intermediate zone), and SVZ. Parvalbumin- (PV) and somatostatin- (SST) expressing interneurons arise predominantly from the MGE: the dorsal MGE generates preferentially SST-, while the ventral MGE generates preferentially PV-expressing interneurons. Calbindin-expressing interneurons arise from the MGE (Figure 3; Anderson et al., 2001; Wichterle et al., 1999; Wichterle et al., 2001; Polleux et al., 2002; Xu et al., 2004; Wonders et al., 2008; Ciceri et al., 2013; Marín et al., 2013).

Caudal ganglionic eminence

The CGE is the secondary source of cortical interneurons, producing around 30% of cortical interneurons. Most of the CGE-derived interneurons are born at around E15.5. CGE-derived interneurons migrate tangentially to the neocortex through the MZ, lower IZ and SVZ. Calretinin- (CR) and/or vaso-intestinal peptide- (VIP), neuropeptide Y- (NPY) and/or reelin, and calbindin-expressing interneurons arise from the CGE (Figure 3; Anderson et al., 2001;

Nery et al., 2002; Xu et al., 2004; Miyoshi et al., 2007; Ciceri et al., 2013; Marín et al., 2013).

Preoptic area

The POA is the tertiary source of cortical interneurons, producing around 10% of cortical interneurons. Interneurons that are mostly born in the POA by E12.5 migrate tangentially to the neocortex through the SVZ and MZ. POA produces PV-, SST-, NPY-, and reelin-expressing interneurons (Figure 3; Gelman et al., 2009; Gelman et al., 2011).

Lateral ganglionic eminence

The LGE is a minor source of cortical interneurons. Interneurons that are born in the LGE after E14.5 migrate tangentially to the neocortex through the SVZ. The LGE also produces projection neurons (ventral LGE) that migrate radially to the striatum and interneurons (dLGE) that migrate rostrally to the olfactory bulb (OB; Anderson et al., 2001; Wichterle et al., 2001; Polleux et al., 2002; Stenman et al., 2003). The dLGE produces CR-expressing olfactory interneurons (Figure 3; Waclaw et al., 2006).

1.1.2.2. Mechanisms regulating neurogenesis: transcription factors

Neural progenitor cells are located in the VZ of the ventral telencephalon. This progenitor cell pool generates interneurons and oligodendrocytes (He et al., 2001; Yung et al., 2002). The generation of these cells depends on transcription factors expressed within the ventral telencephalon. Here, I focus on the transcription factors Dlx2 and Olig2. These factors are essential for the formation of interneurons and oligodendrocytes in the ventral telencephalon. Within the ventral telencephalon, these factors are co-expressed in neural progenitor cells; their expression become exclusive as cells developed (Petryniak et al., 2007).

Dlx2 is a homeobox transcription factor, which belongs to the Dlx family composed of six members, Dlx1 to Dlx6. Dlx2 protein is mostly co-expressed with Dlx1 in the VZ/SVZ of LGE and MGE at E12.5 (Eisenstat et al., 1999).

In accordance with their expression patterns, Dlx1/2 have been reported to modulate the formation of interneurons versus oligodendrocytes. Mice lacking Dlx1/2 exhibit an increase of oligodendrocyte precursor cells in the VZ/SVZ of MGE/AEP (anterior entopeduncular area). Inversely, overexpressing Dlx1/2 decreases the expression of Olig2, a marker for oligodendrocyte precursor cells. Therefore, interneurons are generated by a common progenitor cell, shared with

oligodendrocytes, in which Olig2 is downregulated in the MGE/AEP as development proceeds (Petryniak et al., 2007).

Olig2 is a basic helix-loop-helix (bHLH) transcription factor, which localizes in the VZ/SVZ of the ventral telencephalon during development. Olig2 is mostly expressed in the MGE and weakly in the LGE at E12.5, and then strongly in both MGE and LGE at E14.5 (Takebayashi et al., 2000; Ono et al., 2008; Magalhães and Rivera, 2014). A few Olig2-labeled cells are detected in the CP at E14.5; later, Olig2 labeling is widely found throughout the telencephalon (Ono et al., 2008).

Olig2-labeled precursor cells give rise to different types of cells depending on the time and location of origin: early Olig2-labeled precursor cells give rise to interneurons in the ventral telencephalon (Miyoshi et al., 2007; Ono et al., 2008); then, to interneurons and oligodendrocytes in the ventral telencephalon (Miyoshi et al., 2007; Parras et al., 2007; Petryniak et al., 2007); and at later stages, to macroglial cells in the neocortex (Figure 4; Ono et al., 2008).

1.1.2.3. Tangential migration

Newborn interneurons migrate from their original sources to their final destinations via distinct modes of cell migration in the developing telencephalon: the tangential migration and the rostral stream migration (Figure 3). During development, interneurons born in the dorsal LGE (dLGE) migrate along the rostral migratory stream to the OB, while interneurons born in the other ventral regions migrate tangentially to the neocortex (Figure 3; Marín and Rubenstein, 2001, 2003; Batista-Brito and Fishell, 2009; Marín, 2013). Here, I will focus on the primary source of cortical interneurons, their migratory pathways to reach the neocortex, and the factors involved in tangential migration.

Newborn interneurons migrate tangentially from the ventral territories to occupy the different layers of neocortex (Figure 3; Anderson et al., 1997; Wichterle et al., 1999; Anderson et al., 2001). Several waves of migrating interneurons enter the neocortex through distinct migratory pathways: a superficial pathway within the MZ occurring early in neurogenesis, and later a deep pathway within the lower IZ and SVZ (Anderson et al., 2001).

- At E11.5, MGE-derived interneurons migrate through the MZ.
- At around E13.5, even more MGE-derived interneurons migrate through the lower IZ.
- After E14.5, interneurons migrate from the MGE through the SVZ.

Once interneurons have reached the neocortex, they display several modes of migration to invade the CP in formation. Interneurons disperse tangentially in a lateral-to-medial direction in the lower IZ/SVZ, while some interneurons ascend radially or obliquely into the CP and MZ. Within the MZ, interneurons are dispersed in multiple directions and move in a lateral-to-medial direction, while some of these interneurons descend radially into the CP (Wichterle et al., 2001; Polleux et al., 2002; Ang et al., 2003; Tanaka et al., 2003). Within the cortical anlage, interneurons undergo ventricle-oriented migration: they descend towards the ventricular surface, remained in the VZ for an extended time, and then migrate radially into the CP (Nadarajah et al., 2002; Nadarajah et al., 2003).

