THE FUNCTION OF CONNEXIN43 IN NEURONAL MIGRATION AND CORTICAL DEVELOPMENT

by

Cima Cina

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate Studies

(CELL AND DEVELOPMENTAL BIOLOGY)

THE UNIVERSITY OF BRITISH COLUMBIA

(VANCOUVER)

April 2010

Abstract

During brain development, young neurons closely associate with radial glia while migrating from the ventricular zone (VZ) to the cortical plate (CP) of the neocortex. It has previously been shown that gap junctions are needed for this migration to occur properly, but the precise mechanism responsible is unclear. Using reverse transcription-polymerase chain reaction, western blot analysis, and immunohistochemistry, we found that among the family of gap junction proteins, connexin (Cx) 26, Cx36, Cx37, Cx43, and Cx45 were expressed in the mouse neocortex. In addition Cx43 and Cx26 were highly expressed in the radial glia and migrating neurons. Therefore, to examine the role of Cx43 in neuronal migration, we used Cre recombinase, driven by the nestin promoter, to conditionally knock-out a floxed coding DNA of the *Cx43* gene in mice. Radial glia in the VZ normally express Cx43. They undergo divisions that produce neurons, and serve as migratory guides for the daughter cells that they produce. Based on histological analysis, we proposed that removing Cx43 from radial glia alters the normal lamination of the mouse neocortex. To monitor newborn neurons, we introduced a plasmid containing green fluorescent protein driven by a neuronal (Tα1 tubulin) promoter into the embryonic neocortex using *in utero* electroporation. The majority of transfected migrating neurons remained in the VZ/intermediate zone (IZ) of the Cx43 conditional knock-out (*Cx43cKO*) animals, whereas in $Cx43^{fl/fl}$ mice, neurons migrated through the IZ into the CP, indicating that deletion of Cx43 from nestin-positive cells disrupts neuronal migration. We were able to rescue migration of Cx43cKO neurons by electroporating a cytomegalovirus-Cx43 expression plasmid into the embryonic cortex. In contrast, a C-terminal truncated form of Cx43 failed to rescue migration. In addition, $Cx43^{K258stop/-}$ mice, in which Cx43 lacks the last 125 amino acid residues of the C-terminal tail, gave results similar to those seen with the *Cx43cKO* mice. Furthermore, we conducted experiments with more specific deletions within the C-terminal tail, and found that a narrow region of 47 amino acid residues is required in directing neuronal migration. This study illustrates that full length Cx43 is required for neuronal migration in the neocortex.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ATP	adenosine 5'-triphosphate
BLBP	brain lipid binding protein
BrdU	5-bromo-4-chloro-3-indoly-β-galactosidase
cAMP	3,5'-cyclic adenosine monophosphate
cKO	conditional knockout
Ca^{2+}	calcium
CL	cytoplasmic loop
CNS	central nervous system
CP	cortical plate
CT	carboxy terminal
Cx	connexin
kDa	kiloDalton
Dab1	Disabled homolog 1
DAPI	4',6-diamidine-2-phenylindole
DCX	doublecortin
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlirate
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
EL	extracellular loop
ED	embryonic day
ER	endoplasmic reticulum
FGF-2	fibroblast growth factor-2
FLNA	filamin A
GABA	gamma-aminobutyric acid
GAD-67	glutamate decarboxylase 67
GAPDH	glyceraldehyde-3-phophate dehydrogenase
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GLAST	astrocyte–specific glutamate transporter

Hela	human adenocarcinoma cell line
INM	interkinetic nuclear migration
IP3	inositol triphosphate
IZ	intermediate zone
KO	knockout
LGE	lateral ganglionic eminence
LIS-1	lissencephally 1
LRP8	low-density lipoprotein receptor-related protein 8
MAGUK	membrane associated guanylate kinase
MAP-2	microtubule associated protein-2
MAPK	mitogen activated protein kinase
MGE	medial ganglionic eminence
MZ	marginal zone
NGn2	neurorogenin-2
NT	amino terminal
ODDD	oculo-dentodigital dyslasia
PDGF	platelet-derived growth factor
PKA	protein kinase A
PKC	protein kinase C
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SH2	src homology 2
SH3	src homology 3
SVZ	sub ventricular zone
TM	transmembrane
Tbr2	T box brain 2
VLDLR	very-low-density lipoprotein receptor
VZ	ventricular zone
ZO-1	zona occludens-1

Acknowledgements

First and foremost I offer my sincerest gratitude to my supervisor, Dr. Christian Naus, who has supported me throughout my thesis with his patience and knowledge whilst allowing me the room to work in my own way. I attribute the level of my Ph.D. degree to his encouragement and effort and without him this thesis, too, would not have been completed. One simply could not wish for a better or friendlier supervisor.

I would like to thank the members of my graduate committee, Dr. Vanessa Auld, Dr. Tim O'Connor, and Dr. Victor Viau, for their guidance and suggestions.

I also owe a huge debt of gratitude to Mr. John Bechberger. Without his knowledge and valuable technical advice this study would not have been successful.

As well as keeping me stocked with general supplies, Lynne Bechberger has also inadvertently, and without fail, provided something much greater in all the years I've known her: a friendly smile and a cheerful attitude.

My sincere thanks go to Dr. Wun Chey Sin for teaching me everything I know about PCR cloning, and for her comments that improved my manuscripts.

In my daily work I have been blessed with a friendly and cheerful group of fellow labmates: Charles Lai, Mike Kozoriz, Steve Bond, Annie Aftab, Max Levasseur, and Dr. Vincent Chen. Thank you for your encouragements, insightful comments, and for all the fun we had in the last six years.

Thanks to my husband, Saeed, for his patience and understanding throughout my thesis. Thanks for funding my many many shopping trips so that I unstress during hard times.

My deepest gratitude goes to my parents, Aqdas and Mohammad-hossein, who have always encouraged me to continue and strive for something better. They have always stressed the importance of education and provided me, always at great personal sacrifice but without question, the opportunity to pursue my goals.

I can not end without thanking my children, Fatemeh and Reza, for their love, and endless patience. Thank you for the inspiration during hard times.

The work of this thesis is dedicated to:

My children, Fatemeh and Reza, who have been patient with my studying, and have supported me throughout

Co-authorship Statement

The following thesis contains materials from previously published articles co-authored by Cima Cina, John F. Bechberger, Dr. Mark A. Ozog, Dr. Karen Maass, Dr. Martin Theis, Dr. Klaus Willecke, and Dr. Christian C. Naus. All experimental work, data analysis, and manuscript preparation presented in this thesis was performed by Cima Cina. Identification and design of the program was provided by Dr. Christian Naus and John F. Bechberger, and Cima Cina.

John F. Bechberger, Dr. Mark A. Ozog, Dr. Karen Maass, Dr. Martin Theis, Dr. Klaus Willecke, and Dr. Christian C. Naus provided consultation with preparation of the manuscripts. Dr. Wun Chey Sin provided technical consultation with the preparation of the truncated plasmids.

Dr. Karen Maass provided $Cx43^{K258stop}$ mice; Dr. Martin Theis and Dr. Klaus Willecke provided $Cx43^{fl/fl}$;nestin-Cre mice.

Chapter 1

Historical Review, Objectives, Rationale & Hypothesis

1.1 Historical Review

In the "*Historical Review*" section of this thesis, I will first discuss the development of the cerebral cortex, the different regions of the forebrain and the process of neurogenesis. This will be followed by a discussion of the importance of radial glial cells in neurogenesis, how neurons are made, the modes of neuronal migration, and the formation of the neocortex. Gap junctions have been proven to be important factors during brain development. Therefore, I will discuss the structure and regulation of gap junctions, and then the role of the most abundant gap junction protein, known as connexin43 in proliferation and neuronal migration, then following up by introducing the objectives, rationale, and the hypothesis of this thesis. Questions regarding the role of connexin43 in neuronal migration are raised, followed by the approaches that are taken to address these questions.

1.1.1 Development of the Cerebral Cortex

The forebrain is the most fascinating and complex part of the mammalian nervous system. The mature mammalian forebrain is composed of about 200 billion neurons and many times more glial cells. The precise anatomical structure of the forebrain including the six-layered neocortex is the result of sophisticated and highly organized cell proliferation, neuronal migration, and differentiation. Intriguingly, such an intricate cellular structure develops during a brief span, seven to ten days in rodents for instance, with variations among different species (Shimada and Langman, 1970; Caviness, 1982; Smart and Smart, 1982; Miller, 1986).

In early development, the forebrain, known as the prosencephalon, is generated from the most rostral part of the brain (Eagleston et al., 1990). The diencephalon and the telencephalon comprise the two major regions of the prosencephalon. Furthermore, the telencephalon is composed of the dorsal telencephalon and the ventral telencephalon. As the forebrain matures, the cererbral cortex and the hippocampus develop from the dorsal telencephalon. At the same time, the striatum and globus pallidus, known as the basal ganglia, arise from the ventral telencephalon. At the final stage of forebrain development, bilateral foldings of the prosencephalon give rise to the olfactory bulbs and cortical domains (Le Douarin, 1986; Cobos et al., 2001; reviewed in Rubenstein et al., 1998).

Recent molecular genetic studies have provided evidence for the involvement of a number of control genes in the process of brain development (Harland, 1997; Hemmati-Brivanlou and Melton, 1994). It has been shown that specific developmental genes are necessary for the generation of the brain's subdivisions. These genes are expressed in committed cells within the forebrain (Rubenstein et al., 1994), midbrain and cerebellum (Joyner, 1996), and hindbrain (Guthrie, 1996). They control the regionalization, proliferation, differentiation and migration of cells along the dorso-ventral axis (DV), and the anterior-posterior (AP) axis. For example, the *Emx* genes are expressed in the proliferative zone of the dorsal telencephalon, and provide boundaries between dorsal and ventral telencephalon (Mallamaci et al., 1998). However, ventral telencephalon contains *Dlx*-expressing post-mitotic cells leaving the ventricular zone. Hence, this gene might be involved in regulating the differentiation and migration in that region (Bang and Goulding, 1996). Furthermore, secreted sonic hedgehog protein is expressed in the mid-ventral cells of the forebrain and is responsible for the differentiation of the basal ganglia, whereas locally, peptide growth factors induce the dorsalization of cells (Doniach, 1995; Roelink et al., 1994; Jessel & Lumsden, 1997). The regionalization within the brain domains takes place at an early embryonic (E) period (E8.5-E13) which is the time of neurogenesis.

The process of brain development is generally the same among species. The neocortex originates from the dorsal telencephalon of the embryonic forebrain, and it is arranged into six separate layers of neurons that run parallel to the cortical surface. At the onset of neurogenesis, the embryonic cerebral ventricles contain a layer of proliferative cells, also known as ventricular zone (VZ) which gives rise to neural precursor cells (Fig. 1.1). The precursor cells later give rise to cortical neurons (Rakic, 1982). Indeed, the neural precursor cells exist in two zones. The first zone is the VZ and the second proliferative zone, called the sub ventricular zone (SVZ), appears above the VZ. Recent advances in molecular and cell biology techniques have identified two neural precursor cell types, which include the radial glial cells that are formed in the VZ, and intermediate progenitor cells that are formed in the SVZ. Both these types of neural precursor cells are responsible for generating all of the neurons in the embryonic cerebral cortex (Walsh and Cepko, 1990; Noctor et al., 2004).

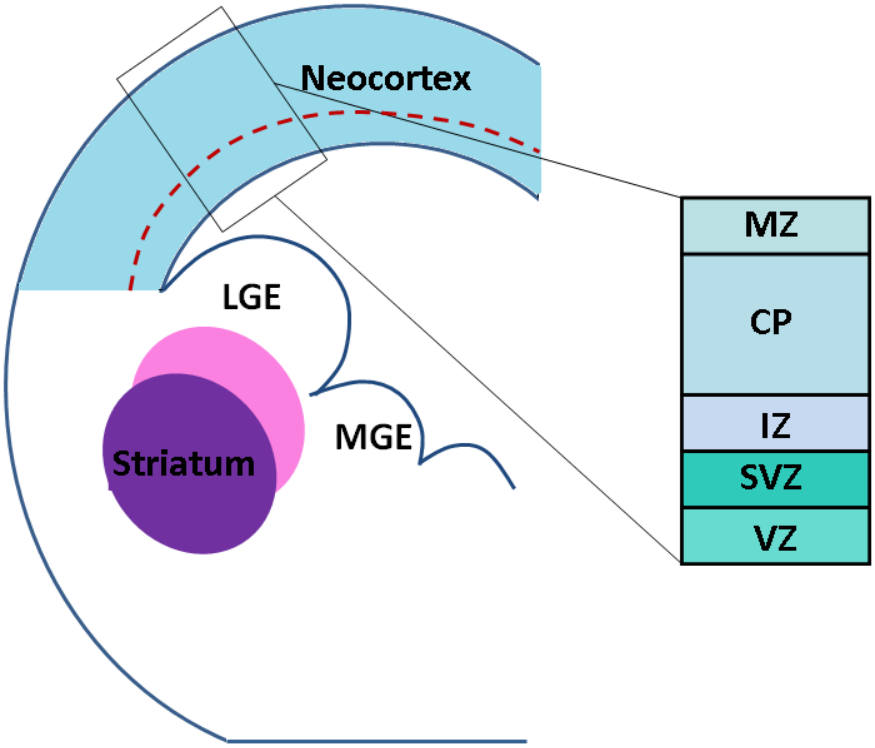


Figure 1.1 The schematic structure of the developing neocortex. The neocortex is divided into five zones: ventricular zone (VZ), subventricular zone (SVZ), intermediate zone (IZ), cortical plate (CP), and the marginal zone (MZ). The medial ganglionic eminence (MGE), the lateral ganglionic eminence (LGE), and the striatum are the major sources for interneurons, which migrate to all regions of the brain, including the cerebral cortex. Modified from Huang, 2009.

1.1.2 Origin of the Radial Glial Cells

Neurons and glial cells are the two major cell types of the mammalian neocortex. They originate from the neuroepithelial cells lining the neural tube (Rakic, 1972). During development, neuroepithelial cells undergo both symmetric and asymmetric cell divisions (Noctor et al., 2001). Before the onset of neurogenesis, neuroepithelial cells go through symmetric divisions to expand in the lateral ventricles. However, during neurogenesis, neuroepithelial cells switch to asymmetric cell divisions in order to generate radial glial cells and intermediate progenitor cells. Later during neurogenesis, both radial glial cells and intermediate progenitor cells divide asymmetrically to give rise to daughter cells which later become neurons (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). The nuclei of neuroepithelial cells undergo interkinetic nuclear migration (INM) while dividing. INM is a phenomenon where the nuclei of neuroepithelial cells translocate up and down the apical-basal axis. Indeed, during the S and G1 phases, the nuclei move to the basal side and move back to the apical side in the G2 phase, and stay at the apical side when undergoing mitosis (Takahashi et al., 1993; Tamai et al., 2007). The purpose of INM during each phase of cell division is to regulate cell fate. INM exposes the cell nuclei to neurogenic and proliferative apical-basal polarity signals. For example, cells with nuclei in the apical location where Notch signals are enriched, divide symmetrically and become proliferative, whereas cells with a basal nuclear location where Delta signals are increased, become neurogenic (Del Bene et al., 2008).

With the onset of neurogenesis, neuroepithelial cells differentiate into the related radial glial cells. Radial glial cells were first identified by Rakic (1971), and have a unique morphology based on Golgi staining (Rakic, 1971; Choi and Lapham, 1978). It was found that radial glia are bipolar cells spanning the entire thickness of the cerebral wall with one attachment at the ventricular surface, and the other attachment projecting to the pial surface. It was also determined that their somas are located close to the ventricular surface of the neural tube (Bentivoglio and Mazzarello, 1999). Similar to neuroepithelial cells, radial glial cells undergo INM during division. In neuroepithelial cells, the nuclei migrate up and down between the apical and pial surface. In contrast, the nuclei of radial glial cells are confined to the portion of the cell in the VZ during INM (Takahashi et al., 1993). A number of studies have provided evidence of the proliferation of the radial glia (Chanas-Sacre et al. 2000;

Misson et al., 1988; Noctor et al., 2008). Studies have shown that radial glial cells can be regarded as neural stem cells because they produce more radial glial cells by symmetric division, and can also produce one radial glia and one neuron through asymmetric division (Miyata et al., 2001; Noctor et al., 2001). *In vitro* studies have shown that signaling molecules such as Notch can determine the fate of these radial glial cells. For example, overexpression of Notch can cause a neurogenic radial glial cell to adopt a gliogenic fate instead (Gaiano et al., 2000). By monitoring the clonal pattern of individual radial glial cells it was observed that these cells undergo three types of cell division from the beginning to the end of neurogenesis. The first type is a symmetric cell division allowing radial glial cells to expand in the VZ, the second type is an asymmetric cell division so that the cells produce neurons, and the last type is a symmetric cell division in order to increase the number of neurons during cortical neurogenesis. This last symmetric cell division expands the size of the neocortex (Cai et al., 2002; Cubelos et al., 2008; Pontious et al., 2008).

During early neurogenesis, 80% of radial glial cell divisions occur in the VZ, and the other 20% occur in the SVZ which is the layer above the VZ that appears at later stages of neurogenesis (Boulder Committee, 1970; Levitt et al., 1983; Rakic, 1988). Furthermore, lineage analysis performed in mammalian striatum and chick optic tectum also indicated that radial glia can not only self-renew, but also they may give rise to other cell types (Gray & Sanes., 1992; Halliday and Cepko., 1992). All major cell types in the CNS including neurons, astrocytes and oligodendrocytes originate from radial glial cells (Reid et al., 1995; Noctor et al., 2001; Götz et al., 2002; Malatesta et al., 2003).

Mutations in genes that are critical for cell division during neurogenesis cause abnormalities in the development of the cerebral cortex. Type I lissencephally, also known as smooth brain is a genetic disorder due to mutations in the *LIS1* gene (Sicca et al., 2003). *LIS1* is normally expressed in the VZ cells and is associated with the dynein and dynactin motor protein complex thus regulating the mitotic cell division (Smith et al., 2000; Dujardin et al., 2003). Any mutation in the *LIS1*gene disrupts normal cell division and migration of projection neurons and interneurons (McManus et al., 2004). Patients with type 1 lissencephally have been reported to have small brains, thus implying a defect in cell proliferation (Norman et al., 1995). In addition, *in utero* RNAi and live cell imaging studies have shown that *LIS1* knockdown causes the migrating multipolar cells to accumulate in the

VZ and the SVZ. Therefore, based on this study, *LIS1* is an important factor for the exit of cells from the multipolar stage so that they return to the normal pattern of neuronal migration (Tsai et al. 2005).

1.1.3 Markers that are Expressed in Radial Glial Cells

At the beginning of neurogenesis, the radial glial cells begin to express specific markers that distinguish them from neuroepithelial cells and can also be used for cell identification. An example of one of these markers is the presence of cytoplasmic glycogen granules, predominantly in the subpial endfeet (Choi and Lapham, 1978; Bruckner and Biesold, 1981). Moreover, glycogen granules are present during neurogenesis and persist into adulthood when radial glial cells differentiate into astrocytes (Gadisseux and Evrard, 1985). Glial-fibrillary acidic protein (GFAP) is an immunohistochemical marker that is used to label radial glial cells in rodents, but it is only present at later stages of differentiation (Dahl et al., 1985; Sancho-Tello et al., 1995). Other markers for radial glial cells include vimentin, which is also present in mature astrocytes of mammals (Pixley and De Vellis, 1984), nestin, present in radial glia and in multipotential neural stem cells (Hockfield and McKay, 1985), monoclonal antibody RC1 (Edwards et al., 1990), and RC2 (Misson et al., 1988), the brain lipid binding protein (BLBP; Feng et al., 1994), and astrocyte–specific glutamate transporter (GLAST; Shibata et al., 1997). Approximately four days before the beginning of astroglial markers GLAST and BLBP expression (Hartfuss et al., 2001), and as early as E9, nestin and RC1-/RC2-immunoreactivity may be present in the radial glial cells of the mouse neocortex and spinal cord (Misson et al., 1988). The expression of nestin is sustained in radial glial cells until their postnatal differentiation into astrocytes. Nestin continues to be expressed in the GLAST and BLBP positive radial glia until their postnatal differentiation into astrocytes. As a result it is evident that there are a number of markers whose expression we can employ to distinguish radial glial cells from their neuroepithelial precursors.

Immunohistochemical studies detected the presence of radial glial cells as early as E9 in the mouse spinal cord and neocortex. One of the reasons that radial glial cells appear early during neurogenesis is that they give rise to neurons. As will be seen in the following sections, radial glial cells act as scaffolding for the migration of neurons from the VZ to the surface of the cerebral cortex (Rakic, 1990; Misson et al., 1991).

In most mammals, following the completion of neuronal migration, radial glial cells differentiate into astrocytes, therefore their presence is limited to the prenatal period (Schmechel and Rakic, 1979; Levitt and Rakic, 1980, Cameron and Rakic, 1991; Misson et al., 1991). This transition is accompanied by distinct morphological changes so that bipolar radial glial cells differentiate into multipolar astrocytes (Pixley and De Vellis, 1984; Marin-Padilla, 1995). In 1989, Voigt labelled radial glial cells in the cerebral cortex of ferrets with fluorescent tracers and detected these tracers in multipolar astrocytes at the completion of the neurogenesis period (Voigt, 1989). In rodents, once the radial glia differentiate into astrocytes at an early postnatal stage, there is a transition in the expression of intermediate filament proteins; nestin, vimentin, RC1, RC2 are lost, and GFAP is expressed instead (Hockfield and McKay, 1985; Culican, 1990). Some radial glial cells never differentiate into astrocytes, thus persisting into adulthood. These exemptions include Bergman glia, located in the developing and adult cerebellum, Müller glia in the retina, and radial glia present in the dentate gyrus of the adult hippocampus (Eckenhoff and Rakic, 1984; Rickmann et al., 1987; Cameron et al., 1993).

Taken together, at around E9, the bipolar radial glial cells originate from the neuroepithelial cells in the developing neocortex. They then begin to express a number of markers that separates them from their precursor cells. At the end of neurogenesis, the radial glial cells differentiate into multipolar astrocytes.

1.1.4 Origin of the Intermediate Progenitor Cells

In 1973, Smart conducted a quantitative study on the VZ and the SVZ proliferation, and reported that proliferation in the SVZ appeared as early as E11 (Smart, 1973). In addition, time-lapse imaging, retroviral labelling, and immunohistochemical studies have shown that secondary proliferative populations, known as intermediate progenitor cells originate from the radial glial cells in the VZ (Noctor et al., 2004; Kriegstein et al., 2006; Pontious et al., 2008). These cells are produced by both symmetric and asymmetric cell divisions. In asymmetric cell divisions, a radial glial cell divides to produce another radial glia and an intermediate progenitor cell. In symmetric cell divisions, an intermediate progenitor cell self-renews thus producing two copies of itself (Noctor et al., 2008). Intermediate progenitor cells express different molecular markers than neuroepithelial cells and radial glial cells. They

express T box brain 2 (Tbr2) and neurogenin 2 (Ngn2) (Englund et al., 2005), and they do not express GLAST (Malatesta et al., 2003). At approximately E15 in the mouse dorsal telencephalon, the intermediate progenitor cells that are born in the VZ translocate their nuclei to the SVZ where they divide symmetrically one more time to give rise to neurons. Later in development, these neurons migrate toward the pial surface (Götz and Huttner, 2005; Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Taken together, the intermediate progenitor cells are derived from the radial glial cells in the VZ. They give rise to a smaller population of neurons that migrate to the surface of the brain to settle in the proper layers of the CP.

1.1.5 Origin of Pyramidal Neurons

There are two major types of neurons in the CNS. They include pyramidal neurons and interneurons. Pyramidal neurons are the most abundant, making up almost 80% of the total neuronal population in mammals. They originate from the dorsal telencephalon of the embryonic cortex, and migrate radially to form the cortical layers. Furthermore, these neurons use glutamate as a neurotransmitter and form excitatory synapses with other cortical areas, thalamus, striatum, brainstem, and spinal cord. The other 20% of the neuronal population is composed of interneurons. They arise in the ventral telencephalon and migrate tangentially to the neocortex. These neurons use γ-aminobutyric acid (GABA) as a neurotransmitter and make inhibitory synapses with other neurons of the neocortex (Anderson et al., 1997; Tan et al., 1998; Marín and Rubenstein, 2001).

In 2000, Malatesta and colleagues found that a large proportion of radial glia at E14 and E16 of the mouse neocortex gave rise to neurons (Malatesta et al., 2000). Another group confirmed these results by conducting *in vivo* experiments. They used a combination of time-lapse video imaging with GFP lineage tracers in order to show that radial glial cells divide and generate neurons at mid neurogenesis, E15-E16 (Noctor et al., 2001). The authors characterized the progeny of single GFP-labelled radial glial cells and were able to observe that the majority of the progeny were neurons, as expected. Furthermore, they observed that each radial glial cell divides asymmetrically at the VZ following INM, thus giving rise to one neuron and one radial glial cell per cell cycle (Noctor et al., 2001). Supporting previous findings, Miyata and colleagues (2001) conducted time-lapse video imaging experiments to

monitor individual radial glial cells from E14 cortical cultures that were labelled by a fluorescent tracer called DiI. The study showed that during asymmetric division in the VZ, radial glial cells give rise to pyramidal neurons. They also discovered that radial glial processes are not lost during cell division. This is important during development because newly born neurons use these radial processes to migrate out of the VZ and into their final location (Miyata et al., 2001).

Each population of radial glial cells is destined to generate only neurons, or astrocytes or oligodendrocytes. However, during neurogenesis, there are small proportions of radial glial cells that can give rise to all three of these cell types (Malatesta et al., 2003).

As previously stated, intermediate progenitor cells also give rise to neurons (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Pontious et al., 2008). Intermediate progenitor cells are generated in the VZ, and then they translocate to the SVZ after multiple cell divisions. In the SVZ, intermediate progenitor cells first become multipolar and then undergo one more round of symmetrical cell division resulting in two immature neurons. These immature neurons leave the SVZ and migrate to the CP where they differentiate and form specific cortical layers (Anthony et al., 2004). The SVZ continues to generate neurons and persists into adulthood. The neurons that are generated in the SVZ are GABAergic interneurons that migrate to the olfactory bulb as chains via the rostral migratory stream (Luskin, 1993). Although both radial glial cells and intermediate progenitor cells give rise to neurons, the majority of these neurons are derived from radial glial cells (Anthony et al., 2004).

During neurogenesis, post mitotic neurons leave their birth place in the VZ and migrate to the pial surface where they settle. This neuronal migration plays a vital role in the formation of the very intricate brain as it occurs in a highly ordered fashion. At this stage, any disturbances including environmental factors, detrimental genetic mutations, or proliferation defects can lead to faulty neuronal migration. Mental retardation, epilepsy, and severe learning disabilities can result from these abnormalities. Understanding how cell migration occurs in the forebrain is therefore crucial to discerning the mechanisms underlying its normal and pathological development (Aicardia, 1994; Guerrini and Filippi, 2005).

1.2 Migration Modes of Cortical Neurons

In general, two types of neuronal migration occur during the development of neocortex: i) radial guided neuronal migration, in which immature neurons between E11 and E18 leave the VZ and follow the scaffolding radial fibers to migrate toward their destination in the CP; and ii) tangential migration, in which interneurons do not use the radial fibers to migrate, and instead they follow migrational cues and other axons to migrate parallel to the pial surface and reach their final location (Lavdas et al., 1999; Nadarajah et al., 2001).

1.2.1 Radial Glial Guided Neuronal Migration

At around E11 in the mouse neocortex, the first generation of neurons leave the VZ and migrate above the VZ to form a layer called the preplate (Fig. 1.2). At E13, the next generation of neurons migrate out of the VZ and divide the preplate into the two separate layers called the marginal zone (MZ) and the subplate (Angevine and Sidman, 1961; Rakic, 1972). The MZ forms the layer I of the mature cerebral cortex and it consists of Cajal-Retzius cells (Konig et al., 1981; reviewed in Parnavelas, 2002). These cells secret Reelin, an extracellular matrix glycoprotein that regulates the laminar organization of the cerebral cortex (Hirotsune et al., 1995). At this stage the intermediate zone (IZ) appears, which contains only axons that are perpendicular to radially migrating neurons. Between E13 and E18, multiple generations of neurons leave the VZ and establish themselves in different layers (Layers II-VI) just beneath the MZ and form the cortical plate (CP). This process is completed in an inside-out manner meaning that the first neurons to arrive make up the deeper layers and the newer generations make up the more superficial layers (Hatten, 1999, Nadarajah et al., 2001, 2003). At the end of neurogenesis, most of these migrating neurons differentiate into pyramidal neurons once they have positioned themselves into the specific layers (Chan et al., 2001; Gorski et al., 2002).

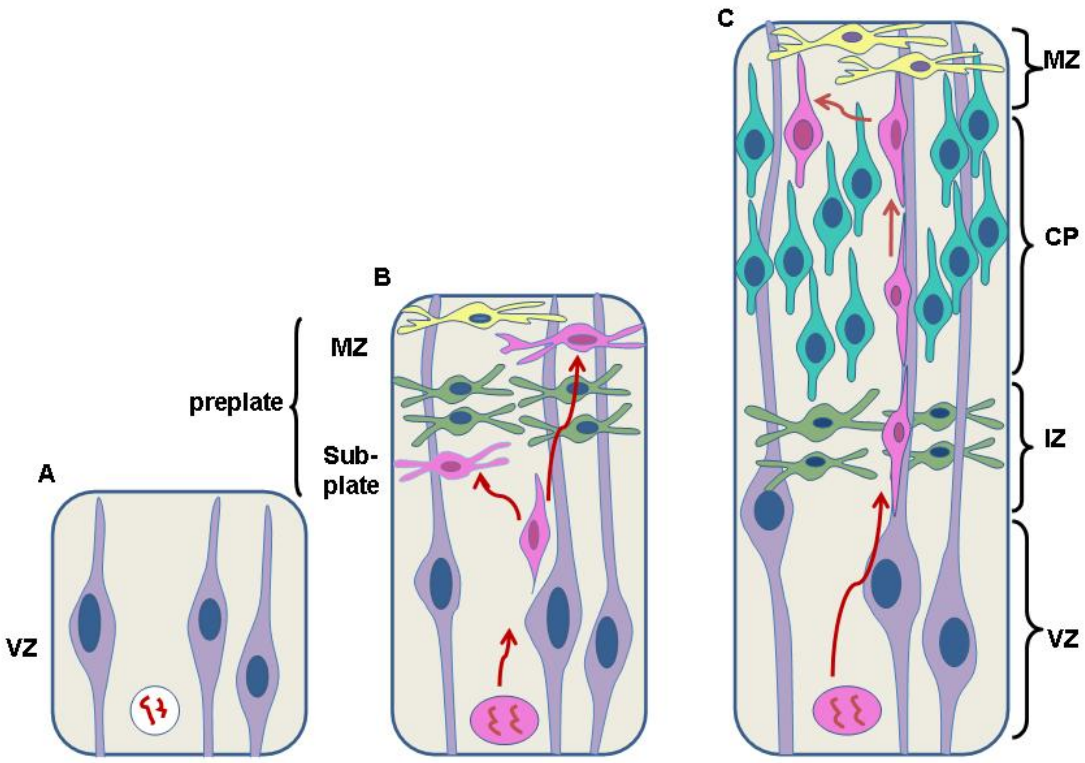


Figure 1.2 Schematic illustration of rodent neocortical formation. **A**, In the ventricular zone (VZ), radial glial cells undergo interkinetic nuclear migration (purple cells), and give rise to daughter cells that become newborn neurons. **B,** Radial migration: at E10, the first generation of neurons migrates away from the VZ along radial glial cells and forms the preplate. The preplate is composed of Cajal-Retzius neurons (yellow cells) which form the marginal zone (MZ), and subplate neurons (pink cells). In the meantime, the intermediate zone (IZ) appears which contains tangentially migrating neurons (green cells). **C,** In the following days, a subsequent set of neurons migrate from the VZ to populate the definitive cortical plate (blue cells). Therefore, at E18, the developing neocortex consists of the VZ, IZ, CP, and the MZ. Modified from Kriegstein and Parnavelas, 2003.

Abnormalities in certain genes that are involved in neuronal migration cause disruptions in the formation of cortical layers. For example, doublecortin *(DCX)* plays an important role in the proper lamination of neurons during migration. *DCX* associates with and stabilizes the dynamics of microtubules in migrating and differentiating neurons (Bielas and Gleeson, 2004; Schaar et al., 2004; LoTurco, 2004). Mutations in *DCX* cause the post-mitotic neurons leaving the VZ to accumulate in the white matter and form a band of neurons beneath the CP. Therefore, this abnormality in the formation of the neocortex causes a disease, known as double cortex (Gleeson et al., 1999; Ramos et al., 2006).

1.2.2 Modes of Radial Migration

There are two types of radial glial guided cell movement in the developing neocortex called 'locomotion' and 'somal translocation'. At early stages of neurogenesis, migrating neurons use a type of cell movement called 'somal translocation' (Miyata et al., 2001; Nadarajah et al., 2001, 2003). In this type of migration, neurons translocate from the VZ to the CP with a long leading process and a short trailing process that becomes shorter as they move up to the CP (Brittis et al., 1995; Morest, 1970; Nadarajah and Parnavelas, 2002).

In 2001, Nadarajah and co-workers used time-lapse video imaging on cortical slices in order to observe the movement of migrating cells. In the study, the authors showed that the migrating neurons undergoing somal translocation have a long leading process that is attached to the pial surface, and a short trailing process that extends from the VZ.
Somal translocation seems to be the prominent mode of migration in different areas of the CNS. For example, in 1998, Meyer and colleagues analyzed the movement of migrating neurons from the VZ to the preplate in the rat cerebral cortex, and observed that the cells used the mechanism of somal translocation in order to migrate toward the preplate. Similar observations have been made in the retina and spinal cord (Brittis et al., 1995; Snow et al., 1995).

In 1974, Rakic used serial section electron microscopy to reconstruct the three-dimensional morphology of migrating neurons present in the IZ at late stage of primate cortical development. These migrating neurons were bipolar with a thick leading process toward the pial surface, and a thin trailing process facing the VZ. Also, they were in close contact with radial glial fibers. As previously mentioned, the bipolar radial glial cells span

the entire thickness of the cerebral wall. Therefore, based on his observations Rakic suggested that the migrating neurons follow the fibers of radial glial cells to reach their final destination in the CP. Many studies have supported this model, with both *in vitro* (Edmindson and Hatten, 1987; Fishel and Hatten, 1991; Hatten, 1993) and *in vivo* experiments (Anton et al., 1996). These studies have shown that the majority of newborn neurons in the cerebral cortex do use radial glial fibers as scaffolding for migration. This type of migration is referred to as 'locomotion', because the short leading process of the migrating neuron is not attached to the pial surface, and can thus move freely. This locomotion of migrating neurons is short and quick, and then it becomes slower as the neurons move close the surface of the brain (Nadarajah et al., 2001).

It should be noted that newer generation of radially migrating neurons use the mechanism of somal translocation at earlier stages of neocortical development when the cerebral wall is thin. However locomotion of later born neurons occurs at later stages. The existence of two modes of radial guided glial migration is due to the expansion of the cerebral wall so that migrating neurons need more than one mechanism to reach their final location (Nadarajah et al., 2001).

In general, the pattern of radial guided neuronal migration in the cerebral cortex can be summarized into four steps: i) post-mitotic neurons leave the VZ and move along the radial fibers to reach the SVZ; ii) in the SVZ, migrating neurons adopt a multipolar morphology to extend their protrusions in all directions while becoming stationary for 24 hours; iii) at this step, the multipolar neurons turn toward the VZ and move back into the VZ. The reason for this retrograde movement might be that these neurons need to obtain informational cues regarding their fate from factors that are present in the VZ. These cues may provide information about which lamina they should settle in once they reach the CP (Bittman et al., 1997); iv) neurons lose their multipolar morphology, extend a long leading process toward the surface of the brain and ascend to the CP along the radial glial fibers (Noctor et al., 2004); v) once the leading processes of migrating neurons reach the MZ, they receive a stop signal from Reelin that is secreted by the cells in the MZ. Subsequently, the migrating neurons dissociate from the radial glial fibers and form the cortical layers (Nadarajah et al. 2002).

1.2.3 Signaling Molecules that Are Involved in Radial Migration in the Neocortex

The formation of the neocortex requires precise migration of neurons from the VZ into the CP. Each wave of newborn neurons migrates past the earlier wave and forms a more superficial lamina until the six-layered neural structure of the CP is properly established in an 'inside-out' manner. In this process, radial glial cells play a critical role in neuronal migration as they are in close contact with the neurons (Rakic, 1972).

Increasing evidence has suggested that a number of signaling molecules allow for the interactions between radial glial cells and migrating neurons in order to facilitate neuronal migration. For example, astrotactin is expressed by migrating neurons and binds to the receptors located at the membrane of the radial glial cells, thus providing neuron-glia adhesion necessary for migration (Zheng et al., 1996). Laminin is expressed in the VZ, subplate and the MZ of the developing neocortex. The expression of laminin in the radial glial cells provides attachment between the cells and the migrating neurons, thus enhancing neuronal migration (Hunter et al., 1992). In addition, neuregulins are membrane proteins that are expressed by cortical neurons and promote neuronal migration (Anton et al., 1997). Neuregulins bind to their receptors which are present on the cortical neurons, and increase the length of leading and trailing processes, thus promoting neuronal migration. Neuregulins also elicit growth and development of the radial glial cells via induction of BLBP on the surface of radial glial cells. BLBP in turn modulates the cell surface of radial glia by elongating the fibers thus facilitating the migration of neurons along these fibers.

Conversely, other signaling molecules cause neuronal migration to stop. For example, Reelin is a glycoprotein that is secreted by the Cajal-Retzius cells of the MZ. Reelin binds to very-low-density lipoprotein receptor (VLDLR) and low-density lipoprotein receptor-related protein 8 (LRP8) on migrating neurons. Through these receptors, Reelin sends a stop signal to migrating neurons. In fact, this action of Reelin depends on the Reelin-α3β1 integrin interactions. Integrins are transmembrane receptors that mediate adhesions between neurons and glia. Reelin binds to α3β1 integrin in a complex including VLDLR and LRP8 to alter neuron-glia adhesion. The formation of this complex enables the dissociation of migrating neurons from the radial glia so that they cease migration and reside in the CP and beneath the MZ (Hirotsune et al., 1995; Dulabon et al., 2000).

Accumulating investigations have shown the requirement of certain genes that are critical for the proper migration of neurons to the cerebral cortex. For example, deletion of Reelin from the Cajal-Retzius cells of the MZ causes a defect in the normal lamination of migrating neurons. In the absence of Reelin, neurons accumulate in an abnormal 'outside-in' pattern in the cerebral cortex. This means that the first wave of neurons accumulates above the CP and the following waves of neurons end up residing beneath the previous neurons (Ogawa et al., 1995; D'Arcangelo et al., 1995). One of the best animal models to study this abnormal migration is the *Reeler* mice in which the *Reelin* gene is mutated, and therefore the CP appears inverted (Caviness, 1982).

The Filamin A (*FLNA*) gene encodes a protein that is expressed in the VZ cells. FLNA has been shown to be associated with F-actin cytoskeleton and stress fibers, thus crucially involved in the regulation of cell shape, formation of filopodia and migration (Fox and Walsh, 1990). Following mutations in the *FLNA* gene, postmitotic neurons fail to leave the VZ, and instead they accumulate in that region thus leading to an abnormal condition, known as periventricular heterotopia (Fox et al. 1998). It should be noted however, that a fraction of neurons migrates normally out of the VZ and forms a proper multi-layered CP. In humans, the mutation in *FLNA* is associated with epilepsy and reading disorders (Dobyns et al., 1997; Sheen and Walsh, 2003; Chang et al., 2005). Therefore, *FLNA* is a potentially important gene that is required for neuronal migration.

1.2.4 Tangential Migration

Pyramidal neurons that reside in the neocortex use the mode of radial glial guided migration to reach the CP. However, some neurons, especially GABAergic inhibitory interneurons, move tangentially and perpendicular to the radial glial fibers to reach their final position (Walsh and Cepko, 1992; DeDiego et al., 1994; O'Rourke et al., 1995). Interneurons do not migrate along the radial glial fibers, and instead they obtain migrational cues from surrounding axons to reach their destination in different regions of the forebrain (DeDiego et al, 1994; Lavdas et al., 1999). A group of interneurons that originate in the medial ganglionic eminence of the ventral telencephalon migrate tangentially to the SVZ, lower IZ, and the MZ of the developing neocortex (Lavdas et al., 1999; Wichterle et al., 2001, Xu et al., 2003). Then, from these regions, these interneurons change directions and migrate radially toward

the CP where they differentiate (Tanaka et al., 2003; Ang et al., 2003). These findings were also confirmed by an *in vivo* study using real–time imaging of glutamate decarboxylase 67 green fluorescent protein (GAD67–GFP) knock-in mice. GAD-67 was used to visualize the GABAergic neurons. The data showed that at developmental stages E15.5-E17.5, the GFP-positive cortical interneurons first entered the SVZ, the IZ, and the MZ of the neocortex, and then migrated to the CP along the radial glial fibers (Tamamaki et al., 2003). Another group of neurons is derived from the VZ of the lateral ganglionic eminence and the striatum. During neuronal migration, these neurons migrate to the SVZ, and then tangentially migrate toward the olfactory bulb where they differentiate into interneurons (De Carlos et al., 1996; Anderson et al., 1997). Additionally, a group of researchers conducted a time–lapse imaging study on cortical slice cultures and observed that a subset of tangentially migrating neurons undergo a third mode of migration, called 'ventricle–directed migration'. After these neurons reach the SVZ, they turn downward and move back to the VZ, where they pause briefly. Then the neurons reverse direction and migrate radially to the CP. The reason for ventricle-directed migration might be that the VZ contains cues regarding positional information for the cortical migrating neurons (Nadarajah et al., 2002).

There are also different signaling molecules that serve as guidance cues for the axons of the tangentially migrating neurons. For example, Netrin is an attractive cue that binds to the receptors present on the surface of the growth cone of migrating neurons and ensures their migration in a dorsal-ventral direction (Serafini et al., 1996). In contrast to Netrin, a repulsive axonal guidance, known as Slit, binds to the Robo receptors that are expressed in the commissural axons. Slit then prevents the ipsilateral neurons from crossing over the midline to the contralateral side during their migration (Kidd et al., 1998). Additionally, Semaphorin is a chemorepulsive signaling molecule that is expressed in the striatum. Semaphorin-3A sends signals to guide the migrating interneurons that are originated in the medial ganglionic eminence so that they do not go through the striatum, and instead they migrate to the neocortex (Chen et al., 2008).

1.2.5 The Role of Actin Cytoskeleton and Membrane Proteins in Neuronal Migration

During cell migration, the formation of membrane ruffling at the leading edge of the migrating neuron is required in order to move the cell forward. This is accomplished through the coupling of actin filaments to the binding proteins, thus activating the downstream signaling pathway. For example, integrins are single-pass transmembrane proteins that lock the actin cytoskeleton to the extracellular matrix. This action of integrins is mediated via an actin binding protein, known as cortactin. Cortactin has been shown to become tyrosine activated by the SH3 domain of Src tyrosin kinase at its C-terminal domain following integrin activation. Phosphorylation of cortactin translocates this protein from the cytosol to the membrane. Then, cortactin binds to and activates actin polymerization proteins, called WASP and actin related proteins, called ARP2/3. Src, ARP2/3, and WASP complex stimulate Rac and Cdc42 of the Rho GTPase family, which in turn elicit actin remodeling at the leading edge of the migrating neurons, thus creating membrane protrusions such as lamellipodia and filopodia. These membrane protrusions that are produced by Rho GTPase family adhere to the extracellular matrix at the integrin based focal contacts in order to initiate and maintain cell motility (Vuori and Ruoslahti, 1995; Small et al., 2002; Hall, 2005).

Furthermore, another family of actin binding proteins that regulates neuronal migration is myosin II. During cell motility actin and myosin II accumulate in the rear of the nucleus and push the nucleus forward. In addition, the cooperation of actin and myosin II helps the trailing process of the migrating cells to retract, thus facilitating neuronal migration (Bellion et al., 2005).

Accumulating investigations have shown that multi-cellular events such as interactions between radial glia and migrating neurons through adhesion molecules, as well as activation of the receptors that provide signaling pathways are required for the proper migration of neurons to their final destination. For example, cadherins are transmembrane proteins that mediate cell-cell contact. Cadherins bind to a complex of proteins in the cytoplasm, called p120 catenin, which in turn binds to actin cytoskeleton and links it to the extracellular matrix. P120 catenin modulates the activity of Rho GTPase family, and reorganizes the actin filaments at the leading edge, thus enhancing cell motility (Noren et al., 2000). Additionally, a member of tight junction proteins, known as Zona occluden-1(ZO-1) links the actin cytoskeleton to occludin, which is a transmembrane protein. The C-terminal

domain of ZO-1 interacts with the actin cytoskeleton and the N-terminal domain of ZO-1 contains the occludin binding site. Therefore, ZO-1 might have implications in the assembly of actin filaments at the tight junctions (Fannings et al., 1998). Moreover, junctional proteins such as gap junctions are multi-pass transmembrane proteins that are involved in cell migration (Fushiki et al., 2003). Aberrations in gap junctions during development have been associated with CNS abnormalities, such as mental retardation and stroke (Aicardia, 1994; Nakase et al., 2004). Therefore, the work of this thesis is focused on the role of gap junctions in neuronal migration and cortical development.

1.3 Gap Junctions

Gap junctions are specialized intercellular membrane channels that provide for the direct intercellular exchange of small metabolites, ions, nutrients and second messengers less than 1200 Da, such as Na^+, K^+, Ca^{2+}, IP_3, ATP, and cAMP (Bennett et al., 1991; Evan and Martin, 2002; Makowski et al., 1977; Spray et al., 1991). Due to their large pore size gap junctions are not selective with regard to the passage of the small metabolites and ions in each cell (Gilula et al., 1972; Pitts and Simms, 1977; Goodenough, 1980). A group of researchers characterized gap junctions by their appearance in electron micrographs as two adjacent membranes separated by a 2-nm space (Brightman and Reese, 1969). These channel-like structures allow for the direct cytoplasmic continuity between the adjacent cells and, thus, provide a direct intercellular gap junctional communication (Goodenough et al. 1980). Also, different gap junction protein constituents have different physiological roles (Flagg-Newton and Loewenstein, 1980; White et al., 1985, Spray and Bennett, 1985). For example, connexin26 that exists in the cochlear connective tissue of the inner ear, provides the KCl spatial buffering through membrane channels in order to balance the endolymphatic fluid, whereas during a stroke, it has been proposed that reactive astrocytes remove toxins away from cells via connexin43 (Martinez et al., 2009; Nakase et al., 2003).