Similar to cortical projection neurons, MGE-derived interneurons occupy the neocortex in an inside-out manner: early-born interneurons reach the deep layers, whereas late-born interneurons arrive in the superficial layers of the developing neocortex (Miyoshi et al., 2007). MGE-derived interneurons that are PV- and SST-expressing interneurons are distributed throughout the cortical layers: early-born interneurons are located in L5 and L6, while late-born interneurons are located in L2/3 and L4. CR- and/ or VIP-expressing interneurons that are born in the CGE are located in L2/3. CR-expressing interneurons are also located in L5 of the neocortex (Miyoshi et al., 2007; Ciceri et al., 2013).

1.1.2.4. Mechanisms regulating tangential migration: guidance factors

Newborn interneurons migrate along typical migratory pathways throughout the developing telencephalon. The segregation of migrating interneurons into distinct telencephalic regions requires the action of diverse guidance factors, comprising motogenic and chemotactic factors. Migrating interneurons can respond to these factors because they express the complement receptors (Marín and Rubenstein, 2001; Batista-Brito and Fishell, 2009; Marín, 2013).

Motogens

Motogens induce the movement of interneurons away from their original sites.

Several neurotrophic factors have been reported to control the tangential migration of MGE-derived interneurons during development. For instance, BDNF (brain-derived neurotrophic factor) and NT4 (neurotrophin 4) have been shown to promote tangential migration via TrkB receptor. Mice lacking TrkB exhibit a decrease in calbindin-expressing interneurons in the neocortex (Polleux et al., 2002). Polleux et al. (2002) have, further, shown that these neurotrophic

factors activate PI3-kinase in MGE-derived interneurons, and that its inhibition impairs tangential migration. Therefore, BDNF and NT4 induce tangential migration of MGE-derived interneurons via TrkB signaling. Similarly, GDNF (glial cell line-derived neurotrophic factor) stimulates tangential migration via GFRα1 (GDNF family receptor α 1) receptor. Mice lacking either GDNF or GFRα1 exhibit a deficit in calbindin-expressing interneurons in the neocortex (Pozas and Ibáñez, 2005).

Neurotransmitters can also influence tangential migration. For instance, GABA has been reported to control the tangential migration of MGE-derived interneurons during development (Cuzon et al., 2006; Bortone and Polleux, 2009). Cuzon et al. (2006) have shown that ambient GABA promotes the entry of migrating interneurons into the neocortex via $GABA_A$-receptors ($GABA_A$-R). Bortone and Polleux (2009) have, further, shown that ambient GABA induces the migration of interneurons, and then reduces their migration once they occupy the neocortex, via $GABA_A$-R. This responsiveness change is due to the upregulation of KCC2 (K-Cl cotransporter 2) in cortical interneurons. An over-expression of KCC2 stops interneuron migration, while knocking down KCC2 increases the amount of migrating interneurons within the neocortex. Therefore, the responsiveness of migrating interneurons to GABA depends on their maturation: hyperpolarizing signals terminate cortical interneuron migration (Bortone and Polleux, 2009).

Chemotactic factors

Chemotactic factors comprise chemorepellants and chemoattractants, which guide migrating interneurons through migratory routes to enter the neocortex.

Chemorepellants are localized in the ventral telencephalon and serve as guidance cues, which guide migrating cortical interneurons away from a certain region. For instance, the semaphorins (sema) sema 3A and sema 3F have been detected in the striatal anlage, and their receptors neuropilin 1 and 2 in MGE-derived interneurons. Semaphorins repel neuropilin-expressing interneurons that are directed toward the neocortex. In contrast, interneurons lacking neuropilins migrate to the striatum. Therefore, semaphorins and their receptors regulate the segregation of striatal and cortical interneurons during development (Marín et al., 2001).

Chemoattractants are localized in the dorsal telencephalon and serve as guidance cues, which guide migrating cortical interneurons toward a certain location. For instance, the chemokine CXCL12 has been strongly detected in the

SVZ/IZ of neocortex at E14.5, but its expression decreases as development proceeds (Tiveron et al., 2006). Tiveron et al. (2006) have found that when CXCL12 is absent, interneurons disappear from the SVZ/IZ, but more interneurons are located in the MZ, suggesting that interneurons have moved to the MZ. Furthermore, its receptor CXCR4 has been detected in the MZ and SVZ/IZ, which correspond to the interneuron migratory routes. Tiveron et al. (2006) have, further, found that interneurons are dispersed throughout the neocortex of CXCR4 knockout (KO) mice. Therefore, interneurons preferentially migrate in the SVZ/IZ because of CXCL12 and CXCR4, suggesting that the switch of tangential-to-radial migration may occur when CXCL12 is downregulated in the SVZ/IZ of the neocortex.

1.1.3. Gliogenesis and cell migration within the telencephalon

There are three types of glial cells in the mature nervous system: astrocytes, oligodendrocytes, and microglial cells. Contrarily to microglial cells, astrocytes and oligodendrocytes originate in the central nervous system. In the developing telencephalon, macroglial cells derive from RG cells (Figure 4; Parras et al., 2007; Petryniak et al., 2007; Noctor et al., 2004). Here, I will focus on the formation of oligodendrocytes.

1.1.3.1. Original sources of oligodendrocytes

Oligodendrocytes are produced in diverse locations of the telencephalon during development. In the developing telencephalon, oligodendrocytes are generated in three distinct waves (Tekki-Kessaris et al., 2001; Kessaris et al., 2006).

- The first wave of oligodendrocytes originates in the VZ/SVZ of the ventral MGE and AEP at around E12.5.
- The second wave of oligodendrocytes originates in the VZ/SVZ of the LGE and CGE at around E14.5.
- The third wave of oligodendrocytes originates in the VZ/SVZ of the neocortex after birth.

1.1.3.2. Mechanisms regulating oligodendrocyte formation within the ventral telencephalon

The formation of oligodendrocytes depends on transcription factors (section 1.1.2.2.). Olig2 has been reported to be involved in oligodendrocyte formation (Parras et al., 2007; Petryniak et al., 2007). Olig2 cooperates with Mash1, another bHLH transcription factor, for the production of a subset of early-

born oligodendrocytes. Olig2-labeled precursor cells are missing in the VZ/SVZ of MGE/AEP in mice lacking Mash1 (Parras et al., 2007). Petryniak et al. (2007) have, further, shown that Mash1 maintains the pool of Olig2-labeled precursor cells in the MGE/AEP, thereby promoting oligodendrocyte formation.

1.1.3.3. Oligodendrocyte migration within the telencephalon

Similar to interneurons, oligodendrocytes that are born in the ventral telencephalon migrate tangentially to the neocortex. During postnatal development, the early-born oligodendrocytes disappear: oligodendrocytes that are present in the adult cortex arise from the LGE/CGE and neocortex (Kessaris et al., 2006).

2. Na-K-Cl cotransporter 1

2.1. Definition

NKCC1 is a membrane protein that transports Na^+, K^+, and Cl^- ions. NKCC1 is a member of the cation-chloride cotransporter (CCC) family, which comprises another Na-K-Cl cotransporter (NKCC2), four K-Cl cotransporters (KCC1-4), one Na-Cl cotransporter (NCC), CCC8, and CCC9. NKCC1 is characterized by twelve transmembrane domains flanked by prominent intracellular amino and carboxyl termini, and extracellular loop domains (Figure 7; Payne et al., 2003; Blaesse et al., 2009).

The gene *Slc12a2* encoding NKCC1 gives rise to several splice variants. Two splice variants are present in the mouse brain: a longer variant that contains the exon 21 (Delpire et al., 1994) and a shorter variant that lacks the exon 21 encoding for a peptide located in the carboxyl terminus (Randall et al., 1997). These mouse splice variants are analogous to the human NKCC1a and NKCC1b, respectively (Payne et al., 1995; Vibat et al., 2001).

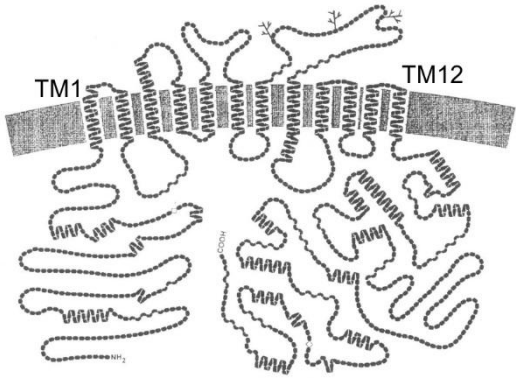

Figure 7. Secondary structure of NKCC1 protein. Na-K-Cl cotransporter 1 has twelve TM domains, large intracellular amino- and carboxy-terminal domains, and extracellular loop domains. TM, transmembrane membrane (Adapted from Haas and Forbush, 1998).

2.2. Expression pattern

NKCC1 is widely distributed in diverse tissues (Haas and Forbush, 1998; Payne et al., 2003). Here, I will focus on its expression pattern in the developing telencephalon.

NKCC1 mRNA is detected in the VZ/SVZ of mouse LGE at E12.5 (Hübner et al., 2001; Li et al., 2002), and in both LGE and MGE at E14.5 (Li et al., 2002). As development proceeds, NKCC1 mRNA expression disappears from the ventral telencephalon, and appears in the neocortex (Hübner et al., 2001).

No study of NKCC1 protein expression has been assessed in the developing mouse telencephalon. In the embryonic rat brain, NKCC1 is expressed in the VZ/SVZ of LGE and MGE at E14.5, and in the neocortex at E17.5. Consistently, NKCC1-expressing cells are neural progenitor cells in the LGE and MGE, and immature neurons in the neocortex (Li et al., 2002).

NKCC1 protein can be detected by different primary antibodies that bind either to the carboxyl terminus (clone T4; Lytle et al., 1995; Li et al., 2002), amino terminus (clone α-wNT; Kurihara et al., 1999; Evans et al., 2000; Mao et al., 2012), or phosphorylated peptide in the amino terminus (clone R5; Flemmer et al., 2002).

2.3. Functions

NKCC1 mediates the accumulation of Cl^- ions into the cells, driven by Na^+ gradient, which is generated by the transporter Na^+/K^+-ATPase (Figure 8). NKCC1 can be inhibited by diuretic loop drugs, bumetanide (1-10 μM) and furosemide (100 μM; Payne et al., 2003).

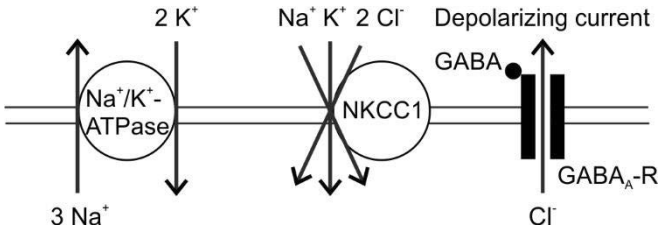

Figure 8. Regulation of intracellular chloride ions by NKCC1. In immature neurons, the Na^+ gradient produced by the Na^+/K^+-ATPase drives the uptake of Cl^- into cells via Na-K-Cl cotransporter 1 (NKCC1), thereby producing a depolarizing Cl^- current via $GABA_A$-receptors ($GABA_A$-R).

NKCC1 cotransport activity is controlled by cell volume- and chloride-sensitive phosphorylation processes. NKCC1 functions in maintaining a high intracellular concentration of Cl^- ions ($[Cl^-]_i$), increasing cell volume due to cell shrinkage, and mediating cell proliferation (Panet et al., 2000; Sung et al., 2000; Flatman et al., 2002; Payne et al., 2003; Yamada et al., 2004; Shiozaki et al., 2006; Achilles et al., 2007; Blaesse et al., 2009; Habela and Sontheimer, 2009).

NKCC1 is involved in the regulation of $[Cl^-]_i$ in immature neurons (Sung et al., 2000; Yamada et al., 2004; Achilles et al., 2007). NKCC1 has been shown to maintain high $[Cl^-]_i$ in immature neurons of the neocortex. As immature cortical neurons have high $[Cl^-]_i$, then the activation of $GABA_A$-R results in GABA-induced Cl^- efflux and neuronal membrane depolarization (Yamada et al., 2004; Achilles et al., 2007). Interestingly, GABA has been reported to modulate cell proliferation in the VZ/SVZ of the neocortex. GABA decreases progenitor cell proliferation in the VZ/SVZ of the rat neocortex at E16-E17 (LoTurco et al., 1995). Haydar et al. (2000) have, further, shown that GABA promotes VZ cell proliferation, and reduces SVZ cell proliferation in the mouse neocortex at E13-E14.

Activation of NKCC1 cotransport is stimulated either by low $[Cl^-]_i$ or cell shrinkage. Therefore, NKCC1 activity increases to restore $[Cl^-]_i$ and normal cell volume via regulatory volume increase. This activation is associated with the phosphorylation of NKCC1 by several kinases, such as SPAK/OSR1 and WNKs (Flatman et al., 2002; Payne et al., 2003; Blaesse et al., 2009).