1.3.1 Structure of Gap Junctions

Each gap junction channel consists of two hemichannels called connexons (Fig. 1.3). In turn, each connexon is composed of six protein subunits termed connexins (Cx). Cxs comprise four transmembrane domains, two extracellular domains, and three cytoplasmic domains [(C-terminal, N-terminal, cytoplasmic loop) Kumar and Gilular, 1996]. The third transmembrane domains of the six Cxs come in close contact, and make up the wall of the pore in each connexon. Each extracellular loop contains three cystein residues forming intramolecular disulfide bonds that link the two extracellular loops. One extracellular loop (E1) connects the first and the second transmembrane domains (M1 to M2); the other extracellular loop (E2) connects the M3 to M4. In addition the extracellular loops are responsible for the docking of connexons located at the two adjacent cells (Yeager and Nicholson, 1996). In general, the size of the cytoplasmic loop region, and the length of the carboxy terminal tail in each Cx is unique, although Cxs share many similar features (White

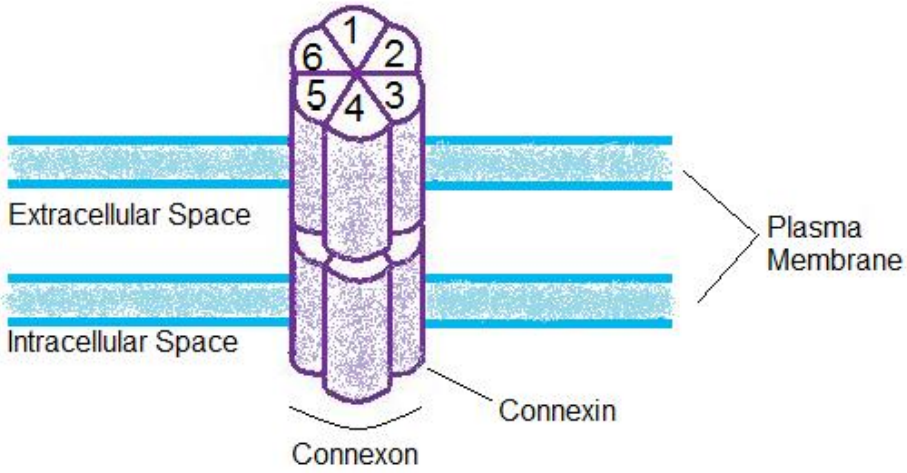
et al, 1995). Cxs are encoded by a multi-gene family consisting of 21 members in the human and 20 members in the mouse genome (Willecke et al., 2002). Cxs are abundantly expressed in vertebrates, during development and in adulthood. Cxs are named according to cDNA cloning from the tissue of origin and their approximate molecular weight in kiloDaltons. For example, Cx26 and Cx43 refer to the Cxs with molecular weight of 26 kDa and 43 kDa, respectively (Beyer et al., 1987). Cxs are expressed in all tissues with the exception of spermatocytes, thrombocytes, and adult skeletal muscle cells.

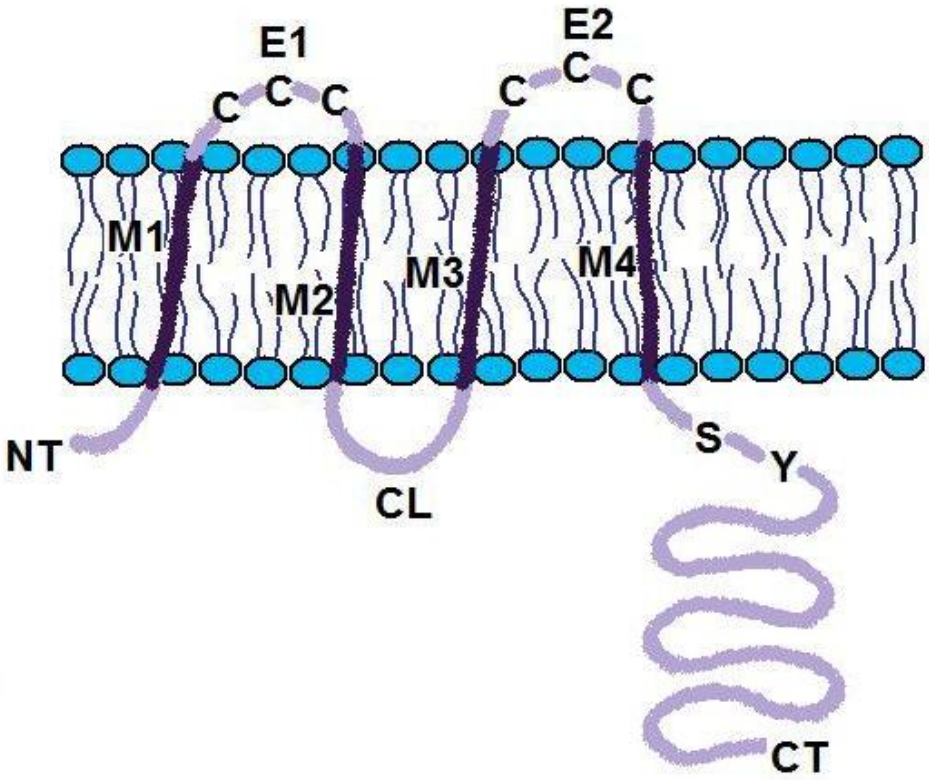
Connexons containing only a single isoform of Cx are called homomeric, while those containing different Cxs are called heteromeric. Two connexons comprised of the same types of Cx proteins can form a homotypic channel between cells, whereas two connexons comprised of different Cx proteins can form a heterotypic gap junction channel (Elenes et al., 2001; Elfgang et al., 1995; White et al., 1995; reviewed in Bruzzone et al., 1996). Heterotypic channels consisting of diverse Cxs have distinct permeabilities, which may affect the function of the gap junction channels in a particular cell type compared to the corresponding homotypic channels (Elfgang et al., 1995).

1.3.2 Regulation of Gap Junctions by Phosphorylation

Except for Cx26, all Cxs are phosphoproteins. Phosphorylation of Cxs is necessary to regulate their formation and function (Saez et al., 1990; Traub et al. 1989). A variety of kinases and phosphotases including sarcoma tyrosine kinase (Src), protein kinase C (PKC), protein kinase A (PKA), and mitogen-activated protein kinases (MAPKs) are needed to regulate the trafficking, assembly and disassembly, degradation, and channel gating of Cxs (Berthoud et al., 2000; Hossain et al., 1998; Lamp and Lau, 2000; Musil and Goodenough, 1991). Cx43, which is the most studied Cx, contains several sites for phosphorylation in its C-terminal region (Fig. 1.4). For example, studies have shown that phosphorylated tyrosine residues in the C-terminal region of Cx43 form binding sites for the SH2 and SH3 domains of Src tyrosine kinase. First, Src binds to Cx43 through an SH3 domain and phosphorylates Cx43 on the proline residues including P274, P277, P280, and P283. Then, Src subsequently binds to Cx43 via an SH2 domain and phosphorylates Cx43 on the Tyr265 site. These series of phosphorylation events lead to the closure of gap junction channels (Lin et al., 2001). Therefore, Src appears to be an important physiological factor to regulate Cx43.

Figure 1.3 Schematic diagram of gap junction proteins. Top: Gap junctions are formed by the association of two hemichannels (connexons) in the apposing plasma membranes of neighboring cells. Each connexon is composed of six protein subunits called connexins. Bottom: Each connexin is composed of four transmemebrane domains (M1-M4), two extracellular loops (E1-E2), each containing three cystein residues which are important for docking, and a cytoplasmic loop (CL). The amino (NT) and the carboxy terminus (CT) are located at the cytoplasmic side. Modified from Bruzzone et al., 1996.


1
2
3
4
5
6
Extracellular Space
Intracellular Space
Plasma
Membrane
Connexin
Connexon


E1
E2
C
C
C
C
C
C
M1
M2
M3
M4
NT
CL
S
Y
CT

Protein kinase C (PKC) has also been shown to phosphorylate Cx43 on its Ser368 binding site and to inhibit the function of gap junctions (Lampe et al., 2000; Lampe and Lau, 2000). Similar findings have been reported in the analysis of cardiomyocytes where co-immunopercipitation experiments showed association between PKC and Cx43. In those experiments, a mitogen and cardioprotective protein, fibroblast growth factor 2 (FGF-2), induces PKC phosphorylation of Cx43 which leads to the closure of gap junctions (Doble et al., 2000). In contrast to PKC, cAMP-dependent protein kinase A (PKA) has been reported to phosphorylate Cx43 on the Ser364 residue and enhance gap junction assembly (Tenbroek et al., 2001).

Moreover, mitogen-activated protein kinsase (MAPK) has been demonstrated to modulate the activity of Cx43 following phosphorylation. Epidermal growth factor (EGF), FGF, and platelet-derived growth factor (PDGF) have been shown to activate MAPK via an intracellular kinase pathway (ERK1/2) which is followed by phosphorylation of Cx43 and concomitant inhibition of gap junction channels (Doble et al., 1996; Hossain et al., 1998; Yao et al., 2000).

1.3.3 Gap Junctions and Interacting Proteins

There are several interacting proteins at gap junctions, any of which could be involved in the gating, assembly, formation, and function of gap junctions. These interacting proteins can be found at the cell-cell junctions, and include: Zona occludens-1 protein (ZO-1), cadherins, claudin, NOV/CCN3, and cytoskeletal proteins (Hertig et al., 1996; Giepmans and Moolennar, 1998; Giepmans et al., 2001; McLeod et al., 2001; Perez-Moreno et al., 2003). Co-localization of actin filament fibers and Cxs has been shown in a variety of cell types (Lo et al. 1996; Wall et al., 2007; Yamane et al., 1999). Also, co-immunopercipitation experiments have linked Cx43 to α-actinin at regions of cell-cell contact in cardiac neural crest cells (Xu et al., 2006). Thus, α-actinin might be a good scaffolding candidate to link gap junctions to the cytoskeleton. Furthermore, as mentioned above, several Cx protein isoforms bind to the membrane associated guanylate kinase (MAGUK) family, such as ZO-1. In fact, ZO-1 has been shown to co-localize and interact with the C-terminal region of Cx43.

It has been demonstrated that removal of the four amino acids (amino acids 379-382) inhibits the binding (Giepmans et al., 2001).

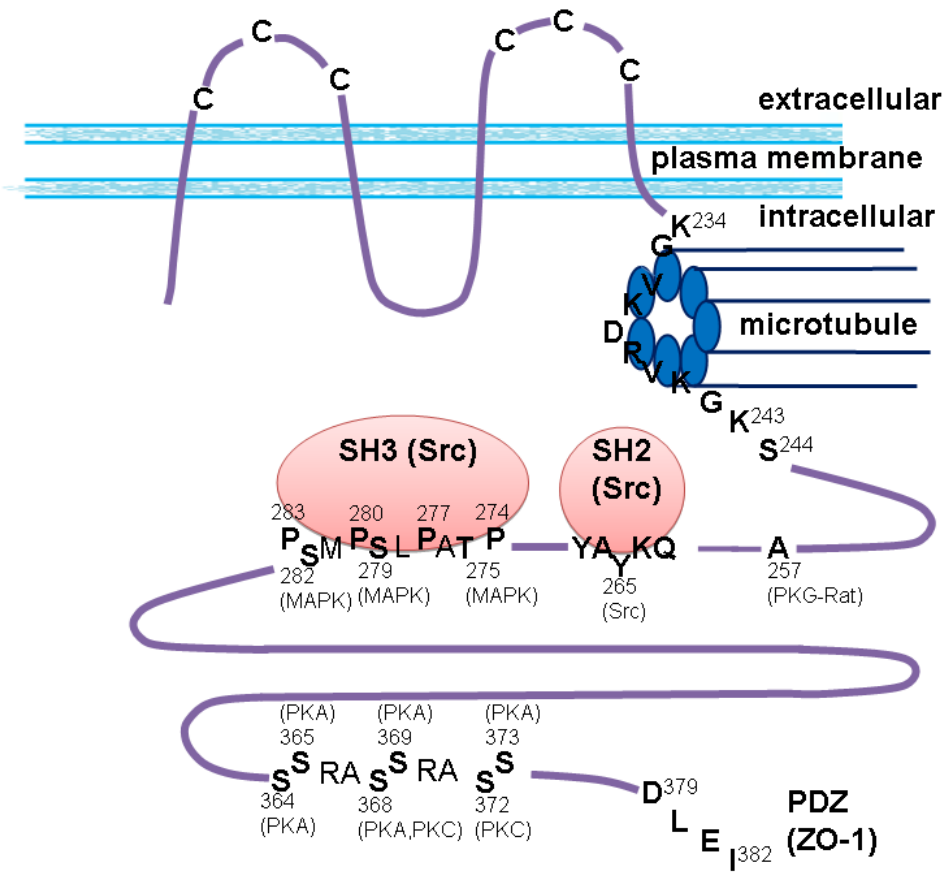


Figure 1.4 Schematic diagram of Cx43 protein and interacting protein modules. There are three cystein residues that link the extracellular loops. Microtubule binding sites on the C-terminal tail are K, V, G, P, and R. Src homology 2 domain (SH2) binds phospho-tyrosine residues. Src homology 3domain (SH3) is another binding site that binds phospho-tyrosine residues of the C-terminal domain. ZO-1 homology domain (PDZ) binds the last four amino acid residues of the C-terminal domain of Cx43. Modified from Giepmans, 2004.

Co-immunopercipitation studies have shown that ZO-1 provides an interaction between gap junctions and the membrane cytoskeletal protein, α-spectrin (Toyofuku et al. 1998).

1.3.4 Gap Junctions in the Developing Cerebral Cortex

Gap junctions have been demonstrated to be highly expressed during the development of the brain, and are implicated in the proliferation of radial glial cells, differentiation and migration of neurons (Lo, 1996). Of the 20 Cx genes expressed in the mouse genome (Willecke et al, 2002), five of them have been shown to be highly expressed in the embryonic brain including Cx26, Cx36, Cx37, Cx43, and Cx45 (Nadarajah et al., 1997). The most important gap junction protein isoforms in the developing brain are Cx43 and Cx26. *In vitro* and *in vivo* studies have shown that during early neurogenesis, proliferative radial glial cells that are located in the VZ are extensively coupled by Cx43 and Cx26 to exchange signaling molecules (Bittman et al., 1997; LoTurco and Kriegstein, 1991; Miragall et al., 1997; Nadarajah et al., 1998, Duval et al., 2002). Immunocytochemical studies have shown Cx26 and Cx43 to be implicated in the coupling of neuroepithelial cells in the VZ of the embryonic brain (Dermietzel et al., 1989; Bittman and LoTurco, 1999). Of note, gap junctional coupling decreases after two weeks postnatally, to be replaced by chemical synapses which emerge once neurons become differentiated (Arumugam et al., 2005; Kandler and Thiels, 2005). In addition, the expression pattern for each Cx is different during the development of the cerebral cortex. The expression of Cx26 and Cx43 increases at the peak of neurogenesis (E14-E16), and decreases as neurogenesis progresses (Dermietzel et al., 1989; Nadarajah et al., 1997). Therefore, it seems appropriate to consider that the expression of gap junctions is developmentally regulated.

In the embryonic stage, Cx26 is expressed only in radial glial cells as demonstrated previously by using double immuno-labelling for Cx26 and radial glial markers such as nestin. However its expression is confined to MAP-2 positive neurons postnatally (Bittman and LoTurco, 1999; Nadarajah et al., 1997). In contrast, radial glial cells sustain Cx43 expression during embryonic stages and after differentiating into astrocytes in postnatal stages (Dermietzel et al., 1989). As newborn neurons reach their final location in the CP and differentiate, they no longer express Cx43, rather they begin to express Cx36. The expression

of Cx36 increases in neurons for the first two postnatal weeks, and then decreases substantially (Prime et al., 2000; Bahrey and Moody, 2003). Cx45 is also expressed in neurons during the embryonic gestation period, and its expression peaks at postnatal day (P) 1 with a considerable decrease at P14 (Maxeiner et al., 2003). Together, these studies suggest that Cxs are highly expressed at early stages of neocortical development, and the expression of each Cx subunit appears to be regulated throughout the development of the cerebral cortex.

1.3.4.1 Function of Gap Junctions in the Developing Cerebral Cortex

During the past couple of decades, accumulating evidence has allowed investigators to shed light on understanding the properties of gap junctions. In general, the functional properties of gap junctions may be classified into four categories including: intercellular communication, hemichannel-mediated release of ions and small metabolites into the extracellular space under physiological conditions, cell-cell adhesion, and intracellular signaling via the C-terminus of the Cx subunit (Lin et al., 2002; Elias et al., 2007; Belliveau et al. 2006).

During neurogenesis, gap junctional coupling in radial glial cells appears to be regulated at all phases of the cell cycle. For instance, VZ cells uncouple when they undergo M-phase. They re-couple through S-phase and G_2-phase, and stay coupled until the M-phase (Bittman et al., 1997). In addition, coupling is high in S-phase in early neurogenesis, but low in late neurogenesis. Therefore, it is reasonable to consider that in late neurogenesis, uncoupling decreases the number of cells that enter S-phase because they are required to stop proliferating, become postmitotic, and subsequently migrate away from the VZ into the CP. Interestingly, the expression of Cx26 is seen mostly in dividing VZ cells during M-phase to G_1-phase, but in contrast, during S-phase to G_2-phase dividing cells mostly express Cx43. Although, cells uncouple during M-phase, the expression of Cxs is not reduced (Bittman and LoTurco, 1999). Together, these studies suggest that during neurogenesis, Cxs may control cell division, since the coupling is regulated through all phases of the cell cycle.

In addition to gap junctional coupling, the presence of gap junction hemichannels which mediate intercellular Ca^{2+} propagation has been demonstrated in neighboring radial glial cells (Weissman et al., 2004). Weissman and colleagues showed the propagation of spontaneous Ca^{2+} waves among cortical radial glia. In the developing cortical VZ, Cx43

hemichannels release ATP into the extracellular space which binds to purinergic $P2Y_1$ receptors on adjacent radial glial membranes. This event causes an activation of IP_3 mediating release of Ca^{2+} from intracellular stores. The majority of radial glial cells initiating Ca^{2+} wave propagation were undergoing DNA synthesis (Weissman et al., 2004). Blocking of the Ca^{2+} wave propagation by gap junction blockers reduced the rate of proliferation (Cotrina et al., 1998; Fillipov et al., 2003; Weissman et al., 2004). Thus, during neurogenesis, the hemichannel activity of Cx43 may regulate proliferation in radial glial cells. Furthermore, over expression of Cx43 and Cx32 in PC12 cell treated with NGF has been shown to enhance neurite outgrowth (Belliveau et al., 2006). The presence of hemichannels in PC12 cells causes the release of ATP into the extracellular space which binds to the $P2Y_1$ receptors. This event leads to an increase in the intracellular Ca^{2+} concentration as mentioned above, and a subsequent increase in neurite length in PC12 cells. Therefore, hemichannels might have a functional role in mediating neurite extension and migration.

Increasing lines of evidence have reported that adhesion between neighboring cells is another factor by which Cx43 exerts its action. In a study by Lin et al. (2002), the implication of Cx43 expression on the adhesiveness and the invasion of malignant C6 glioma cells was investigated. An aggregation assay conducted by the authors showed that C6 glioma cells transfected with Cx43 formed substantially larger aggregates compared to untransfected cells, which indicated that Cx43 expression was associated with an increase in the adhesiveness between C6 glioma tumor cells and the astrocytes. Furthermore, transplantation of glioma aggregates expressing Cx43 into the striatum of a normal brain resulted in an increased migration of glioma cells on the astrocytic syncytia compared to normal glioma cells. Thus, overexpression of Cx43 in brain tumor cells acted on adhesion sites to enhance their invasiveness (Lin et al., 2002).

Based in part on its expression in a variety of tissues, Cx43 is the most studied Cx protein. For this reason, many researchers have examined the interactions of its C-terminal region with cytoplasmic proteins, including cell signaling molecules, scaffolding proteins, and membrane receptors (Ai et al., 2000; Xu et al., 2001; Fu et al., 2004; Giepmans, 2006). For example, Cx43 has been shown to interact with N-cadherins of adhesion junctions and modulate neural crest cell motility. Cx43 and N-cadherin form a complex at the plasma membrane of neural crest cells. The complex binds to P-120 catenin, also involved in

modulating cell motility (Noren et al., 2000). In turn, through a series of signaling cascades, P-120 catenin modulates the activity of Rho GTPase, and neural crest cell motility by rearranging the actin cytoskeleton (Xu et al., 2001). These findings, which indicate the ability of Cx43 to modulate catenin signaling and thereby regulate cell growth, represent another role of Cx43 independent of their classical channel forming role.

1.3.4.2 Gap Junction Association with CNS Disturbances

Accumulating evidence has suggested the involvement of gap junctions in CNS diseases, including stroke, epilepsy, mental retardation, Alzheimer's disease, and Parkinson's disease (Aicardia, 1994; Nagy et al., 1996; Rufer et al., 1996; Nakase et al., 2004; Guerrini and Filippi, 2005).

1.3.4.2.1 The Association of Cx43 with Neuronal Migration

Furthermore, over the past several years, investigators have used *in vitro* models to elucidate the close relationship between cell migration and Cx43 expression during development, which is also the main focus of this thesis. For example, studies on the expression of Cx43 in cardiac neural crest cells have reported a close association between their migratory properties and Cx43 expression (Li et al., 2002; Lo et al., 1999; Reaume et al., 1995). Cardiac neural crest cells of Cx43 knockout mice show reduced motility and directionality. In contrast, overexpression of Cx43 in the same cells showed increased directionality and speed (Xu et al., 2006).

A study by Fushiki and co-workers showed that embryonic mice with the total deletion of Cx43 expression display significant accumulation of BrdU labelled cells in the IZ of the neocortex. This finding demonstrated an aberrant neuronal migration in these mice, whereas in wild-type littermates, migrating neurons establish themselves in the CP (Fushiki et al., 2003). Therefore, Cx43 was shown to be critical for the process of neuronal migration during brain development.

1.3.4.2.2 The Association of Cx43 with Cell Proliferation

Disturbances in neuronal migration have also been shown in mice with conditional deletion of Cx43 driven by Cre expression under the human GFAP promoter (Wiencken-

Barger et al., 2007). Neuronal migration was disturbed in these mice and this defect was mostly dependent on their genetic background and the lack of Cx43 expression. The authors reported that the conditional knockout mice on a 129SVEV background had a smaller cortex, cerebellum, and hippocampus as a result of decreased proliferation in the VZ and SVZ. However, mice on a C57Bl/6J background appeared normal. The phenotype dependence on the genetic background might be explained by epigenetic factors. In mice with 129SVEV background, the epigenetic changes on the other Cx genes such as Cx30 might prevent them from being used to make proteins. Therefore, following deletion of Cx43 from the radial glial cells, the very low level of compensation by other Cx proteins may result in a more sever defect in these mice. In cerebellum, the delamination of Purkinji cells was suggestive of the failure to form radial fibers needed for neuronal migration. Additionally, ectopic granule cells were incapable of migrating radially to the internal granule layer, resulting in the increased thickness of the external granular layer. Thus, the authors suggested Cx43 as an important factor in the development of the cortex, cerebellum, and hippocampus (Wiencken-Barger et al., 2007).

Total knockout of Cx43 is neonatal lethal due to abnormalities in heart development, preventing researchers from conducting a comprehensive study of the developmental effects of such a deletion. In addition, in these mice, Cx43 is deleted from all cell types, making it difficult to investigate the effect of cell-specific deletion of Cxs in neuronal migration and neocortical development. Therefore, in this thesis, to shed light on the role of Cx43 in neuronal migration, and to avoid problems of the neonatal lethality associated with Cx43 null mice, we have made conditional knock-out (*Cx43cKO*) mice, produced by crossing *Cx43* $^{fl/fl}$ mice (Theis et al., 2001), with *nestin–Cre* mice, driving the expression of Cre recombinase in radial glia (Bérubé et al., 2005).

Furthermore, most investigators have used rat models to study the expression of Cxs during development. Thus, in this thesis, it became imperative that the expression of Cx protein isoforms in the mouse model be established prior to analysis of complete and conditional knockout studies.

1.4 Objectives, Rationale and Hypothesis

During embryonic development, extensive gap junctional coupling exists between cells suggesting that direct cell to cell communication is involved in many processes including proliferation, neuronal migration, differentiation, and development of the cerebral cortex (Lo., 1996; Nadarajah and Parnavelas, 1999; Kriegstein et al., 2006). Connexins that make up gap junction channels are differentially expressed during embryonic and postnatal maturation of the nervous system suggesting that gap junction communication plays an important role during various stages of development. While many Cxs are expressed in the developing brain, regional distribution and function of these Cxs remain to be determined. A proper understanding of the role of these Cxs in the developing brain requires an unambiguous characterization of their spatial and temporal pattern of expression. Thus, the general purpose of these studies was to first characterize the expression of developmentally associated Cxs, and to then examine the role of Cx43 (the most abundantly expressed Cx in the developing brain) in the murine model during neuronal migration and cortical development. Based on the previous works of others (see *Historical Review*), it was generally hypothesized that *functional gap junctions contribute to the proper development of the cerebral cortex; thus deletion of Cx genes expressed in radial glia and newborn neurons will perturb neuronal migration and cortical development.*

The purpose of the first study in this thesis was to examine and characterize the temporal and spatial expression of Cxs at the time points E14, E16, and E18 of mouse neocortex. In the VZ of the developing neocortex, radial glia, the cells that give rise to and then guide the newborn neurons to their final destination in the CP (Rakic, 1972), are strongly coupled by gap junctions (LoTurco and Kriegstein, 1991; Bittman et al, 1997). Additionally, newborn neurons are in close contact with radial glia as they migrate to the CP, suggesting that this Cx is important during neuronal migration. Furthermore, it has been shown that the expression of Cx43 increases until mid neurogenesis, then decreases as neurogenesis terminates (Nadarajah et al., 1997), suggesting that Cx43 plays a critical role in cortical development. It was hypothesized that *Cx43 is expressed in all cortical layers of the cerebral cortex at E14-E18, and that this Cx is expressed in radial glial cells and newborn neurons.*

The general purpose of the second and third projects was to investigate how neuronal migration and cortical development are affected by deletion of Cx43 from the radial glia and newborn neurons. As newborn neurons leave the VZ and migrate to the CP, they directly interact with radial glia. Previous studies have shown that gap junctions are needed for this migration to occur properly (Fushiki et al., 2003; Xu et al., 2006; Elias et al., 2007; Wiencken-Barger et al., 2007), but the exact mechanism responsible for this migration is still in question. Thus, it is crucial to determine the effects of deletion of Cx43 from the radial glia and newborn neurons in cortical development. Homozygous Cx43 null mutant mice die shortly after birth due to cardiac malformation. Therefore, we used the Cre/loxP recombinase system to be able to assess the effects of cell-specific Cx-knockouts on migration and development of neurons in mouse neocortex. The methodology used in the following studies employed *in utero* electroporation with wild type and transgenic mice during embryonic stages. The mice have unique advantages and include: Cx43 wild-type; Cx43 null mutant (Reaume et al., 1995), in which Cx43 is deleted from all cell types; nestin-Cre (Bérubé et al., 2005); floxed Cx43 (Theis et al., 2001); and $Cx43^{K258stop}$ (Maass et al., 2004) transgenic mice. Nestin-Cre mice were crossed with floxed Cx43 mice. Therefore, Cx43 was deleted from radial glial cells expressing nestin. $Cx43^{K258stop}$ mice were used since the C-terminal domain of Cx43 is truncated in these mice. It was hypothesized that *in conditional knockout animals, deletion of Cx43 from radial glia and newborn neurons will result in disruption of neuronal migration and cortical lamination.* Furthermore, when re-introduced to a lateral ventricle of conditional knockout mice it was hypothesized that *disruption in neuronal migration and cortical development can be corrected by transfecting the brain of embryonic mice with Cx43 constructs.*

The ability of Cx43 deletion to affect neuronal migration and cortical lamination was tested on embryonic mouse neocortex. In addition, the domain of Cx43 which is critical for neuronal migration was determined. Since Cx43 is extensively expressed in embryonic neocortex, it should influence neuronal migration during development. Moreover, there is increasing evidence that the C-terminal domain of Cx43 interacts with kinases, phosphatases, adhesion molecules, and cytoskeletal anchorage (see *Historical Review*) suggesting the involvement of this region of Cx43 in various developmental events like neuronal migration.

It was hypothesized that *the C-terminal domain of Cx43 is required for proper neuronal migration*.

The general purpose of the last study was to investigate the mechanism by which Cx43 regulates neuronal migration in developing neocortex. As mentioned above, Cx43 interacts with cytoskeletal proteins, NOV/CCN3, kinases, phosphotases, tight junctions and adherence junctions via interacting with its C-terminal domain. These interacting proteins regulate the action of Cx43 in a variety of cellular events such as cell proliferation and migration. Therefore, we conducted experiments with specific deletions within the C-terminal region of Cx43. It was hypothesized that *a central region within the C-terminal domain of Cx43 is involved in regulating neuronal migration.*

1.5 References

Ai Z, Fischer A, Spray DC, Brown AM, Fishman GI (2000) Wnt-1 regulation of connexin43 in cardiac myocytes. J Clin Invest 105:161-171.

Aicardia J (1994) The place of neuronal migration abnormalities in child neurology. Can J Neurol Sci 21:185-193.

Ahmed S, Evans WH (2002) Post-translational integration and oligomerization of connexin26 in plasma membranes and evidence of formation of membrane pores implications for the assembly of gap junctions. Biochem J 65:693699.

Alvarez-Buylla A (1997) Mechanism of migration of olfactory bulb interneurons. Semin Cell Dev Biol 8:207-213.

Alvarez-Buylla A, Garcia-Verdugo JM, Tramontin AD (2001) A unified hypothesis on the lineage of neural stem cells. Nat Rev Neurosci 2:287-293.

Anderson SA, Elsenstat DD, Shi L, Rubenstein JLR (1997) Interneurons migrate from basal forebrain to neocortex: dependence on *Dlx* genes. Science 278:474-476.

Ang ES Jr, Haydar TF, Gluncic V, Rakic P (2001) Four-dimensional migratory coordinates of the GABAergic interneurons of the developing mouse neocortex. J Neurosci 23:5805-815.

Anton ES, Marchionni MA, Lee K-F, Rakic P (1997) Role of GGF/neuregulins signaling in interactions between migrating neurons and radial glia in the developing cerebral cortex. Development 124:3501-3510.

Anthony TE, Klein C, Fishell G, and Heintz N (2004) Radial glia serve as neuronal progenitors in all regions of the central nervous system. Neuron 41**:** 881-890.

Arumugam H, Li X, Colombo PJ, Corriveau RA, Belousov AB (2005) NMDA receptors regulate developmental gap junctions uncoupling via CREB signaling. Nat Neurosci 8:1720-1726.

Austin CP, Cepko CL (1990) Cellular migration patterns in the developing mouse cerebral cortex. Development 110:713-732.

Bahrey HL, Moody WJ (2003) Voltage-gated currents, dye and electrical coupling in the embryonic mouse neocortex. Cereb Cortex 13:239-251.

Bang A, Goulding M (1996) Regulation of vertebrate neural cell fate by transcription factors Curr Opin Neurobiol 6:25-32.

Belliveau D, Bani-Yaghoub M, McGirr B, Naus CG, Rushlow WJ (2006) Enhanced neurite outgrowth in PC12 cells mediated by Connexin hemichannels and ATP. J Biol Chem 281:20920-20931.

Beardslee MA, Laing JG, Beyer EC, Saffitz JE (1998) Rapid turnover of connexin43 in the adult rat heart. Circ Res 83:29-635.

Bellion A, Baudoin JP, Alvarez C, Bornens M, Métin C (2005) Nucleoinesis in tangentially migrating neurons comprises two alternating phases: forward migration of Golgi/centrosome associated with centrosome splitting and myosin contraction at the rear. J Neurosci 25:5691-5699.

Belliveau DJ, Bani-Yaghoub M, McGirr B, Naus CC, Rushlaw WJ. 2006. Enhanced neurite outgrowth in PC12 cells mediated by connexin hemichannels and ATP. J Biol Chem 281:20920-20931.

Bennett MV, Barrio LC, Bargielo TA, Spray DC, Hertzberg E, Saez JC (1991) Gap junctions: New tools, new answers, new questions. Neuron 6:305-320.

Bentivoglio M, Mazzarello P (1999) The history of radial glia. Brain Res Bull 49:305-315.

Berthoud VM, Westphale EM, GrigoryevaA, Beyer EC (2000) PKC isoenzymes in the chicken lens and TPA-induced effects on intercellular communication. Invest Ophtalmol Vis Sci 41:850-858.

Bérubé NG, Mangelsdorf M, Jagla M, Vanderluit J, Garrick D, Gibbons RJ, Higgs DR, Slack RS, Picketts DJ (2005) The chromatin-remodeling protein ATRX is critical for neuronal survival during corticogenesis. J Clin Invest 115:258-267.

Beyer EC, Paul DL, Goodenough DA (1987) Connexin43: a protein from rat heart homologous to a gap junction protein from liver. J Cell Biol 105:2621-2629.

Bielas S, Higginbotham H, Koizumi H, Tanaka T, Gleeson JG (2004) Cortical neuronal migration mutants suggest separate but intersecting pathways. Annu Rev Cell Dev Biol 20:593-618.

Bittman K, Owens DF, Kriegstein AR, LoTurco JJ (1997) Cell coupling and uncoupling in the ventricular zone of the developing neocortex. J Neurosci 17:7037-7044.

Bittman KS, LoTurco JJ (1999) Differential regulation of connexin26 and 43 in murine neocortical precursors. Cereb Cortex 9:188-195.

Boulder Committee (1970) Embryonic vertebrate central nervous system: revised terminology. Anat Rec 166: 257-261.

Brightman MW, Reese TS (1969) Junctions between intimately apposed cell membranes in the vertebrate brain. J Cell Biol 40:648-677.

Brittis PA, Meiri K, Dent E, Silver J (1995) The earliest patterns of neuronal differentiation and migration in the mammalian central nervous system. Exp Neurol 134:1-12.

Bruckner G, and Biesold D (1981) Histochemistry glycogen deposition in perinatal rat brain: importance of radial glial cells. J Neurocytol 10:749-757.

Bruzzone R, White TW, Paul DL (1996) Connections with connexins: the molecular basis of direct intercellular signaling. Eur J Biochem 238:1-27.

Cai L, Hayes NL, Takahashi T, Caviness VS Jr. Nowakowski RS (2002) Size distribution of retrovirally marked lineage matches prediction from population measurements form cell cycle behavior. J Neurosci Res 69:731-744.

Cameron RS, Rakic P (1991) Glial cell lineage in the cerebral cortex: a review and synthesis. Glia 4:124-137.

Cameron HA, Woolley CS, McEwen BS, Gould E (1993) Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience 56:337-344.

Caviness VS Jr. (1982) Neocortical histogenesisin normal and reeler mice: a developmental study based upon [3H]thymidine autoradiography. Brain Res 256:293-302.

Chan CH., Godinho LN., Thomaidou D, Tan SS., Gulisano M, Parnavelas JG (2001) *Emx1* is a marker for pyramidal neurons of the cerebral cortex. Cereb Cortex 11:1191-1198.

Chanas-Sacre G, Rogister B, Moonen G, Leprince P (2000) Radial glia phenotype: origin, regulation and transdifferentiation. J Neurosci Res 61:357-363.

Chang BS, Ly J, Appignani B, Bodell A, Apse KA, Ravenscroft RS, Sheen VL, Doherty MJ, Hackney DB, O'Connor M, Galaburda AM, Walsh CA (2005) Reading impairment in the neuronal migration disorder of periventricular nodular heterotopia. Neurology 64:799-803.

Chen G, Sima J, Jin M, Wang KY, Xue XJ, Zheng W, Ding YQ, Yuan XB (2008) Semaphorin-3A guides radial migration of cortical neurons during development. Nat Neurosci 11:36-44.

Choi BH, Lapham LW (1978) Radial glia in the human fetal cerebrum: a combined Golgi, immunofluorescent and electron microscopic study. Brain Res 148:295-311.

Cobos I, Shimamura K, Rubenstein JL, Martinez S, Puelles L (2001) Fate map of the avian anterior forebrain at the four-somite stage, based on the analysis of quail-chick chimeras. Dev Biol 239:46-67.

Choi BH, Lapham LW (1978) Radial glia in the human fetal cerebrum: a combined Golgi, immunfluorescent and electron microscopy study. Brain Res 148:295-311.

Cotrina ML, Lin JH, Alves-Rodrigues A, Liu S, Li J, Azmi-Ghadimi H, Kang J, Naus CC, Nedergaard M (1998) Connexin regulate calcium signaling by controlling ATP release. Proc Natl Acad Sci USA 95:15735-15740.

Cubelos B, Sebastián-Serrano A, Kim S, Moreno-Ortiz C, Redondo JM, Walsh CA, Nieto M (2008) Cux-2 controls the proliferation of neuronal intermeidate precursors of the cortical subventricular zone. Cereb Cortex 18:1758-1770.

Culican SM, Baumrind NL, Yamamoto M, Pearlman AL (1990) Cortical radial glia: identification in tissue culture and evidence for their transformation to astrocytes. J Neurosci 10:684-692.

D'Arcangelo G, Miao GG, Chen SC, Soares HD, Morgan JI, Curran T (1995) A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. Nature 374:719-723.

Dahl D, Crosby CJ, Sethi JS, Bignami A (1985) Glial fibrillary acidic (GFA) protein in vertebrates: immunofluorescence and immunoblotting study with monoclonal and polyclonal antibodies. J Comp Neurol 239:75-88.

Das Sarma J, Wang F, Koval M (2002) Targeted gap junction protein constructs reveal connexin-specific differences in oligomerization. J Biol Chem 277:20911-20918.

De Carlos JA, López-Mascaraque L, Valverde F (1996) Dynamics of cell migration from the lateral ganglionic eminence in the rat. J Neurosci 16:6146-6156.

DeDiego I, Smith-Fernandez A, Fairen A (1994) Cortical cells that migrate beyond area boundaries: characterization of an early neuronal population in the lower intermediate zone of prenatal rats. Eur J Neurosci 6:983-997.

Del Bene F, Wehman AM, Link BA, Baier H (2008) Regulation of neurogenesis by interkinetic nuclear migration through an apical-basal Notch gradient. Cell 134:1055-1065.

Dermietzel R, Traub O, Hwang TK, Beyer E, Bennett MV, Spray DC, Willecke K (1989) Differential expression of three gap junction proteins in developing and mature brain tissues. Proc Natl Acad Sci USA 8610148-10152.

Dermietzel R, Kremer M, Paputsoglu G, Stang A, Skerrett IM, Gomes D, Srinivas M, Janssen-Bienhold U, Weiler R, Nicholson BJ, Bruzzone R, Spray DC (2000) Molecular and functional diversity of neural connexins in retina. J Neurosci 20:8331-8343.

Doble BW, Chen Y, Bose DG, Litchfield DW, Kardami E (1996) Fibroblast growth factor-2 decreases metabolic coupling and stimulates phosphorylation as well as masking of connexin43 epitopes in cardiac myocytes. Circ Res 79:647-658.

Doble BW, Ping P, Kardami E (2000) The epsilon subtype of protein kinase C is required for cardiomyocyte connexin43 phosphorylation. Circ Res 86:93-301.

Dobyns WB, Guerrini R, Czapansky-Beilman DK, Pierpont ME, Breningstall G, Yock DH Jr, Bonanni P, Truwit CL (1997) Bilateral periventricular nodular heterotopia with mental retardation and synactyly in boys: a new X-linked mental retardation syndrome. Neurology 49: 1042-1047.

Doniach T (1995) Basic FGF as an inducer of an anteroposterior neural pattern. Cell 83:1067-1070.

Dujardin DL, Barnhart E, Stehman SA, Gomes ER, Gundersen GG, Vallee RB (2003) A role for cytoplasmic dynein and Lis1 in directed cell movement. J Cell Biol 163:1205-1211.

Dulabon L, Olson EC, Taglienti MG, Eisenhuth S, McGath B, Walsh CA, Kreidberg JA, Anton ES (2000) Reelin binds to alpha3beta1 integrin and inhibits neuronal migration. Neuron 27:33-44.

Duval N, Gomes D, Calaora V, Calabrese A, Meda P, Bruzzone R (2002) Cell coupling and Cx43 expression in embryonic mouse neural progenitor cells. J Cell Sci 115:3241-3251.

Eagleston GW, Harris WA (1990) Mapping of the presumptive brain region in the neural plate of xenopus laevis. J Neurosci 21:427-440.

Eckenhoff MF, Rakic P (1984) Radial organization of the hippocampal dentate gyrus: a Golgi, ultrastructural, and immunocytochemical analysis in the developing rhesus monkey. J Comp Neurol 223:1-21.

Edmondson JC, Hatten ME (1987) Glial guided granule neuron migration in vitro: a high-resolution time-lapse video microscopy study. J Neurosci 7:1928-1934.

Edwards MA, Yamamoto M, Caviness VS Jr. (1990) Organization of radial glia and related cells in the developing murine CNS. An analysis based upon a new monoclonal antibody marker. Neuroscience 36:121-144.

Elenes S, Martines AD, Delmar M, Beyer EC, Moreno AP (2001) Heterotypic docking of Cx43 and Cx45 connexons blocks fast voltage gating of Cx43. Biophys 81:1406-1418.

Elfgang C, Eckert R, Lichtenberg-Fraté H, Butterweck A, Traub O, Klein RA, Hülser DF, Willecke K (1995) Specific permeability and selective formation of gap junction channels I connexin-transfectd Hela cells. J Cell Biol 129:805-817.

Englund C, Fink A, Lau C, Pham D, Daza RA, Bulfone A, Kowalczyk T, Hevner RF (2005) Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. J Neurosci 25:247-251.

Evans WH, Martin PE (2002) Gap junctions: structure and function. Mol Membr Biol 19:121-136.

Fallon RF, Goodenough DA (1981) Five hour half-life o mouse liver gap junction protein. J Cell Biol 90:521-526.
Fanning AS, Jameson BJ, Jesaitis LA, Anderson JM (1998) The tight junction protein ZO-1 establishes a link between the transmembrane protein occluding and the actin cytoskeleton. J Biol Chem 273:29745-29753.

Feng L, Hatten ME, Heintz N (1994) Brain lipid-binding protein (BLBP): a novel signaling system in the developing mammalian CNS. Neuron 12:895-908.

Fillipov MA, Hormuzdi SG, Fuchs EC, Monyer H (2003) A reporter allele for investigating connexin26 gene expression in the mouse brain. Eur J Neurosci 18:3183-3192.

Fishell G, Hatten ME (1991) Astrotactin provides a receptor system for CNS neuronal migration. Development 113:755-765.

Flagg-Newton JL, Loewenstein WR (1980) Asymmetrically permeable membrane channels in cell junction. Science 207:771-773.
Fox JW, Walsh CA (1990) Periventricular heterotopia and the genetics of neuronal migration in the cerebral cortex. Am J Hum Genet 65:19-24.

Fox JW, Lamperti ED, Ekşioğlu YZ, SE, Feng Y, Graham DA, Scheffer IE, Dobyns WB, Hirsch BA, Radtke RA, Berkovic SF, Huttenlocher PR, Walsh CA (1998) Mutations in filamin 1 prevent migration of cerebral cortical neurons in human periventricular heterotopia. Neuron 21:1315-1325.

Fu CT, Bechberger JF, Ozog MA, Perbal B, Naus CC (2004) CCN3 (NOV) interacts with connexin43 in C6 glioma cells: possible mechanism of connexin-mediated growth suppression. J Biol Chem 279:36943-36950.

Fushiki S, Perez Velazquez JL, Zhang L, Bechberger JF, Carlen PL, Naus CC (2003) Changes in neuronal migration in neocortex of connexin43 null mutant mice. J Neuropathol Exp Neurol 62:304-314.

Gadisseux JF, Evrard P (1985) Glial-neuronal relationship n the developing central nervous system. A histochemical-electron microscope study of radial glial cell particulate glycogen in normal and reeler mice and the human fetus. Dev Neurosci 7:12-32.

Gaiano N, Nye JS, Fishell G (2000) Radial glial identity is promoted by Notch1 signaling in the murine forebrain. Neuron 26:395-404.

Giepmans BN, Moolennar WH (1998) The gap junction protein connexin43 interacts with the second PDZ domain of the zona occludens-1 protein. Curr Biol 8:931-934.

Giepmans BN, Hengeveld T, Postma FR, Moolenaar WH (2001) Interaction of c-Src with gap junction protein connexin-43. Role in the regulation of cell – cell communication. J Biol Chem 276:8544-8549.

Giepmans BN (2006) Role of connexin43 interacting proteins at gap junctions. Adv Cardiol 42:41-56.

Gilula NB, Reeves OR, Steinbach A (1977) Metabolic coupling, ionic coupling, and cell contacts. Nature 235:262-265.

Gleeson JG, Lin PT, Flanagan LA, Walsh CA (1999) Doublecortin is a microtubule associated protein and is expressed widely by migrating neurons. Neuron 23:257-271.

Goodenough DA (1980) Intercellular junctions. Soc Gen Physiol Ser 34:167-178.

Goodenough DA, Revel JP (1980) A fine structural analysis of intercellular junctions in the mouse liver. J Cell Biol 45:272-290.

Goodenough DA, Dick JS, Lyons JE (1980) Lens metabolic cooperation: a study of mouse lens transport and permeability visualized with freeze-substitution autoradiography and electron microscopy. J Cell Biol 86:576-589.