NKCC1 has been reported to control normal cell proliferation *in vitro*, using immortalized cell lines and primary cell cultures. Inhibiting NKCC1 either with bumetanide or furosemide reduces cell proliferation due to cell cycle reentry impairment (Panet et al., 2000; Shiozaki et al., 2006). Inversely, overexpressing

NKCC1 stimulates uncontrolled cell proliferation, which is prevented either by bumetanide or furosemide treatment (Panet et al., 2000). NKCC1 has also been suggested to accumulate Cl^- ions into glioma cells: glioma cells produce smaller daughter cells that have low $[Cl^-]_i$ at the mitotic phase, and subsequently these cells restore their volume, via regulatory volume increase, and $[Cl^-]_i$ during the growth phase (Habela and Sontheimer, 2009). Thus, NKCC1 cotransport activity contributes to the increase of cell volume, which is important for the cell division. Although these studies have been performed on non-neuronal cells, the expression of NKCC1 in the VZ/SVZ of the ventral telencephalon suggests a similar role in progenitor cell proliferation during embryonic mouse brain development. In newborn mice, knocking down NKCC1 reduces progenitor cell proliferation in the SVZ, which leads to a decrease in neuronal density in the OB (Young et al., 2012).

NKCC1 has been reported to be involved in cell migration *in vitro* and *in vivo*. Inhibiting NKCC1 either via bumetanide treatment or genetic knockdown alters the migration of glioma (Haas and Sontheimer, 2010) and neuronal cells (Meija-Gervacio et al., 2011; Koyama et al., 2012).

2.4. NKCC1 knockout mice

NKCC1 KO mice display growth retardation: they are significantly smaller than wild-type (WT) mice as adults. Mice lacking NKCC1 exhibit head tossing and circling behavior (Delpire et al., 1999; Flagella et al., 1999; Pace et al., 2000). This shaker/waltzer phenotype is characteristic of inner ear dysfunction. These mice are deaf, due to structural damages of the cochlea and therefore disrupted endolymph secretion (Delpire et al., 1999; Flagella et al., 1999). These mice also exhibit cecum and intestine bleeding, and intestinal transit issues, due to decrease intestinal secretion (Flagella et al., 1999). Lack of NKCC1 impairs blood pressure (Flagella et al., 1999), saliva secretion (Evans et al., 2000), pain perception (Sung et al., 2000), spermatogenesis (Pace et al., 2000). Males that lack NKCC1 are sterile (Pace et al., 2000).

3. Glial cell line-derived neurotrophic factor

3.1. Definition

GDNF was isolated from rat glial cell line, and found to promote the survival of dopaminergic neurons in embryonic midbrain cultures (Lin et al., 1993). GDNF is a neurotrophic factor of the GDNF family ligands (GFLs), which belong to the transforming growth factor β (TGFβ) superfamily. The family of

GFLs includes four members: GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN). GFLs bind to a receptor complex, which is composed of a transmembrane receptor tyrosine kinase (RTK) rearranged during transfection (RET) and a cell surface-bound GFRα. The family of GFRα co-receptors comprises four members: GFRα1-4. GFLs bind to a preferred co-receptor: GDNF binds preferentially to GFRα1, NRTN to GFRα2, ARTN to GFRα3, and PSPN to GFRα4. Moreover, GDNF can bind to GFRα2; NTRN, ARTN, and PSPN to GFRα1 (Figure 9; Airaksinen and Saarma, 2002).

GDNF can activate either RET or NCAM (neural cell adhesion molecule) via GFRα1 (Airaksinen and Saarma, 2002; Bespalov and Saarma, 2007). GDNF signaling can require heparan sulfate proteoglycans (HSPGs): syndecans concentrate GDNF to be presented to GFRα1 and RET (Sariola and Saarma, 2003).

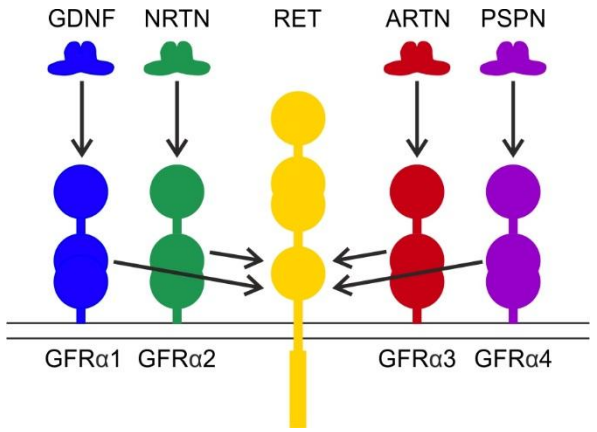

Figure 9. Binding of GDNF-family ligands and receptors. GDNF-family ligands bind to a receptor complex, which includes a transmembrane receptor tyrosine kinase RET and a cell surface-bound GFRα. GDNF binds to GFRα1, NRTN to GFRα2, ARTN to GFRα3, and PSPN to GFRα4. GFRα, GDNF family receptor α; GDNF, glial cell line-derived neurotrophic factor; NRTN, neurturin; ARTN, artemin; PSPN, persephin; RET, rearranged during transfection.

3.2. Expression pattern

GDNF mRNA is present in the VZ/SVZ of ventral and dorsal telencephalon at E12.5. At E15.5, GDNF mRNA is detected in the VZ/SVZ, MZ, and CP of the dorsal telencephalon (Pozas and Ibáñez, 2005).

3.3. Functions

GFLs are involved in the survival, proliferation, differentiation, and migration of many types of neurons (Airaksinen and Saarma, 2002; Sariola and Saarma, 2003); here, I will focus on the effect of GDNF on neuronal migration. Pozas and Ibáñez (2005) have shown that GDNF stimulates morphology differentiation and tangential migration of immature interneurons through GFRα1, but independently of RET and NCAM. The function of GDNF is consistent with their expression pattern during development: GDNF mRNA and GFRα1 mRNA are detected in the MGE, along the tangential migration pathway of immature interneurons, and in the developing neocortex; RET and NCAM are not detected in the telencephalon (Pozas and Ibáñez, 2005). GDNF has been reported to stimulate the migration of enteric neuronal precursor cells in the developing gut by binding the receptor complex composed of RET and GFRα1, and the migration of neuronal precursor cells in the rostral migratory stream by binding the receptor complex composed of NCAM and GFRα1 (Paratcha and Ledda, 2008).

4. Syndecan-3

4.1. Definition

Syndecan-3, also known as N-syndecan, is a transmembrane HSPG, which belongs to the syndecan family. The syndecan family includes four members: syndecan-1 to -4. Syndecan-3 has an intracellular domain, a transmembrane domain, and an extracellular domain that carries heparan sulfate (HS) chains (Figure 10; Carey et al., 1992; Gould et al., 1992).

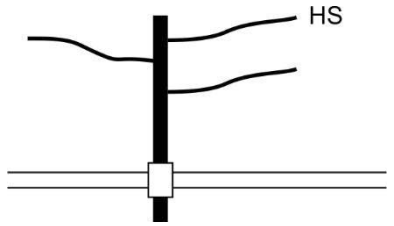

Figure 10. Schematic illustration of syndecan-3. Syndecan-3 has intracellular and transmembrane domains, and an extracellular domain that harbors HS chains. HS, heparan sulfate.

4.2. Expression pattern

The expression pattern of syndecan-3 is developmentally regulated: syndecan-3 is highly detected in neonatal brain, while its expression is lower in

embryonic brain, and barely found in adult brain (Carey et al., 1992). At E14, syndecan-3 is found in the ventral telencephalon, and in the VZ/SVZ of neocortex. At E16, syndecan-3 is expressed in the SP, and then in the SP/IZ region of neocortex at E19-E20 (Kinnunen et al., 1999). In embryonic rodent brain, syndecan-3 is prominently found in neurons, and localizes in neurites (Kinnunen et al., 1998).

4.3. Functions

As previously mentioned (section 3.1.), syndecans bind ligands to present them to their conventional receptors (Sariola and Saarma, 2003). Syndecans can interact with diverse ligands, including neurotrophic factors, extracellular matrix (ECM) molecules, axon guidance molecules, cell adhesion molecules, and many other ligands (Maeda, 2015). This large variety of ligands is involved in brain development, such as neuronal cell migration. Syndecan-3 has been reported to be involved in radial migration of cortical neurons, via its interaction with the ECM-associated heparin binding growth associated molecule (HB-GAM; Hienola et al., 2006). The interaction of immobilized HB-GAM with syndecan-3 has been previously reported to induce neurite outgrowth and cell spreading via SFK (Src family kinase) activation (Kinnunen et al., 1998): the oligomerization of syndecan-3 may bring together its cytoplasmic domain and intracellular molecules leading to their transactivation.

4.4. Syndecan-3 knockout mice

Syndecan-3 KO mice are viable, and display no apparent developmental defects (Reizes et al., 2001). However, mice lacking syndecan-3 have altered food behavior (Reizes et al., 2001), impaired memory (Kaksonen et al., 2002), and impaired radial migration in the neocortex (Hienola et al., 2006).

FORMULATION OF THE RESEARCH QUESTION AND OBJECTIVES

The aims of this study were to develop an immunohistochemistry protocol to investigate the expression of oligodendrocyte precursors and to further study the mechanisms involved in neural development.

The specific aims of the study were to:

- Develop an immunohistochemistry protocol using heat-induced epitope retrieval method for improving the visualization of Olig2 marker in paraffin-embedded sections of embryonic mouse telencephalon and for its quantitative analysis

- Investigate the implication of NKCC1 in neural development in embryonic mouse telencephalon

- Study the implication of syndecan-3 in the migration of immature interneurons in embryonic mouse telencephalon

MATERIALS AND METHODS

Methods personally used in the three publications are presented in the table below. Roman numerals indicate publication(s) in which it was applied. Details can be found in the respective publications.

Method	**Used in**
Brain preparation	I, II, III
DNA isolation	II
Polymerase chain reaction	II
Brain sectioning: microtome	I, II
Brain sectioning: cryostat	III
Immunohistochemistry for paraffin-embedded sections	I, II
Immunohistochemistry for free-floating sections	III

RESULTS AND DISCUSSION

1. Use of decloaking chamber-based heat-induced epitope retrieval method improves the detection of Olig2-labeled cells in the ventral telencephalon (I)

It has been previously reported that Olig2 is expressed in the ventral telencephalon by IHC (immunohistochemistry) studies on frozen sections from embryonic rodent brain (Takebayashi et al., 2000; Parras et al., 2007; Miyoshi et al.,2007; Petryniak et al., 2007; Ono et al., 2008; Zhong et al., 2011; Ueno et al., 2012). However, no study of Olig2 expression was performed on paraffin-embedded sections from embryonic mouse brain. To investigate the distribution of Olig2-labeling on paraffin-embedded sections, we compared the effectiveness of two HIER (heat-induced epitope retrieval) methods: the commonly used microwave oven- and the presently established decloaking chamber-based HIER methods. In agreement with previous reports, the immunostaining pattern of Olig2 was found in the ventral telencephalon of both microwave oven- and decloaking chamber-samples, at E12.5 (I: Figure 1) and E14.5 (I: Figure 2). However, differences appeared in the intensity and quality of staining. Microwave oven-samples displayed higher staining intensity than decloaking chamber-samples. Despite the enhancement of staining intensity in microwave oven-samples, the morphology of Olig2-labeled cells was lost compared with decloaking chamber-samples. We observed a more diffuse labeling of Olig2 in microwave oven-samples, as if the antigen had moved from the nucleus leading to an over-staining of the tissue section and therefore to high background. This data contrasted with the decloaking chamber-samples, in which Olig2-labeled cells were individually discernible; but the overall staining was decreased in tissue sections. This observation was quantified by measuring the area of Hoechst-positive cells: cell area was larger in microwave oven- than in decloaking chamber-samples by 22% (I: Table 1). These results are consistent with previous reports: microwave oven heat can increase background staining (Shi et al., 1991; Jiao et al., 1999), and impair cell morphology (Hunt et al., 1996). Hunt et al. (1996) have reported that microwave oven-samples revealed high staining intensity compared to autoclave-samples, but with alteration in cell morphology.

The damage in cell morphology may lead to difficult visualization of labeled cells for quantitative analyses. We, then, compared the amount of Olig2-labeled cells in the ventral telencephalon between microwave oven- and decloaking chamber-samples. Positive cells were quantified manually and automatically. The number of Olig2-labeled cells was inferior in microwave

oven- to decloaking chamber-samples, by nearly 60% in manual and automated counting (I: Figure 3M). We also determined the amount of Olig2-labeled cells that were quantifiable using Hoechst staining in both HIER methods. Almost 50% of cells in the VZ were labeled with Olig2 in microwave oven-samples, whereas more than 90% of cells in the VZ were labeled with Olig2 in decloaking chamber-samples, in both manual and automated quantifications. Thus, the amount of quantifiable cells was inferior in microwave oven- to decloaking chamber-samples, by nearly 50% in manual and automated counting (I: Figure 3N). These data confirmed that the damage in cell morphology observed in microwave oven-samples was reflected in the reduction of quantifiable cells.