Gorski JA, Talley T, Qiu M, Puelles L, Rubenstein JL, Jones KR (2002) Cortical excitatory neurons and glia, but not GABAergic neurons are produced in the Emx-1 expressing lineage. J Neurosci 22:6309-6314.

Götz M, Hartfuss E, Malatesta P (2002) Radial glial cells as neural precursors: a new perspective on the correlation of morphology and lineage restriction in the developing cerebral cortex of mice. Brain Res Bull 57:777-788.

Gray GE, Sanes JR (1992) Lineage of radial glia in the chicken optic tectum. Development 114:271-283.

Guerrini R, Filippi T (2005) Neuronal migration disorders, genetics, and epileptogenesis. J Child Neurol 4:287-299.

Guthrie S (1996) Patterning the hindbrain. Curr Opin Neurobiol 6:41-48.

Hall A (2005) Rho GTPases and the control of cell behavior. Biochem Soc Trans 33:891-5.

Halliday AL, Cepko L (1992) Generation and migration of cells in the developing striatum. Neuron 9:15-26.

Harland RM (1997) Neural induction in xenopus. New York: Oxford Univ. Press 1-25.

Hartfuss E, Galli R, Heins N, Götz M (2001) Characterization of CNS precursor subtypes and radial glia. Dev Biol 229:15-30.
Hatten ME (1993) The role of migration in central nervous system neuronal development. Curr Opin Neurobiol 3:38-44.

Hatten ME (1999) Central nervous system neuronal migration. Annu Rev Neurosci 22:551-239.

Haubensak W, Attardo A, Denk W, and Huttner WB (2004) Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. Proc Natl Acad Sci USA 101: 3196-3201.

Hemmati-Brivanlou A, Melton DA (1994) Inhibition of activin receptor signaling promotes neutralization in xenopus. Cell 77:273-281.

Hertig CM, Butz S, Koch S, Eppenberger-Eberhardt M, Kemler R, Eppenberger HM (1996) N-Cadherin in adult rat cardiomyocytes in culture: II. Spatio temporal appearance of proteins involved in cell– cell contact and communication. Formation of two distinct N-cadherin/catenin complexes. J Cell Sci 109:11-20.

Hirotsune S, Takahara T, Sasaki N, Hirose K, Yoshiki A, Ohashi T, Kusakabe M, Murakami Y, Muramatsu M, Watanabe S (1995) The reeler gene encodes a protein with an EGF-like motif expressed by pioneer neurons. Nature Genet 10:77-83.

Hockfield S, McKay RD (1985) Identification of major cell classes in the developing mammalian nervous system. J Neurosci 5:3310-3328.

Hossain MZ, Ao P, Boyton AL (1998) Platelet-derived growth factor-induced disruption of gap junctional communication and phosphorylation of connexin43 involves protein kinases C and mitogen-activated protein kinase. J Cell Physiol 176:332-341.

Huang Z (2009) Molecular regulation of neuronal migration during neocortical development. Mol Cell Neurosci 42:11-22.

Hunter DD, Llinas R, Ard M, Merlie JP, Sanes JR (1992) Expression of s-laminin and laminin in the developing rat central nervous system. J Comp Neurol 23:238-251.

Jessel T, Lumsden A (1997) Inductive signals and the assignment of cell fate in the spinal cord and hindbrain. New York: Oxford Univ. Press 290-333.

Joyner AL (1996) Engrailed, Wnt, and Pax genes regulate midbrain-hindbrain development. Trends Genet 12:15-20.

Kandler K, Thiels E (2005) Flipping the switch from electrical to chemical communication. Nat Neurosci 8:1633-1634.

Kidd, T, Brose K, Mitchell KJ, Fetter RD, Tessier-Lavigne M,Goodman CS, Tear G (1998) Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. Cell 92:205–215.

Kriegstein AR, Noctor SC (2004) Patterns of neuronal migration in the embryonic cortex. Trends Neurosci 27:392-399.

Kriegstein A, Noctor S, Martínez-Cerdeño V (2006) Pattern of neural stem and progenitor cell division may underlie evolutionary cortical expansion. Nat Rev Neurosci 7:883-890.

Kriegstein A, Parnavelas JG (2003) Changing concepts of cortical development. Cereb Cortex. 13:i-ii.

Konig N, Hornung JP, Van der Loos H (1981) Identification of Cajal-Retzius cells in immature rodent cerebral cortex: a combined Golgi-EM study. Neurosci Lett 27:225-229.

Kumar NM, Gilula NB (1996) The gap junction communication channel. Cell 84:381-388.

Laird DW, Puranam KL, Revel JP (1991) Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. Biochem J 273:67-72.

Laird DW (2006) Life cycle of connexins in health and disease. Biochem J 394:527-543.

Lampe PD, Lau AF (2000) Regulation of gap junctions by phosphorylation of connexins. Arch Biochem Biophys 384:205-215.

Lampe PD, Tenbroek EM, Burt JM, Kurata WE, Johnson RG, Lau AF (2000) Phophorylation of Cx43 on serine 368 by protein kinase C regulates gap junction communication. J Cell Biol 149:1503-1512.

Lauf U, Giepmans BN, Lopez P, Braconnot S, Chen SC, Falk MM (2002) Dynamic trafficking an delivery of connexons to the plasma membrane and accretion to gap junctions in living cells. Proc Natl Acad Sci USA 99:10446-10451.

Lavdas AA, Grigoriou M, Pachnis V, Parnavelas JG (1999) The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. J Neurosci 19:7881-888.

Le Douarin N (1986) Cephalic placodes and neurogenesis. Trends Neurosci 9:175-180.

Leithe E, Brech A, Rivedal E (2006) Endocytic processing of connexin43 gap junctions: A morphological study. Biochem J 393:59-67.

Levitt P, Rakic P (1980) Immunoperoxidase localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the developing rhesus monkey brain. J Comp Neurol 193:815-840.

Levitt P, Cooper M. L, and Rakic P (1983) Early divergence and changing proportions of neuronal and glial precursor cells in the primate cerebral ventricular zone. Dev Biol 96:72-484.

Li WE, Waldo K, Linask KL, Chen T, Wessels A, Parmacek MS, Kirby ML, Lo CW (2002) An essential role for connexin43 gap junctions in mouse coronary artery development. Development 129:2031-2042.

Lin R, Warn-Cramer BJ, Kurata WE, Lau AF (2001) v-Src-mediated phosphorylation of connexin43 on tyrosine disrupts gap junctional communication in mammalian cells. Cell Commun Adhes 8:265-269.

Lin JH, Takano T, Cotrina ML, Arcuino G, Kang J, Liu S, Gao Q, Jiang L, Li F, Lichtenberg-Frate H, Haubrich S, Willecke K, Goldman SA, Nedergaard M (2002) Connexin43 enhances the adhesivity and mediates the invasion of malignant glioma cells. J Neurosci 22:4302-4311.

Lo WK, Shaw AP, Takemoto LJ, Grossniklaus HE, Tigges M (1996) Gap junction structures and distribution patterns of immunoreactive connexins 46 and 50 in lens regrowths of Rhesus monkeys. Exp Eye Res 62:171-180.

Lo CW, Waldo KL, Kirby ML (1999) Gap junction communication and the modulation of cardiac neural crest cells. Trends Cardiovasc Med 9:63-69.

LoTurco JJ, Kriegstein AR (1991) Clusters of coupled neuroblasts in embryonic neocortex. Science 252:563-566.

LoTurco JJ (2004) Doublecortin and a tale of two serines. Neuorn 41:175-177.

Luskin MB (1993) Subventricular zone-derived neuronal progenitors migrate into the subcortical forebrain of postnatal mice. Neuron 11:173-189.

Maass K, Ghanem A, Kim J, Saathoff M, Ursche S, Kirfel G, Grummer R, Kertz M, Lewalter T, Tiemann K, Winterhager E, Herzog V, Willecke K (2004) Defective epidermal barrier in neonatal mice lacking the C-terminal region of coneixn43. Mol Bio Cell 15:4597-4608.

Malatesta P, Hartfuss E, Götz M (2000) Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. Development 127:5253-5263.

Malatesta P, Hack MA, Hartfuss E, Kettenmann H, Klinkert W, Kirchhoff F, Götz M (2003) Neuronal or glial progeny: regional differences in radial glia fate. Neuron 37**:**751-764.

Marín O, Rubenstein JL (2001) A long remarkable journey: tangential migration in the telencephalon. Nat Rev Neurosci 2:780-790.

Marín-Padilla M (1995) prenatal development of fibrous (white matter), protoplasmic (gray matter), and layer 1 astrocytes in the human cerebral cortex: a Golgi study. J Comp Neurol 357:554-572.

Martin PE, Blundell G, Ahmad S, Errington RJ, Evans WH (2001) Multiple pathways in the trafficking and assembly of connexin 26, 32 and 43 into gap junction intercellular communication channels. J Cell Sci 114:3845-3855.

Makowski L, Caspar DL, Phillips WC, Goodenough DA (1977) Gap junction structures. Ll. Analysis of the X-ray diffraction data. J Cell Biol 74:629-645.

Mallamaci A, Iannoe R, Briata P, Pintonello L, Mercurio S, Boncinelli E, Corti G (1998) EMX2 protein in the developing mouse brain and olfactory area. Mech Dev 77:165-172.

Maxeiner S, Kruger O, Schilling K, Traub O, Urschel S, Willecke K (2003) Spatiotemporal transcription of connexin45 during brain development results in neuronal expression in adult mice. Neuroscience 119:689-700.

McLeod TL, Bechberger JF, Naus CC (2001) Determination of a potential role of the CCN family of growth regulators in connexin43 transfected C6 glioma cells. Cell Commun Adhes 8:441-445.

McManus MF, Nasrallah IM, Pancoast MM, Wynshaw-Boris A, Golden JA (2004) Lis1 is necessary for normal non-radial migration of inhibitory interneurons. Am J Pathol 165:775-784.

Meyer G, Soria JM, Martínez-Galán JR, Martín-Clemente B, Fairén A (1998) Different regions and developmental histories of transient neurons in the marginal zone of the fetal and neonatal rat cortex. J Comp Neurol 397:493-518.

Miller MW (1986) The migration and neurochemical differentiation of gamma-aminobutyric acid (GABA)-immunoreactive neurons in rat visual cortex as demonstrated by a combined immunocytochemical-autoradiographic technique. Brain Res 393:41-46.

Miragall F, Albiez P, Bartels H, Vries U de, Dermietzel R (1997) Expression of the gap junction protein connexin43 in the subependymal layer and the rostral migratory stream of the mouse: evidence for an inverse correlation between intensity of connxin43 expression and cell proliferation activity. Cell Tissue Res 287:243-253.

Misson JP, Edwards MA, Yamamoto M, Caviness VS Jr. (1988) Identification of radial glial cell within the developing murine central nervous system: studies based upon a new imunohistochemical marker. Brain Res Dev Brain Res 44:95-108.

Misson JP, Austin CP, Takahashi T, Cepko CL, Caviness VS Jr. (1991) The alignment of migrating neural cells in relation to the murine neopallial radial glial fiber system. Cereb Cortex 1:221-229.

Miyata T, Kawaguchi A, Okano H, Ogawa M (2001) Asymmetric inheritance of radial glial fibers by cortical neurons. Neuron 31:727-741.

Miyata T, Kawaguchi A, Saito K, Kawano M, Muto T, and Ogawa M (2004) Asymmetric production of surface-dividing and nonsurface-dividing cortical progenitor cells. Development 131:3133-3145.

Mizutani K, & Saito T (2005) Progenitors resume generating neurons after temporary inhibition of neurogenesis by Notch activation in the mammalian cerebral cortex. Development 132, 1295-1304.

Morest DK (1970) A study of neurogenesis in the forebrain of opossum pouch young. Z Anat 130:265-305.

Musil LS, Goodenough DA (1991) Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. J Cell Biol 115:1357-1374.

Musil LS, Goodenough DA (1993) Multisubunit assembly of an integral plasma membrane channel protein, gap junction connexin43, occurs after exit from the ER. Cell 74:1065-1077.

Nadarajah B, Jones AM, Evans WH, Parnavelas JG (1997) Differential expression of connexins during neocortical development and neuronal circuit formation. J Neurosci 17:3096-3111.

Nadarajah , Makarenkova H, Becker DL, Evans WH, Parnavelas JG (1998) Basic FGF increases communication between cells of the developing neocortex. J Neurosci 18:7881-7890.

Nadarajah B, Parnavelas JG (1999) Gap junction-mediated communication in the developing and adult cerebral cortex. Novartis Found Symp 219:157-170.

Nadarajah B, Brunstrom JE, Grutzendler J, Wong RO, Pearlman AL (2001) Two modes of radial migration in early development of the cerebral cortex. Nat Neurosci 2:143-150.

Nadarajah B, Alifragis P, Wong RO, Parnavelas JG (2002) Ventricle-directed migration in the developing cerebral cortex. Nat Neurosci 5:218-224.

Nadarajah B, Parnavelas JG (2002) Modes of neuronal migration in the developing cerebral cortex. Nat Rev Neurosci 3:423-432.

Nadarajah B, Alifragis P, Wong RO, Parnavelas JG (2003) Neuronal migration in the developing cerebral cortex: Observations based on real-time imaging. Cereb Cortex 13:607-611.

Nagy JI, Li W, Hertzberg EL, Marotta CA (1996) Elevated connexin43 inmmunoreactivity at sites of amyloid plaques in Alzheimer's disease. Brain Res 717:173-178.

Martinez AD, Acuna R, Figueroa V, Maripillan J, Nocholson B (2009) Gap-junction channels dysfunction in deafness and hearing loss. Antioxid Redox Signal 11:309-322.

Nakase T, Fushiki S, Naus CC (2003) Astrocytic gap junctions composed of connexin43 reduce apoptotic neuronal damage in cerebral ischemia. Stroke 34:1987-1993.

Nakase T, Söhl G, Theis M, Willecke K, Naus CC (2004) Increased apoptosis and inflammation after focal brain ischemia in mice lacking conexin43 in astrocytes. Am J Pathol 164:2067-2075.

Naus CC, Hearn S, Zhu D, Nicholson BJ, Shivers RR (1993) Ultrastructural analysis of gap junctions in C6 glioma cells transfected with connexin43 cDNA. Exp Cell Res 206:72-84.

Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstein AR (2001) Neurons derived from radial glial cells establish radial units in neocortex. Nature 409:714-720.

Noctor SC, Martínez-Cerdeño V, Ivic L, Kriegstein AR (2004) Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci 7:136-144

Noctor SC, Martínez-Cerdeño V, Kriegstein AR (2008) Distinct behaviors of neural stem and progenitor cells underlie cortical neurogenesis. J Comp Neurol 508:28-44.

Noren NK, Liu BP, Burridge K, Kreft B (2000) P120 catenin regulates the actin cytoskeleton via Rho family GTPases. J Cell Biol 150:567-579.

Norman M, McGillivray B, Kalousek D, et al (1995) Congenital malformations of the brain: Pathological, embryological, clinical, radiological and genetic aspects. New York: Oxford University Press.

Ogawa M, Miyata T, Nakajima K, Yagyu K, Seike M, Ikenaka K, Yamamoto H, Mikoshiba K (1995) The reeler gene associated antigen on Cajal-Retzius neurons is a crucial molecule for laminar organization of cortical neurons. Neuron 14: 899-912.

O'Rourke NA, Sullivan DP, Kaznowski CE, Jacobs AA, McConnell SK (1995) Tangential migration of neurons in the developing cerebral cortex. Development 12:2165-2176.

Parnavelas JG, Nadarajah B (2001) Radial glial cells. are they really glia? Neuron 31:881-884.

Parnavelas JG (2002) The origin of cortical neurons. J Med Biol Res 35:1423-1429.

Pitts JD, Simms JW (1977) Permeability of junctions between animal cells. Intercellular transfer of nucleotides but not of macromolecules. Exp cell Res 104:153-163.
Perez-Moreno M, Jamora C, Fuchs E (2003) Sticky business: orchestrating cellular signals at adherence junctions. Cell 112:535–548.

Pixley SK, de Vellis J (1984) Transition between immature radial glia and mature astrocytes studied with a monoclonal antibody to vimentin. Brain Res 317:201-209.

Pontious A, Kowalczyk T, Englund C, Hevner RF (2008) Role of intermediate progenitor cell in cerebral cortex development. Dev Neurosci 30:24-32.

Prime G, Horn G, Sutor B (2000) Time-related changes in connexin mRNA abundance in the rat neocortex during postnatal development. Brain Res Dev Brain Res 3:111-125.

Qin H, Shao Q, Igdoura SA, Alaoui-Jamali MA, Laird DW **(**2003**)** Lysosomal and proteasomal degradation play distinct roles in the life cycle of Cx43 in gap junctional intercellular communication deficient and competent breast tumor cells. J Biol Chem 278:30005-30014.

Rakic P (1971) Guidance of neurons migrating to the fetal monkey neocortex. Brain Res 33:471-476.

Rakic P (1972) Mode of cell migration to the superficial layers of fetal monkey neocortex. J Comp Neurol 145:61-83.

Rakic P (1974) Neurons in rhesus monkey visual cortex: systemic relation between time of origin and eventual disposition. Science 183:425-427.

Rakic P (1982) Early developmental events: cell lineages, acquisition of neuronal positions, and areal and laminar development. Neurosci Res Program Bull 20:439-451.

Rakic P (1988) Specification of cerebral cortical areas. Science 241:170-176.

Rakic P (1990) Principles of neural cell migration. Experientia 46:882-91.

Ramos RL, Bai J, LoTurco JJ (2006) Heterotopia formation in rat but not mouse neocortex after RNA interference knockdown of DCX. Cereb Cortex 16:1323-1331.

Reaume AG, de Sousa PA, Kulkarni S, Langille BL, Zhu D, Davies TC, Juneja SC, Kidder GM, Rossant J (1995) Cardiac malformation in neonatal mice lacking connexin43. Science 267:1831-1834.

Reid CB, Liang I, Walsh C (1995) Systematic widespread clonal organization in cerebral cortex. Neuron 15:299-310.

Rickmann M, Amaral DG, Cowan WM (1987) Organization of radial glial cells during the development of the rat dentate gyrus. J Comp Neurol 264:449-479.

Roelink H, Auqsburger A, Heemskerk J, Korzh V, Norlin S, Ruiz i Altaba A, Tanabe Y, Placzek M, Edlund T, Jessel TM, Dodd J (1994) Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the nothocord. Cell 76:761-775.

Rubenstein JL, Martinez S, Shimamura K & Puelles L (1994) The embryonic vertebrate forebrain: the prosomeric model. Science 266:578-580.

Rubenstein JL, Shimamura K, Martines S, Puelles L (1998) Regionalization of the prosencephalic neural plate. Annu Rev Neurosci 21:445-477.

Rufer M, Wirth SB, Hofer A, Dermietzel R, Pastor A, Kettenmann H, Unsicker K (1996) Regulation of connexin-43, GFAP, and FGF-2 is not accompanied by changes in astroglial coupling in MPTP-lesioned FGF-2 treated parkinsonian mice. J Neurosci Res 46:606-617.

Saba R, Nakatsuji N, & Saito T (2003) Mammalian BarH1 confers commissural neuron identity on dorsal cells in the spinal cord. J Neurosci 23:1987-1991.

Saez JC, Nairn AC, Czernik AJ, Spray DC, Hertzberg EL, Greengard P, Bennett MV (1990) Phosphorylation of connexin 32, a hepatocyte gap-junction protein, by cAMP-dependent protein kinase, protein kinase C and $Ca2^{+}$/calmodulin-dependent protein kinase II. Euro J Biochem 192:263-273.

Saez JC, Berthoud VM, Branes MC, Martinez AD, Beyer EC (2003) Plasma membrane channels formed by connexins: their regulation and functions. Physiol Rev 83:1359-1400.

Saito T, & Nakatsuji N (2001) Efficient gene transfer into the embryonic mouse brain using in vivo electroporation. Dev Biol 240:237-246.

Sancho-Tello M, Vallés S, Montoliu C, Renau-Piqueras J, Guerri C (1995) Developmental pattern of GFAP and vimentin gene expression in rat brain and in radial glial cultures. Glia 15:157-166.

Sasaki T, Garante PR (1986) Fate of annular gap junctions in the papillary cells of the enamel organ in the rat incisor. Cell Tissue Res 246:523-530.

Segretain D, Falk MM (2004) Regulation of connexin biosynthesis, assembly, gap junction formation, and removal. Biochem Biophys Acta 1662:3-21.

Serafini T, Colamarino SA, Leonardo ED, Wang H, Beddington R, Skarnes WC, Tessier-Lavigne M (1996) Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell 87:1001-1014.

Schaar BT, Kinoshita K, McConnell SK (2004) Doublecortin microtubule affinity is regulated by a balance of kinase and phosphatases activity at the leading edge of migrating neurons. Neuron 41:203-213.

Schmechel DE, Rakic P (1979) A Golgi study of radial glial cells in developing monkey telencephalon: morphogenesis and transformation into astrocytes. Anat Embryol 16:115-152.

Sheen VL, Walsh CA (2003) Developmental genetic malformations of the cerebral cortex. Curr Neurol Neurosci Rep 3:433-441.

Shibata T, Yamada K, Watanabe M, Ikenaka K, Wada K, Tanaka K, Inoue Y (1997) Glutamate transporter GLAST is expressed in the radial glia-astrocyte lineage of developing mouse spinal cord. J Neurosci 17:9212-9219.

Shimada M, Langman J (1970) Cell proliferation, migration, and differentiation in the cerebral cortex of the golden hamster. J Comp Neurol 139:227-244.

Sicca F, Kelemen A, Genton P, Das S, Mei D, Moro F, Dobyns WB, Guerrini R (2003) Mosaic mutations of the LIS1 gene cause subcortical band heterotopia. Neurology 61:1042-1046.

Small JV, Stradal T, Vignal E, Rottner K (2002)The lamellipodium: where motility begins. Trends Cell Biol 12:112-20.

Smart IH (1973) Proliferative characteristics of the ependymal layer during the early development of the mouse neocortex: a pilot study based on recording the number, location, and plane of cleavage of mitotic figures. J Anat 116:67-91.

Smart IH, Smart M (1982) Growth pattern in the lateral wall of the mouse telencephalon: 1. Autoradiographic studies of the histogenesis of the isocortex and adjacent areas. J Anat 134: 273-298.

Smith DS, Niethammer M, Ayala R, Zhou Y, Gambello MJ, Wynshaw-Boris A, Tsai LH (2000) Regulation of cytoplamic dynein behavior and microtubule organization by mammalian Lis1. Nat Cell Biol 2:767-775.

Snow RL, Robson JA (1995) Migration and differentiation of neurons in the retina and optic tectum of the chick. Exp Neurol 134:13-24.

Willecke K, Eiberger J, Degen J, Eckardt D, Romualdi A, Guldenagel M, Deutsch U, Sohl G. (2002) Structural and functional diversity of connexin genes in the mouse and human genome. Biol Chem 383:725-737.

Söhl G, Maxeiner S, Willecke K (2005) Expression and functions of neuronal gap junctions. Nat Rev Neurosci 6:191-200.

Spray DC, Bennett MW (1985) Physiology and pharmacology of gap junctions. Annu Rev Physiol 47:281-303.

Spray DC, Moreno AP, Kessler JA, Dermietzel R (1991) Characterization of gap junctions between cultures leptomeningeal cells. Brain Res 568:1-14.

Takahashi T, Nowakowski RS, Caviness VS Jr (1993) Cell cycle parameter and patterns of nuclear movement in the neocortical proliferative zone of the fetal mouse. J Neurosci 13:820-833.

Tamai H, Shinohara H, Miyata T, Saito K, Nishizawa Y, Nomura T, Osumi N (2007) Pax6 transcription factor is required for interkinetic nuclear movement of neuroepithelial cells. Genes Cells 12:983-996.

Tamamaki N, Yanagawa Y, Tomioka R, Miyazaki J, Obata K, Kaneko T (2003) Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. J Comp Neurol 467:60-79.

Tan SS, Kalloniatis M, Sturm K, Tam PP, Reese BE, Faulkner-Jones B (1998) Separate progenitors for radial and tangential cell dispersion during development of the cerebral neocortex. Neuron 21:295-304.

Tanaka D, Nakaya Y, Yanagawa Y, Obata K, Murakami F (2003) Multimodal tangential migration of neocortical GABAergic neurons independent of GPl anchored proteins. Development 130:5803-5813.

TenBroek EM, Lampe PD, Solan JL, Raynhout JK, Johnson RG (2001) Ser364 of connnexin43 and upregulation of gap junction assembly by cAMP. J Cell Biol 155:1307-1318.

Theis M, de Wit C, Schlaeger TM, Eckardt D, Krüger O, Döring B, Risau W, Deutsch U, Pohl U, Willecke K (2001) Endothelium-specific replacement of the connexin43 coding region by a lacZ reporter gene. Genesis 29:1-13.

Toyofuku T, Yabuki M, Otsu K, Kuzuya T, Hori M, Tada M (1998) Direct association of the gap junction protein connexin-43 with ZO-1 in cardiac myocytes. J Biol Chem 273:12725-12731.

Traub O, Look J, Dermietzel R, Brummer F, Hülser D, Willecke K (1989) Comparative characterization of the 21-kD and 26-kD gap junction proteins in murine liver and cultured hepatocytes. J Cell Biol 108:1039-1051.

Tsai JW, Chen Y, Kriegstein AR, Vallee RB (2005) LIS1 RNA interferences blocks neural stem cell division, morphogenesis, and motility at multiple stages J Cell Biol 170:935-945.

Voigt T (1989) Development of glial cells in the cerebral wall of ferrets: direct tracing of their transformation from radial glia into astrocytes. J Comp Neurol 289:74-88.

Vuori K, Ruoslahti E (1995) Tyrosin phosphorylation of p130Cas and cortactin accompanies integrin-mediated cell adhesion to extracellular matrix. J Bio Chem 270:2259-2226.

Wall ME, Otey C, Qi J, Banes AJ (2007) Connexin 43 is localized with actin in tenocytes. Cell Motil Cytoskeleton 64:121-130.

Walsh C, Cepko CL (1990) Cell lineage and cell migration in the developing cerebral cortex. Experimentia 46:940-947.

Walsh C, Cepko CL (1992) Widespread dispersion of neuronal clones across functional regions of the cerebral cortex. Science 255:434-440.

Weissman TA, Riquelme PA, Ivic L, Flint AC, Kriegstein AR (2004) Calcium waves propagate through radial glial cells and modulate proliferation in the developing neocortex. Neuron 5:647-661.

White RL, Spray DC, Campos de Carvalho AC, Wittenberg BA, Bennett MV (1985) Some electrical and pharmacological properties of gap junctions between adult ventricular myocytes. Am J Physiol 249:447-455.

White TW, Bruzzone R, Paul DL (1995) The connexin family of intercellular channel forming. Kidney Int 48:1148-1157.

Wichterle H, Turnbull DH, Nery S, Fishell G, Alvarez-Buylla A (2001) In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain, Development 128:375935-71.

Wiencken-Barger AE, Djukic B, Casper KB, McCarthy KD (2007) A role for connexin43 during neurodevelopment. Glia 55:675-686.

Xu X, Li WE, Huang GY, Meyer R, Chen T, Luo Y, Thomas MP, Radice GL, Lo CW (2001) Modulation of mouse neural crest cell motility by N-cadherin and connexin43 gap junctions. J Cell Biol 154:217-230.

Xu Q, de la Cruz E, Anderson SA (2003) Cortical interneuron fate delamination: diverse sources for distinct subtypes? Cereb Cortex 13:670-676.

Xu X, Francis R, Wei CJ, Linask KL, Lo CW (2006) Connexin43-mediated modulation of polarized cell movement and the directional migration of cardiac neural crest cells. Development 133:3629-3639.

Yamane Y, Shiga H, Asou H, Haga H, Kawabata K, Abe K, Ito E (1999) Dynamics of astrocyte adhesion as analyzed by a combination of atomic force microscopy and immuno-cytochemistry: The involvement of actin filaments and connexin43 in the early stage of adhesion. Arch Histol Cytol 62:355-361.

Yao J, Morioka T, Oite T (2000) PDGF regulates gap junction communication and connexin43 phosphorylation by PI 3-kinase in mesangial cells. Kidney Int 57:1915-1926.

Yeager M, Nicholson BJ (1996) Structure of gap junction intercellular channels. Current Opinion in Structural Biology 6:183-192.

Zhang JT, Chen M, Foote CI, Nicholson BJ (1996) Membrane integration of in vitro-translated gap junctional proteins: co-and post-translational mechanisms. Mol Biol Cell 7:471-482.

Zheng C, Heintz N, Hatten ME (1996) CNS gene encoding astrotactin, which supports neuronal migration along radial fibers. Science 272:417-419.

Chapter 2

Expression of Connexins in Embryonic Mouse Neocortical Development

A version of this chapter has been previously published; Cima Cina, John F. Bechberger, Mark A. Ozog, and Christian G, Naus (2007) Expression of connexins in embryonic mouse neocortical development. *J Comp. Neurol* **504**: 298-313.

2.1 Introduction

In the embryonic brain of mammals, projection neurons originate from the neuroepithelium which lines the dorsolateral wall of the telencephalon in the ventricular zone (VZ). At the onset of neurogenesis (≈E11) the neuroepithelial cells transform into radial glial cells that span the entire wall of the cerebral cortex. These neural precursor cells eventually give rise to the cells of the cerebral cortex (Anthony et al., 2004; Gotz et al., 2005). Radial glial precursors located in the dorsal telencephalon generate only pyramidal excitatory neurons, whereas non-pyramidal inhibitory interneurons originate from their parental radial glial cells located in the ventral telencephalon. In the mouse model, these neurons can be seen migrating into the cortical plate (CP) between E11 and E18 (Takahashi et al., 1996). Two predominant migrational routes exist for the cortical neurons: 1) between E11 and E18 neurons proceed radially from the VZ to the CP along radial glial fibers in a process called radial migration (Rakic, 1972); 2) interneurons originating from the ganglionic eminence proceed perpendicular to the radial fibers and parallel to the pial surface, in a process called tangential migration (Marin and Rubenstein, 2001). In the early stage of neurogenesis, the first postmitotic cortical neurons migrate radially from the VZ to form the preplate (E11-E13). At this time, the intermediate zone (IZ) becomes evident which contains fibers extending from the ganglionic eminence. The early preplate neurons migrate independently of radial glial fibers via somal translocation (Nadarajah et al., 2001). The subsequently generated neurons (between E13-E18) leave the VZ and enter the preplate to form the CP, dividing this region into the subplate and the marginal zone (MZ). The MZ forms lamina I and contains Cajal-Retzius cells (Parnavelas, 2002). At the same time, the subventricular zone (SVZ) containing progenitor cells generated in the VZ and then dividing in the SVZ becomes apparent. The CP develops in an inside-out manner to become laminae II-VI of the mature cortex. The earlier-born neurons reside in the deeper layers (layers V, VI), whereas later-born neurons occupy the most superficial layers (layers II, III) (Haubensak et al., 2004). These neurons that are born during later neurogenesis rely more on another form of radial migration, locomotion (Nadarajah et al., 2001). When migrating neurons reach their final destination, they detach from the radial glia, stop migrating and subsequently start to differentiate. It is believed that perturbations in neuronal migration into the cerebral cortex are associated with numerous neurological diseases and disorders in humans, including

mental retardation and epilepsy (Aicardia, 1994; Guerrini and Filippi, 2005). A possible etiology for such migrational deficiencies, and thus cerebral cortical malformation, may be due to aberrant gap junctional intercellular coupling during development. Indeed, a study by Cepeda et al. (1993) provided indirect evidence to highlight the role of the gap junctions during brain development. In that study, neocortical tissue was obtained from epileptic human postnatal brains with different degrees of abnormality, and then the transfer of the low-molecular-weight fluorescent dye Lucifer Yellow (LY) which readily passes through gap junctions was measured among neurons. The authors observed a significant developmental decrease in the occurrence of dye coupling. Therefore, in developing human neocortex, with an increase in age the frequency of dye coupling decreased. Furthermore, among the youngest group, the incidence of dye coupling was higher in the least abnormal cerebral cortex and lower in the brains with the highest degree of epilepsy.

Furthermore, in early stages of brain development gap junctions provide direct transfer of vital ions and signaling molecules between neurons until postnatal stages in which synaptic formation occurs (Yuste et al, 1992; Peinado et al., 1993). For instance, Connors and Long (2004) review findings that demonstrate that Cx36 provides gap junctional coupling among inhibitory interneurons and facilitates the synchronous neocortical activity in later stages of development. Therefore, these findings provide support for the hypothesis that gap junctions have a vital role in brain development.

Gap junctions are intercellular membrane channels that provide cytoplasmic continuity between adjacent cells and permit the exchange of molecules less than 1,200 Da in size (Simon and Goodenough, 1998). The structural unit of the gap junction is the connexon, a proteinaceous cylinder with a hydrophilic channel that is equally contributed in formation by each of the closely-apposed cells. Connexons, themselves, are composed of proteins termed connexins (Cx), which are encoded by a multi-gene family consisting of at least 20 members in mammals and are named according to their relative size (Willecke et al., 2002).

In adult mammals, mature neurons show limited to no gap junctional coupling (Sotelo and Korn, 1978; Peinado et al., 1993). However, during development, neurons can be seen coupled to one another and to other cell types. While the isoforms Cx26, Cx30, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, and Cx47 have been identified in the developing central nervous system (CNS), neurons themselves have been reported to express Cx36 exclusively,

as well as the isoforms Cx45, Cx26, Cx43, and Cx32 (Nadarajah et al., 1997; Sohl et al., 1998; Nadarajah and Parnavelas, 1999; Nagy and Rash, 2000; Rozental et al.., 2000; Alvarez-Maubecin et al., 2001; Maxeiner et al., 2003). Intercellular coupling between neurons, via Cx26 and or Cx36 interactions, can be extensive during development. For instance, neurons have been shown to be coupled with up to 60 other neurons within the VZ (Connors et al., 1983; LoTurco and Kriegstein, 1991; Yuste et al., 1992; Peinado et al., 1993).

Other cell types in the developing CNS that demonstrate gap junctional coupling include astrocytes and radial glia, both of which express Cx43, and Cx26, oligodendrocytes, which express the isoforms Cx32, Cx29, and Cx47, as well as endothelial cells, which express Cx37, Cx40, and Cx43 (Dermietzel et al., 1991, 2000; Giaume et al., 1991; Bruzzone et al., 1993; Traub et al., 1998; Bittman and LoTurco, 1999; Nagy et al., 1999, 2004; Rash et al., 2001; Theis et al, 2005; Isakson et al., 2006). During development, neurons have been identified to be coupled to these other cell types via Cx26 and Cx43 interactions. Specifically, neuronal progenitor cells are coupled to the radial glia in the VZ, lose this heterocellular coupling as they migrate up the radial glia processes in the CP, where they then form homocellular coupling and become mature neurons (Nadarajah et al., 1997; Bittman and LoTurco, 1999; Bittman et al, 2002).

Functional intercellular coupling and Cx expression appears to play an important role in neuronal migration and cerebrocortical development. Indeed, embryonic mice lacking Cx43 expression phenotypically present an accumulation of BrdU-positive cells in the intermediate zone, whereas these cells establish themselves normally in the CP of wild-type littermates (Fushiki et al., 2003). The availability of null-mutant mice of other Cx isoforms will allow the role of Cx-specificity on cortical development to be determined. However, as most studies regarding Cx expression during development have focused on the rat model, it becomes imperative that the expression of these gap junction proteins in the mouse model be established prior to detailed analysis of transgenic and knockout studies. Thus, the purpose of this study was to characterize the expression of developmentally associated Cxs in the murine neocortex during the time of neuronal migration.

2.2 Materials and Methods

2.2.1 Animals

C57 black/6J mice were maintained in an animal facility with a 12:12-hour light:dark cycle and were provided food and water *ad libitum*. The animals were maintained according to Canadian Council on Animal Care (CCAC) guidelines for the care and use of laboratory mice at the University of British Columbia. Following mating of adult mice, the day on which a vaginal plug was identified was considered embryonic day 0 (E0).

2.2.2 RNA Isolation and Reverse Transcription Polymerase Chain Reaction (PCR)

Cortices of adult and embryonic mice at time points E14, E16, and E18 were isolated, freed of meninges, and chopped into small pieces. Total RNA was extracted using TRIzol reagent (Invitrogen, Burlington, ON), rinsed twice with 75% ethanol, and quantified using spectrophotometry at 260 nm. Two micrograms of the RNA was DNase treated and subsequently reverse-transcribed in a thermocycler. The RT product was amplified (30 cycles) using primers specific for each Cx isoform (Table 1). Glyceraldehydephosphate dehydrogenase (GAPDH) was used as a positive control to confirm the isolation and RT of the RNA and to demonstrate relative equal loadings of RT product. The amplified products were characterized by electrophoresis in a 2% agarose gel containing 0.1% ethidium bromide and were run in parallel with a 1KB standard (Invitrogen).

2.2.3 Antibodies

The Cx antibodies used in this study are listed in Table 2.2. The Cx26 antibody (Zymed, San Francisco, CA) was produced in rabbit. The cDNA was originally isolated from mouse liver cells. The immunogen was a 13 amino acid (aa) synthetic peptide, 214–226 from the C-terminus of the mouse Cx26 protein. Specificity of the Cx26 antibody has been demonstrated previously by conducting immunohistochemistry, Western blot, and freeze-fracture replica immunogold labeling (FRIL) on embryonic and adult liver and brain tissue (Nagy et al., 2001). The Cx26 antibody detected a single band of 26 kDa molecular weight in brain tissue using Western blot (Nagy et al., 2001). No band was seen when 250 µL of diluted primary antibody was preincubated with 50 µg of the immunizing peptide using Western blot. We

Table 2.1 Primer Sequences Used for RT-PCR Analysis and Mouse Genotyping

Targer	Primer Sequences Sense/Antisense	Expected Product Size (bp)	References
Cx45	5′-TTTGTGTGCAACACAGAGCA-3′/ 5′-CCCACCTCAAACACAGTCCT-3′	350	Theis et al. (2004)
Cx43	5′-CCCCACTCTCACCTATGTCTCC-3′/ 5′-ACTTTTGCCGCCTAGCTATCCC-3′	500	Naus et al. (1997)
Cx40	5′-CTGTCCCCACCCAGTCAACT-3′/ 3′-CGATGGTATCACTGTTTGCC-3′	460	Naus et al. (1997)
Cx37	5′-TGTACCTTGGCTGCCTCTCT-3′/ 5′-AGCCTGCATCAGTGCTTTTT-3′	396	Simon et al. (2003)
Cx36	5′-GTAGGGGAGACGGTGTACGA-3′/ 5′-TCGAAACACCACTTGGATGA-3′	498	Schubert et al. (2005)
Cx32	5′-CCATAAGTCAGGTGTAAAGGAGC-3′/ 5′-AGATAAGCTGCAGGGACCATAGG-3′	550	Sohl et al. (2001)
Cx30	5′-GGTACCTTCTACTAATTAGCTTGG-3′/ 5′-AGGTGGTACCCATTGTAGAGGAAG-3′	544	Guldenagel et al. (2000)
Cx26	5′-AGGAAGGTGCCACTGAGAAA-3′/ 5′-ACGAGACGCTTCCAGTTTGT-3′	312	Filippov et al. (2003)
GAPDH	5′-AATGCATCCTGCACCACCAA-3′/ 5′-GTAGCCATATTCATTGTCATA-3′	515	Yang et al. (2001)

RT-PCR, reverse transcription polymerase chain reaction; bp, base pairs.

Table 2.2 Connexin Antibodies Used for Western Blotting and Immunohistochemistry

Antibody	Type	Designation	Source
Connexin26	polyclonal	c-terminus	Zymed, San Francisco, CA Cat. no. 51-2800
Connexin36	polyclonal	cytoplasmic loop	Zymed Cat. no. 51-6300
Connexin37	polyclonal	c-terminus	Alpha Diagnostics, San Antonio, TX Cat. no. Cx37A11-A
Connexin43	polyclonal	c-terminus	Sigma-Aldrich, Oakville, ON Cat. no. C6219
Connexin45	polyclonal	c-terminus	Chemicon, Temecula, CA Cat. no. AB1745

showed the specificity of Cx26 by using homogenates of liver and heart. Cx26 was found in homogenates of liver (positive control) but not in homogenates of heart (negative control) (Fig. 2.2A).

The Cx36 antibody (Zymed) was produced in rabbit. The cDNA was originally isolated from mouse brain cells. A 9-aa synthetic peptide, 296–304, derived from the C-terminal region of the mouse Cx36 protein, was used as immunogen. The antibody detects a single band of ≈36 kDa molecular weight in developing mouse brain homogenates by Western blot analysis (Zymed). The specificity of the antibody has been confirmed by using tissue homogenates of brain and heart. Cx36 was found in homogenates of mouse brain (positive control) but not in homogenates of heart (negative control) (Fig. 2.2B). For a negative control experiment, no band was seen in homogenates of brain tissue when 3 µg of the primary antibody was diluted in 100 µg of Cx36 peptide using Western blot (Rash et al., 2000). In addition, specificity of Cx36 antibody has been confirmed by lack of immunoreactivity in the retina of Cx36 knockout mice (Guldenagel et al., 2001).

The Cx37 antibody (Alpha Diagnostics, San Antonio, TX) was produced in rabbit. The cDNA was originally isolated from mouse blood endothelial cells. The synthetic peptide used as immunogen covers aa 318–333 of the C-terminus. The antibody detects a single band of 37 kDa molecular weight in developing mouse brain homogenates by Western blot (manufacturer's technical information). The Cx37 immunogenic peptide of mouse is specific for Cx37 and no significant homology is seen with other Cxs (Hill et al., 2002; manufacturer's technical information). We also showed the specificity of Cx37 antibody by using tissue homogenates of brain and heart (positive control, Fig. 2.2C). For a negative control experiment, no band was seen when 250 µL of diluted primary antibody was preincubated with 50 µg of the immunizing peptide using Western blot. In addition, no staining was seen in blood vessels when Cx37 antibody was incubated with excess immunogen (Earley et al., 2004).

The Cx43 antibody (Sigma-Aldrich, Oakville, CA) was produced in rabbit using a synthetic peptide Lys-Pro-Ser- Ser-Arg-Ala-Ser-Ser-Arg-Pro-Asp-Asp-Leu-Glu-Ile conjugated to KLH. The peptide corresponds to a C-terminal fragment of the cytoplasmic domain of human and rat Cx43, aa residues 363–382, with an N-terminal added lysine. This Cx43 antibody has been previously studied in adult and developing cerebral cortex (Nadarajah et al., 1996, 1997). This antibody detects multiple bands of a 43-kDa region by Western blot analysis on whole brain homogenates (Hossain et al., 1994; Rash et al., 2001; manufacturer's technical information). Cx43 was found in tissue homogenates of brain and

heart (positive control, Fig. 2.2D). Brain sections with preadsorbed antibodies showed no staining by immunogold labeling (Hofer et al., 1998).

The Cx45 antibody (Chemicon, Temecula, CA) was produced in rabbit. The cDNA was originally isolated from mouse cardiac cells. The peptide sequence for the immunogen corresponded to aa 354–367 of human Cx45 (manufacturer's technical information). The antibody detects a single 46-kDa band with molecular weight in homogenates of different regions of developing and adult brain by Western blot (Maxeiner et al., 2003). The specificity of the Cx45 antibody was demonstrated by comparing embryonic brain homogenates of wildtype and Cx45-deficient mouse. Cx45 was found in brain homogenates of wildtype mouse but not in brain homogenates of Cx45-deficient mouse using Western blot (Maxeiner et al., 2003). In addition, Cx45 was found in tissue homogenates of brain and heart (positive control, Fig. 2.2E). No band was seen when Cx45 antibody was preincubated with the respective epitope specific peptide fragments (Siu Yi Leung et al., 2002).

The monoclonal nestin (Rat-401) antibody (Cat. no. 556309, BD Biosciences, ON), was produced in mouse, using aa residues 544–776 as the immunogen (Hockfield and McKay, 1985). Immunohistochemistry and Western blot analysis on neural tube of E15 rats showed that the nestin antibody only recognizes radial glial cells and dividing neural stem cells in the embryonic but not adult rat CNS (Lendahl et al., 1990). This antibody recognizes a single band of ≈198 kDa molecular weight in homogenates of developing neural tube cells by Western blot (Hockfield and McKay, 1985). In addition, similar results were obtained using lysates from PC 12 cells probed with antirat nestin at concentrations of 2, 1, and 0.5 µg/mL.

The monoclonal MAP-2 antibody (Cat. no. M 4403, Sigma-Aldrich), was produced in mouse. Rat brain microtubule-associated proteins (MAPs) were used as the immunogen to prepare this antibody. The antigenic site for the immunogen was identified as Lys-Asn-Val-Arg-Ser-Lys-Val-Gly-Ser-Thr-Glu-Asn-Ile-Lys-His-Gln-Pro-Gly-Gly-Gly-Arg-Ala-Lys (Aizawa et al., 1989). While this antibody reacts with all forms of MAP-2, it does not cross-react with other MAPs or tubulin. Anti-MAP-2 shows elective labeling of dendrites throughout the brain (Huber and Matus, 1984; manufacturer's technical information). While immunoprecipitation and antibody gel staining techniques did not show any crossreacting

antigen in nonneuronal cells including mouse kidney cells, 3T3, and Hela cells, this antibody has been shown to be specific for differentiated neurons (Izant and McIntosh, 1980).

2.2.4 Protein Isolation and Western Blot Analysis

Protein was isolated from cerebral cortices of E14, E16, and E18 embryonic mice using radioimmune precipitation lysis buffer (50 mM Tris-HCl, pH 8.0, 1% IGEPAL) supplemented with Mini Complete protease inhibitors (Roche Applied Science, Laval, QC) and phosphatase inhibitors (Sigma). Protein concentrations were determined using the BCA protein quantification kit (Pierce, Rockford, IL). Protein samples (30 µg) were boiled for 2 minutes in SDS sample buffer (pH 8.0) and separated on a 12% polyacrylamide gel in parallel with molecular weight markers (Bio-Rad Laboratories, Hercules, CA). Subsequently, the electrophoresed proteins were transferred onto a nitrocellulose membrane (Bio- Rad Laboratories) at 100 V for 1 hour. The blots were then blocked with 5% dry milk in PBS (pH 7.4, with 1% Tween 20) for 1 hour and subsequently incubated with antibodies against Cx26, Cx36, Cx43, and Cx45 at 4°C overnight. Following rinsing with phosphate-buffered saline (PBS), the blots were incubated in horseradish peroxidase-tagged secondary antibody (Cedarlane Laboratories, Hornby, ON) for 1hour at room temperature followed by incubation in Super- Signal chemiluminescent substrate (Pierce). The labelled blots were then exposed to Kodak X Omat x-ray film to visualize antibody binding. To ensure equal loading of protein samples, the blots were stripped of their Cx antibodies and reprobed for GAPDH (Cedarlane Laboratories).