Appropriate cell identification for accurate cell quantification is crucial in diagnostic pathology. Then, increased sensitivity is essential in the evaluation of prognostic markers (Shi et al., 1991). In this study, we have used two different IHC protocols. In the IHC protocol using the microwave oven, detergents were used to increase the tissue permeability to antibodies: SDS, tween20, triton, and saponin. In contrast, no detergents were used in the IHC protocol using the decloaking chamber. The use of these detergents could be the reason of impaired cell morphology observed in microwave oven-samples, in comparison with the decloaking chamber-samples. Furthermore, different buffers were used in both IHC protocols. Because the IHC protocols used, here, are different, we tested both devices and both epitope retrieval buffers in each protocol to investigate the effectiveness of decloaking chamber-based HIER method. Despite the similar immunostaining pattern of Olig2 in each experiment, the best result was obtained using the decloaking chamber with its buffer in both IHC protocols (I: Figure 4). Thus, the decloaking chamber-based HIER method is a more sensitive technique than the microwave oven. Since the first characterization of epitope retrieval based on a simple method of boiling the samples in distilled water using microwave oven heat (Shi et al., 1991), several approaches have been described to improve IHC staining: the IHC protocol has been modified at every possible step, including devices, heating and buffer conditions. The heating conditions correspond to the temperature and time of heating, which are important factors for retrieval of epitopes masked by the fixative (Shi et al., 1991). Different heating devices have been used for epitope retrieval, and compared with the microwave oven, such as autoclave, pressure cooker, water-bath, and steamer. These devices were adjusted to produce a similar intensity of staining to microwave oven. A correlation between the heating parameters appears: the higher the temperature of heating is, the shorter the time is required to obtain the same IHC staining (Taylor et al., 1996). The intensity of staining has also been compared by using different

buffers. The buffer conditions correspond to the composition and the pH value of the buffer used for epitope retrieval. Shi et al. (1995) have tested the influence of pH on the intensity of staining of several antigens. Three distinct pattern of staining were observed under the influence of pH: several antigens exhibit a minor variation of staining with pH, other antigens have strong IHC staining at high or low pH, and some antigens have improved IHC staining with increasing pH. Furthermore, Shi et al. (1995) have tested seven buffer solutions: similar intensities of staining were detected, indicating that the pH is a more critical parameter than the composition of epitope retrieval buffer. Here, the composition of the buffer used in decloaking chamber-based HIER method is not known, but the pH value is lower than the pH of buffer used in microwave oven-based HIER method. We observed that the decloaking chamber-buffer gave better results than the microwave oven-buffer in both IHC protocols: single Olig2-labeled cells were identified in decloaking chamber-samples when the decloaking chamber-buffer was used with the decloaking chamber. Thus, the IHC protocol using the decloaking chamber-based HIER method is preferentially used for Olig2 detection in paraffin-embedded sections from embryonic mouse brain.

In summary, the reduced number of steps and incubation time lead to a decrease in the duration of IHC protocol: this protocol is therefore fast and easy to use. Simplifying IHC protocol constitutes a potential approach for standardization. IHC standardization is essential for obtaining reproducible and reliable data, and also for quantitative analyses. Here, both manual and automated quantifications gave similar results, thereby verifying the efficiency of the latter method. The decloaking chamber-based HIER method constitutes a more sensitive technique for epitope retrieval: single Olig2-labeled cells were discernible and therefore easily quantifiable in the developing ventral telencephalon. Thus, the decloaking chamber-based HIER method is a valuable tool in basic research.

2. NKCC1 regulates cell proliferation in the ventral telencephalon (II)

It has been previously reported that NKCC1 mRNA is found in the VZ/SVZ of LGE in E12.5-E14.5 mice (Hübner et al., 2001; Li et al., 2002). During development, the expression pattern of NKCC1 protein has been assessed in the rat brain, using NKCC1 T4 antibody: NKCC1 protein localizes in the VZ/SVZ of LGE and MGE at E14.5. However, its labeling has not been validated in NKCC1 KO brains (Li et al., 2002). Here, we studied the expression pattern of NKCC1 protein in the mouse brain at E12.5, using the NKCC1 α-wNT antibody. NKCC1 was detected in the VZ/SVZ of LGE (II: Figure 1A). In the LGE, no positive labeling was found in NKCC1 KO mice (II: Figure 1B), which confirms the specificity of NKCC1 α-wNT antibody. Its specificity has been previously confirmed in NKCC1 KO mice (Mao et al., 2012). Therefore, this is the first study of NKCC1 detection in the mouse brain, at E12.5. At E12.5, we defined the identity of cells that express NKCC1 using nestin, a marker for neural progenitor cells, and clone TuJ1, a marker for immature neurons. In accordance with its expression pattern, we found that NKCC1 co-localized with nestin, and its labeling was opposite to TuJ1-labeling region (II: Figure 1C-D). Therefore, NKCC1-expressing cells are neural progenitor cells in the LGE at E12.5. These results could be confirmed by using other markers for RG cells, such as vimentin, RC2, BLBP, and GLAST (Hartfuss et al., 2001; Noctor et al., 2002; Pilz et al., 2013). During development, neural progenitor cells divide in the telencephalon (Noctor et al., 2001; Brown et al., 2011; Pilz et al., 2013). We, further, identified the identity of NKCC1-expressing cells using Ki67, a marker for actively dividing cells. Consistently, NKCC1 co-localized with Ki67 in the VZ/SVZ, indicating that NKCC1-expressing cells are actively dividing in the VZ/SVZ at E12.5 (II: Figure 2B). This result could be confirmed by using the marker 4A4 for mitotic RG cells (Noctor et al., 2002; Pilz et al., 2013). Together, these results suggested a possible role of NKCC1 in cell proliferation.

We studied the role of NKCC1 in cell proliferation using NKCC1 KO mice. Lack of NKCC1 impaired the mitotic activity of VZ cells in the LGE at E12.5 (II: Figure 3A-C), which suggested that NKCC1 maintained the proliferation of progenitor cells in the LGE. To determine whether this result is due to an alteration in the cell cycle, we, first, analyzed the cell cycle length. No change in the estimated cell cycle length was observed in the LGE of NKCC1 KO relative to WT mice at E12.5 (II: Figure 3D-F). This result could be confirmed by using two thymidine analogues, Idu and BrdU, to define the length of the cell cycle (Friocourt et al., 2008). We, further, found that the cell cycle reentry was reduced in the LGE of NKCC1 KO relative to WT mice at E13.5 (II:

Figure 3G-I), indicating that NKCC1 influenced the cells to reenter the cell cycle to proliferate instead of exiting the cell cycle. These results are consistent with previous *in vitro* studies, using non-neuronal cells and bumetanide treatment (Panet et al., 2000; Shiozaki et al., 2006). Moreover, no change appeared in cleaved-caspase 3 staining in the LGE of NKCC1 KO relative to WT mice (data not shown), thereby excluding that impairment in cell proliferation is caused by increased cell death. Furthermore, NKCC1 has been suggested to increase the volume of cells before entering the S-phase (Habela and Sontheimer, 2009). Lowering $[Cl^-]_i$ or cell shrinkage that occur during cell division activate NKCC1. NKCC1 activation is associated with phosphorylation (Flatman et al., 2002; Payne et al., 2003; Blaesse et al., 2009). It would be interesting to use also NKCC1 R5 antibody to know whether NKCC1 that localizes at the VZ/SVZ of the LGE is phosphorylated, and therefore activated. NKCC1 has been reported to regulate cell ion homeostasis. NKCC1 functions in maintaining high $[Cl^-]_i$ in immature cortical neurons, which leads to GABA-induced membrane depolarization via $GABA_A$-R (Yamada et al., 2004; Achilles et al., 2007). GABA is known to modulate cell proliferation depending on the region and stage of development. At E16-E17, GABA reduces cell proliferation in the VZ/SVZ of rat neocortex (LoTurco et al., 1995). In mouse neocortex, GABA modulates differently cell proliferation in the VZ and SVZ at E13-E14 (Haydar et al., 2000). In contrast, in postnatal day 6-7 rat cerebella, GABA increases proliferation of immature granule cells that is regulated through MAPK cascade (Fiszman et al., 1999). Through ERK/MAPK pathway, NKCC1 has been reported to control cell proliferation *in vitro* (Panet et al., 2002). In the NKCC1 KO mice, it would be interesting to monitor the phosphorylation of MAPK, and also the expression of cell-cycle regulated proteins such as cyclin D1 and cdk4 that are involved in G1- to S-phase progression, and p21, an inhibitor of cdk, that is involved in cell cycle arrest to provide a molecular mechanism for NKCC1-mediated cell cycle progression. Therefore, NKCC1 can function by regulating cell volume or target proteins in signaling cascades to control progenitor cell proliferation in the LGE *in vivo*.

Neural progenitor cells produce interneurons and oligodendrocytes in the LGE (He et al., 2001; Yung et al., 2002). In accordance with its expression pattern, we examine the function of NKCC1 in the production of interneurons and oligodendrocytes in NKCC1 KO mice. We studied the expression of Dlx2, a marker for interneuron progenitor cells. We found that Dlx2 expression was reduced in the LGE of NKCC1 KO relative to WT mice at E12.5, due to impaired mitotic activity of Dlx2-labeled cells (II: Figure 4A-C). Once cells become

postmitotic, they start migrating to their final destinations. We used Sp8, a marker for migrating dLGE-derived interneurons. We found that fewer Sp8-immature interneurons were migrating from the dLGE of NKCC1 KO at E14.5, compared with WT mice (II: Figure 4G-I). Together, these results suggested a role of NKCC1 in interneuron development. At E14.5, oligodendrocyte progenitor cells originate in the LGE (Kessaris et al., 2006). We studied the expression of Olig2, a marker for oligodendrocyte progenitor cells. We found that Olig2 expression was reduced in the LGE of NKCC1 KO relative to WT mice at E14.5, which suggested a role of NKCC1 in oligodendrocyte formation. Thus, NKCC1 is involved in the formation of interneurons and oligodendrocytes in the LGE. To corroborate these findings, it would be interesting to examine the labeling of different markers for interneurons and oligodendrocytes at their final destinations in NKCC1 KO mice. The dLGE generates interneurons that migrate either tangentially to the neocortex or rostrally to the OB (Anderson et al., 2001). We could examine CR-labeling in the OB; however, no subtypes of interneurons that are born in the LGE are currently known to reach the neocortex. The LGE generates oligodendrocytes that migrate tangentially to the neocortex (Kessaris et al., 2006). We could examine the labeling of APC or CNPase in the cortex. Moreover, Young et al. (2012) have shown that knocking down NKCC1 impaired progenitor cell proliferation in the SVZ of newborn mice, thereby reducing neuronal density in the OB. It would be interesting to knock down NKCC1 to confirm its implication in progenitor cell proliferation, and also to study its implication in cell migration. NKCC1 has been previously reported to alter cell migration (Haas and Sontheimer, 2010; Meija-Gervacio et al., 2011; Koyama et al., 2012). In agreement with its expression pattern (Hübner et al., 2001; Li et al., 2002), NKCC1 could be involved in radial migration, and therefore in positioning interneurons within the neocortex. Then, GAD65-GFP mice could be crossed with NKCC1 KO mice to observe GFP-labeled interneurons in adult NKCC1 KO cortex.

In summary, this study shows that ion homeostasis in neural progenitor cells of the LGE is essential in normal progenitor cell proliferation, thereby maintaining the pool of interneuron and oligodendrocyte progenitor cells. Precise timing of neural development is essential for the normal brain formation. In the developing brain, NKCC1 mediates Cl^- homeostasis, which is important for GABA-induced depolarization and cell volume control. Impairment of Cl^- homeostasis in mice lacking NKCC1 may be involved in neurodevelopmental disorders, as deficiencies in neocortical interneuron formation have been reported to contribute to epilepsy and schizophrenia (Lewis, 2000; Powell et al., 2003).

3. GDNF binds to syndecan-3, and promotes the tangential migration of immature interneurons in the ventral telencephalon (III)

Neuronal migration depends on diverse guidance factors, which bind HS chains (Maeda, 2015). It has been previously reported that the extracellular domain of syndecan-3 harbors HS chains (Gould et al., 1992), and that GDNF binds to HS (Rickard et al., 2003). Consistently, GDNF, NRTN, and ARTN were found to interact with syndecan-3 (III: Figure 2A), and more specifically via their HS chains: heparinase III inhibited the interaction of GDNF and syndecan-3 (III: Figure 2B). Additionally, GDNF was shown to induce syndecan-3 oligomerization on the plasma membrane (III: Figure 2D). Thus, GDNF binds syndecan-3, leading to its oligomerization. These results suggested that GDNF and syndecan-3 might interact in the developing telencephalon.

In the developing telencephalon, syndecan-3 has been reported to promote neurite outgrowth by binding to ECM-associated HB-GAM (Kinnunen et al., 1998; Hienola et al., 2006). GDNF was found on the cell membrane and in the ECM (III: Figure 2C). To mimic the association of ECM and ligands, GDNF, NRTN, and ARTN were immobilized on plastic microplates to study their functions. These GFLs were shown to promote cell adhesion and spreading, dependently of HSPG (III: Figure 3B-D): these biological processes were impaired by the use of either heparinase III or GDNF mutant. This mutant has a lower affinity to GFRα1, but can still activate the receptor complex GFRα1 and RET (Eketjäll et al., 1999): it was revealed to be biologically active (III: Figure S2B). These results suggested the necessity of immobilized GDNF and HS binding for cell adhesion and spreading. Moreover, cell spreading was found to be dependent on SFK activation (III: Figure 3E-G): syndecan-3 has been reported to signal by SFK activation (Kinnunen et al., 1998). Together, GDNF, NRTN, and ARTN can act directly via syndecan-3.