2.2.5 Immunohistochemistry

Adult brains as well as embryonic brains obtained from E14, E16, and E18 mice were fixed by immersion in 4% paraformaldehyde (in PBS, pH 7.4) overnight at 4°C. The fixed brains were cryoprotected in 30% sucrose, mounted in OCT, and cut into 10 µm coronal sections using a cryostat. Cut brain sections were mounted on coverslips, blocked for nonspecific antibody binding with IgG blocking solution (M.O.M. kit, Vector Laboratories, Burlington, ON) and subsequently incubated in antibodies for Cx26, Cx36, Cx37, Cx43, and Cx45 at 4°C overnight. Selected samples were double-labelled with the neuronal marker microtubule associated protein-2 (MAP-2) (Sigma) and nestin (a radial glia marker) (BD

Biosciences). Laminae II/III of the mature neocortex and the VZ, the IZ, and the CP of embryonic cerebral cortex were processed for imaging. Specificity of the primary antibodies was assessed by omitting them from the labeling protocol on selected slices. Slices were then incubated in Alexa Fluor-tagged IgG antibodies (Molecular Probes, Eugene,OR) for 1 hour and mounted in ProLong Gold containing DAPI (Molecular Probes). Labeled sections were examined and analyzed with a Zeiss Axioskop fluorescent microscope and AxioVision 4.2 software (Carl Zeiss, Thornwood, NY). In order to show the distribution pattern of Cxs in E14-E18, representative figures were produced by combining images into a montage to cover the thickness from the VZ to the MZ. To generate the final figures, all images were stored as TIFF files and processed in Adobe Photoshop 7.0 (San Jose, CA). The images were adjusted to optimize brightness, contrast, and sharpness and then assembled into figures.

2.2.6 Densitometric Evaluations

The density of protein expression Cx26, Cx36, Cx37, Cx43, and Cx45 in four separate Western blot experiments was measured using Scion image software (Scion Image, Frederick, MD). The density value for each Cx was normalized to GAPDH. Arbitrary units were chosen to show the relative density of each Cx relative to other Cxs.

2.2.7 Data Analysis

All experiments were performed at least four times with two repeats per sample. All values are presented in relative density as mean ± SEM and the results were analyzed by one way ANOVA followed by the Tukey test. A P value < 0.05 was considered statistically different.

2.3 Results

2.3.1 Connexins Expressed in the Embryonic Cerebral Cortex

As the majority of neuronal migration in the mouse cerebral cortex occurs between the developmental stages of E14–E18, we examined the expression of several brain expressed Cxs at the specific timepoints E14, E16, and E18. RNA was isolated from the cortices at the three timepoints and subjected to RT-PCR using the primers listed in Table 2.1. Analysis of amplified RT products revealed that transcripts for Cx26, Cx36, Cx37, Cx43, and Cx45 were expressed at all three timepoints examined (Fig. 2.1). Transcripts for Cx40, however, were only observed at the E14 stage and appeared comparatively lower in quantity than transcripts for most of the other Cxs examined, with the exception of Cx30 and Cx32, both of which were absent from the embryonic mouse brain at these three stages of development (Fig. 2.1). Functionality of the Cx30 and Cx32 primers was confirmed using RNA isolated from adult mouse cerebral cortex and liver, respectively (data not shown). In RT-PCR experiments, confirmation of RNA isolation, RT, and relative equal loading of the RT product was confirmed using primers for GAPDH (Vandesompele et al., 2002) (Fig. 2.1). To determine if the transcripts of the various Cxs identified in the brains of embryonic mice (see above) translated into protein expression, we performed immunoblot analysis on protein isolated from the cerebral cortices of E14, E16, and E18 mice (Fig. 2.2A–E). The isoforms of Cx26, Cx36, Cx37, Cx43, and Cx45 were all expressed in the cortices of mice during the three stages of development and showed a relatively consistent level of expression (when compared to each other and GAPDH levels) (Fig. 2.2A–E). Cx26 was detected in cerebral cortex and the level of this protein appeared similar at all three time points examined. Cx26 was expressed in embryonic liver tissue used as a positive control, whereas embryonic heart tissue used as negative control was devoid of Cx26 expression (Fig. 2.2A). Cx36 was expressed at all time points and the level of this Cx protein appeared to be similar at all time points (Fig. 2.2B). Embryonic heart tissue was used as a negative control for the expression of Cx36 (Fig. 2.2B). The isoform of Cx37 was detected at a similar level in cerebral cortex at all three time points examined. The expression of this Cx was also shown in heart tissue used as a positive control (Fig. 2.2C). Cx43 was identified in cerebral cortex at all time points as well as in heart (positive control), while it showed an increase from E14 to E18 (Fig. 2.2D).

Figure 2.1 RNA transcripts of connexins detected in the developing mouse cortex from E14-E18. RNA was isolated from cortices of mouse embryos and subjected to RT-PCR for various Cx transcripts. Cx26, Cx36, Cx37, Cx43, and Cx45 were readily detected at E14-E18, while Cx40 was present at a low level at E14.

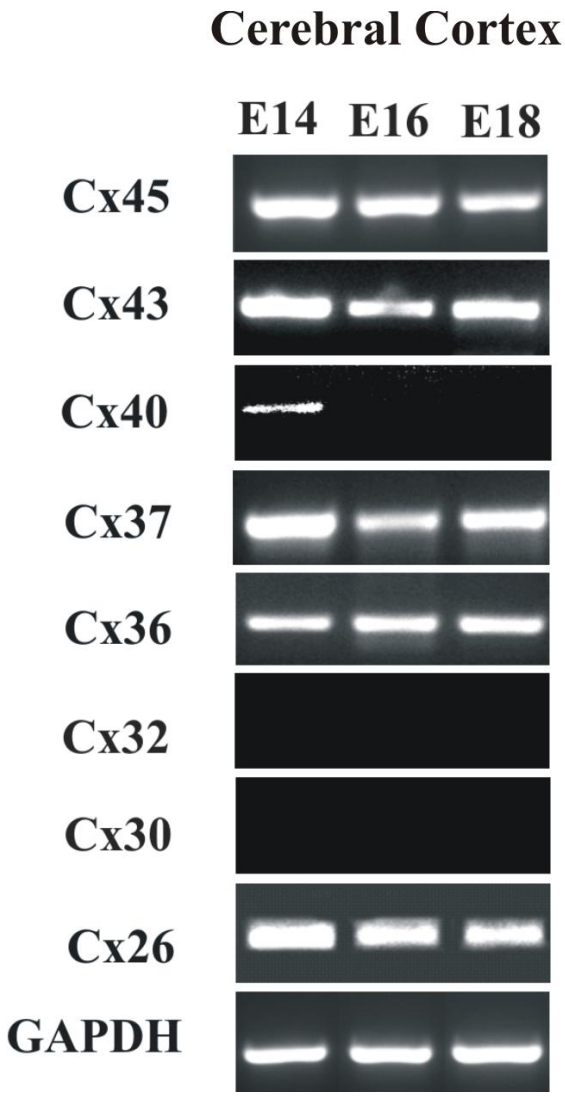
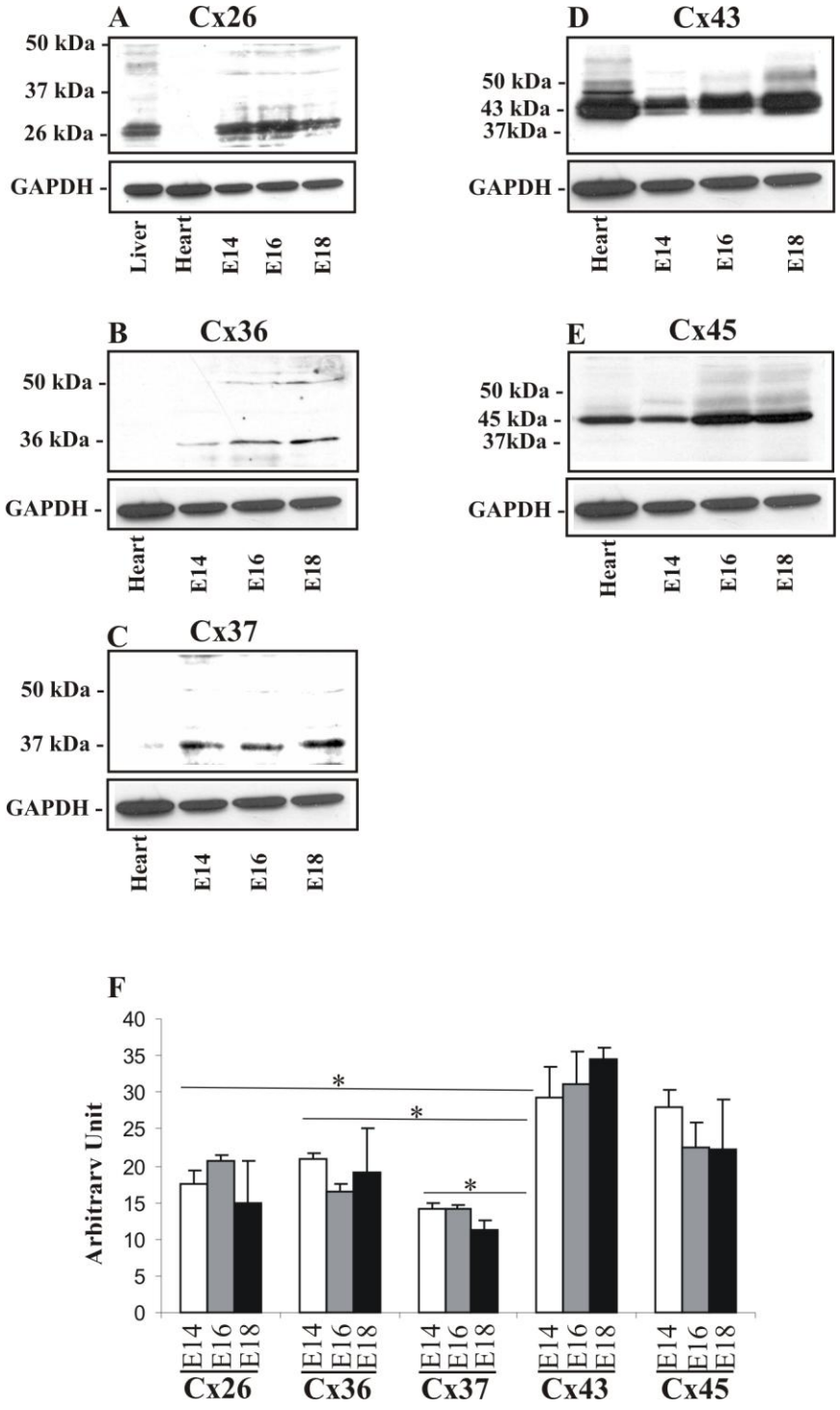

Cerebral Cortex
E14
E16
E18
Cx45
Cx43
Cx40
Cx37
Cx36
Cx32
Cx30
Cx26
GAPDH

Figure 2.2 Representative immunoblots showing the detection of Cx proteins expressed in the developing mouse cortex. **A–E:** Equal amounts of embryonic liver, heart, and cortical tissue lysates were immunoblotted and probed with Cx26 (26 kDa), Cx36 (36 kDa), Cx37 (37 kDa), Cx43 (43 kDa), and Cx45 (46 kDa) antibodies, respectively. **A:** Cx26 was expressed in liver (positive control) and cortical tissue; however, this Cx was absent in heart tissue (negative control). The expression of Cx26 appeared to be similar at all timepoints. **B:** Cx36 was expressed in all cortical tissue but not in heart (negative control). This Cx expression appeared to be similar at all timepoints. **C**: Cx37 was expressed equally at all timepoints. This Cx was also expressed in heart (positive control). **D:** Cx43 was expressed in all cortical tissue and its expression was shown to be more abundant at E18. Heart tissue was used as a positive control. **E:** Cx45 was expressed at all timepoints. Heart tissue was used as a positive control. GAPDH was used as a loading control. **F:** Densitometric evaluation of Cxs in developing cerebral cortex. The graph represents the quantification of Cx26, Cx36, Cx37, Cx43, and Cx45 in four independent Western blot experiments for each of the Cxs as compared with GAPDH. The arbitrary unit shows the density of each Cx relative to other Cxs. The expression level of Cx43 was significantly higher than Cx26, Cx36, and Cx37 ($n = 4$, $*P < 0.05$).


A
Cx26
50 kDa
37 kDa
26 kDa
GAPDH
Liver
Heart
E14
E16
E18
D
Cx43
50 kDa
43 kDa
37kDa
GAPDH
Heart
E14
E16
E18
B
Cx36
50 kDa
36 kDa
GAPDH
Heart
E14
E16
E18
E
Cx45
50 kDa
45 kDa
37kDa
GAPDH
Heart
E14
E16
E18
C
Cx37
50 kDa
37 kDa
GAPDH
Heart
E14
E16
E18
F
Arbitrarv Unit
40
35
30
25
20
15
10
5
0
*
*
*
E14
E16
E18
Cx26
Cx36
Cx37
Cx43
Cx45

Cx43 appeared to be the most abundant Cx isoform expressed in the developing cerebral cortex compared to Cx26, Cx36, and Cx37 (Fig. 2.2F). Cx45 was detected in cerebral cortex and the level of this protein was similar at all developmental time points examined (Fig. 2.2E). The expression level of this Cx was not significantly different from Cx43 (Fig. 2.2F). The Western blot data are in agreement with our RT PCR findings showing the expression of Cx26, Cx36, Cx37, Cx43, and Cx45 in the cerebral cortex at different time points (E14, E16, and E18). Taken together, these findings
indicate the expression of Cx26, Cx36, Cx37, Cx43, and Cx45 in the embryonic mouse neocortex during development from E14–E18.

2.3.2 Variations in the spatial expression of Cxs

Examination of Cx expression during development was conducted in the dorsal telencephalic wall of the E14, E16, and E18 mouse neocortex by immunofluorescent labeling using antibodies against Cx26, Cx36, Cx37, Cx43, and Cx45. Cx30 and Cx32 were not detected in any of the embryonic stages examined by immunolabelling, consistent with our RT-PCR results (data not shown). The expression of Cx26 was observed in the cerebral cortex throughout development and this Cx was generally evenly distributed among all cerebral cortical layers (the VZ, the IZ, and the CP), although there were areas with more intense labelling in the VZ (Fig. 2.3A–C). The expression pattern of Cx36 in cerebral cortex appeared to be higher in the CP and the VZ compared to the IZ at E16 and E18, whereas at E14 the distribution of this Cx was generally even in the three layers of the cerebral cortex (Fig. 2.4A–C). The results of immunofluorescent labelling for Cx37 revealed a generally even distribution with a slight decrease from E14 to E18 in the three layers of cerebral cortex examined (Fig. 2.5A–C). As shown in Figure 2.6, while the expression of Cx43 was identified in the three stages of development, the labelling pattern of this Cx appeared in a gradient manner (higher in the VZ and CP and lower in the IZ) at E16 and E18 (Fig. 2.6A–C). The expression of Cx45 was also examined in the cerebral cortex of embryonic mouse by immunolabeling. As shown in Figure 2.7A–C, Cx45 was expressed in the VZ, the IZ, and the CP at all three stages of development examined. The labelling pattern of this Cx appeared to be comparatively higher at E18 than the other two timepoints (E14 and E16) (Fig. 2.7A–C). The results obtained from the immunofluorescent experiments are in agreement with the RT-

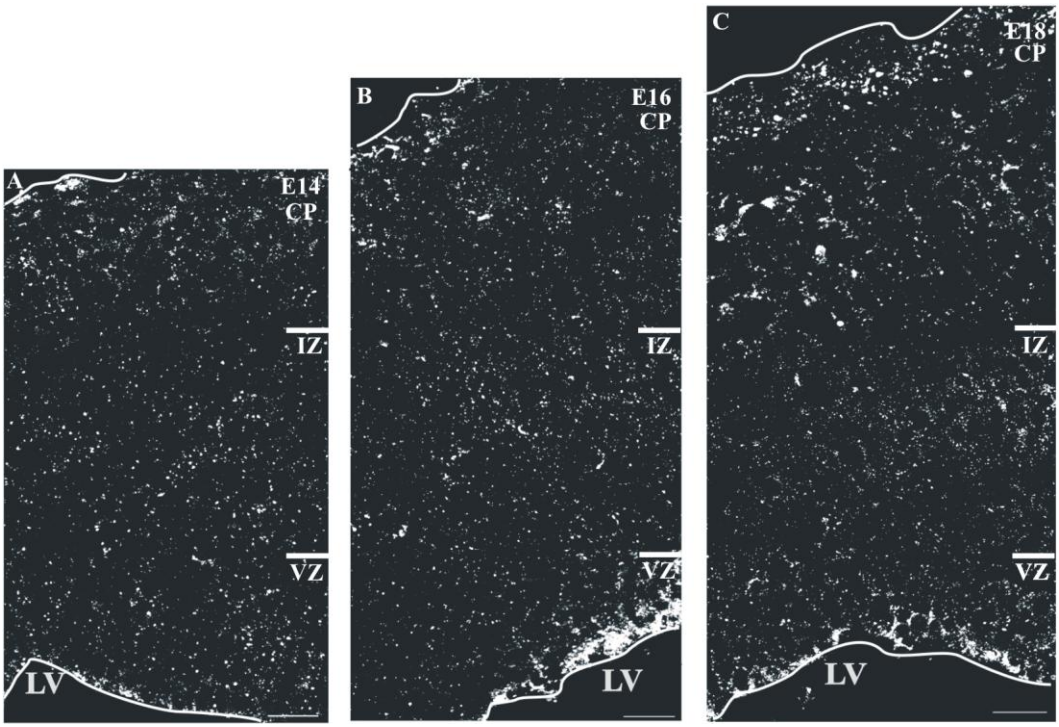


Figure 2.3 Immunohistochemical analysis shows that the distribution of Cx26 appears to be similar throughout development. A representative of the distribution pattern of Cx26 in cerebral cortical layers. **A-C:** 10 μm cortical slices at different timepoints (E14-E18) were stained against Cx26 antibody. Cx26 appeared evenly distributed in cerebral cortical layers. The distribution pattern was similar at all timepoints examined. VZ, ventricular zone; IZ, intermediate zone; CP, cortical plate; LV, lateral ventricle. Scale bar = 20 μm.

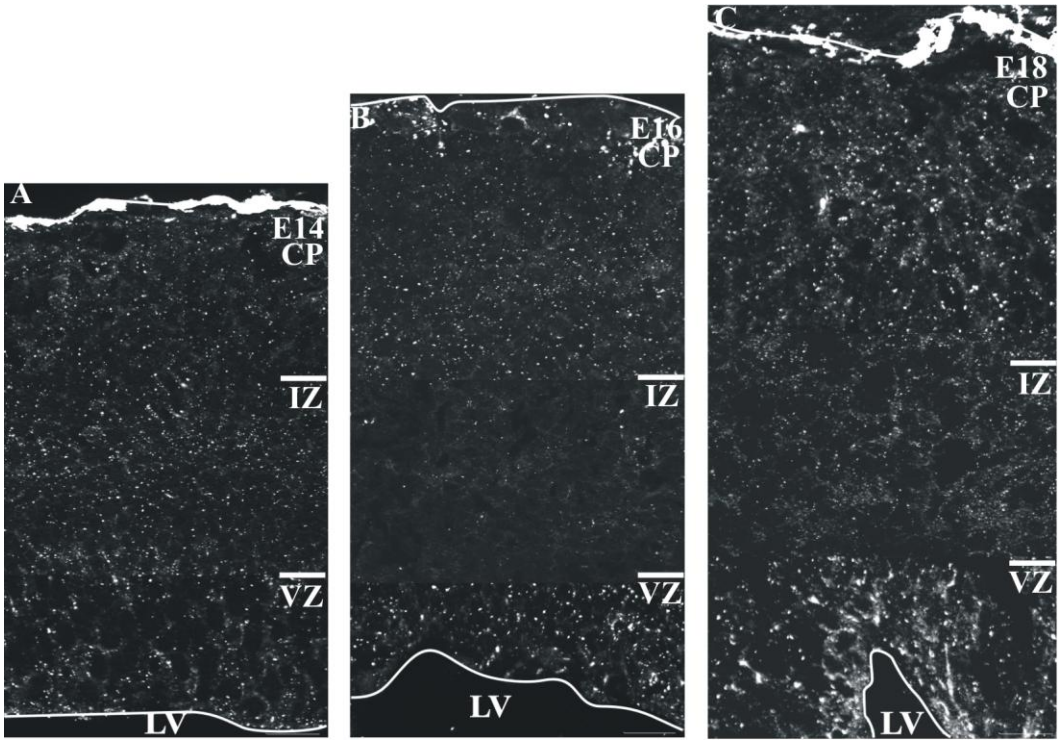


Figure 2.4 Cx36 is expressed in a gradient manner in the cerebral cortex. A representative of the distribution pattern for Cx36 in cerebral cortical layers from E14-E18. **A-C:** 10 μm cortical slices at different timepoints (E14-E18) were stained with the Cx36 antibody. The distribution pattern of Cx36 at E14 appeared to be similar in all cortical layers. Labeling for Cx36 is distinctly lower in the IZ than in the VZ and the CP at E16 and E18. Scale bar = 20 μm.

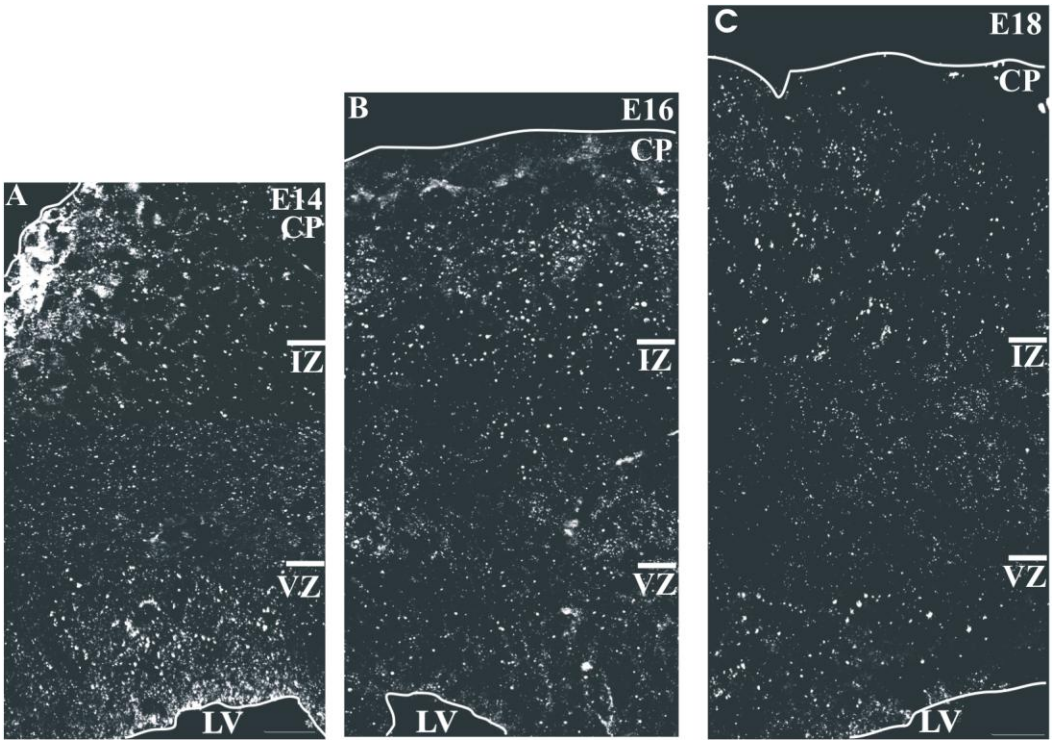


Figure 2.5 Cx37 is slightly decreased from E14-E18 of embryonic mouse neocortex. A representative of the distribution pattern for Cx37 in cerebral cortical layers from E14-E18. **A-C:** 10 μm cortical slices at different timepoints (E14-E18) were stained against Cx37 antibody. The distribution pattern of Cx37 appeared to be similar, with a slight decrease from E14-E18 in all cortical layers. This Cx was expressed at all timepoints examined. Scale bar = 20 μm.

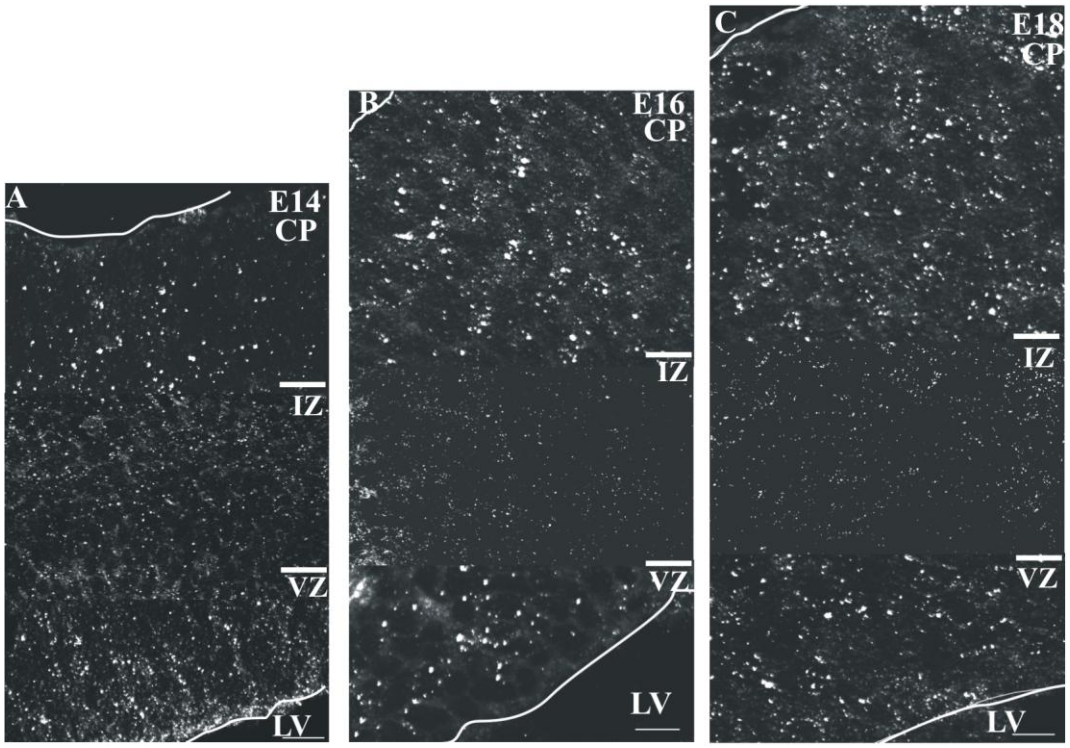


Figure 2.6 Cx43 is expressed in a gradient manner in the cortex. A representative of the distribution pattern for Cx43 in cerebral cortical layers from E14-E18. **A-C:** 10 μm cortical slices at different timepoints (E14-E18) were stained with the Cx43 antibody. The distribution pattern of Cx43 at E14 appeared to be similar in all cortical layers. Labeling for Cx43 is distinctly lower in the IZ than in the VZ and the CP at E16 and E18. Scale bars = 20 μm.

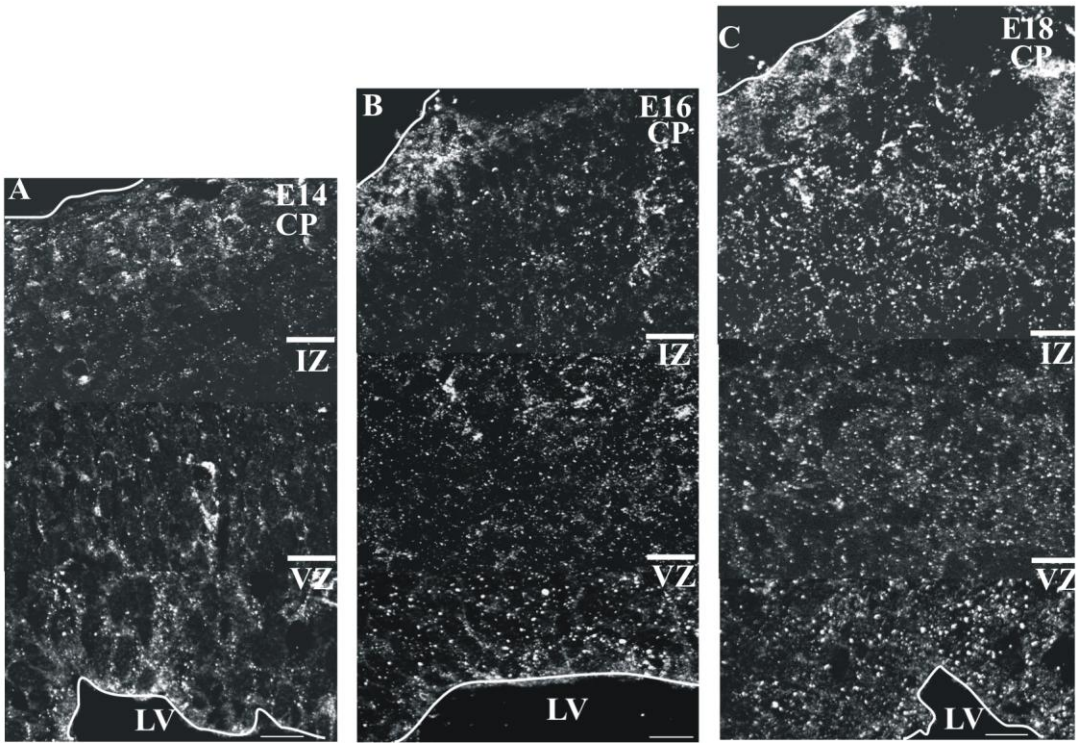


Figure 2.7 Cx45 immunoreactivity in the cortical layers of E14-E18 of mouse embryo increases as cortex develops. Cortical slices stained using an antibody against Cx45 showing punctate immunostaining in the VZ, the IZ, and the CP. The distribution pattern of Cx45 appeared to be increased from E14 to E18 **(A-C).** Scale bars = 20 μm.

PCR and the Western blot analysis. These findings indicate that Cx26, Cx36, Cx37, Cx43, and Cx45 are expressed in the neocortex at E14, E16, and E18 during development.

2.3.3 Distribution of Cxs and their Relation to MAP-2-positive Neurons in the Cortical Plate of the Developing Mouse Cortex at E18

To determine the cellular localization of various Cxs with regard to the neurons in the CP, we performed double immunofluorescent labelling experiments using MAP-2 (a neuronal marker) and antibodies against Cx26, Cx36, Cx37, Cx43, and Cx45 in tissue sections of mouse cerebral cortex at E18. As depicted in Figure 2.8A1–C5, confocal microscopy images with high magnification revealed punctuate staining of Cx26, Cx36, Cx37, Cx43, and Cx45 in the CP of E18 mouse cerebral cortex. Cx36 and Cx26 showed high immunoreactivity associated with MAP-2 positive neurons located in the CP (Fig. 2.8A4–C5). In contrast, Cx45, Cx43, and Cx37 showed lower immunoreactivity to MAP-2 positive neurons (Fig. 2.8A1–C3).

2.3.4 Association of Cx26 and Cx43 with Nestin-positive Radial Glia in the Cortical Plate of Mouse Neocortex Throughout Development

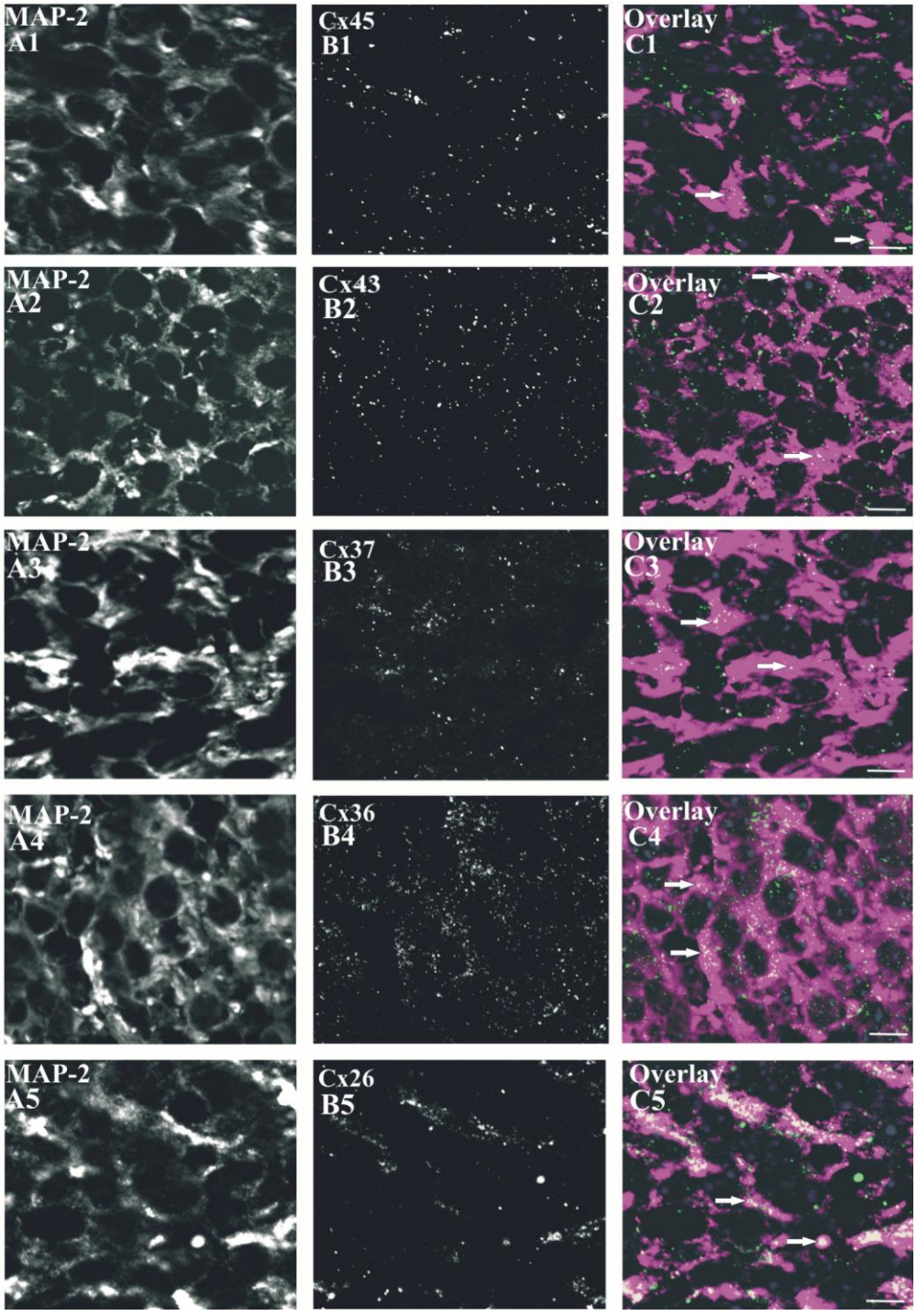
The relation of Cx26 and Cx43 with radial glial fibers in the CP at all three stages of development was examined. Double immunofluorescent labelling experiments using nestin and antibodies against Cx26 and Cx43 were performed on tissue sections of mouse cerebral cortex at E14, E16, and E18. Confocal microscopy images with high magnification showed punctuate staining of Cx26 and Cx43 with immunoreactivity associated with nestin-positive radial glia located in the CP at all timepoints (Fig. 2.9A1–B3).

2.3.5 Distribution of Cxs and their Relation to MAP-2-positive Neurons in the Laminae II/III of Adult Mouse Neocortex

To evaluate the localization of various Cxs with regard to the adult neocortex, we conducted double immunofluorescent labelling procedures to co-localize MAP-2 with Cx26, Cx36, Cx37, Cx43, and Cx45 in tissue sections of mature neocortex. As depicted in Figure 2.10, confocal microscopy images with high magnification showed punctuate staining of Cx26, Cx36, Cx37, Cx43, and Cx45 in the laminae II/III of the adult mouse neocortex. The expression of Cxs was significantly lower compared to the embryonic cortical plate sections

(Fig. 2.8). Cx36 showed a high association with MAP-2-positive neurons (Fig. 2.10A4–C4), whereas Cx45, Cx43, Cx37, and Cx26 revealed no localization to neurons (Fig. 2.10A1–C1, A2–C2, A3–C3, A5–C5).

Figure 2.8 MAP-2 labelling of neurons in the cortical plate is also immunoreactive for Cx26, Cx36, Cx37, Cx43, and Cx45. Double-immunolabeled confocal microscope images of 10 μm cortical tissue sections at E18 labeled with MAP-2 (magenta) (left panel: **A1-A5**) and antibodies against various Cxs (green) (middle panel: **B1-B5**). The nuclei of the cells were visualized by DAPI (blue) and are shown in the overlay images (right panel: **C1-C5**). Within the cortical plate, Cx26, Cx36, Cx37, Cx43, and Cx45 punctate immunostaining showed their distribution associated with MAP-2 positive cells. While Cx36 and Cx26 showed high immunoreactivity in association with MAP-2 positive cells (**A4-C5**), Cx45, Cx43, and Cx37 showed very low association with these cells (**A1-C3**) (arrows). Scale bars = 5 μm.


MAP-2
A1
Cx45
B1
Overlay
C1
MAP-2
A2
Cx43
B2
Overlay
C2
MAP-2
A3
Cx37
B3
Overlay
C3
MAP-2
A4
Cx36
B4
Overlay
C4
MAP-2
A5
Cx26
B5
Overlay
C5

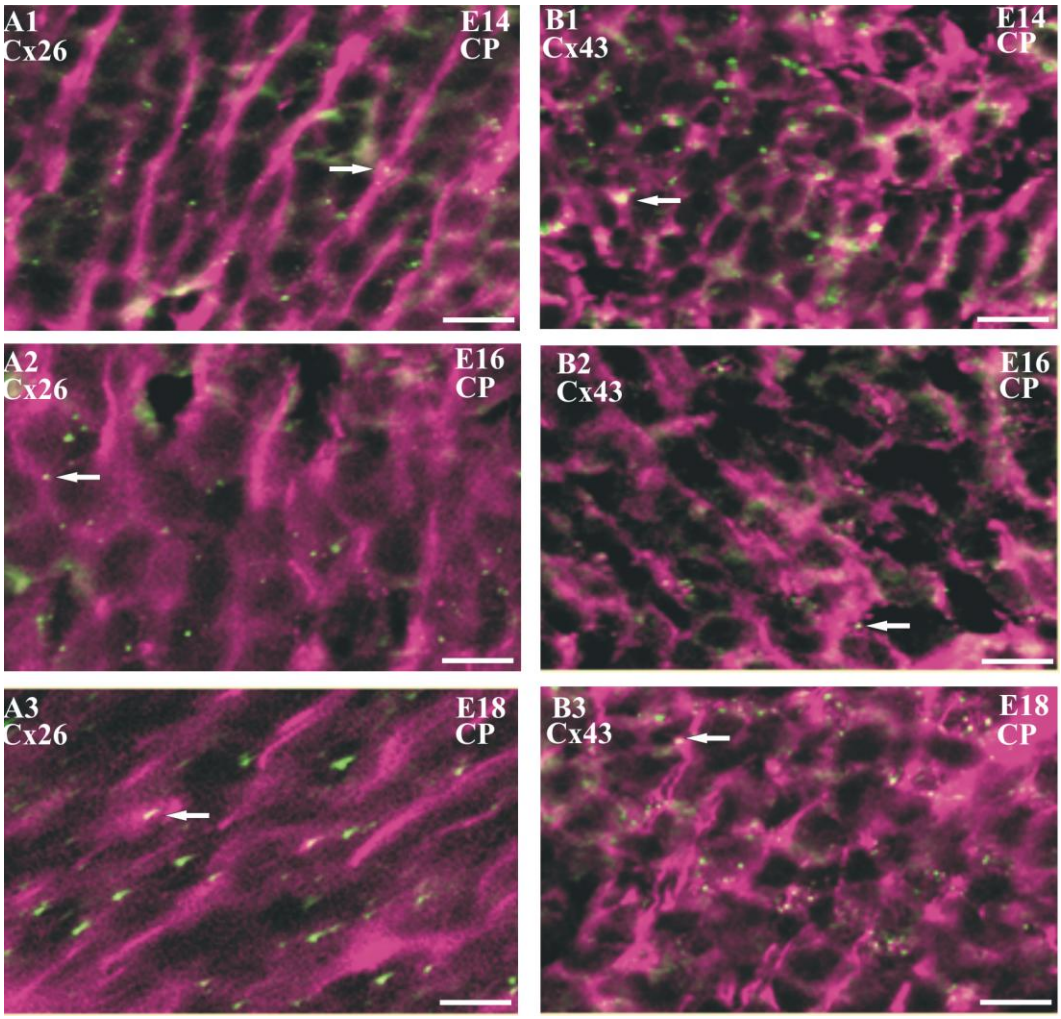
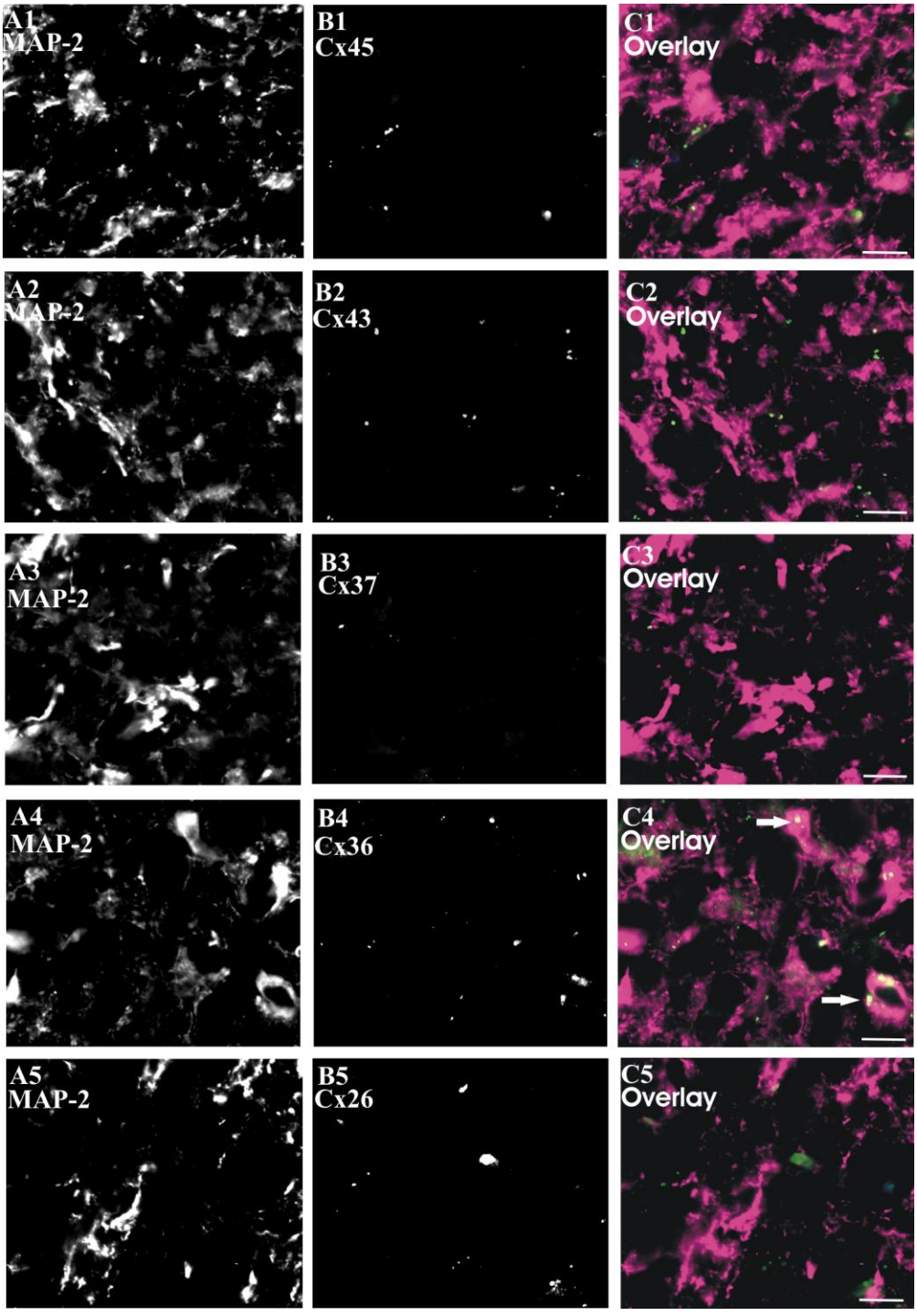


Figure 2.9 Nestin labeling of radial glia in the cortical plate is also immunoreactive for Cx26 and Cx43. Double-immunolabelled confocal microscope images of 10 μm cortical tissue sections labeled with nestin (shown in magenta) and antibodies against Cx26 (left panel: **A1-A3**) and Cx43 (right panel: **B1-B3**) (shown in green). Punctate immunostaining of Cx26 and Cx43 (arrows) in the cortical plate of E14, E16, and E18 mouse neocortex showed their expression associated with nestin-positive radial glia. Scale bars = 5 μm.