Cell adhesion and spreading are essential steps for neurite outgrowth. Diffusible GFLs promotes neurite outgrowth in several types of neurons (Airaksinen and Saarma, 2002). It has been previously reported that ECM-associated HB-GAM interacting with syndecan-3 promotes neurite outgrowth via SFK activation (Kinnunen et al., 1998). Here, immobilized GDNF was shown to induce the formation of neurites (III: Figure 4A). Furthermore, neurite outgrowth was impaired in neurons treated with either heparinase III or SFK inhibitor, and also in neurons grown on GDNF mutant (III: Figure 4B). These results suggested that the interaction between immobilized GDNF and HS is required for neurite outgrowth, and that SFK activation is involved in the formation of neurites. Consistently, neurite outgrowth was impaired in syndecan-3-deficient neurons

(III: Figure 3D). Thus, the interaction of immobilized GDNF with syndecan-3 induces neurite outgrowth via SFK activation.

The interaction of GDNF with syndecan-3 could be crucial for brain development. Cortical development depends on the radial migration of projection neurons within the neocortex, and the tangential migration of immature interneurons from the ventral telencephalon to the neocortex (Marín and Rubenstein, 2003). It has been previously reported that GDNF regulates the tangential migration of immature interneurons via GFRα1, but independently of RET and NCAM (Pozas and Ibáñez, 2005), which suggests that GDNF may have different receptor complexes to regulate neuronal migration. Interestingly, syndecan-3 was found to be involved in radial migration (Hienola et al., 2006). Here, we proposed that GDNF could signal via syndecan-3 in cortical interneurons. This hypothesis was supported by the expression pattern studies: GDNF is expressed in tangential migration routes (Pozas and Ibáñez, 2005), and syndecan-3 is expressed in embryonic rodent brain neurons (Carey et al., 1992; Kinnunen et al., 1998). Here, GDNF was found to stimulate the migration of embryonic cortical neurons. This migration was impaired in GDNF mutant, suggesting the involvement of syndecan-3 in neuronal migration. Consistently, GDNF-induced migration was impaired in embryonic cortical neurons deficient in syndecan-3 (III: Figure 5A). Interestingly, the highest concentration of GDNF could stimulate the migration of deficient neurons, suggesting the involvement of other GDNF receptors, such as GFRα1 (Pozas and Ibáñez, 2005). These results were supported with further studies using embryonic brain explants: immature interneurons from WT explants were attracted by GDNF (III: Figure 5D), but not by GDNF mutant (III: Figure S4). Consistently, GDNF did not attract immature interneurons from syndecan-3 KO brains (III: Figure 5D). Together, these results suggested that GDNF promotes the migration of immature interneurons via syndecan-3. HB-GAM was also found to induce radial migration via syndecan-3 (Hienola et al., 2006). Hienola et al. (2006) have shown that syndecan-3 KO mice have delayed radial migration in the neocortex. These syndecan-3 KO mice display normal cell proliferation and differentiation (Hienola et al., 2006). Here, we studied the tangential migration of immature interneurons within the telencephalon. We found that syndecan-3 KO also exhibited migration deficiency of immature interneurons in embryonic mouse brain: migrating interneurons were accumulated in the ventral telencephalon of syndecan-3 KO mice (III: Figure 6). Immature interneurons migrate from the ventral telencephalon to the neocortex through the MZ, lower IZ, and SVZ (Anderson et al., 2001). Here, we found that the amount of calbindin-labeled cells tended to decrease in the IZ/SP layer of

syndecan-3 KO neocortex. However, it would have been interesting to quantify within the MZ, because this layer seemed to have fewer calbindin-labeled cells in syndecan-3 KO than in WT mice. Newborn interneurons migrate tangentially through the MZ at early stage of neurogenesis. Later, newborn interneurons migrate tangentially through the lower IZ and SVZ. In these layers, migrating interneurons continue to move tangentially in a lateral-to-medial direction, while some interneurons migrate radially towards the MZ. In the MZ, interneurons disperse tangentially in all directions, while some of these interneurons descend subsequently into the CP (Wichterle et al., 2001; Polleux et al., 2002; Ang et al., 2003; Tanaka et al., 2003). Therefore, as we observed an accumulation of tangentially migrating interneurons within the ventral telencephalon, fewer interneurons had reached the neocortex. This impairment in migrating interneurons would, then, lead to a deficit in interneurons in the adult cortex. Consistently, fewer interneurons were detected in the dorsomedial cortex of syndecan-3 KO than in the WT mice (III: Figure 5B). Thus, GDNF interacts with syndecan-3 to promote the migration of immature interneurons in the developing telencephalon. In concordance with these findings, Pozas and Ibáñez (2005) have reported that GFRα1 mutant mice display altered migration route of migrating interneurons, and that fewer interneurons are found in the cortex of both GDNF and GFRα1 mutant mice. Thus, syndecan-3 may act as a co-receptor of GFRα1 in GDNF-induced migration of immature interneurons within the telencephalon.

In summary, syndecan-3 is a receptor for immobilized GDNF, NRTN, and ARTN: these GFLs bind to syndecan-3. The interaction of immobilized GDNF with syndecan-3 leads to the activation of SFK, important for cell spreading, the formation of neurites, and the tangential migration of immature interneurons. Additionally, as syndecan-3 harbors several HS chains, then a single syndecan-3 can bind several GFLs. Therefore, syndecan-3 can concentrate these GFLs in the ECM and neuronal cell surface in the surrounding area of their conventional receptors. This model suggests the cleavage of syndecan-3 extracellular domain to release GFLs, which can activate their receptors.

CONCLUSIONS

The main conclusions from this work are:

I.
We have developed an immunohistochemistry protocol using the device decloaking chamber for heat-induced epitope retrieval.
The use of decloaking chamber for epitope retrieval improves the detection of Olig2-labeled oligodendrocyte precursor cells within the developing ventral telencephalon.
Olig2-labeled cells are, then, easily quantifiable within the ventral telencephalon.

II.
NKCC1 is detected in the VZ/SVZ of LGE at early stage of neurogenesis.
NKCC1-expressing cells are neural progenitor cells in the LGE.
NKCC1 influences the progenitor cells to reenter the cell cycle.
Mice lacking NKCC1 exhibit fewer migrating interneurons and Olig2-labeled cells in the LGE at later stage, compared with controls.
Thus, ion homeostasis in neural progenitor cells of the LGE is essential in normal cell proliferation, thereby maintaining the pool of interneuron and oligodendrocyte progenitor cells.

III.
Syndecan-3 is a receptor for immobilized GDNF, NRTN, and ARTN.
Immobilized GDNF interacting with syndecan-3 mediates the tangential migration of immature interneurons within the developing telencephalon.

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-Ana Cathia

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