Figure 2.10 MAP-2 labeling of neurons in the adult cerebral cortex is immunoreactive for Cx36. Double-immunolabeled confocal microscope images of 10 μm cortical tissue sections from laminae II/III of mature neocortex labeled with MAP-2 (magenta) (left panel: **A1-A5**) and antibodies against various Cxs (green) (middle panel: **B1-B5**). The nuclei of the cells were visualized by DAPI (blue) and are shown in the overlay images (right panel: **C1-C5**). **A4-C4**: Within the laminae II/III of the mature neocortex Cx36 punctate immunostaining showed high association with MAP-2 positive cells (arrows), whereas Cx45 (**A1-C1**), Cx43 (**A2-C2**), Cx37 (**A3-C3**), and Cx26 (**A5-C5**) did not show immunoreactivity with MAP-2 positive neurons. Scale bars = 5 μm.


A1
MAP-2
B1
Cx45
C1
Overlay
A2
MAP-2
B2
Cx43
C2
Overlay
A3
MAP-2
B3
Cx37
C3
Overlay
A4
MAP-2
B4
Cx36
C4
Overlay
A5
MAP-2
B5
Cx26
C5
Overlay

2.4 Discussion

By using RT-PCR, immunoblotting, and immunohistochemistry, we investigated the expression level and distribution pattern of several connexins at three stages of embryonic development of the mouse cerebral cortex. The main findings from this analysis are as follows: 1) Cx26, Cx36, Cx37, Cx43, and Cx45 were highly expressed in the neocortex at E14–E18, while Cx30 and Cx32 were absent and Cx40 was detected at a low level. 2) Cx26 and Cx37 showed an even distribution in all three cortical layers of neocortex, Cx36 and Cx43 were more abundant in the VZ and the CP than in the IZ of all timepoints examined, and Cx45 appeared to increase in expression during development.

2.4.1 Cx26 Expression in Embryonic Mouse Neocortex

In the present study we detected both Cx26 RNA and protein throughout the cerebral cortex of the developing mouse embryo, as measured by RT-PCR and Western blot analysis. Also, our immunofluorescent labelling experiments showed generally even distribution of Cx26 in the VZ, the IZ, and CP during embryonic development, with high immunoreactivity in neurons based on our double immunofluorescent labelling. In contrast, Cx26 showed no association with neurons in the adult mouse neocortex. Many studies have investigated the distribution of Cx26 utilizing a variety of techniques including RT-PCR, Western, and Northern blot analysis as well as immunohistochemistry. However, there exist conflicting results regarding the expression of this Cx. A study conducted by Fillipov et al. (2003) showed that in E16 and postnatal stages of mouse neocortex, Cx26 was expressed only in meninges and not in neurons or astrocytes. This difference may be due to the techniques we used. Even though extreme care was taken when removing meninges during dissection, we could still observe the expression of Cx26 in the neocortex. Our results are in agreement with studies by Dermietzel et al. (1989) and Bittman and LoTurco (1999), demonstrating the presence of Cx26 during embryonic stages and its expression in the VZ of E14–E17 mouse neocortex. In another study by Nadarajah et al. (1997) the expression of Cx26 at E12, E14, E16, and E19 in the rat cerebral cortex was examined and the results revealed that this Cx was present at all timepoints, with a peak at E16, diminished by the end of E19. Here, Cx26 has also been shown to be colocalized with the neuronal marker (MAP 2) and the radial glia marker (nestin) during development, in agreement with previous studies

showing the expression of this Cx in neurons, astrocytes, and radial glia (Nadarajah et al., 1997; Bittman and LoTurco, 1999; Nagy et al., 2001; Bittman et al., 2002; Condorelli et al., 2003). The expression of Cx26 increases during mid to late neurogenesis (around E16), suggesting its participation in important developmental events such as proliferation and migration. Furthermore, our immunofluorescent labeling experiments showed low expression of Cx26 in laminae II/III of adult mouse neocortex with no immunoreactivity in neurons.

2.4.2 Cx30 and Cx32 are Absent From the Embryonic Neocortex

In the present study we were unable to detect Cx30 and Cx32 in the mouse neocortex during embryonic development between E14 and E18. Siu Yi Leung et al. (2001), however, detected Cx32 in cultured midbrain neurons from rat at E14. Differences between the two studies may reflect different animal models and brain tissues since neocortex is known to develop extensively after birth. Previous studies have been able to detect Cx30 in astrocytes and Cx32 in oligodendrocytes and along myelinated fibers only in postnatal and adult brain. These studies suggest a function for Cx30 and Cx32 in the mature neocortex, including their role in gap junctional coupling between astrocytes and oligodendrocytes along myelin fibers to exchange ions and small metabolites (Nadarajah et al., 1997; Nagy et al., 1999, 2003; Nagy and Rash, 2000; Alverez- Meubecin et al., 2000; Rash et al., 2001).

2.4.3 Expression of Cx36 in Embryonic Mouse Neocortex

We were able to detect both Cx36 RNA and protein expression in the embryonic mouse neocortex. Previously, by RT-PCR and Northern blot analysis, Cx36 was found in embryonic mouse forebrain at E9.5–E18.5, with a peak at E15.5 (Gulisano et al., 2000). We have also shown that this Cx is expressed more abundantly in the VZ and the CP than in the IZ at E16 and E18. Furthermore, Cx36 immunoreactivity was seen associated with MAP-2-positive neurons in the CP of cerebral cortex at E18 and laminae II/III of mature neocortex. However, the expression of this Cx was significantly lower in mature neocortex compared to the embryonic cerebral cortex. These findings are in agreement with many previous reports revealing the presence of Cx36 only in neurons of developing and adult rodent and human brain and retina (Sohl et al., 1998; Belluardo et al., 2000; Condorelli et al., 2000; Teubner et al., 2000; Venance et al., 2000; Rash et al., 2001). The presence of Cx36 during embryonic

stages and its expression exclusively in neurons in the VZ and the CP during embryonic development suggests that this Cx is involved in coupling between neuroepithelial cells in the VZ during neurogenesis and between migrating neuron in the CP during corticogenesis (Gulisano et al., 2000).

2.4.4 Expression of Cx37 in the Embryonic Mouse Neocortex

In the present study we detected both Cx37 RNA and protein at E14, E16, and E18 of embryonic mouse neocortex. This Cx also showed an even distribution in the cerebral cortical layers at E14–E18, with low immunoreactivity localized to MAP-2-positive neurons in the CP of E18 mouse neocortex. This Cx was expressed at low levels in mature neocortex, and showed no association with MAP-2-positive neurons. This finding is confirmed by previous studies showing the expression of Cx37 in the developing and adult rodent brain. While some investigators have shown a meningeal and endothelial localization, others found this Cx to be expressed in spinal cord motoneurons, suggesting their involvement in synaptic connections between motoneurons during embryonic stages (Willecke et al., 1991; Traub et al., 1998; Chang et al., 1999; Condorelli et al., 2003).

2.4.5 Cx40 Expression in the Embryonic Mouse Neocortex

We were able to detect Cx40 RNA at a low level in the embryonic mouse neocortex at E14. A previous study by Chang et al. (1999) showed the expression of this Cx in embryonic rat spinal cord motoneurons at the mRNA and protein level; thus, Cx40 may be expressed in spinal cord neurons and not in cortical neurons. Some investigators also showed this Cx in hippocampal progenitor cells while others found Cx40 in astrocytes, in the meninges, large blood vessels, or in the retina of postnatal and adult rodent brain (Dermietzel, 1996; Rozental et al., 1998; Guldenagel et al., 2000; Condorelli et al., 2003; Matesic et al., 2003).

2.4.6 Expression of Cx43 in the Embryonic Mouse Neocortex

In the present study we detected both Cx43 RNA and protein in the embryonic mouse neocortex from E14–E18. Our immunolabelling experiments show an even distribution of Cx43 at E14 and a gradient distribution pattern in the VZ and the CP at E16

and E18. This finding is supported by previous studies (Nadarajah et al., 1997; Fushiki et al., 2003) that show more Cx43 in the VZ and the CP of E16 mouse neocortex. Our results reveal an apparent association of Cx43 with some MAP-2-positive cells, as well as expression in radial glial cells in the cortical plate of mouse at E14, E16, and E18. This Cx was expressed in the laminae II/III of the adult mouse neocortex and showed no association with MAP-2-positive neurons. This finding is confirmed by previous studies that used a variety of techniques including immunohistochemistry, freeze fracture replica, electron microscopy, and Southern and Western blot analysis to show the spatial and temporal expression of Cx43 in developing and adult brain. The majority of these investigations used rats as animal models. The expression of Cx43 has been reported in the radial glia contacting neurons migrating toward the CP, migratory neural crest cells, astrocytes, cortical neurons, spinal cord motoneurons, and embryonic rat midbrain floor (Nadarajah et al., 1996; Naus et al., 1997; Bittman and LoTurco, 1999; Chang et al., 1999; Nadarajah and Parnavelas, 1999; Dermietzel et al., 2000; Rash et al., 2001; Siu Yi Leung et al., 2001; Leung et al., 2002; Cheng et al., 2004). Based on our findings on the distribution of Cx43 at E14–E18 and the aforementioned studies, it is suggested that Cx43 is involved in important developmental functions including cell cycle, neurogenesis, and neuronal migration. There is increasing evidence indicating the differential changes in the number of cells expressing Cx43 through the cell cycle during early and late neurogenesis (Nadarajah et al., 1998; Bittman and LoTurco, 1999). At the onset of neurogenesis, all neural precursors in the VZ in the S, G1, and G2 phases are highly coupled by Cx43 and then uncoupled in M. However, during late neurogenesis (E17) the number of cycling neocortical cells expressing Cx43 decreases from the S phase to G1 phase of the cell cycle. This decrease correlates with cell uncoupling at this stage of development when cells become postmitotic in the VZ and start to differentiate or migrate (Bittman and LoTurco, 1999). Thus, participation of Cx43 in regulating the cell cycle may be a way of controlling neurogenesis during brain development.

Furthermore, one possible elucidation regarding the involvement of Cx43 in neuronal migration might be that the spread of intercellular Ca^{2+} waves are propagated via gap junctions. Ca^{2+} waves provide a mechanism for coordination of activities like cell migration and proliferation (Sanderson et al., 1996). This process has been demonstrated in a number of cell types, including cortical radial glia, astrocytes, and striatal precursors

(Dermietzel et al., 2000; Scemes et al., 2003; Weissman et al., 2004). Gap junctions are also permeable to ATP and they release this signaling molecule into the extracellular space (Guthrie et al., 1999), which in turn stimulates the metabotropic purinergic receptors (P2YRs) on the adjacent cells. P2Yrs are coupled to phospholipase C production, which leads to increased production of IP3 and subsequent Ca^{2+} release from intracellular stores (Duval et al., 2002). Recent studies have shown the release of ATP through hemichannels, suggesting that Cx43 might also be involved in Ca^{2+} wave propagation (Belliveau et al., 2006). Furthermore, the expression of P2Y1 receptors appears to be tied to Cxs, as a dramatic reduction of these receptors is observed in the neural progenitors of Cx43 null mice (Scemes et al., 2003). Scemes et al. (2003) provided direct evidence for the participation of P2Y1 receptors in the migration of neural progenitors, and have shown that Cx43 null cells migrated a shorter distance per unit time than wild-type cells in vitro. Since the level of P2Y1 receptors is dependent on Cx43 expression, it is very possible that these two molecules are part of the same pathway controlling neuronal migration. Interestingly, in support of a role for Cx43 in neuronal migration in vivo, Fushiki et al. (2003) reported disruption of migration in Cx43 knockout mice.

2.4.7 Cx45 Expression in the Embryonic Mouse Neocortex

In the present study we were able to detect Cx45 RNA and protein in the mouse neocortex at E14–E18. Using immunohistochemistry, we suggest that the distribution pattern of this Cx is increased from E14 to E18. Indeed, based on previous reports higher expression of Cx45 in E18 of embryonic mouse neocortex might be due to developing oligodendrocytes, which are known to express high levels of Cx45 (Dermietzel et al., 1997; Kunzelmann et al., 1997).

Our double immunofluorescent staining experiments showed Cx45 immunoreactivity with very few MAP-2-positive neurons as well as nonneuronal cells. This is confirmed by previous results from Condorelli et al. (2003), in which Cx45 was found at high levels in both neuronal and nonneuronal clusters in the developing cerebral cortex. Furthermore, our immunofluorescent labelling experiments on mature neocortex showed that Cx45 was expressed at a lower level compared to the embryonic stage, and this Cx was not associated with MAP-2 neurons in laminae II/III.

Maxeiner et al. (2003), using a Cx45 LacZ-reporter mouse and immunoblot analysis experiments, found high protein levels of Cx45 in the cerebral cortex at E18.5 to postnatal day 8 in almost all brain regions; however, the expression of this Cx was decreased during the second postnatal week and then became restricted in adult brain to the thalamus. Higher expression of this Cx during embryonic stages compared to the postnatal period suggests that this Cx might participate in corticogenesis. The expression of this Cx showed no immunoreactivity to neurons in adult mouse neocortex.

In conclusion, we detected the expression of Cx26, Cx36, Cx37, Cx43, and Cx45 at the RNA and protein level in the developing neocortex. We have also shown a Cx-specific distribution pattern in the cortical layers during embryonic development. Furthermore, our double immunofluorescent labelling experiments using MAP-2 antibody revealed that Cx26 and Cx36 are highly immunoreactive with neurons located in the CP at E18, whereas only Cx36 is localized to MAP-2-positive cells in the mature neocortex. Similar experiments with nestin antibody showed that Cx26 and Cx43 are highly associated with radial glia located in the CP at all timepoints examined (E14, E16, and E18).

At the present time, there has not been a study on the expressions of all the Cxs investigated in this study on developing mouse neocortex. Thus, this analysis of basal Cx expression level has a potential to serve as a foundation for studies examining the effect of Cx deletion in transgenic mice. Indeed, it will be challenging to clarify which specific Cx is vital for the development of neocortex and migration of neurons.

2.5 References

Aicardia J (1994) The place of neuronal migration abnormalities in child neurology. Can J Neurol Sci 21:185-193.

Aizawa H, Kawasakki H, Murofushi H, Kotani S, Suzuki K, Sakai, H (1989) A common amino acid sequence in 190-KDa microtubule-associated protein and Tau for the promotion of microtubule assembly. J Biol Chem 10:5885-5890.

Alvarez-Maubecin V, Garcia-Hernandez F, Williams JT, Van Bocks-Taele EJ (2000) Functional coupling between neurons and glia. J Neurosci 20:4091-4098.

Anthony TE, Klein C, Fishell G, Heintz N (2004) Radial glia serve as neuronal progenitors in all regions of the central nervous system. Neuron 41:881-890.

Belliveau DJ, Bani-Yaghoub M, McGirr B, Naus CC, Rushlaw WJ (2006) Enhanced neurite outgrowth in PC12 cells mediated by connexin hemichannels and ATP. J Biol Chem 281:20920-20931.

Belluardo N, Mudo G, Trovato-Salinaro A, LeGurun S, Charollais A, Serre-Beinier V, Amato G, Haeflinger JA, Meda P, Condorelli PF (2000) Expression of connexin36 in the adult and developing rat brain. Brain Res 865:121-138.

Bennett MV, Zukin RS (2004) Electrical coupling and neuronal synchronization in the Mammalian brain. Neuron 41:495-511.

Bittman KS, LoTurco JJ (1999) Differential regulation of connexin26 and 43 in murine neocortical precursors. Cereb Cortex 9:188-195.

Bittman KS, Becker DL, Cicirata F, Parnavelas JG. 2002. Connexin expression in homotypic and heterotypic cell coupling in the developing cerebral cortex. J Comp Neurol 443:201–212.

Bruzzone R, Haefliger JA, Gimlich RA, Paul DL (1993) Connexin40, a component of gap junctions in vascular endothelium, is restricted in its ability to interact with other connexins. Mol Biol Cell 4:7-20.

Cepeda C, Walsh JP, Peacock W, Buchwald NA, Leuine MS (1993) Dyecoupling in human neocortical tissue resected from children with intractable epilepsy. Cereb Cortex 3:95-107.

Chang Q, Gonzales M, Pinter MJ, Balice-Gordon RJ (1999) Gap junctional coupling and patterns of connexin expression among neonatal rat lumbar spinal motor neurons. J Neurosci 19:10813-10828.

Cheng A, Tang H, Cai J, Zhu M, Zhang X, Rao M, Mattson MP (2004) Gap junctional communication is required to maintain mouse cortical neural progenitor cells in a proliferative state. Dev Biol 272:203-216.

Cohen-Salmon M, Ott T, Michel V, Hardelin JP, Perfettini I, Eybalin M, Wu T, Marcus DC, Wangemann P, Willecke K, Petit C (2002) Targeted ablation of connexin26 in the inner ear epithelial gap junction network causes hearing impairment and cell death. Curr Biol 12:1106-1111.

Condorelli DF, Belluardo N, Trovato-Salinaro N, Mudo G (2000) Expression of Cx36 in mammalian neurons. Brain Res Brain Res Rev 32:72-85.

Condorelli DF, Trovato-Salinaro A, Mudo G, Mione MB, Belluardo N (2003) Cellular expression of connexins in the rat brain: neuronal localization, effects of kainate-induced seizures and expression in apoptotic neuronal cells. Eur J Neurosci 18:1807-1827.

Connors BW, Long MA (2004) Electrical synapses in the mammalian brain. Annu Rev Neurosci 27:393-418.

Connors BW, Benardo LS, Prince DA (1983) Coupling between neurons of the developing rat neocortex. J Neurosci 3:773-782.

Dermietzel R (1996) Molecular diversity and plasticity of gap junctions in the nervous system. In: Dermietzel R, Spray D, editors. Gap junctions in the nervous system. New York: Chapman and Hall 13-38.

Dermietzel R, Traub O, Hwang T, Beyer E, Bennett M, Spray D (1989) Differential expression of three gap junction proteins in developing and mature brain tissues. Proc Natl Acad Sci U S A 86:10148-10152.

Dermietzel R, Hertberg EL, Kessler JA, Spray DC (1991) Gap junctions between cultured astrocytes: immunocytochemical, molecular, and electrophysiological analysis. J Neurosci 11:1421-1432.

Dermietzel R, Farooq M, Kessler JA, Althaus H, Hertzberg EL, Spray DC (1997) Oligodendrocytes express gap junction proteins connexin32 and connexin45. Glia 20:101-114.

Dermietzel R, Gao Y, Scemes E, Vieira D, Urban M, Kremer M, Bennett MV, Spray DC. (2000) Connexin43 null mice reveal that astrocytes express multiple connexins. Brain Res Brain Res Rev 32:45-56.

Duval N, Gomes D, Calaora V, Calabrese A, Meda P, Bruzzone R (2002) Cell coupling and Cx43 expression in embryonic mouse neural progenitor cells. J Cell Sci 115:3241-3251.

Earley S, Resta TC, Walker BR (2004) Disruption of smooth muscle gap junctions attenuates myogenic vasoconstriction of mesenteric resistance arteries. Am J Physiol Heart Circ Physiol 287:2677-2686.

Fillipov MA, Hormuzdi SG, Fuchs EC, Monyer H (2003) A reporter allele for investigating connexin26 gene expression in the mouse brain. Eur J Neurosci 18:3183-3192.

Fushiki S, Prezz Velazquez JL, Zhang L, Bechberger JF, Carlen PL, Naus CC (2003) Changes in neuronal migration in neocortex of connexin43 null mutant mice. J Neuropathol Exp Neurol 62:304-314.

Giaume C, Marin P, Cordier J, Glowinski J, Premont J (1991) Adrenergic regulation of intercellular communication between cultured striatal astrocytes from the mouse. Proc Natl Acad Sci U S A 88:5577-5581.

Gotz M, Sommer L (2005) Cortical development: the art of generating cell diversity. Development 132:3327-3332.

Guerrini R, Filippi T (2005) Neuronal migration disorders, genetics, and epileptogenesis. J Child Neurol 4:287-299.

Guldenagel M, Sohl G, Plum A, Traub O, Teubner B, Weiler R, Willecke K (2000) Expression patterns of connexin genes in mouse retina. J Comp Neurol 425:193-201.

Gulisano M, Parenti R, Spinella F, Cicirata F (2000) Cx36 is dynamically expressed during early development of mouse brain and nervous system. Neuroreport 11:3823-3828.

Guthrie PB, Knappenberger J, Bennett MV, Charles AC, Kater SB (1999) ATP release from astrocytes mediates glial calcium waves. J Neurosci 2:520-528.

Hattori M, Adachi H, Tsujimoto M, Arai H, Inoue K (1994) Miller-Dieker lissencephaly gene encodes a subunit of brain platelet-activating factor acetylhydrolase. Nature 370:216-218.

Haubensak W, Attardo A, Denk W, Huttner WB (2004) Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. Proc Natl Acad Sci U S A 101:3196-3201.

Hill CE, Rummery N, Hickery H, Sandow SL (2002) Heterogeneity in the distribution of vascular gap junctions and connexins: implications for function. Clin Exp Pharmacol Physiol 7:620-625.

Hockfield S, McKay RD (1985) Identification of major cell classes in the developing mammalian nervous system. J Neurosci 12:3310-3328.

Hofer A, Dermietzel R (1998) Visualization and functional blocking of gap junction hemichannels (connexons) with antibodies against external loop domains in astrocytes. Glia 24:141-154.

Hossain MZ, Murphy LJ, Hertzberg EL, Nagy JI (1994) Phosphorylated forms of connexin43 predominate in rat brain: demonstration by rapid inactivation of brain metabolism. J Neurochem 62:2394-2403.

Huber G, Matus A (1984) Differences in the cellular distribution of two microtubule-associated proteins MAP1 and MAP2 in rat brain. J Neurosci 4:151-160.

Isakson BE, Damon DN, Day KH, Liao Y, Duling BR (2006) Connexin40 and connexin43 in mouse aortic endothelium: evidence for coordinated regulation. Am J Physiol Heart Circ Physiol 290:1199-1205.

Izant JG, McIntosh R (1980) Microtubule-associated proteins: a monoclonal antibody to MAP2 binds to differentiated neurons. Proc Natl Acad Sci U S A 77:4741-4745.

Kumar M, Gilula NB (1996) The gap junction communication channel. Cell 84:381-388.

Kunzelmann P, Blumcke I, Traub O, Dermietzel R, Willecke K (1997) Coexpression of connexin45 and -32 in oligodendrocytes of rat brain. J Neurocytol 26:17-22.

Lendahl U, Zimmerman LB, McKay RD (1990) CNS stem cells express a new class of intermediate filament protein. Cell 60:585-595.

Leung DS, Unsicker K, Reuss B (2002) Expression and developmental regulation of gap junction connexins cx26, cx32, cx43 and cx45 in the rat midbrain-floor. Int J Dev Neurosci 20:63-75.

LoTurco JJ, Kriegstein AR (1991) Clusters of coupled neuroblasts in embryonic neocortex. Science 252:563-566.

Marin O, Rubenstein JL (2001) A long remarkable journey: tangential migration in the telencephalon. Nat Rev Neurosci 2:780-790.

Matesic D, Tillen T, Sitaramayya A (2003) Connexin 40 expression in bovine and rat retinas. Cell Biol Int 27:89-99.

Maxeiner S, Kruger O, Schilling K, Traub O, Urschel S, Willecke K (2003) Spatiotemporal transcription of connexin45 during brain development results in neuronal expression in adult mice. Neuroscience 119:689-700.

Nadarajah B, Parnavelas JG (1999) Gap junction-mediated communication in the developing and adult cerebral cortex. Novartis Found Symp 219:157-170.

Nadarajah B, Thomaidou D, Evans WH, Parnavelas JG (1996) Gap junctions in the adult cerebral cortex: regional differences in their distribution and cellular expression of connexins. J Comp Neurol 376:326-342.

Nadarajah B, Jones AM, Evans WH, Parnavelas JG (1997) Differential expression of connexins during neocortical development and neuronal circuit formation. J Neurosci 17:3096-3111.

Nadarajah B, Makarenkova H, Becker DL, Evans WH, Parnavelas JG (1998) Basic FGF increases communication between cells of the developing neocortex. J Neurosci 19:7881-7890.

Nadarajah B, Brunstrom JE, Grutzendler J, Wong RO, Pearlman AL (2001) Two modes of radial migration in early development of the cerebral cortex. Nat Neurosci 2:143-150.

Nagy JI, Rash JE (2000) Connexins and gap junctions of astrocytes and oligodendrocytes in the CNS. Brain Res Brain Res Rev 32:29-44.

Nagy JI, Patel D, Ochalski PA, Stelmack GL (1999) Connexin30 in rodent, cat and human brain: selective expression in gray matter astrocytes, co-localization with connexin43 at gap junctions and late developmental appearance. Neuroscience 88:447-468.

Nagy JI, Li X, Rempel J, Stelmack G, Patel D, Staines WA, Yasumura T, Rash JE (2001) Connexin26 in adult rodent central nervous system: demonstration at astrocytic gap junctions and colocalization with connexin30 and connexin43. J Comp Neurol 441:302-323.

Nagy JI, Ionescu AV, Lynn BD, Rash JE (2003) Connexin29 and connexin32 at oligodendrocyte and astrocyte gap junctions and in myelin of the mouse central nervous system. J Comp Neurol 464:356-370.

Nagy JI, Dudek FE, Rash E (2004) Update on connexins and gap junctions in neurons and glia in the mammalian nervous system. Brain Res Brain Res Rev 47:191-215.

Naus CC, Bechberger JF, Zhang Y, Venance L, Yamasaki H, Juneja SC, Kidder GM, Giaume C (1997) Altered gap junctional communication, intercellular signaling, and growth in cultured astrocytes deficient in connexin43. J Neurosci Res 49:528-540.

Parnavelas JG (2002) The origin of the cortical neurons. Braz J Med Biol Res 12:1423-1429.

Peinado A. 2001. Immature neocortical neurons exist as extensive syncitial networks linked by dendrodendritic electrical connections. J Neurophysiol 85:620–629.

Peinado A, Yuste R, Ratz LC (1993) Extensive dye coupling between rat neocortical neurons during the period of circuit formation. Neuron 10:103-114.

Rakic P (1972) Mode of cell migration to the superficial layers of fetal monkey neocortex. J Comp Neurol 145:61-84.

Rash JE, Staines WA, Yasumura T, Patel D, Furman CS, Stelmack GL, Nagy JI (2000) Immunogold evidence that neuronal gap junctions in adult rat brain and spinal cord contain connexin-36 but not connexin-32 or connexin-43. Proc Natl Acad Sci U S A 97:7573-7578.

Rash JE, Yasumura T, Dudek FE, Nagy JI (2001) Cell-specific expression of connexins and evidence restricted gap junctional coupling between glial cells and between neurons. J Neurosci 21:1983-2000.

Rozental R, Morales M, Mehler MF, Urban M, Kremer M, Dermietzel R, Kessler JA, Spray DC (1998) Changes in the properties of gap junctions during neuronal differentiation of hippocampal progenitor cells. J Neurosci 18:1753-1762.

Rozental R, Giaume C, Spray DC (2000) Gap junctions in the nervous system. Brain Res Rev 32:11-15.

Sanderson MJ (1996) Intercellular waves of communication. News Physiol Sci 11:262-269.

Scemes E, Duval N, Meda P (2003) Reduced expression of P2Y1 receptors in connexin43-null mice alters calcium signaling and migration of neural progenitor cells. J Neurosci 23:11444-11452.

Schubert T, Degen J, Willecke K, Hormuzdi SG, Monyer H, Weiler R (2005) Connexin36 mediates gap junctional coupling of alpha-ganglion cells in mouse retina. J Comp Neurol 485:191-201.

Simon AM, Goodenough DA (1998) Diverse functions of vertebrate gap junctions. Trends Cell Biol 8:477-483.

Simon AM, McWhorter AR (2003) Decreased intercellular dye-transfer and downregulation of non-ablated connexins in aortic endothelium deficient in connexin37 or connexin40. J Cell Sci 116:2223-2236.

Siu Yi Leung D, Unsicker K, Reuss B (2001) Gap junctions modulate survival-promoting effects of fibroblast growth factor-2 on cultured midbrain dopaminergic neurons. Mol Cell Neurosci 18:44-55.

Siu Yi Leung D, Unsicker K, Bernhard R (2002) Expressional and developmental regulation of gap junction connexins Cx26, Cx32, Cx43, and Cx45 in the rat midbrain-floor. Int J Dev Neurosci 20:63-75.

Sohl G, Degen J, Teubner B, Willecke K (1998) The murine gap junction gene connexin36 is highly expressed in mouse retina and regulated during brain development. FEBS Lett 428:27-31.

Sohl G, Theis M, Hallas G, Brambach C, Dahl E, Kidder G, Willecke K (2001) A new alternatively spliced transcript of the mouse connexin32 gene is expressed in embryonic stem cells, oocytes, and liver. Exp Cell Res 266:177-186.

Sotelo C, Korn H (1978) Morphological correlates of electrical and other interactions through low-resistance pathways between neurons of the vertebrate central nervous system. Int Rev Cytol 55:67-107.

Takahashi T, Nowkowski RS, Kaviness VS Jr. (1996) Interkinetic and migratory behavior of a cohort of neocortical neurons arising in the early embryonic murine cerebral wall. J Neurosci 16:5762-5776.

Teubner B, Degen J, Sohl G, Guldenagel M, Bukauskas FF, Trexler EB, Verselis VK, De Zeeuw CI, Lee CG, Kozak CA, Petrasch-Parwez E, Dermietzel R, Willecke K (2000) Functional expression of the murine connexin36 gene coding for a neuron-specific gap junctional protein. J Member Biol 176:249-262.

Theis M, Mas C, Doring B, Degen J, Brink C, Caille D, Charollais A, Kruger O, Plum A, Nepote V, Herrera P, Meda P, Willecke K (2004) Replacement by a lacZ reporter gene assigns mouse connexin36, 45 and 43 to distinct cell types in pancreatic islets. Exp Cell Res 294:18-29.

Theis M, Sohl G, Eiberger J, Willecke K (2005) Emerging complexities in identity and function of glial connexins. Trends Neurosci 28:188-195.

Traub O, Hertlein B, Kasper M, Eckert R, Krisciukaitis A, Hulser D, Willecke K (1998) Characterization of the gap junction protein connexin37 in murine endothelium, respiratory epithelium, and after transfection in human HeLa cells. Eur J Cell Biol 77:313-322.

Venance L, Rozov A, Blatow M, Burnashev N, Feldmeyer D, Monyer H (2000) Connexin expression in electrically coupled postnatal rat brain neurons. Proc Natl Acad Sci U S A 97:10260-10265.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by genometric averaging of multiple internal control genes. Genome Biol 18 RESEARCH0034.

Weissman TA, Riquelme PA, Ivic L, Flint AC, Kriegstein AR (2004) Calcium waves propagate through radial glial cells and modulate proliferation in the developing neocortex. Neuron 5:647-661.

Willecke K, Heynkes R, Dahl E, Stutenkemper R, Hennemann H, Jungbluth S, Suchyna T, Nicholson BJ (1991) Mouse connexin37: cloning and functional expression of a gap junction gene highly expressed in lung. J Cell Biol 114:1049-1057.

Willecke K, Eiberger J, Degen J, Eckardt D, Romualdi A, Guldenagel M, Deutsch U, Sohl G. (2002) Structural and functional diversity of connexin genes in the mouse and human genome. Biol Chem 383:725-737.

Yang CW, Lim SW, Han KW, Ahn HJ, Park JH, Kim YH, Kirsh M, Cha JH, Kim YS, Kim J, Bang BK (2001) Upregulation of ciliary neurotrophic factor (CNTF) and CNTF receptor or in rat kidney with ischemia-reperfusion injury. J Am Soc Nephrol 12:749-757.

Yuste R, Peinado A, Katz LC (1992) Neuronal domains in developing neocortex. Science 257:665-669

Chapter 3

Involvement of the Cytoplasmic C-Terminal Domain of Connexin43 in Neuronal Migration

---

A version of this chapter has been previously published; Cima Cina, Karen Maass, Martin Theis, Klaus Willecke, John F. Bechberger, and Christian C. Naus (2009) Involvement of the Cytoplasmic C-Terminal domain of Connexin43 in Neuronal Migration. *J Neuronsci*. **29**: 2009-2021.

3.1 Introduction

During embryonic development, radial glial cells divide and give rise to astrocytes and neurons in the cerebral cortex (Noctor et al., 2001; Malatesta et al., 2003; Casper and McCarthy, 2006; Noctor et al., 2008). Radial glia are bipolar cells originating in the ventricular zone (VZ) of the dorsal telencephalon, with one process extending to the ventricular surface, whereas the other process projects outward to the pial surface. As the neocortex forms, the radial glia undergo divisions that produce neurons and build a dynamic scaffold that facilitates migration from the VZ to their location in the cortical plate (CP) (Anthony et al., 2004; Götz and Sommer, 2005). Within the developing brain, neural progenitor cells are extensively coupled by gap junctions (LoTurco and Kriegstein, 1991; Bittman et al, 1997). Gap junctions are intercellular membrane channels that provide cytoplasmic continuity between adjacent cells and permit the exchange of molecules <2 kDa in size (Simon and Goodenough, 1998). Gap junction channels are formed by docking of two connexons in contacting plasma membranes of adjacent cells. The protein subunits of these hemichannels are called connexins (Cx) (Goodenough and Paul, 2003). Two gap junction proteins, Cx43 and Cx26, are widely expressed at the points of contact between radial glia and migrating neurons (Nadarajah et al., 1997; Cina et al., 2007), suggesting their role in neuronal migration. Indeed, a recent study showed that Cx43 and Cx26 are involved in neuronal migration by mediating adhesion and not by formation of intercellular gap junction channels (Elias et al., 2007).

The targeted deletion of *Cx* genes in mice (accomplished through homologous recombination) has provided preliminary insights into the role of gap junctions in neuronal migration. For instance, we have shown previously that embryonic mice lacking Cx43 expression phenotypically present significant accumulation of bromodeoxyuridine-positive ($BrdU^+$) pulse-labeled cells in the intermediate zone (IZ), consistent with a disruption in neuronal migration, whereas these cells establish themselves in the CP of wild-type littermates (Fushiki et al., 2003). Unfortunately, Cx43 is widely expressed both temporally and spatially, making it difficult to attribute the migrational phenotype to radial glia alone when presented in a complete knock-out. To further complicate the situation, complete knock-out of Cx43 is neonatal lethal attributable to abnormalities in heart development, preventing a comprehensive study of the long-term effects of such a deletion. To circumvent

the lethality associated with total Cx43 knock-out, we have made conditional knock-out (*Cx43cKO*) mice produced by crossing *Cx43* $^{fl/fl}$ mice (Theis et al., 2001), with *nestin–Cre* mice, driving the expression of Cre recombinase in radial glia (Bérube´ et al., 2005).

In addition to *Cx43cKO* mice, *Cx43*^{K258stop} mice, in which the last 125 amino acid residues of the cytoplasmic C-terminal domain of Cx43 are lacking (Maass et al., 2004), have been used in this study to examine the significance of Cx43 and its C terminus in neuronal migration.

Our data demonstrate that conditional knock-out of Cx43 in radial glia disrupts neuronal migration, but this defect is rescued by reconstitution of full-length Cx43, but not by C-terminal truncated Cx43, into the VZ of the neocortex.

3.2 Materials and Methods

3.2.1 Animals

The production of the Cx43 null mutant (Reaume et al., 1995), *nestin–Cre* (Bérube´ et al., 2005), floxed Cx43 (Theis et al., 2001), and $Cx43^{K258stop}$ (Maass et al., 2004) transgenic mice have been reported previously. *Nestin–Cre* mice were crossed with floxed Cx43 mice, maintained in an animal facility with a 12 h light/dark cycle, and were provided food and water *ad libitum*. The animals were maintained according to Canadian Council on Animal Care guidelines for the care and use of laboratory mice at the University of British Columbia. After mating of adult mice, the day on which a vaginal plug was identified was considered embryonic day 0 (E0).

3.2.2 Genotype Analysis by PCR

For detection of the *Cx43* floxed ($Cx43^{fl}$) allele and the *Cx43* wild type ($Cx43^{+}$) allele, forward primers (5'-TCA TGC CCG GCA CAA GTG AGA C-3') and reverse primers (5'-TCA CCCCAAGCTGACTCAACCG-3') were applied to generate a product of 1 kb floxed amplicon and a 900 bp wild-type amplicon (Theis et al., 2001). To detect the deleted *Cx43* ($Cx43^{-}$) allele, forward primers (5'-AT TTT GCC GCC GCC TAG CTA TCC C-3') and reverse primers (5'-GCT TGC CGA ATA TCA TGG TGG A-3') were used to generate a product of 1 kb for the deleted amplicon and 500 bp for the wild-type amplicon (Perez Velazquez et al., 1996). For the detection of the *nestin–Cre* transgene, forward primers (5'-TGA CCA GAG TCA TCC TTA GCG-3') and reverse primers (5'-AATGCTTCTGTCCGTTTGCC-3') were applied, generating a 300–500 bp product (Tronche et al., 1999). To detect the C-terminally truncated *Cx43* allele, forward primers (5'-GCA TCC TCT TCA AGT CTG TCT TCG-3') and reverse primers (5'-CAA AACACCCCCCAAGGAACCTAG-3') were used to generate of 851 bp amplicon for the *Cx43* allele and 452 bp amplicon for the $Cx43^{K258stop}$ allele (Maass et al., 2004).

3.2.3 Immunohistochemistry

Brains obtained from embryonic day 14 and 18 and postnatal day 16 (P16) (the birth date is defined as P0) mice were fixed by immersion in 4% paraformaldehyde (in PBS, pH 7.4) overnight at 4°C. The fixed brains were cryoprotected in 30% sucrose, mounted in OCT (Tissue Tek), and cut into 10μm coronal sections using a cryostat. Cut brain sections were mounted on coverslips and blocked for nonspecific antibody binding with IgG blocking solution (M.O.M. kit; Vector Laboratories), and embryonic sections were subsequently incubated with polyclonal antibodies for wild-type Cx43 (Nadarajah et al., 1997) (1:200; epitope spanning amino acids 363–382 located at the C-terminal region of Cx43; Sigma-Aldrich) or C-terminally truncated Cx43 (1:50; epitope spanning amino acids 120–140; Abgent), at 4°C overnight. Selected samples were also double labelled with antibodies to the neuronal marker microtubule associated protein- 2 (MAP-2) (1:400; Sigma-Aldrich) (Huber and Matus, 1984), nestin (a radial glia marker) (1:400; BD Biosciences) (Hockfield and McKay, 1985), green fluorescent protein (GFP) (1:800; Millipore Bioscience Research Reagents) (Paquin et al., 2005), or β-galactosidase (β-gal) (1:200; Biogenesis) (Mizutani and Saito, 2005). Antibodies used on P16 tissue sections were specific for neuronal-specific nuclear protein (NeuN) (a neuronal marker) (1:200; Millipore Bioscience Research Reagents), Ki67 (a proliferation marker) (1:1000; Vector Laboratories), and myelin basic protein (MBP) (1:250; Millipore Bioscience Research Reagents) (Groome et al., 1986). The VZ, the IZ, and the CP of embryonic cerebral cortex as well as the subventricular zone (SVZ), the white matter (WM), and the cortex of postnatal forebrain, were processed for imaging. Specificity of the primary antibodies was assessed by omitting them from the labeling protocol on selected slices. The specificity of antibodies recognizing C-terminally truncated Cx43 was confirmed by conducting immunostaining on brain tissue sections of Cx43 null mice. Slices were then incubated in Alexa Fluor-tagged goat antimouse, goat anti-rabbit, and goat anti rat IgG antibodies (Invitrogen) for 1 h and mounted in ProLong Gold containing 4',6'- diamidino-2- phenylindole (DAPI) (Invitrogen). Labelled sections were examined and analyzed using a Zeiss Axioskop2 epifluorescent microscope and Axio- Vision 4.2 software (Carl Zeiss). To generate the final figures, all images were stored as TIFF files and processed in Adobe Photoshop 7.0 (Adobe Systems).

3.2.4 5-Bromo-4-chloro-3-indoly-β-galactoside Staining

For 5-bromo-4-chloro-3-indoly-β-galactoside (X-Gal) staining, sections were processed as described previously (Akagi et al., 1997), and embryos were X-Gal stained using established procedures (Brenner et al., 1994; Hogan et al., 1994).

3.2.5 Protein Isolation and Western Blot Analysis

Western blot analysis on embryonic brain tissue was conducted as described previously (Cina et al., 2007). Briefly, protein was isolated from cerebral cortices of E18 mice using radioimmune precipitation lysis buffer (50 mM Tris-HCl, pH 8.0, and 1% IGEPAL) supplemented with Mini Complete protease inhibitors (Roche Applied Science) and phosphatase inhibitors (Sigma). Protein concentrations were determined using the BCA protein quantification kit (Pierce). Protein samples (30 μg) were boiled for 2 min in SDS sample buffer, pH 8.0, and separated on a 12% polyacrylamide gel in parallel with molecular weight markers (Bio-Rad). Subsequently, the electrophoresed proteins were transferred onto a nitrocellulose membrane (Bio-Rad) at 100 V for 1 h. The blots were then blocked with 5% dry milk in PBS (pH 7.4, with 1% Tween 20) for 1 h and subsequently incubated with an antibody against Cx43 at 4°C overnight. After rinsing with PBS, the blots were incubated in horseradish peroxidase-tagged secondary antibody (Cedarlane Laboratories) for 1 h at room temperature, followed by incubation in SuperSignal chemiluminescent substrate (Pierce). The labeled blots were then exposed to Kodak X-Omat x-ray film to visualize antibody binding. To ensure equal loading of protein samples, the blots were stripped of their Cx43 antibody and reprobed for glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) (Cedarlane Laboratories).

3.2.6 Cortical Layer Thickness

Cortical layer thickness was measured on 10 μm coronal sections of at least four of E18 or P16 control and *Cx43cKO* littermates. Sections were stained with DAPI and NeuN, and the outline of each zone was drawn. Pictures were analyzed in AxioVision 4.2 software. Mean values of three measurements per section were used for calculating statistical significance.

3.2.7 BrdU labelling, proliferation, and migrational analysis

Male *Cx43* $^{fl/fl}$*; nestin–Cre* mice were crossed with female *Cx43* $^{fl/fl}$ mice, and pregnant mice at gestational day 14 were used for conducting BrdU experiments. For injections, 200 mg/kg BrdU was dissolved in saline and injected intraperitoneally (Redila et al., 2006). Pregnant female mice were injected with BrdU once, and animals were allowed to survive for either 2 or 96 h. Embryonic brains obtained from either E14 or E18 mice were sectioned and mounted on coverslips, and DNA was denatured by incubating the slides in HCl for 30 min at 37°C. The acid was neutralized by immersing the slides in 0.1 M borate buffer, pH 8.5 (Sigma), for 20 min. Brain sections were then blocked for nonspecific antibody binding with IgG blocking solution (M.O.M. kit; Vector Laboratories) and subsequently processed for immunohistochemical staining using a mouse monoclonal antibody against BrdU (Sigma). Selected samples were also double labelled with polyclonal antibodies for Ki67 (1:1000; Vector Laboratories). From each animal, 10 coronal sections were assessed spanning the rostral to the caudal end of the neocortex. Based on DAPI staining, the sections of the neocortex were divided into three zones: the VZ, the IZ, and the CP. To define each zone, the outline of the VZ, the IZ, and the CP of each section was traced using the AxioVision 4.2 software. Immunopositive cells were manually counted with the investigator blinded as to group identity (control and *Cx43cKO*). To measure a migration change, the number of $BrdU^+$ labeled cell nuclei was counted in a 16X optical view of each zone per 10 μm section, whereas $BrdU^+$ or $Ki67^+$ cells were counted in the VZ of each section to assess proliferation. $BrdU^+$ cells were counted if they had round nuclei that were evenly stained; thus elongated endothelial nuclei were excluded on the basis of shape (Fushiki et al., 2003). Punctated nuclei were also not counted because these cells may have entered or exited the S phase at the time of labeling (Fushiki et al., 2003). The mean number of $BrdU^+$ or $Ki67^+$ cells per section was calculated for both proliferation and migration. These numbers were then analyzed for statistical significance using an ANOVA.

3.2.8 In utero *Electroporation*

A plasmid expressing nuclear-localized GFP driven by a neuronal promoter (Tα1 tubulin) (gift from Dr. Freda D. Miller, University of Toronto, Toronto, Ontario, Canada) (Gloster et al., 1994) was used either alone or with each of the following plasmids: (1) full-

length Cx43 driven by the cytomegalovirus (CMV) promoter (*CMV–Cx43*) (Fu et al., 2004); (2) C terminally truncated Cx43 tagged with GFP [*Cx43Δ244–382GFP* (Cx43t–GFP)] (Bates et al., 2007); (3) U6–Cx43siRNA [sequence, CAATTCCTCCTGCCGCAAT (Iacobas et al., 2008); gift from Dr. Eliana Scemes, Albert Einstein College of Medicine, Bronx, NY]; and (4) U6-control–siRNA (sequence, CTCCTTTTTT; gift from Dr. Timothy O'Connor, University of British Columbia, British Columbia, Canada). The introduction of DNA by *in utero* electroporation was conducted as described previously (Tabata and Nakajima, 2002; Barnabé Heider et al., 2005). Briefly, pregnant female *Cx43cKO* mice, *Cx43* null mice, or $Cx43^{K258stop}$ mice were anesthetized with isoflurane and nitrous oxide. A midline incision was performed to reach the embryos, and 1 μl of DNA was injected into the E14 lateral ventricle. After injection, electroporation was conducted using a square electroporator CUY21 EDIT (Protech) to deliver five 50 ms pulses of 50 V with 950 ms intervals per embryo. Embryos were reinserted *in utero*, and the incision was closed. Four days later, at E18, the embryonic brains were harvested and fixed in 4% paraformaldehyde at 4°C overnight. The fixed brains were cryoprotected in 30% sucrose, mounted in OCT, cryosectioned (10 μm), and analyzed.

3.2.9 Intensity Measurements of GFP Across Neocortical Layers

NIH Image J software (http://rsb.info.nih.gov/ij/) was used to quantify GFP fluorescent intensity of neocortical layers and data analyzed in Microsoft Excel. For each image, the outlines of the VZ, the IZ, and the CP layers were drawn, and then a threshold was set to isolate GFP labelling to match the size and distribution of cells perceived by eye in the original grayscale image. The average pixel intensity from each layer was measured and subtracted from the background intensity. Six sections from at least three brains per condition were used, and the results were expressed as the percentage of total GFP intensity, for each condition, within each layer of the neocortex (Mizuno et al., 2007).

3.2.10 Data Analysis

All experiments were performed at least three times. When appropriate, values are presented as mean ± SEM, and the results were analyzed by one-way ANOVA, followed by the Student's *t* test or Mann–Whitney test. A p value of <0.05 was considered statistically significant.

3.3 Results

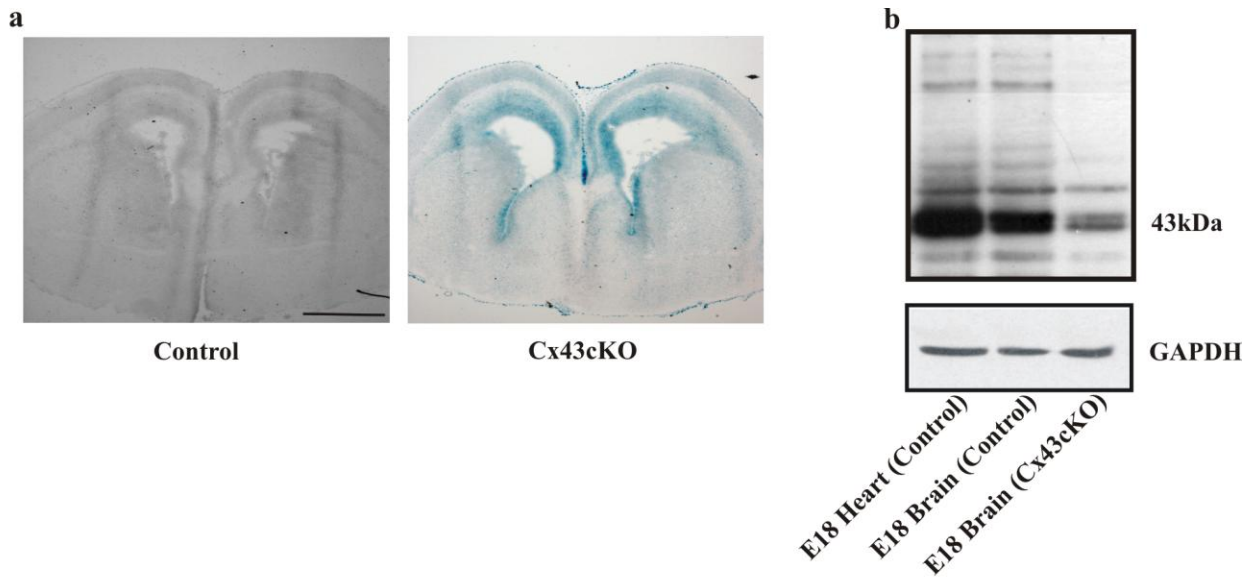
3.3.1 Cre is Active in *Cx43 $^{fl/fl}$; nestin–Cre* Mice and Deletes Cx43 from VZ Radial Glia and Immature Neurons

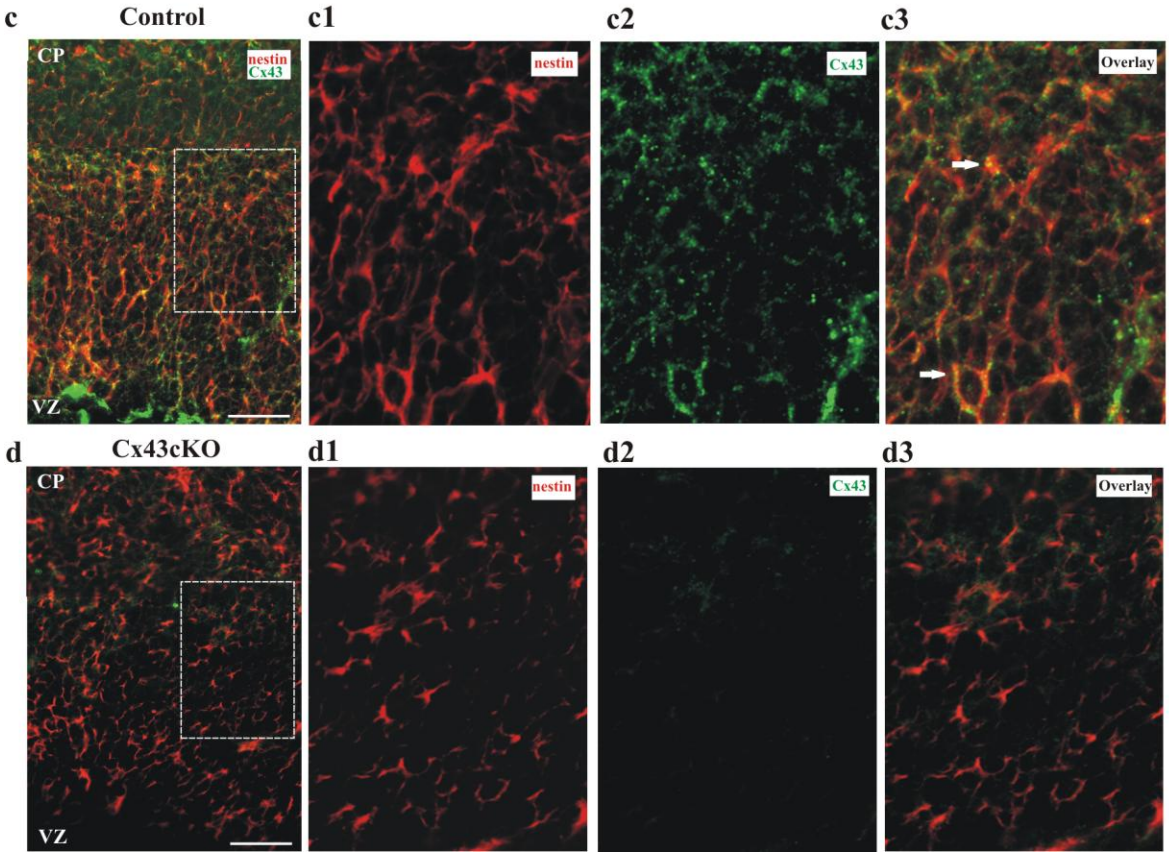
During early neurogenesis, nestin-expressing radial glia are the foundation of the developing neocortex and give rise to neurons, astrocytes, and oligodendrocytes as the cortex matures (Anthony et al., 2004; Götz and Sommer, 2005). Nestin is highly expressed in radial glial progenitor cells located in the VZ and is expressed from E10 onward (Malatesta et al., 2003). Taking advantage of this expression profile, we specifically inactivated a floxed *Cx43* allele (*Cx43^{fl}* allele) (Theis et al., 2001) in radial glial progenitors at the beginning of neurogenesis using the Cre/LoxP system in *Cx43 $^{fl/fl}$; nestin–Cre* (*Cx43cKO*) mice. Activation of Cre resulted in the complete knock-out of several floxed target genes expressed in the CNS (Tronche et al., 1999; Bérubé et al., 2005). The *Cx43 $^{fl/fl}$* mice are phenotypically indistinguishable from wild-type littermates and are born at predicted Mendelian frequency (Theis et al., 2001). They are referred to as "control" in the following. By conducting β-gal staining, immunohistochemistry, and Western blotting, we confirmed activity of the Cre recombinase in the VZ; additionally, we showed localization of Cre recombinase in radial glial cells in embryonic brain of *Cx43cKO* mice. β-Gal staining (β-galactosidase is expressed instead of Cx43 after Cre-mediated recombination) of the neocortex of E14 embryos confirms Cre is being expressed in the brain of *Cx43cKO* mice but not in control mice (Fig. 3.1a).

To demonstrate the removal of Cx43 from the brain, we used immunoblot analysis. Tissue lysates from E18 cortex and heart were probed with anti-Cx43 and GAPDH antibodies. Cx43 protein was strongly decreased in lysates of E18 *Cx43cKO* cortex relative to control (Fig. 3.1b). Residual expression of Cx43 was most likely attributable to *Cx43* gene activity in cells not targeted by *nestin–Cre*. Embryonic heart tissue was used as a positive control for the expression of Cx43 (Fig. 3.1b).

Double-immunofluorescent labeling with Cx43 and nestin antibodies further confirmed deletion of Cx43 from the radial glia. As can be seen in Figure 3.1, c and d, there was a dramatic reduction in the classical punctate Cx43 signals in the plasma membrane of nestin^{+} cells in the *Cx43cKO* animals. Having verified efficient Cre activity in *Cx43cKO*

Figure 3.1 Cre activity in *Cx43*$^{fl/fl}$; *nestin–Cre* (*Cx43cKO*) mice. **a,** Coronal sections of E14 embryonic forebrain from control *Cx43*$^{fl/fl}$ (left) and *Cx43cKO* (right) mice were stained for lacZ, indicating Cre-mediated loss of Cx43 expression. Cre activity was observed in Cx43cKO mice but not in control mice. n = 4. **b**, Representative immunoblot showing the reduction of Cx43 protein in the neocortex of *Cx43cKO* mice. Equal amounts of embryonic heart and cortical tissue protein were immunoblotted and probed with antibodies recognizing Cx43 (43 kDa). GAPDH was used as a loading control. n = 3. **c, d**, *Cx43cKO* mice display a strong decrease in the level of Cx43 expression throughout the neocortex compared with control mice. Coronal brain sections of E18 littermates were stained for nestin and Cx43. Cx43 showed association (arrows) with nestin-expressing cells in control animals (**c**) and was absent in Cx43cKO sections (**d**). **c1–d3** are higher-magnification micrographs of the areas outlined in **c** and **d**. n = 4. Scale bars: **a**, 1 mm; **c, d**, 100 µm; **c1–d3**, 40 µm.


a
Control
Cx43cKO
b
43kDa
GAPDH
E18 Heart (Control)
E18 Brain (Control)
E18 Brain (Cx43cKO)


c
Control
c1
c2
c3
CP
nestin
Cx43
Overlay
VZ
d
Cx43cKO
d1
d2
d3

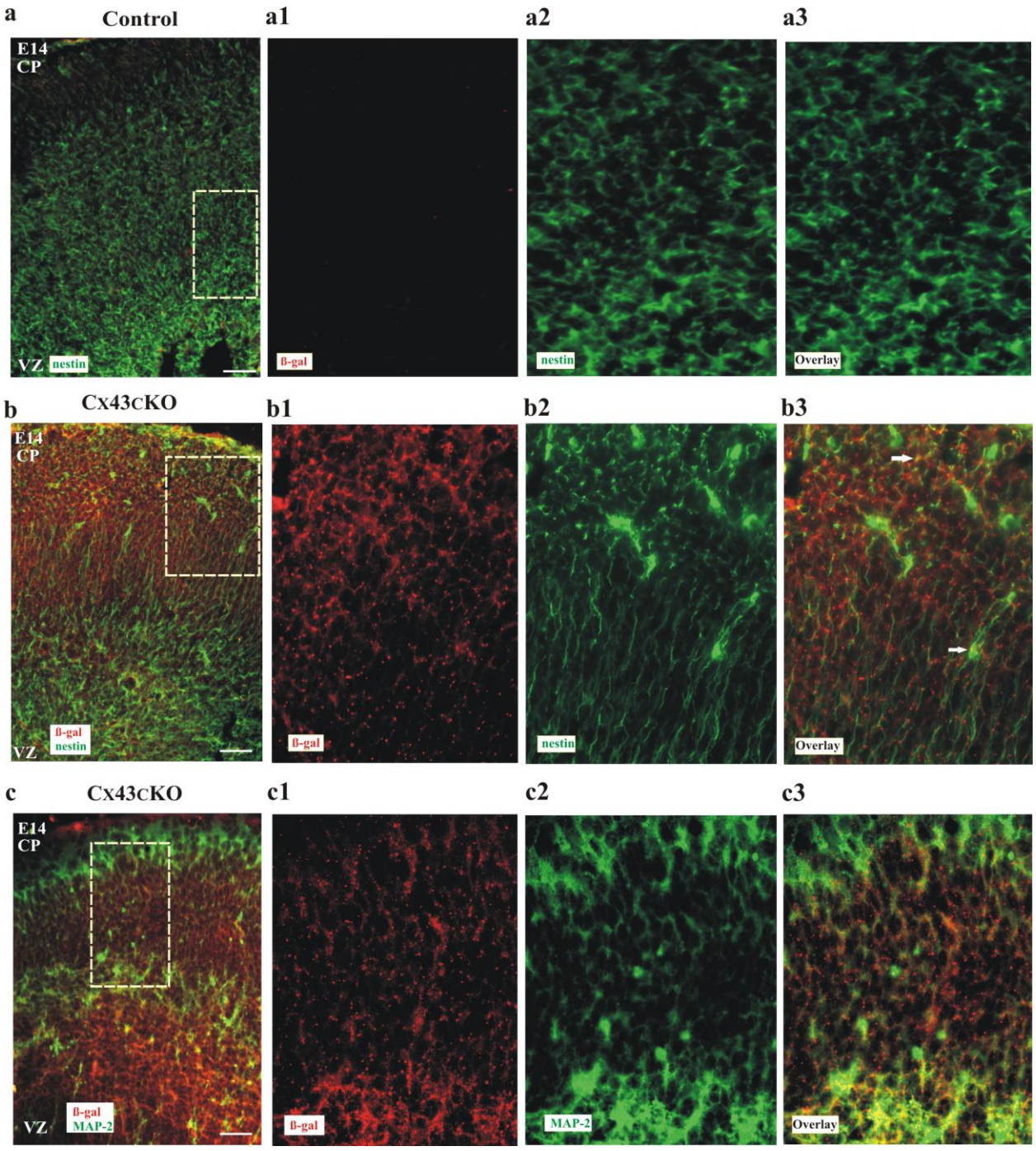
mice, we then addressed the question whether Cre was expressed in both cell types of interest, the radial glia and the early born neurons. Therefore, double-immunofluorescence labelling experiments were performed on tissue sections of control and *Cx43cKO* cortices at E14, using antibodies against β-gal together with either nestin or MAP-2 antibodies, specific for radial glia and neurons, respectively. Brain sections of control mice were devoid of β-gal labelling (Fig. 3.2a), whereas brain sections of *Cx43* $^{fl/fl}$*; nestin–Cre* mice showed extensive β-gal labelling, indicating Cre-mediated loss of Cx43 expression, which was associated with nestin^{+} and MAP-2^{+} cells (Fig. 3.2b,c). Thus, in *Cx43cKO* mice, Cre is expressed in radial glia and early born neurons.

3.3.2 Cx43 is required for normal lamination of the neocortex

The majority of neuronal migration in the mouse cerebral cortex occurs between developmental stages E14 and E18. Therefore, any abnormalities in the laminar formation of this structure in *Cx43cKO* mice should be observed by E18. Cortical sections of *Cx43cKO* (*Cx43*$^{fl/fl}$*; nestin–Cre*) and control (*Cx43*$^{fl/fl}$) E18 mice were treated with the fluorescent nuclear stain DAPI, to measure the entire thickness of the cortical hemispheres, as well as that of the VZ, the IZ, and the CP sublayers of each hemisphere. In addition, the thickness of the cortical layers was measured and compared between control and *Cx43cKO* mice. As shown in Figure 3.3, there was a significant change in the thickness of all layers except the VZ. The thickness of the CP was greater than the thickness of the IZ in the control neocortex, whereas this pattern was reversed in the Cx43cKO neocortex, consistent with the accumulation of cells in the IZ (Fig. 3.3a,b). The total cortical thickness of cerebral hemisphere in *Cx43cKO* mice appeared to be unchanged when compared with control mice (control, 799 ± 12 μm vs *Cx43cKO*, 756 ± 11 μm; $p > 0.05$). Therefore, these findings suggest that absence of Cx43 from radial glial fibers and immature neurons interrupts normal formation of the neocortical layers by accumulation of cells in the IZ.

To address the question whether the phenotype that was seen in the neocortex of E18 *Cx43cKO* mice persists into the postnatal stage or whether it recovers as the neocortex matures, the entire thickness of the postnatal cerebral hemisphere, as well as that of the sublayers of each hemisphere, was measured and compared between control and *Cx43cKO*

Figure 3.2 Cre activity is observed in radial glia and MAP-2-positive neurons in *Cx43cKO* brains. Coronal sections of control (**a**) and *Cx43cKO* (**b, c**) neocortices were stained for β-gal (red), MAP-2, and/or nestin (green). Control brains were devoid of β-gal labeling compared with *Cx43cKO* littermates (**a–c**). **a1–c3** represent higher-magnification micrographs of the section in ***a–c***. Note that β-gal staining is associated with both nestin and MAP-2 expression in *Cx43cKO* mice. Arrows represent association of nestin- or MAP-2-expressing cells with β-gal. n = 3. Scale bars: **a–c**, 100 μm; **a1–c3**, 40 μm.


a
Control
a1
a2
a3
E14
CP
VZ
nestin
ß-gal
nestin
Overlay
b
Cx43cKO
b1
b2
b3
E14
CP
ß-gal
nestin
VZ
ß-gal
ß-gal
nestin
Overlay
c
Cx43cKO
c1
c2
c3
E14
CP
ß-gal
MAP-2
VZ
ß-gal
MAP-2
Overlay

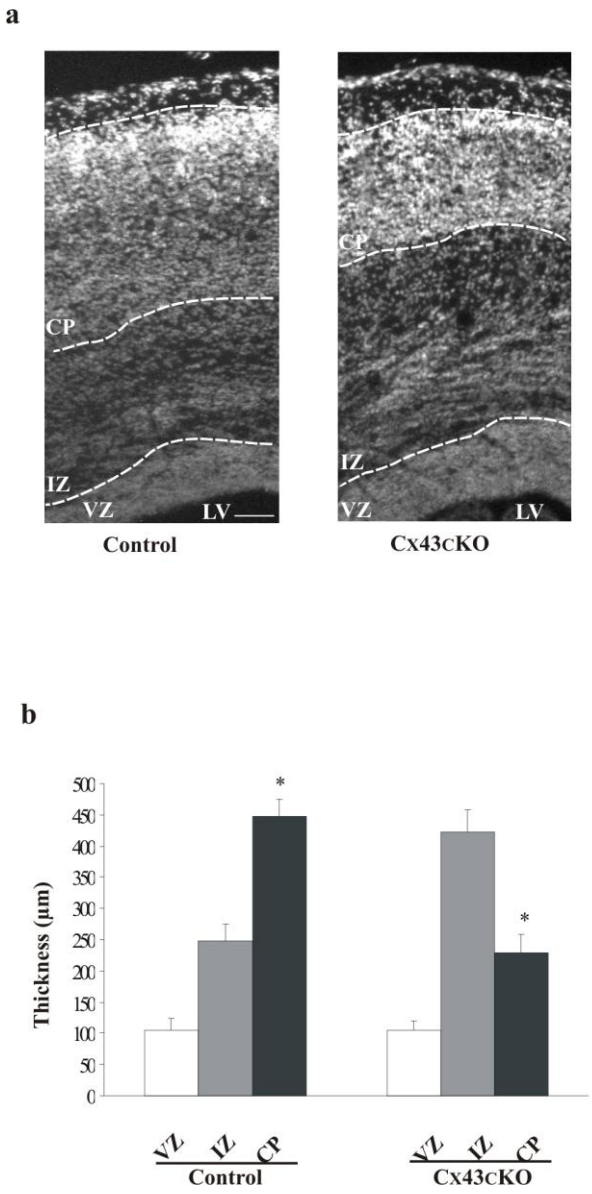


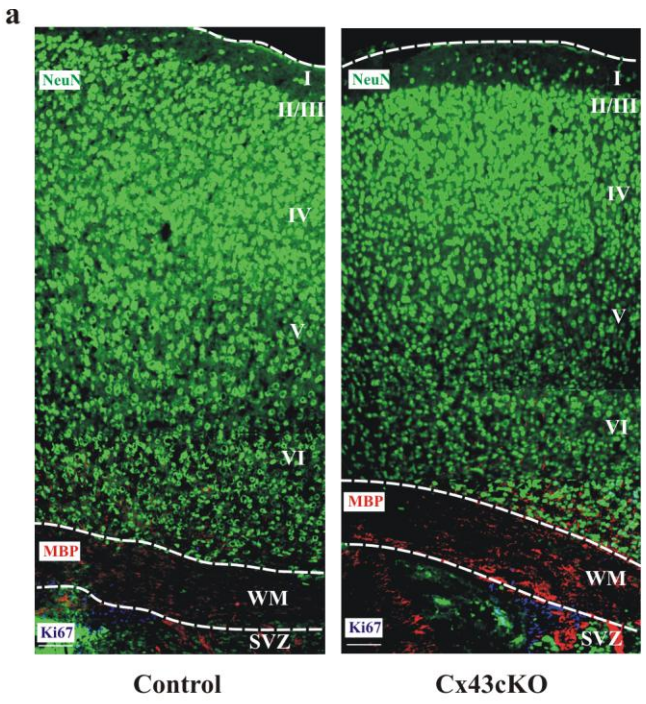
Figure 3.3 Deletion of Cx43 in the developing neocortex perturbs normal cortical lamination. **a**, Thickness of the VZ, IZ, and CP was measured in the neocortices of control and *Cx43cKO* mice. The cortical sections were stained with DAPI, and the cortical layers were identified based on their anatomical cell distribution. Using AxioVision software, the identified border lines between layers were labeled. In the cerebral cortices of E18 *Cx43cKO* mice, the thickness of the IZ was increased and the CP decreased compared with the IZ of control mice. **b**, The representative graph shows that the IZ in the *Cx43cKO* mice is increased in size compared with the control mice, whereas in control mice the CP is larger. *$p < 0.05$, Student's *t* test. n = 4. LV, Lateral Ventricle. Scale bar, 100 µm.

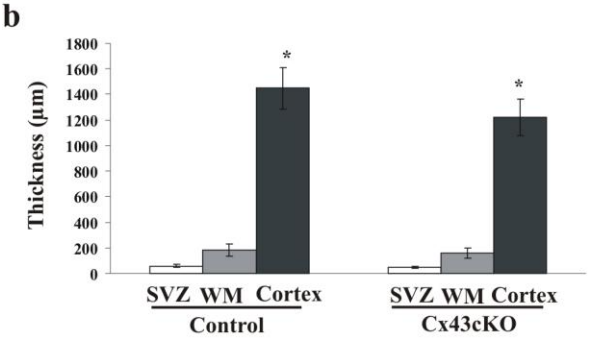
littermates. To define the SVZ, the WM, and the cortex of postnatal forebrain, we performed immunohistochemistry by using a cell proliferation marker (Ki67), MBP, and NeuN, respectively. From each animal, 10 coronal sections spanning the rostral to the caudal end of the cerebral cortex were assessed, and the thickness of each lamina was measured. As seen in Figure 3.4a, in general, the lamination of the cerebral cortex in *Cx43cKO* mice seemed to be normal, but compared with the control littermates at P16, the cortex and cerebral hemisphere were significantly thinner (15.5, 16, and 17% respectively; $p < 0.05$) (Fig. 3.4b). The thickness of the neocortex (including the cortical layers, white matter, and the subventricular zone) in *Cx43cKO* mice was smaller than that of the control littermates (*Cx43cKO*, 1410 ± 61μm vs control, 1692 ± 60 μm; $p < 0.05$). Furthermore, the thickness of the upper layers (I–IV) and the lower layers (V–VI) in *Cx43cKO* mice was defined, measured, and then compared with the control littermates. As shown in Figure 3.4c, in the control forebrain, the thickness of the layers I–IV was significantly greater than the thickness of the layers V–VI (36%), whereas this pattern was reversed in the *Cx43cKO* mice, consistent with the greater thickness of the layers V–VI compared with the layers I–IV (30%). Collectively, these findings suggest that the deletion of Cx43 from radial glia causes an accumulation of cells in the IZ during development, which leads to a smaller cortex in postnatal mice.

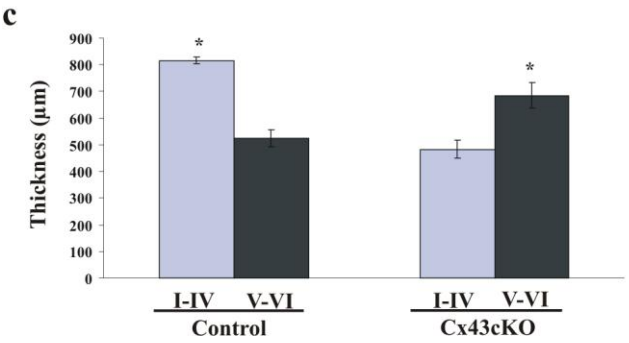
3.3.3 Differences in Cortical Formation between Cx43cKO and Control Mice are a Result of Neuronal Migration but not Altered Cellular Proliferation

BrdU integration into newly synthesized DNA occurs in the VZ and the SVZ of the developing cortex as a result of proliferation of progenitor cells and intermediate progenitor cells, respectively. Here, we performed a 2 h labelling of BrdU to look at the effect of Cx43 in the neural progenitor cell population. Immunohistochemistry for BrdU and Ki67 was conducted to show the proliferative cells during S-phase and all phases of the cell cycle, respectively (Fig. 3.5a,b). As shown in the representative pictures, a normal distribution of proliferative cells was observed in *Cx43cKO* mice. Based on DAPI staining, the sections of the neocortex were divided into three zones: the VZ, the IZ, and the CP. To define each zone, the outline of the VZ, the IZ, and the CP of each section was traced using the AxioVsion 4.2 software. The number of $BrdU^+$ or $Ki67^+$ cells was calculated in the VZ of 10 rostral, medial, and caudal sections from four brains per group. There was no significant difference in the

Figure 3.4 Loss of Cx43 in the developing neocortex leads to a reduction in the thickness of the postnatal neocortex. **a,** Thickness of the SVZ, WM, and the cortex was measured in the P16 cortices of control and *Cx43cKO* mice. From each animal, 10 cortical sections spanning rostral end to the caudal end were stained for NeuN (green) to label mature neurons in the cortex (lamina I–VI), MBP (red) to label myelin fibers in the WM, and Ki67 (blue) to label proliferating cells in the SVZ. Using AxioVision software, the outline of each layer was drawn and the thickness was measured. **b,** The representative graph shows that, compared with the control littermates at P16, the cortex of the *Cx43cKO* mice was thinner. **c,** The representative graph shows significantly reduced thickness of upper cortical layers and increased thickness of lower cortical layers in *Cx43cKO*, but the normal layering is maintained. $*p < 0.05$, Mann–Whitney test. n = 5. Scale bars, 100 μm.


a
NeuN
I
II/III
IV
V
VI
MBP
WM
Ki67
SVZ
Control
Cx43cKO


b
Thickness (µm)
1800
1600
1400
1200
1000
800
600
400
200
0
*
SVZ WM Cortex
Control
SVZ WM Cortex
Cx43cKO


c
Thickness (µm)
900
800
700
600
500
400
300
200
100
0
*
I-IV V-VI
Control
I-IV V-VI
Cx43cKO

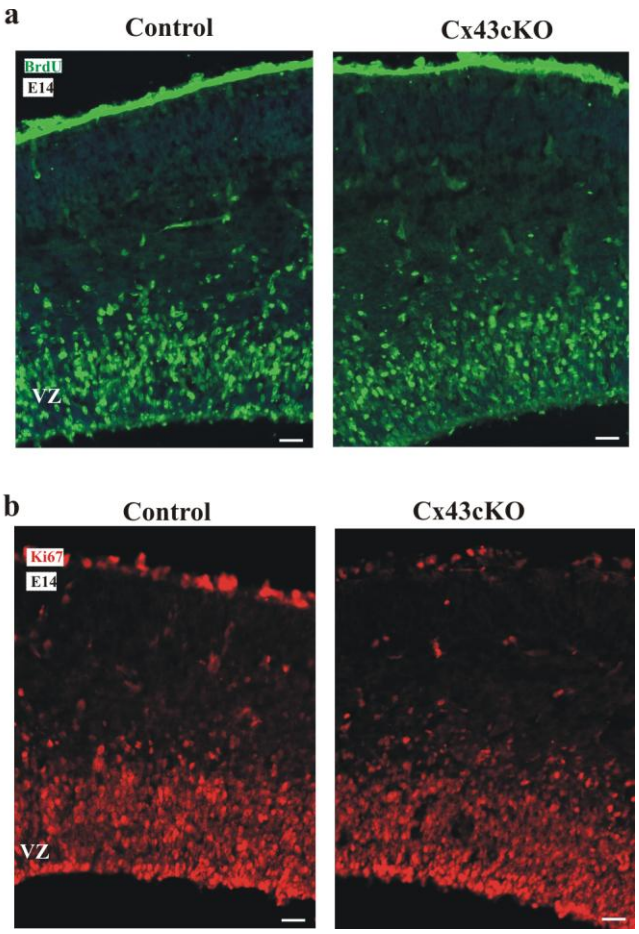


Figure 3.5 Deletion of Cx43 in radial glia of *Cx43cKO* mice does not produce a change in the rate of proliferation. Representative sections of BrdU labelling at E14 are shown in **a**. BrdU was administered at E14, and brains were fixed 2 h after labelling. Immunohistochemistry for BrdU was performed. From at least four brains per group, 10 coronal sections were assessed spanning the rostral to the caudal end of the neocortex. Based on DAPI staining, the sections of the neocortex were divided into three zones: the VZ, the IZ, and the CP. To define each zone, the outline of the VZ, the IZ, and the CP of each section was traced using the AxioVsion 4.2 software. Selected samples were also double labeled with polyclonal antibodies for Ki67 and showed a normal distribution of proliferative cells in the *Cx43cKO* mice (**b**). n = 4. Scale bars, 100 µm.

number of $Brdu^+$ or $Ki67^+$ cells in the VZ during proliferation between control and *Cx43cKO* mice (BrdU: control, 1386 ± 75 vs *Cx43cKO*, 1295 ± 91 cells per section; Ki67: control, 1870 ± 56 vs *Cx43KO*, 1650 ± 130 cells per section; $p > 0.05$). These findings indicate that the accumulation of cells in the IZ of the *Cx43cKO* mice is not a result of disrupted cellular proliferation.

Our previous BrdU studies investigating embryonic neocortical cell migration in Cx43 knock-out mice *in vivo* showed an accumulation of immature neurons in the outer IZ and fewer in the inner CP, suggesting delayed neuronal migration (Fushiki et al., 2003). Here, in a similar approach, embryos were exposed to BrdU on E14 and analyzed at E18 in control and *Cx43cKO* mice. In control mice, the majority of labeled cells were detected in the CP, whereas in *Cx43cKO* mice, more BrdU-labeled cells were observed in the IZ (Fig. 3.6a). In control animals, >60% of the labeled cells resided in the CP and only 38% in the IZ. In contrast, the CP of the *Cx43cKO* mice contained only 30% of all labeled cells, with 65% residing in the IZ (Fig. 3.6b).

3.3.4 Exogenous Wild-type Cx43 is Necessary for Neuronal Migration *In vivo*

We predicted that the accumulation of cells in the VZ/IZ is attributable to disrupted neuronal migration. To test this prediction, we monitored the migration of newborn neurons leaving the VZ in *Cx43cKO* mice. We made use of a transgene expressing GFP under control of a Tα1 tubulin promoter, because the majority of cells expressing Tα1 tubulin are neuronal progenitor cells (Gloster et al., 1999). The transgene was injected into the lateral ventricle of E14 control and *Cx43cKO* mice, followed by *in utero* electroporation; the embryos were allowed to develop until E16 or E18. Subsequently, the embryos were removed, and the brains were isolated and sectioned. One day after electroporation, only cells within the VZ or the IZ displayed GFP expression (data not shown), as reported previously (Ohtsuka et al., 1999; Barnabé-Heider et al., 2005). We quantified and compared the distribution of GFP-labeled cells as they migrate from the VZ through the IZ and into the CP between control and *Cx43cKO* mice. The neocortex was divided into three zones (the VZ, the IZ, and the CP), and the percentage intensity of GFP labelling within each zone was determined (see Materials and Methods). At E16, most cells accumulated in the IZ with no significant difference in the fraction of GFP intensity between control and *Cx43cKO*, as shown in the representative

images (76 and 73%, respectively; $p > 0.05$) (Fig. 3.7a,c). At E18, however, GFP^+ cells had migrated out of the VZ, passed through the IZ (15.34% GFP intensity), and into the CP (79.9% GFP intensity) of control animals, in which they differentiate into mature neurons. This pattern was not observed in the cortex of *Cx43cKO* mice, in which GFP^+ cells accumulate in the IZ (65.09% GFP intensity), with very few cells reaching the CP (18.17% GFP intensity) by E18 ($p < 0.05$) (Fig. 3.7a,c). Furthermore, the majority of cells expressing GFP were positive for NeuN and MAP-2, confirming those as neurons (Fig. 3.7b and Fig. 3.10, respectively). These results indicate that Cx43 is necessary for proper neuronal migration.

3.3.5 The C-terminal Domain of Cx43 is Required for Neuronal Migration

We have shown previously that, during embryonic development, several connexins, including Cx43, are expressed not only in the VZ but also throughout the IZ and the CP (Cina et al., 2007). Furthermore, we found that Cx43 expression is closely associated with the radial glia and the migrating neurons (Cina et al., 2007), suggesting that Cx43 might play a significant role in the interactions between migrating neurons and the radial glial scaffold.

Additionally, in a study by Elias et al. (2007), Cx43 was demonstrated to provide necessary adhesion between migrating neurons and radial glial fibers for proper neuronal migration. Thus, to rescue the Cx43-dependent migration/development paradigm, we introduced Cx43 into the cortices of Cx43-deficient mice by *in utero* electroporation. Cx43 was expressed under the control of the CMV promoter (CMV–Cx43).

As demonstrated in Figure 3.8a, when the CMV–Cx43 plasmid was coinjected with a plasmid encoding Tα1–GFP and electroporated into the lateral ventricle of E14 *Cx43cKO* embryos, the migrating neurons were able to reach the CP at E18. In addition, brain sections of *Cx43cKO* mice subjected to this *in utero* electroporation were immunostained for Cx43 and showed extensive labeling as seen in Figure 3.8b. These results strongly suggest that the reconstitution of full-length Cx43 into the lateral ventricle of *Cx43cKO* embryos at E14 rescued the defective neuronal migration.

Previous studies have investigated the consequence of deleting the C-terminal domain of Cx43 on cell motility. In C6 glioma cells, the C-terminal region of Cx43 has been

Figure 3.6 Loss of Cx43 in immature neurons causes accumulation of $BrdU^+$ cells in the VZ/IZ of *Cx43cKO* forebrains. **a,** Representative sections of BrdU labelling in E18 cortex of mice exposed to BrdU at E14. In *Cx43cKO* mice, there were still BrdU-labelled cells positioned in the VZ compared with the control mice. **b**, Distribution of BrdU-labelled cells in the IZ and the CP of the neocortex at E18. In the *Cx43cKO* mice, the number of BrdU-labelled cells in the CP was decreased and in the IZ increased compared with the control mice. Total number of cells in all zones was counted, and no significant difference was found among all the animals studied. $*p < 0.05$, Student's *t* test. n = 6. Scale bars, 100 µm.

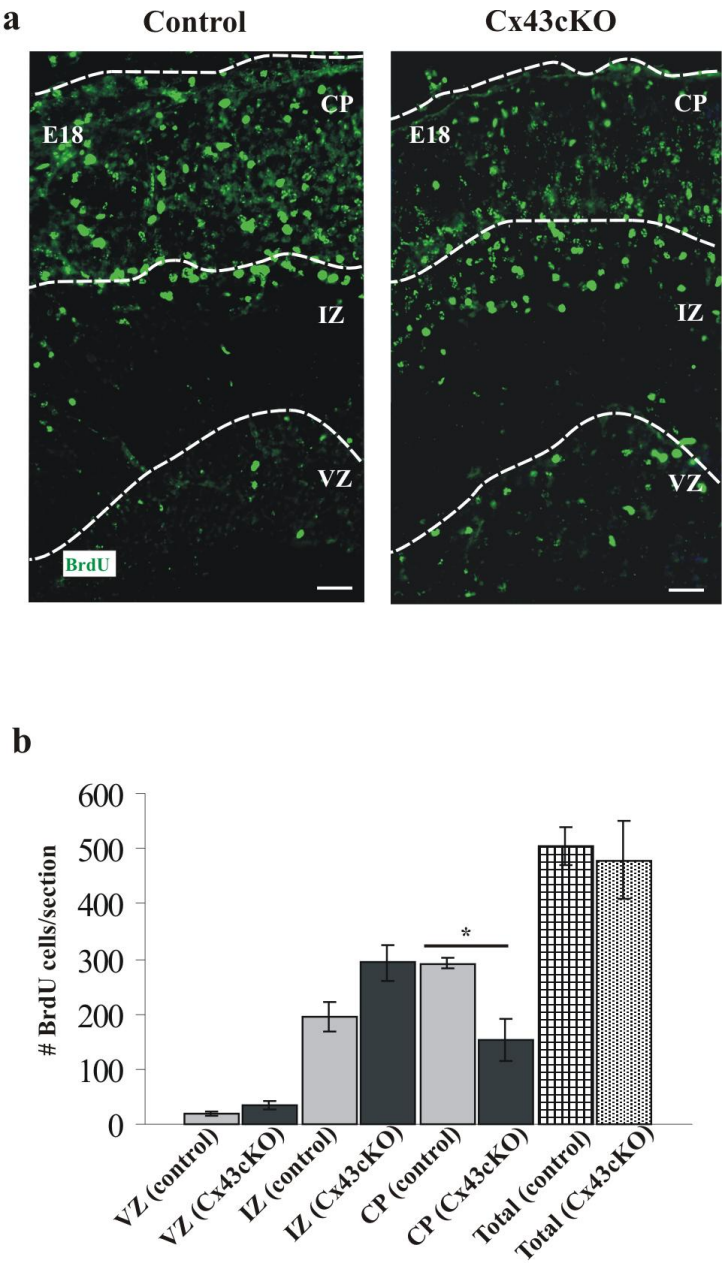
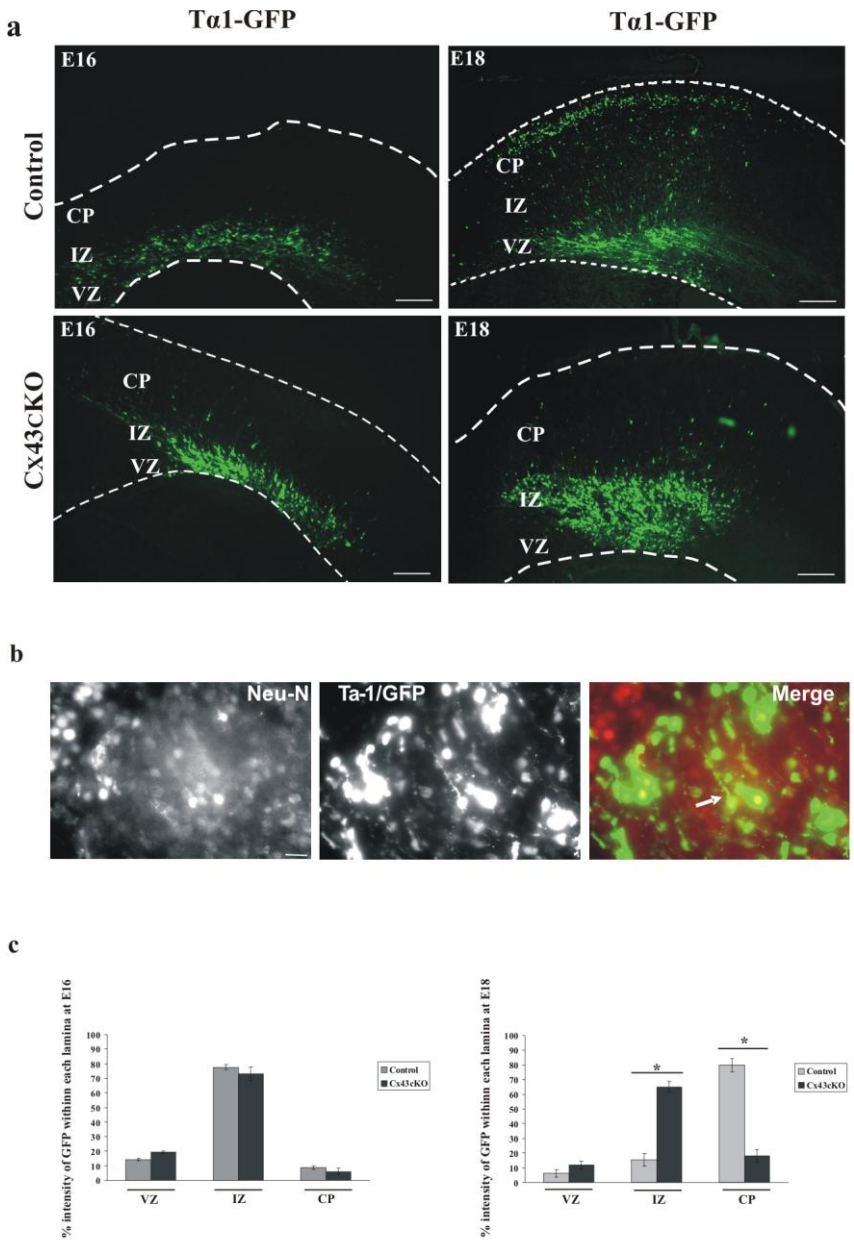

a
Control
Cx43cKO
CP
E18
IZ
VZ
BrdU
CP
E18
IZ
VZ
b
BrdU cells/section
600
500
400
300
200
100
0
*
VZ (control)
VZ (Cx43cKO)
IZ (control)
IZ (Cx43cKO)
CP (control)
CP (Cx43cKO)
Total (control)
Total (Cx43cKO)

Figure 3.7 Deletion of Cx43 in radial glial cells and newborn neurons of *Cx43cKO* mice interrupts neuronal migration. **a,** At E14, a transgene expressing GFP under control of a neuronal promoter (Tα1 tubulin) was injected into the lateral ventricle of control or *Cx43cKO* forebrains, followed by *in utero* electroporation. The brains were removed at E16 or E18, 10 µm sections of cerebral cortices were collected, and subsequently the GFP^+ cells were visualized under an epifluorescent microscope. At E16, most GFP^+ cells were still accumulated in the IZ with no significant difference between control and *Cx43cKO*. In control mice, at E18, GFP^+ cells were able to migrate to their destination in the CP, whereas in *Cx43cKO* cerebral cortices, the neurons failed to migrate to the CP. Four days after electroporation, coronal sections of the embryonic cerebral cortex were analyzed for coexpression of GFP and NeuN. Note that the majority of GFP^+ cells also express NeuN, confirming them as neurons (**b**). The percentage intensity of GFP-labeled cells in the VZ, the IZ, and the CP of the E16 and E18 forebrain sections was calculated and plotted as the mean ± SEM as shown in **c**. $*p < 0.05$, Student's *t* test. n = 5. Scale bars: **a**, 200 µm; **b**, 50 µm.


a
Tα1-GFP
Tα1-GFP
E16
E18
Control
CP
IZ
VZ
E16
E18
CX43CKO
CP
IZ
VZ
b
Neu-N
Ta-1/GFP
Merge
c
% intensity of GFP withinn each lamina at E16
Control
Cx43cKO
VZ
IZ
CP
% intensity of GFP withinn each lamina at E18
Control
Cx43cKO
VZ
IZ
CP

shown to play a significant role in enhancing motility (Moorby, 2000; Bates et al., 2007), but Elias et al. (2007) found the C-terminal of Cx43 not to be essential for this role, because their experiments showed that truncated Cx43 was able to rescue the neuronal migration defect in developing rat neocortex after small interfering RNA (siRNA) knockdown of endogenous Cx43. To elucidate the contribution of the C terminus in Cx43– mediated neuronal migration, we electroporated E14 *Cx43cKO* embryos with a construct coding for a C-terminally truncated form of Cx43 tagged with GFP (Cx43t–GFP). Interestingly, the truncated form of Cx43 failed to rescue migration; the cells remained in the VZ/IZ, suggesting that the C-terminal domain of Cx43 is necessary for radial migration in *Cx43cKO* mouse neocortex (Fig. 3.8c). We performed immunohistochemistry for Cx43 using an antibody directed against the N terminal. The punctate expression of Cx43t and its association with the Tα1–GFP transgene in the VZ is shown in Figure 3.8d. Immunostaining for Cx43 N-terminal domain on *Cx43KO* tissue sections was negative, whereas wild-type *Cx43* tissue sections showed punctate staining demonstrating the specificity of the antibody (data not shown).

We next quantified the effect of the CMV–Cx43 transgene in *Cx43cKO* mice and compared it with the effect of the Cx43t–GFP transgene in the same genotype. By E18, 68% of the intensity of GFP^+ cells was observed in the CP when electroporated with CMV–Cx43, whereas in embryos electroporated with Cx43t–GFP, the majority of the cells (65% cell intensity) accumulated in the IZ with significantly less GFP intensity (10.28%) in the CP ($p < 0.05$) (Fig. 3.8e), as shown in the representative images (Fig. 3.8a,c). These data suggest that the C terminal domain of Cx43 is required for neuronal migration.

To further examine the contribution of the C-terminal domain of Cx43 in neuronal migration, we performed *in utero* electroporation experiments using $Cx43^{K258stop/-}$ mice that endogenously lack the last 125 amino acid residues of the Cx43 C-terminal domain (Maass et al., 2004). E14 cortices were electroporated with the Tα1–GFP plasmid, and embryos were analyzed 4 d later. The $Cx43^{K258stop/-}$ cortices showed results similar to *Cx43cKO* mice. Specifically, the vast majority of the Tα1–GFP-expressing neurons remained in the VZ/IZ at E18 (Fig. 3.9a). In contrast, in $Cx43^{K258stop/+}$ mice, neurons were able to reach their final location in the CP when electroporated with the Tα1–GFP plasmid at E14 (Fig. 3.9b). Quantitative analysis demonstrated that, in $Cx43^{K258stop/-}$ mice, 71.16% intensity of GFP^+ cells was seen in the IZ with only 3.55% cell intensity in the CP. In contrast, the majority of the

GFP^+ cells (64.59% cell intensity) reached the CP in $Cx43^{K258stop/+}$ mice, implying that neuronal migration was not perturbed in these mice as shown in the representative image ($p < 0.05$) (Fig. 3.9b). Thus, we propose that the C-terminal tail of Cx43 is essential to promote neuronal migration in the embryonic mouse neocortex.

To further confirm the role of Cx43 in neuronal migration, we conducted *in utero* electroporation experiments on *Cx43KO* ($Cx43^{-/-}$) mice. Tα1–GFP was expressed alone or co-expressed with CMV–Cx43 in E14 neocortices, and embryos were analyzed at E18. The data demonstrated that, although newborn neurons electroporated with Tα1–GFP plasmid failed to migrate to the CP (Fig. 3.9c), exogenous Cx43 was able to rescue neuronal migration (Fig. 9d). The results were confirmed by quantitative analysis demonstrating that, in *Cx43KO* mice, 58.99% GFP intensity was present in the IZ and 8.16% in the CP when electroporated with Tα1 GFP, whereas the fraction of GFP intensity in the IZ decreased to 34.64% and in the CP increased to 50.79% when *Cx43KO* mice were electroporated with CMV–Cx43 transgene ($p < 0.05$) (Fig. 3.9d). Additionally, either a siRNA directed against Cx43 (U6–Cx43–siRNA) or a control–U6–siRNA plasmid was cointroduced using electroporation with Tα1–GFP into the developing neocortex of E14 wild-type embryos. By E18, an accumulation of GFP^+ cells was observed in the VZ and the IZ of the wild-type mice electroporated with Cx43–siRNA (Fig. 3.9e), whereas mice electroporated with U6–control–siRNA showed normal neuronal migration (Fig. 3.9f). As shown in Figure 3.9, quantitative analysis confirmed that there was a significant change in the fraction of intensity of GFP in the CP when $Cx43^{+/+}$ embryos were electroporated with Cx43–siRNA plasmid compared with control–siRNA plasmid. Most clearly, there was a loss of GFP intensity in the cortical plate (3.15% intensity), whereas the majority of the intensity was seen in the VZ and the IZ (26.41 and 68.70%, respectively). However, in mice electroporated with control–siRNA, the fraction of GFP intensity was more abundant in the CP (87.41%) than in the VZ or the IZ.

Because exogenous expression of Cx43 in radial glial progenitors of *Cx43cKO* and *Cx43KO* mice restores neuronal migration to levels seen in control mice, our study strongly supports the concept that Cx43 plays a vital role in neuronal migration. Furthermore, because a C-terminally truncated Cx43 failed to rescue migration, the C-terminal region appears necessary for this function of Cx43.

Figure 3.8 Rescue of radial migration phenotype by wild-type Cx43**. a,** CMV–Cx43 and Tα1–GFP plasmids were co-injected into the lateral ventricle of control and *Cx43cKO* mice, followed by *in utero* electroporation at E14. The brains were removed at E18, and the GFP-positive cells were visualized under an epifluorescent microscope. The insertion of wild-type Cx43 into the lateral ventricle resulted in proper neuronal migration. **b,** Coronal sections were stained for Cx43 (shown in red). n = 4. **c,** C-terminal region of Cx43 is necessary for radial migration. Truncated form of Cx43 (Cx43t–GFP) failed to rescue deficient migration. **d,** Frozen sections were stained for Cx43 (N terminal, shown in red). *n* = 3. Scale bars: **a, c,** 200 μm; **b, d**, 50 μm. **e**, The percentage intensity of GFP-labelled cells in the VZ, the IZ, and the CP of the E18 forebrain sections of *Cx43cKO* mice, electroporated with either CMV–Cx43 or Cx43t–GFP, was calculated and plotted as the mean ± SEM. The percentage intensity of GFP in the CP of the mice electroporated with CMV–Cx43 was found to be significantly higher when compared with mice electroporated with Cx43t–GFP. *$p < 0.05$, Student's *t* test. n = 5.

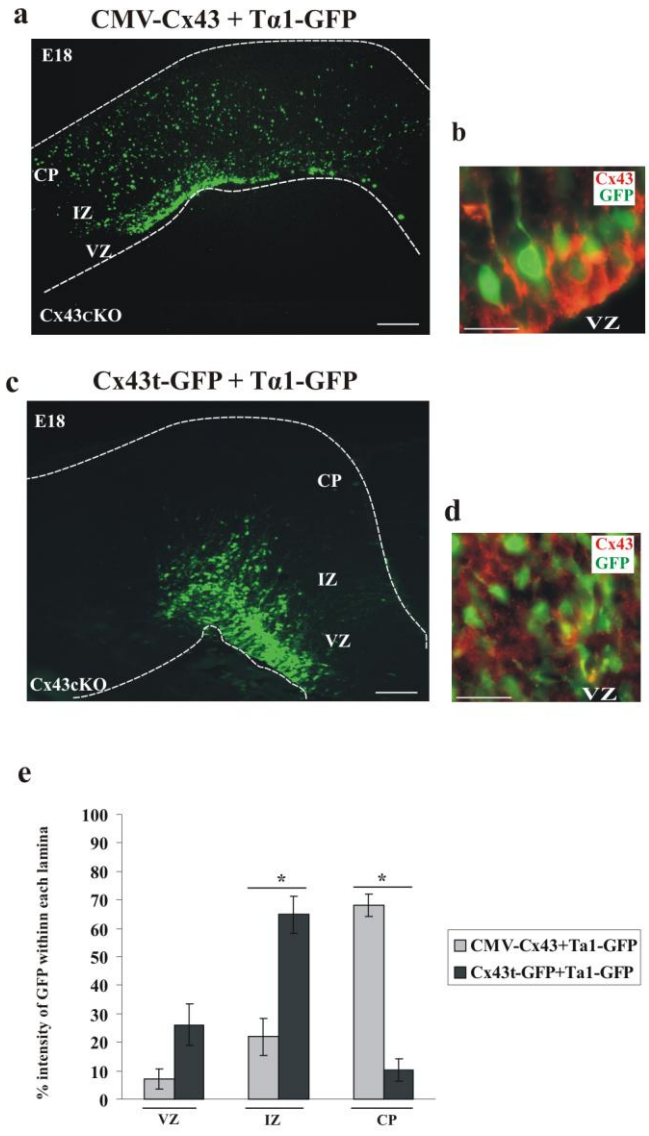
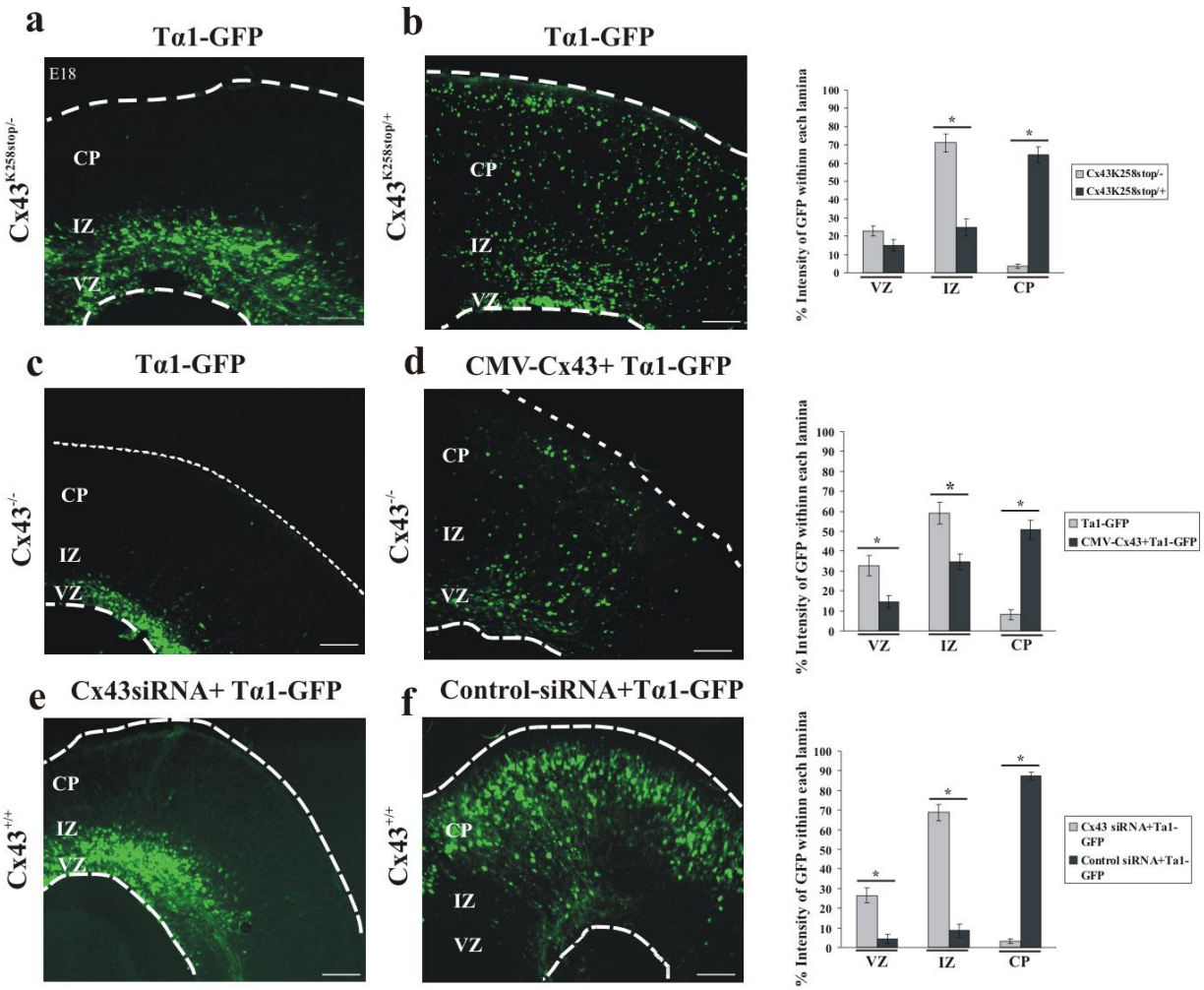

a
CMV-Cx43 + Tα1-GFP
E18
CP
IZ
VZ
Cx43cKO
b
Cx43
GFP
VZ
c
Cx43t-GFP + Tα1-GFP
E18
CP
IZ
VZ
Cx43cKO
d
Cx43
GFP
VZ
e
% intensity of GFP within each lamina
100
90
80
70
60
50
40
30
20
10
0
*
*
VZ
IZ
CP
CMV-Cx43+Ta1-GFP
Cx43t-GFP+Ta1-GFP

Figure 3.9 The C-terminus is necessary for the function of Cx43 during neuronal migration**.** At E14, Tα1–GFP plasmid was injected alone or coinjected with CMV–Cx43 plasmid into the lateral ventricle of *Cx43*$^{-/-}$, *Cx43*$^{K258stop/+}$, or *Cx43*$^{K258stop/-}$ mice, followed by electroporation (**a**–**d**). Four days later, the electroporated brains were removed, 10 µm sections of cerebral cortices were collected, and subsequently the GFP^{+} cells were visualized under an epifluorescent microscope. Although in *Cx43*$^{K258stop/-}$ mice neurons failed to migrate to the CP (**a**), *Cx43*$^{K258stop/+}$ mice showed normal neuronal migration (**b**). The bar graph on the right indicates a significant difference in neuronal migration between *Cx43*$^{K258stop/-}$ and *Cx43*$^{K258stop/+}$ mice. In *Cx43*$^{-/-}$ mice, neurons electroporated with Tα1–GFP failed to migrate to their final location in the CP (**c**), whereas CMV–Cx43 rescued neuronal migration in these mice (**d**). The bar graph on the right shows that the percentage intensity of GFP in the CP of *Cx43*$^{-/-}$ mice, rescued with CMV–Cx43, is significantly higher than of those injected with only Tα1–GFP. Disrupted neuronal migration by Cx43siRNA is shown in **e** and **f**. In Cx43$^{+/+}$ mice, 4 d after electroporation, cells transfected with Cx43–siRNA were positioned in the VZ/IZ, and no cells reached the CP (**e**), whereas cells transfected with control–siRNA were able to migrate to the CP (**f**). The bar graph on the right demonstrates that Cx43–siRNA causes a significant loss in the CP when compared with the control–siRNA. *$p < 0.05$, Student's *t* test. n = 3. Scale bars, 200 µm.


a
Tα1-GFP
E18
Cx43K258stop/-
CP
IZ
VZ
b
Tα1-GFP
Cx43K258stop/+
CP
IZ
VZ
% Intensity of GFP withinn each lamina
Cx43K258stop/-
Cx43K258stop/+
VZ
IZ
CP
c
Tα1-GFP
Cx43-/-
CP
IZ
VZ
d
CMV-Cx43+ Tα1-GFP
Cx43-/-
CP
IZ
VZ
% Intensity of GFP withinn each lamina
Ta1-GFP
CMV-Cx43+Ta1-GFP
VZ
IZ
CP
e
Cx43siRNA+ Tα1-GFP
Cx43+/+
CP
IZ
VZ
f
Control-siRNA+Tα1-GFP
Cx43+/+
CP
IZ
VZ
% Intensity of GFP withinn each lamina
Cx43 siRNA+Ta1-GFP
Control siRNA+Ta1-GFP
VZ
IZ
CP

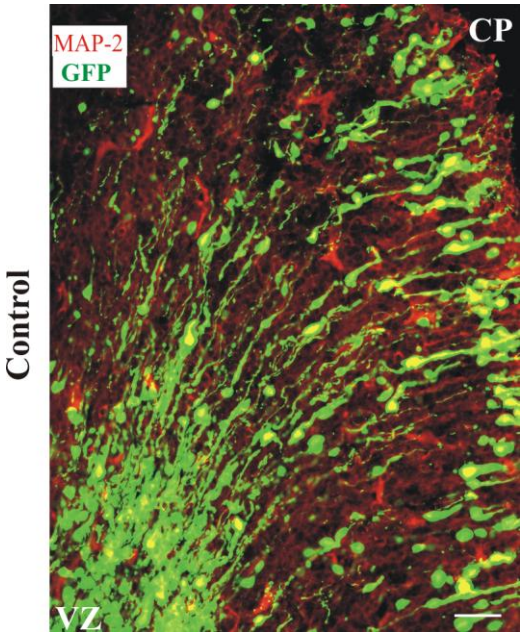


Figure 3.10 Normal neuronal migration in control mice**.** At E14 a transgene expressing GFP under control of a neuronal promoter (Tα1 tubulin) was injected into the lateral ventricle of control forebrains followed by *in utero* electroporation. The brains were removed at E18, 10 µm sections of cerebral cortices were collected, and stained for MAP-2. The coronal sections of the embryonic cerebral cortex were analyzed for co-expression of GFP and MAP-2 under an epi-fluorescent microscope. Note that the Tα1-GFP^{+} cells are associated with MAP-2^{+} neurons. n=5. Scale bar, 100 µm.

3.4 Discussion

Over the past several years, a number of research groups have used *in vitro* models to elucidate the role of Cx43 in cell migration. For example, astrocytes normally form a layer that radiates away from organotypic slice cultures of embryonic mouse brain, but we have shown previously that astrocytes in *Cx43KO* slices remain confined to the central regions suggesting a role for Cx43 in regulating the migration of astrocytes (Perez Velazquez et al., 1996). Aggregation assays have shown Cx43 enhanced adhesion between astrocytes and increased invasiveness of glial tumor cells (Lin et al., 2002), whereas Cx43KO cardiac neural crest cells were observed to migrate slower than normal with reduced directionality (Xu et al., 2006). Together, these studies suggest that Cx43 may contribute to cellular migration during development.

Despite the many advances in our understanding about cellular motility and the role Cx43 has to play in directing it, there have been relatively few successful attempts to document these effects *in vivo*. We have striven to address this issue by investigating the role of Cx43 in the neocortical development of three distinct mouse lines. Cx43 null mice represent a complete knock-out model. Although general homozygous Cx43 ablation is lethal, death occurs postnatally, allowing us to trace the effect of Cx43 on neuronal migration during embryogenesis (Reaume et al., 1995; Fushiki et al., 2003). $Cx43^{K258stop}$ mice lack the last 125 amino acid residues of the cytoplasmic C-terminal domain of Cx43. This is also a homozygous lethal phenotype, resulting in postnatal death from a complete loss of the epidermal permeability barrier (Maass et al., 2004). The *Cx43cKO* mice used in the current study allow selective knock-out of Cx43 only in the radial glial progenitors.

The current work represents significant evidence for Cx43 modulating the motility of the neuronal daughter cells that are produced by the radial glial daughter cells and suggests a possible mechanism by which this occurs.

3.4.1 Deletion of Cx43 from Radial Glial Progenitors Causes Abnormal Cortical Development

Our results demonstrate that expression of Cx43 in radial glial progenitors is necessary for normal neocortical development. The spatial restricted ablation of Cx43 in *Cx43cKO* mice leads to a thickening of the IZ and a concomitant thinning of the CP, both of

which appear to be the result of newly born neurons accumulating in the IZ. To show this is in fact the case, we labeled radial glial progenitor cells in the VZ with BrdU. As expected, the number of BrdU labelled cells in the CP of *Cx43cKO* mice was significantly lower than was observed in the control mice. This was not a result of reduced cellular proliferation, however, because the average number of $BrdU^+$ cells was the same for both groups when a short survival BrdU experiment was conducted to assess the S-phase. Similar results have been reported in our previous studies using *Cx43* null mice, in which the majority of newly born neurons labelled with BrdU in the VZ tended to accumulate in the IZ (Fushiki et al., 2003). This suggests that the migrational defect observed in *Cx43* null mice results from an absence of Cx43 in radial glial progenitor cells. Furthermore, expression of exogenous wild-type Cx43 restores normal migration patterns in *Cx43cKO* mice when introduced *in vivo*. Therefore, our findings strongly suggest that Cx43 acts as a regulator for neuronal migration.

Loss of Cx43 alters the ability of newborn neurons to migrate along the radial fibers extending into the CP. As a result, accumulation of neurons disrupts the normal lamination of the cerebral cortex. To further explore these cortical abnormalities, we analyzed the cortical thickness during postnatal development and demonstrated that, compared with the control littermates at P16, in *Cx43cKO* mice, the upper-layer (I–IV) thickness was markedly reduced; the size of the entire cortical hemisphere was also reduced, but the lamination of the forebrain seemed unchanged. Similarly, a previous study demonstrated that, during postnatal development in *Cx43cKO* mice, removal of Cx43 in GFAP-expressing cells leads to a smaller cerebral cortex by disrupting cellular organization (Wiencken-Barger et al., 2007). Interestingly, this phenotype was dependant on the genetic background of the experimental mice. C57BL/6 mice suffered no ill effects when Cx43 was removed from radial glia, whereas crossing into the 129SEV background resulted in developmental defects even more severe than we observed in our own study. GFAP and nestin are both radial glial markers, but GFAP expression is not observed until E12.5 (Brenner and Messing, 1996; Zhuo et al., 2001; Kriegstein and Gotz, 2003). Nestin, however, is first expressed in neuroepithelial cells surrounding the neural tube at approximately E10, which later mature into radial glia (Hartfuss et al., 2001; Malatesta et al., 2003). By driving Cre expression with the nestin promoter, we block Cx43 expression at the very beginning of neurogenesis. Given that all of our experiments were performed in the C57BL/6 background and we still observed a robust

alteration in neuronal migration, we predict that subsequent experiments in which *nestin–Cre* is crossed into a $Cx43^{fl/fl}$ 129SEV background will further enhance developmental defects beyond those seen in the Wiencken-Barger study.

Although the importance of Cx43 on neuronal migration is fairly established, the mechanisms regulating these effects are still in question. Gap junctional coupling is not likely to be an important factor driving migration, because fluorescent recovery after photo-bleaching and dye-coupling techniques have not detected major differences in gap junctional coupling between wild-type and *Cx43cKO* mice (Wiencken-Barger et al., 2007). It has been suggested that gap junctional plaques act as dynamic adhesive contact points between radial glial fibers and newborn neurons moving into the CP (Elias et al., 2007); the current study demonstrates that the C-terminal tail is another important factor.

3.4.2 The Cytoplasmic C-terminal Tail of Cx43 is Crucial in Cell Migration

Motility of adherent cancer cells is already known to be modulated by the C-terminal tail of Cx43 (Bates et al., 2007), and here we have shown that this is the case for neuronal migration during development as well. Electrophoretic delivery of full-length Cx43 into the VZ of *Cx43cKO* embryos is able to restore normal migration of neurons into the CP. Similar delivery of Cx43 with the C-terminal truncated does not rescue migration. Even more compelling is our observation that neurons in $Cx43^{K258stop}$ embryos also fail to move into the CP normally. These results are in conflict with the recent study from Elias et al. (2007), in which short hairpin RNA (shRNA) was used to knockdown Cx43 expression, and they reported that a C-terminal truncated version of Cx43 was able to return migration rates to normal. It is not entirely clear why the results of their experiments differ from ours, but these discrepancies may actually hold clues as to the mechanism regulating motility. Their method of delivering shRNA against Cx43 via electroporation would have knocked down expression in only a subset of radial glial cells. It is possible then that the cells that retained normal Cx43 levels acted as a support network, facilitating adhesion-mediated migration of truncated Cx43-expressing cells. In our system, in which nearly all radial glia are Cx43 deficient, such a support system would not exist; thus, the expression of truncated Cx43 becomes insufficient to support normal migration.

A previous study from van Rijen et al. (2004), in which an inducible system was used to conditionally delete the expression of cardiac *Cx43* in adult mice, reported that a decrease of Cx43 protein up to 95% is needed to induce cardiac-related death. Thus, one could speculate that the siRNA technique is limited in its ability to reduce Cx43 expression and cause a major defect. Regardless of the reason behind these differences, we have gathered compelling evidence that the C-terminal tail of Cx43 is in fact responsible for directing movement of newborn neurons into the CP.

In summary, our data supports two major conclusions. First, in *Cx43cKO* mice, when *Cx43* is deleted from radial glia, many newborn neurons remain in the IZ, which causes an increase in the thickness of the IZ and a subsequent decrease in the thickness of the CP compared with control mice. Second, Cx43, and its cytoplasmic C-terminal domain, play a functional role in radial guided neuronal migration during neocortical development. The detailed molecular mechanisms behind this activity are still unclear. Many proteins have been shown to interact with the C-terminal domain of Cx43, linking it to cytoskeletal organization. These proteins include CCN3/NOV (McLeod et al., 2001; Fu et al., 2004), N-cadherins (Xu et al., 2001), Src (Lin et al., 2001), β-catenin (Ai et al., 2000), Microtubules (Giepman et al., 2001), and Zona occludens-1 (ZO-1) (Giepmans and Moolenaar, 1998), and each represents a key component of a different cellular structure or function, any one of which could assist in regulating migration. Scaffolding proteins such as actin are linked to tight junctions and adherence junctions via members of the membrane associated guanylate kinase family of proteins such as ZO-1 (Itoh et al., 1997; Perez-Moreno et al., 2003). Furthermore, ZO-1 interacts with the C-terminal domain of Cx43 as removal of the last four amino acids completely abolishes the binding (Giepmans et al., 2001). Therefore, it is tempting to speculate that scaffolding proteins such as ZO-1 stabilize the actin cytoskeleton to the Cx43 C-terminal domain, thus involving Cx43 in cytoskeletal anchorage. A better understanding of these mechanisms will facilitate the study of a number of neurodevelopmental malformations and diseases.

3.5 References

Ai Z, Fischer A, Spray DC, Brown AM, Fishman Jl (2000) Wnt-1 regulation of coneixn43 in cardiac myocytes. J Clin Invest 105:161-171.

Akagi K, Sandig V, Vooijs M, Van der Valk M, Giovannini M, Strauss M, Berns A (1997) Cre-mediated somatic site-specific recombination in mice. Nucleic Acids Res 25:1766-1773.

Anthony TE, Klein C, Fishell G, Heintz N (2004) Radial glia serve as neuronal progenitors in all regions of the central nervous system. Neuron 41:881-890.

Barnabé-Heider F, Wasylnka JA, Fernandes KJ, Porsche C, Sendtner M, Kaplan DR, Miller FD (2005) Evidence that embryonic neurons regulate the onset of cortical gliogenesis via cardiotrophin-1. Neuron 48:253-265.

Bates DC, Sin WC, Aftab Q, Naus CC (2007) Connexin43 enhances glioma invasion by a mechanism involving the carboxy terminus. Glia 55:1554-1564.

Bérubé NG, Mangelsdorf M, Jagla M, Vanderluit J, Garrick D, Gibbons RJ, Higgs DR, Slack RS, Picketts DJ (2005) The chromatin-remodeling protein ATRX is critical for neuronal survival during corticogenesis. J Clin Invest 115:258-267.

Bittman K, Owens DF, Kriegstein AR, LoTurco JJ (1997) Cell coupling and uncoupling in the ventricular zone of the developing neocortex. J Neurosci 17:7037-7044.

Brenner M, Messing A (1996) GFAP transgenic mice. Methods 10:351-364.

Brenner M, Kisseberth WC, Su Y, Besnard F, Messing A (1994) GFAP promoter directs astrocyte-specific expression in transgenic mice. J Neurosci 14:1030-1037.

Casper KB, McCarthy KD (2006) GFAP–positive progenitor cells produce neurons and oligodendrocytes throughout the CNS. Mol Cell Neurosci 31:676-684.

Cina C, Bechberger JF, Ozog MA, Naus CCG (2007) Expression of connexins in embryonic mouse neocortical development. J Comp Neurol 504:298-313.

Elias LA, Wang DD, Kriegstein AR (2007) Gap junction adhesion is necessary for radial migration in the neocortex. Nature 448:901-907.

Fu CT, Bechberger JF, Ozog MA, Perbal B, Naus CCG (2004) CCN3 (NOV) interacts with connexin43 in C6 glioma cells. J Biol Chem 279:36943-36950.

Fushiki S, Perez Velazquez JL, Zhang L, Bechberger JF, Carlen PL, Naus CC (2003) Changes in neuronal migration in neocortex of connexin43 null mutant mice. J Neuropathol Exp Neurol 62:304-314.

Giepmans BN, Moolenaar WH (1998) The gap junction protein connexin43 interacts with the second PDZ domain of the zona occludens-1 protein. Curr Biol 8:931-934.

Giepmans BN, Verlaan I, Hengeveld T, Janssen H, Calafat J, Falk MM, MoolenaarWH (2001) Gap junctions protein connexin-43 interacts directly with microtubules. Curr Biol 11:1364-1368.

Gloster A, Wu W, Speelman A, Weiss S, Causing C, Pozniak C, Reynolds B, Chang E, Toma JG, Miller FD (1994) The Tα1 α-tubulin promoter specifies gene expression as a function of neuronal growth and regeneration in transgenic mice. J Neurosci 14:7319-7330.

Gloster A, El-Bizri H, Bamji SX, Rogers D, Miller FD (1999) Early induction of Tα1 α-tubulin transcription in neurons of the developing nervous system. J Comp Neurol 405:45- 60.

Goodenough DA, Paul DL (2003) Beyond the gap: functions of unpaired connexon channels. Nat Rev Mol Cell Biol 4:285-294.

Götz M, Sommer L (2005) Cortical development: the art of generating cell diversity. Development 132:3327-3332.

Groome NP, Dawkes A, Gales M, Hruby S, Alvord EC Jr (1986) Region specific immunoassays for human myelin basic protein. J Neuroimmunol 12:253-264.

Hartfuss E, Galli R, Heins N, Götz M (2001) Characterization of CNS precursor subtypes and radial glia. Dev Biol 229:15-30.

Hockfield S, McKay RD (1985) Identification of major cell classes in the developing mammalian nervous system. J Neurosci 5:3310-3328.

Hogan B, Beddington R, Costantini F, Lacy E (1994) Manipulating the mouse embryo: a laboratory manual. Plainview, NY: Cold Spring Harbor Laboratory.

Huber G, Matus A (1984) Differences in the cellular distribution of two microtubule-associated proteins MAP1 and MAP2 in rat brain. J Neurosci 4:151-160.

Iacobas DA, Iacobas S, Urban-Maldonado M, Scemes E, Spray DC (2008) Similar transcriptomic alterations in Cx43 knock-down and knock-out astrocytes. Cell Commun Adhes 15:195-206.

Itoh M, Nagafuchi A, Moroi S, Tsukita S (1997) Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to α catenin and actin filaments. J Cell Biol 138:181-192.

Kriegstein AR, Götz M (2003) Radial glia diversity: a matter of cell fate. Glia 43:37-43.

Lin JH, Takano T, Cotrina ML, Arcuino G, Kang J, Liu S, Gao Q, Jiang L, Li F, Lichtenberg-Frate H, Haubrich S, Willecke K, Goldman SA, Nedergaard M (2002)

Connexin43 enhances the adhesivity and mediates the invasion of malignant glioma cells. J Neurosci 22:4302-4311.

Lin R, Warn-Cramer BJ, Kurata WE, Lau AF (2001) v-Src phosphorylation of connexin43 in Tyr247 and Tyr65 disrupt gap junctional phosphorylation. J Cell Biol 154:815-827.

Lo Turco JJ, Kriegstein AR (1991) Clusters of coupled neuroblasts in embryonic neocortex. Science 252:563-566.

Maass K, Ghanem A, Kim JS, Saathoff M, Urschel S, Kirfel G, Grümmer R, Kretz M, Lewalter T, Tiemann K, Winterhager E, Herzog V, Willecke K (2004) Defective epidermal barrier in neonatal mice lacking the C-terminal region of coneixn43. Mol Biol Cell 15:4597-4608.

Malatesta P, Hack MA, Hartfuss E, Kettenmann H, Klinkert W, Kirchhoff F, Götz M (2003) Neuronal or glial progeny: regional differences in radial glia fate. Neuron 37:751-764.

McLeod TL, Bechberger JF, Naus CC (2001) Determination of a potential role of the CCN family of growth regulators in connexin43 transfected C6 glioma cells. Cell Commun Adhes 8:441– 445.

Mizuno H, Hirano T, Tagawa Y (2007) Activity-dependent cortical wiring: Formation of interhemispheric connections in neonatal mouse visual cortex requires projection neuron activity. J Neurosci 27:6760-6770.

Mizutani K, Saito T (2005) Progenitors resume generating neurons after temporary inhibition of neurogenesis by notch activation in the mammalian cerebral cortex. Development 132:1295-1304.

Moorby CD (2000) A connexin43 mutant lacking the carboxyl cytoplasmic domain inhibits both growth and motility of mouse 3T3 fibroblasts. Mol Carcinog 28:23-30.

Nadarajah B, Jones AM, Evans WH, Parnavelas JG (1997) Differential expression of connexins during neocortical development and neuronal circuit formation. J Neurosci 17:3096-3111.

Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstein AR (2001) Neurons derived from radial glial cells establish radial units in neocortex. Nature 409:714-720.

Noctor SC, Martínez-Cerdeño V, Kriegstein AR (2008) Distinct behaviors of neural stem and progenitor cells underlie cortical neurogenesis. J Comp Neurol 508:28-44.

Ohtsuka T, Ishibashi M, Gradwohl G, Nakanishi S, Guillemot F, Kageyama R (1999) Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. EMBO J 18:2196-2207.

Paquin A, Barnabé-Heider F, Kageyama R, Miller FD (2005) CCAAT/enhancer-binding protein phosphorylation biases cortical precursors to generate neurons rather than astrocytes *in vivo.* J Neurosci 25:10747-10758.

Perez-Moreno M, Jamora C, Fuchs E (2003) Sticky business: orchestrating cellular signals at adherence junctions. Cell 112:535-548.

Perez Velazquez JL, Frantseva M, Naus CC, Bechberger JF, Juneja SC, Velumian A, Carlen PL, Kidder GM, Mills LR (1996) Development of astrocytes and neurons in cultured brain slices from mice lacking connexin43. Brain Res Dev Brain Res 97:293-296.

Reaume AG, de Sousa PA, Kulkarni S, Langille BL, Zhu D, Davies TC, Juneja SC, Kidder GM, Rossant J (1995) Cardiac malformation in neonatal mice lacking connexin43. Science 267:1831-1834.

Redila VA, Olson AK, Swann SE, Mohades G, Webber AJ, Weinberg J, Christie BR (2006) Hippocampal cell proliferation is reduced following prenatal ethanol exposure but can be reduced with voluntary exercise. Hippocampus 16:305-311.

Simon AM, Goodenough DA (1998) Diverse functions of vertebrate gap junctions. Trends Cell Biol 8:477-483.

Tabata H, Nakajima K (2002) Neurons tend to stop migration and differentiate along the cortical internal plexiform zones in the reelin signal-deficient mice. J Neurosci Res 69:723-730.

Theis M, de Wit C, Schlaeger TM, Eckardt D, Krüger O, Döring B, Risau W, Deutsch U, Pohl U, Willecke K (2001) Endothelium-specific replacement of the connexin43 coding region by a lacZ reporter gene. Genesis 29:1-13.

Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, Bock R, Klein R, SchützG (1999) Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat Genet 23:99-103.

Van Rijen HV, Eckardt D, Degen J, Theis M, Ott T, Willecke K, Jongsma HJ, Opthof T, de Bakker JM (2004) Slow conduction and enhanced anisotropy increase the propensity for ventricular tachyarrhythmias in adult mice with induced deletion of connexin43. Circulation 109:1048-1055.

Wiencken-Barger AE, Djukic B, Casper KB, McCarthy KD (2007) A role for connexin43 during neurodevelopment. Glia 55:675- 686.

Xu X, Li WE, Huang GY, Meyer R, Chen T, Luo Y, Thomas MP, Radice GL, Lo CW (2001) Modulation of mouse neural crest cell motility by N-cadherin and connexin43 gap junctions. J Cell Biol 154:217-230.

Xu X, Francis R, Wei CJ, Linask KL, Lo CW (2006) Connexin43-mediated modulation of polarized cell movement and the directional migration of cardiac neural crest cells. Development 133:3629-3639.

Zhuo L, Theis M, Alvarez-Maya I, Brenner M, Willecke K, Messing A (2001) hGFAP-Cre transgene mice for manipulation of glial and neuronal function in vivo. Genesis 31:85-94.

Chapter 4

Essential Region of the Cytoplasmic C-terminal Domain of Connexin43 in Neuronal Migration

A version of this chapter will be submitted to a suitable journal; Cima Cina, Wuy C. Sin, John F. Bechberger, and Christian C. Naus. Essential Region of the Cytoplasmic C-terminal Domain of Connexin43 in Neuronal Migration.

4.1 Introduction

During development, radial glial cells give rise to major cell types in the cerebral cortex, including neurons, astrocytes, and oligodendrocytes (Malatesta et al., 2003; Noctor et al., 2008). Radial glial cells originate in the ventricular zone (VZ) of the dorsal telencephalon and following extensive divisions they produce progenitor cells that give rise to neurons. Young neurons closely associate with their parent, radial glial cells while migrating from the VZ to their final location in the cortical plate (CP) of the neocortex (Anthony et al., 2004).

Originally, Nadarajah and co-workers observed that gap junctions are expressed in the VZ, the IZ and the CP of the developing neocortex while mediating contacts between migrating neurons and the radial glial fibers (Nadarajah et al., 1997). Gap junctions are large diameter channels that are formed by docking of two connexons between adjacent cells. The protein subunits of these connexons are called connexins (Cx) (Goodenough and Paul, 2003). Gap junctions have been implicated in a variety of developmental events including proliferation, differentiation, and migration (Weissman et al., 2004; Elias et al., 2007; Xu et al., 2006). In agreement with these findings, we have shown that two gap junction proteins, Cx43 and Cx26, are extensively expressed in both radial glia and migrating neurons suggesting their role in neuronal migration (Cina et al., 2007). Our detailed analysis of embryonic neocortical cell migration in conditional knockout (*Cx43cKO*), and complete knockout (*Cx43KO*) mice *in vivo*, has shown that the C-terminal domain of Cx43 plays a functional role in radial guided neuronal migration during neocortical development (Cina et al., 2009). Furthermore, in support of our work, an *in vitro* study conducted in our laboratory revealed that deletion of the C-terminal domain of Cx43 modulates the mobility of adherent cancer cells (Bates et al., 2007).

Increasing lines of evidence has confirmed that many cytosolic proteins interact with the C-terminal domain of Cx43. These interacting proteins have already been shown to regulate Cx43 in assembly, trafficking and turnover, formation of gap junction plaques, and insertion of Cxs at the membrane. These interacting proteins include: NOV/CCN3, cytoskeletal elements, kinases, phosphatases, and junctional proteins (Giepmans and Moolennar, 1998; Berthoud et al, 2000; McLeod et al., 2001). In addition, the C-terminal domain of Cx43 modulates a variety of cellular events via interacting proteins. For example, in a study by Xu et al. (2006), Cx43 KO cardiac neural crest cells exhibited reduced

migration due to a disruption in the organization of actin stress fibers compared to the wild-type cells. This study indicates the effect of gap junctions on the cytoskeletal arrangements.

In the present study, to determine the specific region of the C-terminal domain of Cx43 that is involved in the neuronal migration, different deletions were made within the C-terminal region to rescue neuronal migration in *Cx43cKO* and *Cx43KO* mice. Based on our results, we suggest that the region between amino acid residues 259-305 located within the C-terminal domain of Cx43 is necessary to promote neuronal migration. These results have implications for understanding the spectrum of human migration disorders.

4.2 Materials and Methods

4.2.1 Animals

The production of the Cx43 null mutant (Reaume et al., 1995), *nestin–Cre* (Bérubé et al., 2005), and floxed Cx43 (Theis et al., 2001) transgenic mice have been reported previously. *Nestin–Cre* mice were crossed with floxed Cx43 mice, maintained in an animal facility with a 12 h light/dark cycle, and were provided food and water *ad libitum*. The animals were maintained according to Canadian Council on Animal Care guidelines for the care and use of laboratory mice at the University of British Columbia. After mating of adult mice, the day on which a vaginal plug was identified was considered embryonic day 0 (E0). In the present study, *Cx43cKO* (*Cx43*$^{fl/fl}$*; nestin–Cre*), control (*Cx43*$^{fl/fl}$), *Cx43*$^{-/-}$, and *Cx43*$^{+/+}$ mice were used to conduct experiments.

4.2.2 Genotype Analysis by PCR

For detection of the *Cx43* floxed (*Cx43*^{fl}) allele and the *Cx43* wild type (*Cx43*$^{+}$) allele, forward primers (5'-TCA TGC CCG GCA CAA GTG AGA C-3') and reverse primers (5'-TCA CCCCAAGCTGACTCAACCG-3') were applied to generate a product of a 1 kb floxed amplicon and a 900 bp wild-type amplicon (Theis et al., 2001). To detect the deleted *Cx43* (*Cx43* $^{-}$) allele, forward primers (5'-AT TTT GCC GCC GCC TAG CTA TCC C-3') and reverse primers (5'-GCT TGC CGA ATA TCA TGG TGG A-3') were used to generate a product of 1 kb for the deleted amplicon and 500 bp for the wild-type amplicon (Perez Velazquez et al., 1996). For the detection of the *nestin–Cre* transgene, forward primers (5'-TGA CCA GAG TCA TCC TTA GCG-3') and reverse primers (5'-AATGCTTCTGTCCGTTTGCC-3') were applied, generating a 300–500 bp product (Tronche et al., 1999).

4.2.3 Plasmid Constructs

Plasmids containing rat Cx43 cDNA were generated by PCR cloning with the following primers: Cx43Δ305: AATTGAATTCACCATGGTGACTGGAGTGCCTTGG (sense), AATTTCTAGATTAGCTAGCTTGCTTGTAATTG (antisense); Cx43Δ376: AATTGAATTCACCATGGTGACTGGAGTGCCTTGG (sense), AATTTCTAGATTAAGGCCTGCTGCTGGCGCGG (antisense), and inserted into the

pEGFP-N1 expression vector under the control of a SV40 promoter using the *Xho1* and *ECoR1* restriction sites.

4.2.4 Cell Culture and Transfection

Mouse fibroblast Cx43 null cells (partially immortalized astrocytes with a fibroblast phenotype; Valiunas et al., 2005), and Hela cells [with no Cx43 expression, (American Type Culture Collection; Manassas, VA)] were used. Cells were maintained in DMEM (Invitrogen Corp., Burlington, Ontario, Canada), supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin, 100 µg/ml penicillin (Sigma-Aldrich, St. Louis, MO). Cells were subcultured two times per week and maintained in a 37°C incubator in a moist atmosphere with 10% CO_2. One day before transfection, 1 x 10^6 cells were seeded per 35–mm dish in DMEM. For generation of Cx43Δ305 or Cx43Δ376 expressing cells transiently, cells were transfected with 2 µg of each plasmid using LipofectAMINE 2000 (Invitrogen) in 200 µl of OPTIMEM (Invitrogen). After 72 hour, cells were fixed using 4% paraformaldehyde and were subsequently processed for immunostaining.

4.2.5 Protein Isolation and Western blot Analysis

Western blot analysis was conducted as described previously (Cina et al., 2009). Briefly, cells were lysed in radioimmune precipitation lysis buffer (50 mM Tris-HCl, pH 8.0, and 1% IGEPAL) supplemented with Mini Complete protease inhibitors (Roche Applied Science) and phosphatase inhibitors (Sigma). Protein concentrations were determined using the BCA protein quantification kit (Pierce). Protein samples (30 µg) were boiled for 2 min in SDS sample buffer, pH 8.0, and separated on a 12% polyacrylamide gel in parallel with molecular weight markers (Bio-Rad). Subsequently, the electrophoresed proteins were transferred onto a nitrocellulose membrane (Bio-Rad) at 100 V for 1 h. The blots were then blocked with 5% dry milk in TBS (pH 7.4, with 1% Tween 20) for 1 h and subsequently incubated with an antibody against Cx43 (N-terminal; Abgent) at 4°C overnight. After rinsing with PBS, the blots were incubated in horseradish peroxidase-tagged anti-rabbit secondary antibody (Cedarlane Laboratories) for 1 h at room temperature, followed by incubation in SuperSignal chemiluminescent substrate (Pierce). The labelled blots were then exposed to Kodak X-Omat x-ray film to visualize antibody binding. To ensure equal loading

of protein samples, the blots were stripped of their Cx43 antibody and reprobed for GAPDH (mouse, 1:20,000 dilution; Cedarlane Laboratories).

4.2.6 Immunocytochemistry

Brains obtained from embryonic day 18 mice were fixed by immersion in 4% paraformaldehyde overnight at 4^{o}C. The fixed brains were cryoprotected in 30% sucrose, mounted in OCT, and cut into 10 µm coronal sections using a cryostat. Cut brain sections were mounted in ProLong Gold containing DAPI. For cell cultures, cells were grown on coverslips, fixed in 4% formaldehyde at room temperature for 10 min, and permeabilized with 0.3% Triton X-100. Cells were incubated with rabbit polyclonal antibodies for wild-type Cx43 (Nadarajah et al., 1997) (1:200; epitope spanning amino acids 363–382 located at the C-terminal region of Cx43; Sigma-Aldrich), or a mouse monoclonal antibody for wild-type Cx43 (1:100; epitope spanning amino acids 252-270 located at the C-terminal region of Cx43; Chemicon), at 4°C overnight. Labelled sections and cells were examined and analyzed using a Zeiss Axioplan2 epifluorescent microscope and AxioVision 4.2 software.

4.2.7 *In utero* electroporation

A plasmid expressing GFP driven by a neuronal promoter (Tα1 tubulin) (gift from Dr. Freda D. Miller, University of Toronto, Toronto, Ontario, Canada) (Gloster et al., 1994) was used either alone or with each of the following plasmids: (1) C-terminally truncated Cx43 (*Cx43*Δ*305–382*); (2) C-terminally truncated Cx43 (*Cx43*Δ*376–382*); and (3) C-terminally truncated Cx43 (*Cx43*Δ*258-382*), in which the last 125 amino acid residues of the cytoplasmic C-terminal domain of Cx43 are lacking (Maass et al., 2004) (a gift from Dr. Karen Maass, Upstate Medical University, Syracuse, NY). The introduction of DNA by *in utero* electroporation was conducted as described previously (Cina et al., 2009). Briefly, pregnant female *Cx43*$^{-/-}$ mice and *Cx43cKO* mice were anesthetized with isoflurane and nitrous oxide. A 3-cm incision was made to expose the embryos, 1 µl of DNA was injected into the lateral ventricle of E14 embryos. Following injection, electroporation was performed using a square electroporator CUY21 EDIT (Protech) to deliver five 50 ms pulses of 50 V with 950 ms intervals per embryo. Embryos were returned to the uterus, and the incision was closed. The embryos were allowed to develop further until E18. The brains were removed

and fixed in 4% paraformaldehyde at 4^{o}C overnight. The fixed brains were placed in 30% sucrose for cryoprotection, mounted in OCT, sectioned (10 μm), and analyzed.

4.3 Results

In our previous study, we showed that the injection of Cx43Δ244-382 into the VZ of the *Cx43cKO* embryos did not rescue migration. In addition, neurons in *Cx43K258stop* embryos also failed to move into the CP. However, the delivery of full-length Cx43 was able to restore normal migration. Although, we concluded that the C-terminal domain of Cx43 was crucial in neuronal migration (Cina et al., 2009), the specific region of the C-terminal domain that is involved in migration remains to be unknown. Hence, in the present study, two deletions were made within the middle and the end part of the C-terminal domain between amino acid residues 258-382 in order to determine the important region that rescues neuronal migration. These two deletions include Cx43Δ305-382 and Cx43Δ376-382.

4.3.1 Mouse Fibroblast Cx43 Knockout Cells Express Cx43Δ305-382 and Cx43Δ376-382

Prior to using Cx43 deletion constructs for *in utero* electroporation, they were first tested in cultured cells. To determine the expression of Cx43Δ305-382 and Cx43Δ376-382 plasmids in Cx43 knockout mouse fibroblast cells, immunofluorescent labelling experiments using a monoclonal Cx43 antibody which recognizes amino acids 252-270 in the Cx43Δ305-382 construct (Fig. 4.1B), and a polyclonal Cx43 antibody which recognize amino acids 363-382 in the Cx43Δ376-382 construct (Fig. 4.1C), were conducted. As shown in Figure 4.1, the images revealed that while control (untransfected) cells were devoid of labelling (Fig. 4.1A), in transfected cells, Cx43Δ305-382 and Cx43Δ376-382 were localized at the plasma membrane as well as the perinuclear regions (Fig. 4.1B,C**).**

4.3.2 Hela Cells Express Cx43-truncated Plasmid cDNAs

To determine the cellular localization of Cx43Δ305-382 and Cx43Δ376-382 in Hela cells, we performed immunofluorescent labelling experiments using antibodies against Cx43 (Fig. 4.2). As depicted in Figure 4.2A, control Hela cells showed no immunoreactivity associated with Cx43 when stained with a Cx43 antibody, however residual background staining was observed in these cells. By contrast, Cx43 imunoreactivity was seen in the plasma membrane and perinuclear region of transfected cells (Fig. 4.2B,C). In addition, western blot analysis was conducted on cells transfected with Cx43Δ305-382 or Cx43Δ376-

382 plasmids. Protein lysates from transfected Hela cells, and E18 cortices of wild type and *Cx43*$^{k258stop/+}$ mice (positive controls), were probed with anti-Cx43 and GAPDH antibodies. Cx43 protein was strongly expressed in the aforementioned lysates except for the untransfected cells (negative control) (Fig. 4.2D).

Immunofluorescent labelling experiments with Cx43 antibodies as well as western blot analysis confirmed deletion and specificity of Cx43 in both cell lines transfected with Cx43Δ305-382 and Cx43Δ376-382 plasmids. Hence the aforementioned constructs were used in the following study to determine the mechanism by which Cx43 is involved in neuronal migration.

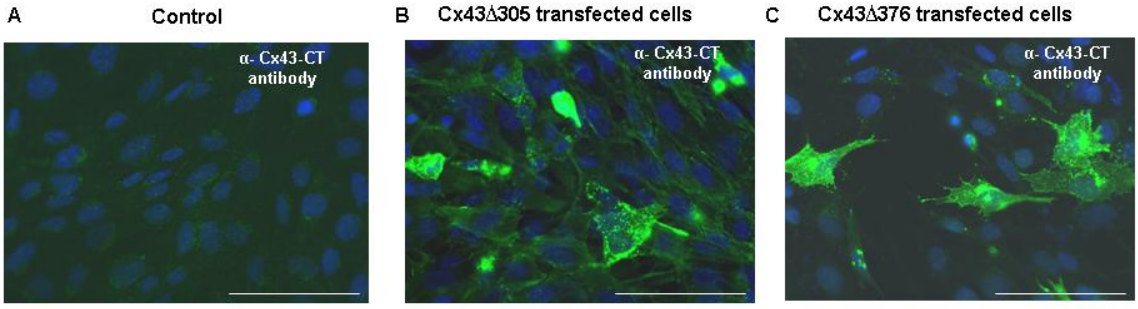
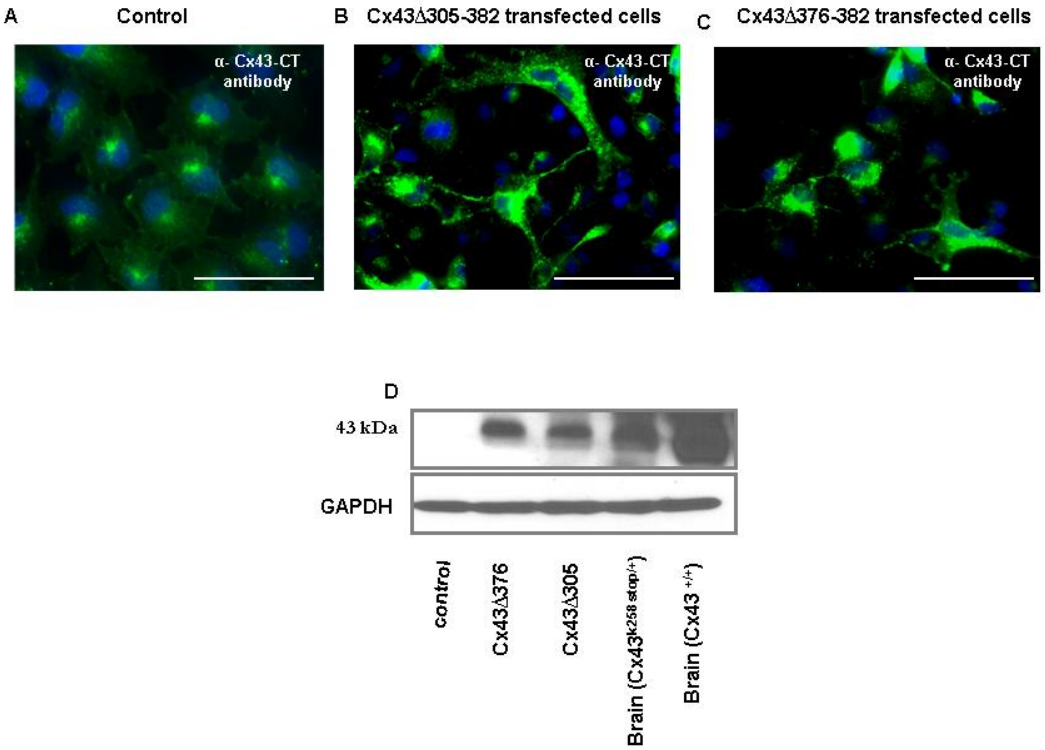


Figure 4.1 Expression of Cx43Δ305-382 and Cx43Δ376-382 in Cx43 knockout mouse fibroblast cells. **A,** In control cells immunofluorescent labelling using an antibody against Cx43 showed no labelling. By contrast, Cx43Δ305-382, and Cx43Δ376-382 transfected cells showed immunoreactivity for Cx43 located at the plasma membrane and the perinuclear region. Expression is shown by green fluorescence (**B,C**). Nuclei are visualized by DAPI (Blue). n=3. Scale bars, 40 µm.

Figure 4.2 Cx43Δ305-382 and Cx43Δ376-382 are expressed in Hela cells. **A,** In control cells, immunofluorescent labelling using an antibody against Cx43 showed no labelling. However, Hela cells transfected with Cx43Δ305-382, and Cx43Δ376-382 showed immunorectivity for Cx43 localized at the plasma membrane and the perinuclear region. Expression is shown by green fluorescence (**B,C**). Nuclei are visualized by DAPI (Blue). **D,** A representative immunoblot showing protein expression of Cx43 in transfected Hela cells at the predicted size. Equal amounts of protein lysates were immunoblotted and probed with antibodies recognizing Cx43 (43kDa). Untransfected Hela cells were used as a negative control. Protein lysates from E18 cortices of wild type and *Cx43*$^{k258stop/+}$ mice were used as positive controls. GAPDH was used as a loading control. n=3. Scale bars, 40 μm.

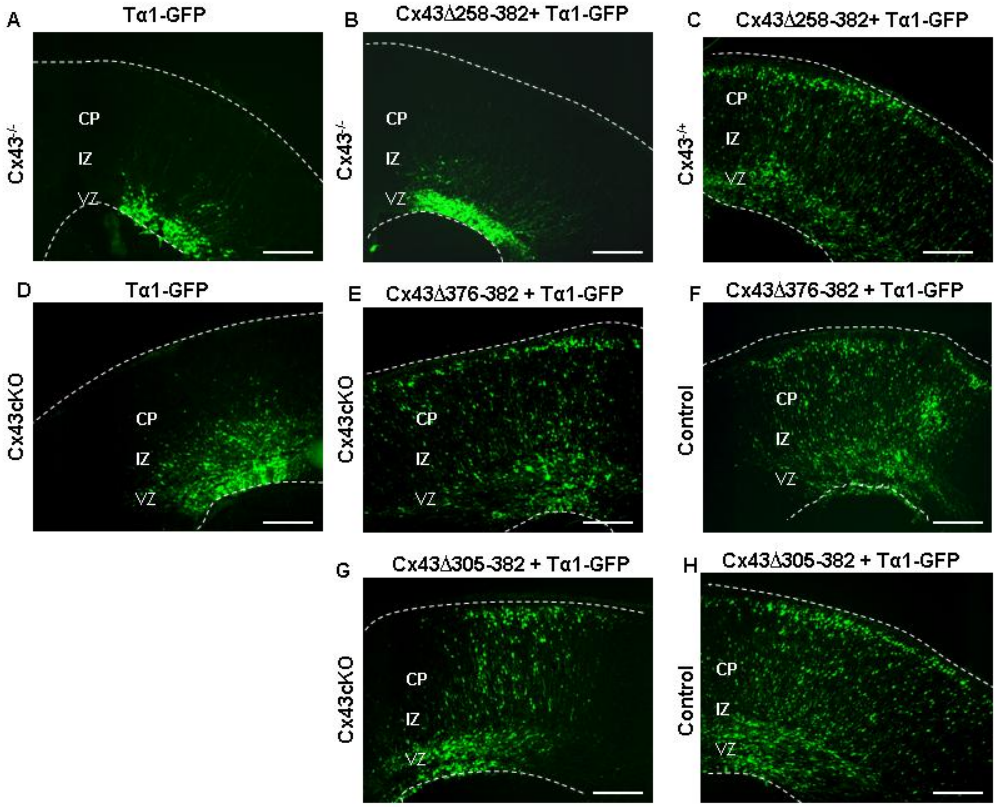

A
Control
α- Cx43-CT antibody
B
Cx43Δ305-382 transfected cells
α- Cx43-CT antibody
C
Cx43Δ376-382 transfected cells
α- Cx43-CT antibody
D
43 kDa
GAPDH
control
Cx43Δ376
Cx43Δ305
Brain (Cx43k258 stop/+)
Brain (Cx43 +/+)

4.3.3 A Central Region in the C-terminal Domain of Cx43 is Required for Neuronal Migration

We have previously shown that Cx43 plays a vital role in neuronal migration. Furthermore, the C-terminal region appears necessary for this migration to occur (Cina et al., 2009). Therefore, in the present study, to further examine the contribution of the C-terminal domain of Cx43 in neuronal migration, specific deletions were made within the C-terminal domain including Cx43Δ258-382, Cx43Δ305-382, and Cx43Δ376-382 plasmids. The aforementioned plasmids were co-injected with Tα1-GFP (a transgene expressing GFP under control of Tα1 tubulin promoter; Gloster et al., 1999) into the lateral ventricle of E14 control, *Cx43cKO*, *Cx43*$^{-/-}$ and *Cx43*$^{+/-}$ mice, and electroporated as previously described (Cina et al., 2009). Subsequently embryos were analyzed at E18. The data revealed that when the Tα1-GFP plasmid alone was electroporated into the lateral ventricle of E14 *Cx43*$^{-/-}$ mice, the vast majority of the Tα1-GFP-expressing neurons remained in the VZ/IZ at E18 (Fig. 4.3A). In addition, in *Cx43*$^{-/-}$ mice, while newborn neurons electroporated with Cx43Δ258-382 plasmid co-expressed with Tα1-GFP failed to migrate to the CP, neuronal migration in Cx43$^{+/-}$ appeared normal (Fig. 4.3B,C). Furthermore, E14 cortices of *Cx43cKO* embryos were electroporated with the Tα1-GFP plasmid, and embryos were analyzed four days later. The results showed that most GFP^{+} cells accumulated in the IZ (Fig. 4.3 D). Interestingly, when either Cx43Δ305-382 or Cx43Δ376-382 plasmids was co-injected with Tα1-GFP and introduced into the developing cortex of E14 *Cx43cKO* mice, neuronal migration was restored to levels seen in control mice (Fig. 4.3E-H).

Because exogenous expression of Cx43Δ305-382 or Cx43Δ376-382, but not the Cx43Δ258-382, in newborn neurons of Cx43cKO restores neuronal migration, our study suggests that the region between amino acid residues 259 and 305 located at the C-terminal domain of Cx43 is sufficient to mediate the action of Cx43 in neuronal migration. In addition, since Cx43Δ376-382-expressing neurons were able to migrate to their final location in the CP, deletion of the last four amino acids of the C-terminal which are the binding sites for interaction with ZO-1 does not have an affect on neuronal migration during cortical development.

Figure 4.3 C-terminal region is necessary for the proper function of Cx43 during neocortical neuronal migration. At E14, Tα1-GFP was injected alone or co-injected with Cx43Δ258-382, Cx43Δ305-382, or CxΔ376-382 into the lateral ventricle of *Cx43cKO*, control, *Cx43*$^{-/-}$, and *Cx43*$^{+/-}$ mice, followed by electroporation (**A**-**H**). Four days later, the electroporated brains were removed, 10 µm sections of cerebral cortices were collected, and subsequently the GFP^{+} cells were visualized under an epifluorescent microscope. Although, in *Cx43*$^{-/-}$ mice, newborn neurons electroporated with Tα1-GFP and Cx43Δ258-382 plasmid failed to migrate to the CP (**A,B**), Cx43$^{+/-}$ mice showed normal neuronal migration (**C**). In Cx43cKO embryos, neurons electroporated with Tα1-GFP alone failed to migrate to the CP (**D**), whereas electroporation of either Cx43Δ305-382, or Cx43Δ376-382 plasmids along with Tα1-GFP in *Cx43cKO* mice rescued neuronal migration to levels seen in control mice (**E**-**H**). n=3. Scale bars, 200 µm.


A
Tα1-GFP
Cx43-/-
CP
IZ
VZ
B
Cx43Δ258-382+ Tα1-GFP
Cx43-/-
CP
IZ
VZ
C
Cx43Δ258-382+ Tα1-GFP
Cx43-/+
CP
IZ
VZ
D
Tα1-GFP
Cx43cKO
CP
IZ
VZ
E
Cx43Δ376-382 + Tα1-GFP
Cx43cKO
CP
IZ
VZ
F
Cx43Δ376-382 + Tα1-GFP
Control
CP
IZ
VZ
G
Cx43Δ305-382 + Tα1-GFP
Cx43cKO
CP
IZ
VZ
H
Cx43Δ305-382 + Tα1-GFP
Control
CP
IZ
VZ

4.4 Discussion

Although increasing evidence indicates that gap junctions mediate the radial migration of neurons to the CP (Cina et al., 2009; Wiencken-Barger et al., 2007; Elias et al., 2007; Fushiki et al., 2003), the exact mechanism behind this activity is still in question. Following our recent work in 2009, which led us to report that the C-terminal domain of Cx43 is crucial in cell migration, we have further investigated this finding in more detail by making specific deletions within the C-terminal tail and using two transgenic mouse lines: *Cx43*$^{-/-}$ mice in which Cx43 is lacking from all cell types (Reaume et al., 1995), and Cx43cKO mice which allow selective deletion of Cx43 only in radial glial cells (Béurbé et al., 2005). The current work presents further progress in understanding the action of Cx43 in modulating the movement of newborn neurons that derive from radial glial cells, and suggests a region in the C-terminal domain of Cx43 containing amino acid residues 259–305 might be an important factor in mediating the role of Cx43 in neuronal migration.

4.4.1 A Crucial Region in the C-terminal Domain of Cx43 Mediates Neuronal Migration

In the present study, electroporetic delivery of Cx43Δ258-382 plasmid into the VZ of *Cx43*$^{-/-}$ embryos failed to restore neuronal migration into the CP. This finding was supported by our previous data showing that newborn neurons in *Cx43*$^{K258stop/-}$ embryos also failed to move into the CP normally (Cina et al., 2009).

Furthermore, similar delivery of Cx43 with the C-terminal truncated versions of Cx43 plasmids, Cx43Δ305-382 or Cx43Δ376-382, was able to rescue migration in *Cx43cKO* mice. With these results, we were able to narrow down the region that is involved in regulating neuronal migration to an area between amino acid residues 259 and 305 located within the C-terminal domain of Cx43. However, it remains to be determined exactly what proteins interact with this region of the C-terminal domain in order to implicate Cx43 in neuronal migration.

The C-terminal domain is located in the cytosol, and this allows for the interaction of this domain with Cx43 interacting proteins that link Cx43 to cytoskeletal anchorage, and therefore support in regulating neuronal migration. These proteins include F-actin, CCN3/NOV, N-cadherins, kinases, and phosphatases (Herve et al., 2007; Fu et al., 2004;

McLeod et al., 2001; Xu et al., 2001). For example, it has been shown that overexpression of Cx43 in C6 glioma cells causes a rearrangement of actin into the stress fibers so that the cells become flattened (Naus et al., 1992). In addition, disorganization of actin stress fibers has been observed in Cx43KO cardiac neural crest cells (Xu et al., 2006). Furthermore, direct association of several actin binding proteins with Cx43 has been reported in a number of studies. For instance, α-actinin which is a binding protein interacts with and links Cx43 to the actin cytoskeleton. It has also been shown to be present at the leading edge of cardiac neural crest cells meaning that it is involved in cell migration (Xu et al. 2006). Another binding protein, known as, cortactin has been shown to interact with the C-terminal domain of Cx43. The phosphorylation of Cx43 by P38 MAPK leads to the activation of cortactin at the leading edge which in turn increases the cell motility (Squecco et al., 2006). Therefore, in the present study, it is possible that during development, the C-terminal domain of Cx43 reorganizes the actin cytoskeleton via interacting with the binding proteins that are localized at the leading edge of the migrating neurons and enhances neuronal migration.

Moreover, the majority of evidence supports the finding that protein kinase Src interacts directly with and phosphorylates Cx43 through SH2 and SH3 domains that are located along the C-terminal amino acid residues 265–283 (Loo et al., 1999; Kanemitsu et al. 1997).

Inhibition of Src has been shown to affect glioma cell invasion by regulating actin cytoskeleton at the leading edge (Angers-Loustau et al., 2004). In that study, the authors indicated that the phosphorylation of the cortactin is required to activate this protein thus inducing the membrane ruffling. Therefore, inhibition of Src could reduce the invasiveness of the glioma cells by inactivating the actin dynamics. Intriguingly, SH2 and SH3 binding sites are located along the region of the C-terminal domain that has been reported in this study to be an important factor in neuronal migration (amino acid residues 259–305). Based on these findings, we hypothesize that Src could link Cx43 to the actin cytoskeleton via interaction with the C-terminal domain, and thereby influence neuronal migration. To test this hypothesis, point mutations in the SH2 and SH3 binding sites and their targets that are located within the C-terminal domain might be potential experimental candidates in order to test the effect of Src tyrosine kinase on neuronal migration. Through collaborating with Dr. Tabernero's laboratory (The University of Salamanca, Spain), our lab has access to a double

Cx43 mutant construct containing the Tyr247 and Tyr65 residues in which tyrosine is substituted by non-phosphorylatable phenylalanine. This construct is prepared in a pIRES2-DsRed2 vector that encodes the red fluorescent protein. To test whether this construct rescues neuronal migration, it could be injected into the lateral ventricle of E14 *Cx43*$^{-/-}$ or *Cx43cKO* embryos followed by electroporation.

In addition to Src tyrosine kinase, mitogen activated kinase (MAPK) phosphorylates Cx43 and down regulates its activity. MAPK phosphorylates Cx43 on its three serine residues, Ser255, Ser279, and Ser282 (Kanemitsu and Lau, 1993). The authors showed that epidermal growth factor (EGF) treatment of rat liver cells activated EGF receptors on the cell membrane which stimulated MAPK. The stimulation of MAPK led to the phosphorylation of Cx43 which in turn diminished the assembly and the activity of Cx43. Interestingly, the amino acid residues Ser279 and Ser282 are located within the same region of the C-terminal domain of Cx43 that has been reported to be involved in neuronal migration in the present study (amino acid residues 259-305). Therefore, MAPK might play a role in regulating neuronal migration by phosphorylating Cx43 on its C-terminal domain.

Protein kinase C (PKC) has also been shown to phosphorylate Cx43 both *in vitro* and *in vivo* (Reynhout et al, 1992; Lampe et al., 2000). These studies showed that PKC activators such as 12-O-tetradecanoylphorbol-13-acetate (TPA) activate PKC which in turn phosphorylates Cx43 on its serine residue, Ser368 within the C-terminal domain. This phosphorylation decreases the gap junction assembly and activation. Also, the results of the present study indicate that Cx43Δ376-382 construct was able to rescue neuronal migration in *Cx43cKO* mice. Therefore, it is possible that PKC affects neuronal migration by phosphorylating Cx43 in its Ser368 residue located within the C-terminal domain.

In contrast to PKC, protein kinase A (PKA) up regulates Cx43 assembly. PKA directly acts on Ser364 within the C-terminal domain and phosphorylates Cx43 thus inducing Cx43 activity (Tenbroek et al., 2001). However, the role of PKA in cell migration via interaction with the C-terminal domain of Cx43 remains to be elucidated.

A better understanding of these mechanisms that are important for regulating neuronal migration could assist the study of a number of neurodevelopmental malformations.

4.5 References

Angers-Loustau A, Hering R, Werbowetski TE, Kaplan DR, Del Maestro RF (2004) SRC regulates actin dynamics and invasion of malignant glial cells in three dimensions. Mol Cancer Res 2:595-605.

Anthony TE, Klein C, Fishell G, Heintz N (2004) Radial glia serve as neuronal progenitors in all regions of the central nervous system. Neuron 41:881-890.

Bates DC, Sin WC, Aftab Q, Naus CC (2007) Connexin43 enhances glioma invasion by a mechanism involving the carboxy terminus. Glia 55:1554-1564.

Bérubé NG, Mangelsdorf M, Jagla M, Vanderluit J, Garrick D, Gibbons RJ, Higgs DR, Slack RS, Picketts DJ (2005) The chromatin-remodeling protein ATRX is critical for neuronal survival during corticogenesis. J Clin Invest 115:258-267.

Berthoud VM, Westphale EM, GrigoryevaA, Beyer EC (2000) PKC isoenzymes in the chicken lens and TPA-induced effects on intercellular communication. Invest Ophtalmol Vis Sci 41:850-858.

Cina C, Bechberger JF, Ozog MA, Naus CCG (2007) Expression of connexins in embryonic mouse neocortical development. J Comp Neurol 504:298-313.

Cina C, Maass K, Theis M, Willecke K, Bechberger JF, Naus CC (2009) Involvement of the cytoplasmic C-terminal domain of connexin43 in neuronal migration. J Neurosci 29:2009-2021.

Elias LA, Wang DD, Kriegstein AR (2007) Gap junction adhesion is necessary for radial migration in the neocortex. Nature 448:901-907.

Fu CT, Bechberger JF, Ozog MA, Perbal B, Naus CCG (2004) CCN3 (NOV) interacts with connexin43 in C6 glioma cells. J Biol Chem 279:36943-36950.

Fushiki S, Perez Velazquez JL, Zhang L, Bechberger JF, Carlen PL, Naus CC (2003) Changes in neuronal migration in neocortex of connexin43 null mutant mice. J Neuropathol Exp Neurol 62:304-314.

Gloster A, Wu W, Speelman A, Weiss S, Causing C, Pozniak C, Reynolds B, Chang E, Toma JG, Miller FD (1994) The Tα1 α-tubulin promoter specifies gene expression as a function of neuronal growth and regeneration in transgenic mice. J Neurosci 14:7319–7330.

Gloster A, El-Bizri H, Bamji SX, Rogers D, Miller FD (1999) Early induction of Tα1 α-tubulin transcription in neurons of the developing nervous system. J Comp Neurol 405:45-60.

Goodenough DA, Paul DL (2003) Beyond the gap: functions of unpaired connexon channels. Nat Rev Mol Cell Biol 4:285-294.

Herve JC, Bourmeyster N, Sarrouilhe D, Duffy HS (2007) Gap junctional complexes: From partners to functions. Prog Biophys Mol Biol 94:29-65.

Kanemitsu MY, Loo LW, Simon S, Lau AF, Eckhart W (1997) Tyrosine phosphorylation of connexin 43 by v-Src is mediated by SH2 and SH3 domain interactions. J Biol Chem 272:22824-22831.

Lampe PD, Tenbroek EM, Burt JM, Kurata WE, Johnson RG, Lau AF (2000) Phophorylation of Cx43 on serine 368 by protein kinase C regulates gap junction communication. J Cell Biol 149:1503-1512.

Loo LW, Kanemitsu MY, Lau AF (1999) In vivo association of pp60v-src and the gap junction protein connexin43 in v-src transformed fibroblasts. Mol Carcinog 3:187-195.

Maass K, Ghanem A, Kim JS, Saathoff M, Urschel S, Kirfel G, Grümmer R, Kretz M, Lewalter T, Tiemann K, Winterhager E, Herzog V, Willecke K (2004) Defective epidermal barrier in neonatal mice lacking the C-terminal region of coneixn43. Mol Biol Cell 15:4597-4608.

Malatesta P, Hack MA, Hartfuss E, Kettenmann H, Klinkert W, Kirchhoff F, Götz M (2003) Neuronal or glial progeny: regional differences in radial glia fate. Neuron 37:751-764.

McLeod TL, Bechberger JF, Naus CC (2001) Determination of a potential role of the CCN family of growth regulators in connexin43 transfected C6 glioma cells. Cell Commun Adhes 8:441-445.

Nadarajah B, Jones AM, Evans WH, Parnavelas JG (1997) Differential expression of connexins during neocortical development and neuronal circuit formation. J Neurosci 17:3096-3111.

Naus CC, Zhu D, Todd SD, Kidder GM. (1992) Characteristics of C6 glioma cells overexpressing a gap junction protein. Cell Mol Neurobiol 12:163-175.

Noctor SC, Martínez-Cerdeño V, Kriegstein AR (2008) Distinct behaviors of neural stem and progenitor cells underlie cortical neurogenesis. J Comp Neurol 508:28-44.

Reaume AG, de Sousa PA, Kulkarni S, Langille BL, Zhu D, Davies TC, Juneja SC, Kidder GM, Rossant J (1995) Cardiac malformation in neonatal mice lacking connexin43. Science 267:1831-1834.

Reynhout JK, Lampe PD, Johnson RG (1992) An activator of protein kinase C inhibits gap junction communication between cultured bovine lens cells. Exp Cell Res 198:337-342.

Squecco R, Sassoli C, Nuti F, Martinesi M, Chellini F, Nosi D, Zecchi-Orlandini S, Francini F, Formigli L, Meacci E (2006) Sphingosine 1-phosphate induces myoblast differentiation

through Cx43 protein expression: A role for a gap junction-dependent and -independent function. Mol Biol Cell 17:4896-4910.

Tenbroek EM, Lampe PD, Solan JL, Reynhout JK, Johnson RG (2001) Ser364 of connexin43 and the upregulation of gap junction assembly by cAMP. J Cell Biol 155:1307-1318.

Theis M, de Wit C, Schlaeger TM, Eckardt D, Krüger O, Döring B, Risau W, Deutsch U, Pohl U, Willecke K (2001) Endothelium-specific replacement of the connexin43 coding region by a lacZ reporter gene. Genesis 29:1-13.

Valiunas V, Bechberger JF, Naus CC, Brink PR, and Goldberg GS (2005) Nontransformed cells can normalize gap junctional communication with transformed cells. Biochem Biophys Res Commun 333:174-179.

Wiencken-Barger AE, Djukic B, Casper KB, McCarthy KD (2007) A role for connexin43 during neurodevelopment. Glia 55:675-686.

Weissman TA, Riquelme PA, Ivic L, Flint AC, Kriegstein AR (2004) Calcium waves propagate through radial glial cells and modulate proliferation in the developing neocortex. Neuron 5:647-661.

Xu X, Li WE, Huang GY, Meyer R, Chen T, Luo Y, Thomas MP, Radice GL, Lo CW (2001) Modulation of mouse neural crest cell motility by N-cadherin and connexin43 gap junctions. J Cell Biol 154:217-230.

Xu X, Francis R, Wei CJ, Linask KL, Lo CW (2006) Connexin43-mediated modulation of polarized cell movement and the directional migration of cardiac neural crest cells. Development 133:3629-3639.

Chapter 5

General Discussion and Conclusions

During neocortical development, radial glial cells in the VZ of the dorsal telencephalon undergo mitosis and give rise to neurons, astrocytes**,** and oligodendrocytes (Noctor et al. 2008). The newborn neurons leave the VZ and migrate along the radial glial fibers to reach their final location in the CP, subsequently forming the highly organized six layers of neurons in the adult cerebral cortex (Nadarajah et al., 2001; Anthony et al., 2004).

A central issue in neuronal migration is in understanding the mechanism that regulates the movement of neurons and their interactions with the scaffolding radial glial fibers. Indeed, among the many processes that encode the instructions for cellular events are the delivery of intercellular communication and signaling molecules to the radial glial cells which then orchestrates neocortical brain development. By way of intercellular communication, gap junctions are implicated in cell-cell interaction in almost all tissues and organs. Gap junctions are intercellular membrane channels that provide the exchange of ions and small metabolites less than 1200 Da, such as Na^+, K^+, Ca^{2+}, IP_3, ATP, and cAMP between adjacent cells (Neyton and Trautmann, 1986; Simon and Goodenough, 1998). Gap junctions have been shown to be involved in the proliferation and differentiation of radial glial cells, and migration of neurons (Lo, 1996; Fushiki et al., 2003; Weissman et al., 2004; Elias et al., 2007). For example, embryonic mice lacking Cx43 expression phenotypically present an abnormal accumulation of BrdU–positive cells in the IZ, whereas these cells establish themselves normally in the CP of wild-type littermates (Fushiki et al., 2003).

The targeted deletion of *Cx* genes in mice is undoubtedly a valuable tool to study the role of gap junctions in neuronal migration. The impact of the results obtained from those transgenic mouse models have however been hampered by the fact that most studies investigating the expression of Cxs during development were performed on rats. Therefore, in order to facilitate the interpretation of results obtained from transgenic mice, our first study aimed at mapping the spatio–temporal distribution of various Cxs in the embryonic mouse brain.

5.1 Expression of Connexins in the Embryonic Mouse Neocortex

In order to investigate the spatial and temporal expression of Cxs during brain development, we combined different approaches. Briefly, we performed RT-PCR, immunoblotting, and immunohisotchemistry experiments on E14-E18 mouse neocortex. These experiments showed that among the 20 Cxs expressed in mammals (Söhl and Willecke, 2003), Cx26, Cx36, Cx37, Cx43, and Cx45 are expressed in the E14-E18 mouse neocortex (see chapter 2). Cx26 was detected throughout the whole neocortex with an even distribution in the VZ, the IZ, and the CP. Cx36 was also expressed in the mouse neocortex. However, its expression was higher in the VZ and the CP than in the IZ. Cx37 was evenly distributed in all cortical layers of the mouse neocortex. Surprisingly, our results demonstrated that the distribution pattern of Cx43 was temporally regulated. The distribution of Cx43 changes from an even expression in all cortical layers at E14 to a gradation pattern in the VZ and CP of the E16 and E18 neocortex. Similarly, our immunofluorescent labelling experiments showed that Cx45 expression was also temporally regulated with an overall increase over the E14–E18 period. The higher expression of Cx45 at E18 might be due to the developing oligodendrocytes that express high levels of Cx45. Another possibility is that at the later stages of neuronal migration the expression of Cx45 might be increased in the migrating neurons compared to the immature neurons at early stages of neuronal migration.

Additionally, our immunofluorescent labelling experiments showed that among the five Cxs expressed in the embryonic mouse neocortex, Cx26 and Cx43 were extensively expressed in the VZ and highly associated with the nestin-positive radial glia and MAP-2-positive neurons (see chapter 2; Cina et al. 2007). These results are in agreement with observations previously reported (Nadarajah et al, 1997; Bittman et al., 2002). Therefore, these results suggest that Cx43 and Cx26 might serve an important role in neuronal migration.

The results presented in this thesis uncover the spatio-temporal distribution of the major Cxs expressed during brain development and could potentially serve as a reference guide for future studies examining the effect of Cx deletion in transgenic mice.

Our first study showed that Cx43 is expressed in the radial glia and newborn neurons of the developing neocortex. These results led us to examine whether Cx43 is implicated in neuronal migration. Because Cx43 is ubiquitously expressed in most tissues, the study of its implication in a specific population of cells is complicated. Therefore, the

strength of our second study is that we used three distinct transgenic mouse models to achieve a tissue–specific deletion of Cx43 and investigate its involvement in neuronal migration. These transgenic mice included a $Cx43^{-/-}$ mice lacking Cx43 in all cell types. These mice die shortly after birth due to abnormalities in heart development. However, as mentioned previously, Cx43 is widely expressed in many tissues and the use of an animal model in which the protein is deleted from all cell types is of limited usefulness for attributing the migrational phenotype to only radial glia. Therefore, in order to refine our strategy and results, we also used *Cx43cKO* mice which allow deletion of Cx43 in radial glial cells selectively. Finally, we also used $Cx43^{K258stop/-}$ mice, which lack the last 125 amino acids residues of the C-terminal domain of Cx43, to study the involvement of the C-terminal tail in neuronal migration.

Our results showed that the deletion of Cx43 in *Cx43cKO* mice lead to a greater thickness of the IZ and a concomitant thinning of the CP, therefore suggesting that newborn neurons failed to migrate properly in the CP (see chapter 3). Interestingly, Wiencken-Barger and co-workers have also reported a decreased size of the neocortical, hippocampal and cerebellar area in *Cx43cKO* mice, in which Cx43 was selectively deleted in GFAP expressing cells. The authors attributed these defects to an altered cellular proliferation in the VZ (Wiencken-Barger et al., 2007).

5.2 The C-terminal Region of Cx43 is Crucial for Neuronal Migration

To investigate more precisely the role of Cx43 in radial glial cells, we took advantage of the *in utero* electroporation technique to deliver a transgene expressing GFP under control of Tα1-tubulin promoter (Tα1-GFP). The majority of Tα1-GFP-expressing cells are radial glial cells. The transgene was injected into the lateral ventricle of E14 control, *Cx43cKO,* and *Cx43*$^{-/-}$ mice, followed by *in utero* electroporation. At E18, while in control mice, GFP^{+} cells had migrated out of the VZ and reached the CP, these cells accumulated in the IZ, with very few cells reaching the CP of *Cx43cKO* and *Cx43*$^{-/-}$ mice. Furthermore, injection of a plasmid exogenously expressing the wild-type form of Cx43 in the lateral ventricle of E14 *Cx43cKO,* and *Cx43*$^{-/-}$ mice, restored neuronal migration. Hence, our results demonstrate that Cx43 regulates the migration of cortical neurons in the embryonic brain. Loss of Cx43 in radial glia or newborn neurons abolishes the ability of neurons to migrate along radial glial fibers. As a consequence, neurons remain in the IZ, and are unable to reach their proper destination into the CP (see chapter 3; Cina et al., 2009).

Gap junctional coupling is probably not an important factor in neuronal migration as dye coupling techniques have not detected major differences in gap junctional coupling between wild type and *Cx43cKO* mice (Wiencken-Barger et al., 2007). Therefore, to investigate the mechanism by which Cx43 mediates neuronal migration, we conducted similar electroporetic delivery of a plasmid expressing a C-terminal truncated form of Cx43 (Cx43Δ244-382) into the ventricle of *Cx43cKO* embryos. Our results showed that the truncated version of Cx43 did not rescue migration. Similarly, newborn neurons in *Cx43*$^{K258stop/-}$ embryos failed to reach their final location in the CP, while neuronal migration in *Cx43*$^{K258stop/+}$ embryos was not perturbed. Thus, we concluded that the C-terminal tail of Cx43 has a crucial role in the migration of newborn neurons into the CP (see chapter 3). These findings however are in conflict with the recent work of Elias and co-workers (2007), in which shRNA was used to knock down Cx43 expression in the cerebral cortex of wild-type rats. From their results, the authors concluded that Cx43 and Cx26 form adhesive contact points between radial glial fibers and migrating neurons which facilitate neuronal migration. Their results did not show involvement of the C-terminal tail of Cx43 in migratory mechanisms. The discordance between the two studies can however be explained by the limitations inherent to the experimental design used by Elias and co-workers. By limiting

their knockdown strategy to the sole use of shRNA against Cx43, Elias and co-workers could only target a subset of radial glial cells while the untargeted population of radial glia cells had normal Cx43 expression levels. Consequently, at no point in their study could the authors prove that the migration of neurons expressing a C-terminal truncated form of Cx43 was not supported or assisted by the untargeted population of radial glia cells. Our strategy constitutes a more valid model since we could successfully knockdown Cx43 in all radial glia cells.

5.3 The Important Region of the C-terminal Domain of Cx43 in Neuronal Migration

Finally, our last objective aimed at targeting more precisely the molecular mechanisms important for Cx43-mediated neuronal migration. We are addressing this question by electroporetic delivery of plasmids expressing different versions of Cx43 with a truncated C-terminal domain into the lateral ventricle of E14 *Cx43*$^{-/-}$ and *Cx43cKO* mice. Exogenous expression of Cx43Δ258-382 plasmid is not able to rescue neuronal migration in *Cx43*$^{-/-}$ embryos. Conversely, Cx43Δ305-382 or Cx43Δ376-382–expressing cells were able to rescue migration in *Cx43cKO* mice. Based on these results, we conclude that the region of the C-terminal tail located between the amino acid residues 259 and 305 is important for neuronal migration during brain development (see chapter 4).

In summary, the significant contributions of this research to the field of gap junctions include: i) Cx26 and Cx43 are extensively expressed in the radial glia and the migrating neurons, indicating their role in neuronal migration during development; ii) In *Cx43cKO* and *Cx43*$^{-/-}$ mice, deletion of Cx43 from radial glial cells leads to an accumulation of newborn neurons in the IZ and causes a greater thickness of the CP compared to the CP of control littermates; iii) The C-terminal domain of Cx43 plays a functional role in glial guided neuronal migration; iv) A central region of the Cx43 comprised between the amino acids 259 and 305 is involved in directing neuronal migration.

It should be noted here that the involvement of Cx26 and Cx36 in neuronal migration and cortical development was also examined in the developing mouse neocortex. We previously showed that Cx26 was highly expressed in the radial glia and newborn neurons during development (Cina et al., 2007). Therefore, we hypothesized that deletion of Cx26 from the radial glial cells will perturb neuronal migration and cortical development. To test this hypothesis, we used *Cx26*$^{fl/fl}$*; nestin-Cre* mice, and measured the thickness of the cortical layers of the neocortex as previously mentioned (Cina et al., 2007). Deletion of Cx26 from radial glial cells and newborn neurons resulted in abnormalities in the cortical layer formation during development. In the neocortex of *Cx26*$^{fl/fl}$*; nestin-Cre* mice, the IZ was the thickest layer, whereas in control littermates, the CP was thicker that the other two layers (the IZ and the VZ). Unfortunately, this study was not explored further because the *Cx26*$^{fl/fl}$; nestin-Cre mice died *in utero* for an unknown reason.

Furthermore, in our first study, we also showed that Cx36 was expressed in the developing mouse neocortex (Cina et al., 2007). In addition, Cx36 has been shown to be expressed in a subset of interneurons (Deans et al., 2001). Hence, we hypothesized that the deletion of Cx36 from interneurons will lead to an aberrant neuronal migration. To assess the effect of deleting Cx36 in immature neurons, Tα1–GFP was injected into the lateral ventricle of *Cx36*$^{-/-}$ embryos at E14 and electroporated. The embryos were allowed to continue development for four days until E18 before being analyzed. The results showed that most GFP^{+} neurons were able to migrate out of the VZ and into the CP. Thus, deletion of Cx36 from immature neurons had no effect in neuronal migration (unpublished work). Based on the results obtained from Cx26 and Cx36 we decided to focus only on the role of Cx43 in neuronal migration and cortical development.

5.4 Potential Mechanisms for the Role of Cx43 in Neuronal Migration

It remains to be determined what proteins in the cytosol interact with this C-terminal region of Cx43 to regulate neuronal migration. The C-terminal tail interacts with many proteins that could link Cx43 to cytoskeletal anchorage. These proteins include kinases, phosphatases, ZO-1, CCN/NOV, and N-cadherin and β-catenin, (Giepmans and Moolennar, 1998; McLeod et al., 2001; Xu et al. 2001). For example, Src is a protein with tyrosine kinase activity that phosphorylates Cx43 on the tyrosine residues of the C-terminal tail (Lin et al., 2001; Kanemitsu et al., 1997). The proposed model for this mechanism is that the Src binding domain, SH2, facilitates the phosphorylation of Cx43 in Tyr265 within the C-terminal domain. This phosphorylation site provides a bridge for the second Src binding domain, SH3, to phosphorylate proline rich residues of Cx43 which are P274, P277, P280, and P283 (see Figure 1.3). Src phosphorylation reduces intercellular communication, thus modulating the function of Cx43 (Lin et al., 2001). In mouse cultured astrocytes, immunopercipitation strategies suggested a direct association between endogenous Cx43 and wild-type Src which was accompanied by increased Tyr265 phosphorylation of Cx43. Moreover, the treatment of astrocytes with chemical ischemia/hypoxia lead to the dephosphorylation of Cx43 followed by internalization and then closure of gap junctions *in vivo* (Li et al., 2005). Moreover, previous studies have shown an upregulation in the activation of MAPK when rat liver cells were treated with EGF. In turn, activation of MAPK led to the phosphorylation of Cx43. In those studies, site-directed mutagenesis and peptide sequencing experiments showed that three serine residues within the C-terminal tail of Cx43 including Ser255, Ser279, and Ser282 are phosphorylation sites for MAPK. The phosphorylation of Cx43 by MAPK subsequently diminishes gap junctional communication (Kanemitsu and Lau, 1993; Lau et al., 1992).

Interestingly, the phosphorylation sites for Src and MAPK are located along the same area of the C-terminal domain that has been proposed in this study to be an important region involved in neuronal migration (amino acid residues 259–305, see Figure 1.3). Therefore, Src or MAPK might potentially be the factors that link Cx43 to neuronal migration through interactions with the C-terminal tail. Mutation constructs of Tyr247, Tyr265, Ser279 and Ser282 are potentially significant candidates to determine whether these residues are required for the effect of Cx43 on neuronal migration. In addition, by

collaborating with Dr. Tabernero's laboratory (The University of Salamanca, Spain), we now have access to a double mutant construct in which Tyr247 and Tyr265 are substituted by non-phosphorylable phenylalanine. The mutant construct could be injected into the lateral ventricle of E14 *Cx43*$^{-/-}$ or *Cx43cKO* embryos followed by electroporation. Furthermore, an alternative approach to investigate the regulation of Cx43 signaling in neuronal migration by Src is through constructing phosphomimetic mutations such as glutamic acid. In the C-terminal tail of Cx43, Tyr265 could be substituted by phosphomimetic residue Glu in order to inactivate the phosphorylation site for the SH2 domain of Src. The mutant construct could be injected into *Cx43* $^{-/-}$ or *Cx43cKO* mouse brains followed by electroporation.

It is unclear exactly how phosphorylation of Cx43 by Src might affect neuronal migration. During phosphorylation of Cx43 along the amino acid residues 259-305, the negative charge of the tyrosine phosphorylation causes a conformational change in the structure of Cx43 (Lin et al., 2001). The result of this event might be that the C-terminal domain of Cx43 begins to interact with and recruit new proteins that are in close proximity. These proteins could include actin cytoskeleton and the binding proteins such as cortactin and drebrin. In fact, both cortactin and drebrin have been shown to interact with Cx43 (Butkevich et al., 2004; Squecco et al., 2006). Upregulation of cortactin by Cx43 might cause cortactin to bind to and activate ARP2/3 complex and WASP proteins, which in turn stimulate the remodeling and polymerization of actin cytoskeleton at the leading edge (Weed et al, 2000). Src, WASP, and ARP2/3 complex simulate the Rho GTPase family including Rac and Cdc42. This activation in turn leads to the elongation of actin filaments at the leading edge, thus producing membrane protrusions in order to move the cell forward (Small et al., 2002). In addition, Ca^{2+} is needed for the activation of the Ca^{2+}-dependent Rho GTPase, which in turn causes the membrane extensions of the migrating neurons to adhere to integrin based focal adhesion site of the radial glial fibers. Since it has been proposed that Cx43 hemichannels propagate Ca^{2+} through the cortical neurons (Weissman et al., 2004), therefore, Cx43 might be responsible for the upregulation of the Ca^{2+}-dependent Rho activity followed by the adhesion of the membrane extension to the radial glial fiber, thus initiating migration. In support of this hypothesis, Elias and colleagues have shown that Cx43 is present at the leading edge of the migrating neurons (Elias et al., 2007). Therefore, it is possible that phosphorylation of Cx43 by Src causes Cx43 to recruit cortactin to the leading edge of the

migrating neurons, thus promoting migration through a downstream signaling pathway. Additionally, it has been shown that cells with Src deficiency have a strong attachment to the extracellular surface, therefore they show decreased capability for migration (Klinghoffer et al., 1999). In fact, inhibition of Src has been shown to cause a rapid disappearance of lamellipodia by regulating actin cytoskeleton at the leading edge of glioma cells (Angers-Loustau et al., 2004). A proposed model for the involvement of Cx43 in neuronal migration is depicted in Figure 5.1.

5.5 Relevance of the Findings to CNS disorders

Increasing evidence has reported that aberrations in the properties of gap junctions might lead to CNS diseases including epilepsy, brain ischemia, schizophrenia, mental retardation, Alzheimer's disease, and Parkinson's disease (Aicardia, 1994; Nagy et al., 1996; Rufer et al., 1996; Nakase et al., 2003; Guerrini and Filippi, 2005). For example, in $Cx43^{fl/fl}$; GFAP-Cre mice following the occlusion of middle cerebral artery, the volume of the stroke was enhanced compared to the control littermates. Thus, Cx43 that is abundant in astrocytes has a neuroprotective role against stroke (Nakase et al., 2003).

Furthermore, single mutations along the coding regions of the human *Cx43* gene including the C-terminal domain leads to a disease, known as oculodentodigital dysplasia (ODDD) (Paznekas et al., 2003). ODDD patients are recognized by facial abnormalities, fused fingers and toes, and abnormal teeth. In addition, some patients suffer from learning disabilities and mental retardation (Loddenkemper et al., 2002). The ODDD mice showed 50% reduction in gap junctional communication as well as decreased amount of Cx43 protein. Furthermore, in these mice, two Cx43 mutations (fs230 and fs260) resulted in sever truncation of C-terminal tail. This is due to the dominant effect of mutant Cx43 on wild type Cx43 (Roscoe et al., 2005). Therefore, cortical slices of these mice could be prepared and examined for possible defects in neuronal migration. It is possible that in ODDD mice, the function of the brain mimics the human disease, including mental retardation where malformation in the brain development is observed. This could be explained by the findings of this thesis, where the deletion of Cx43 in *Cx43cKO* mice leads to abnormal migration and cortical malformation.

The defects in neuronal migration following the deletion of the Cx43 gene from immature neurons in *Cx43cKO* mice are comparable to the defects caused by mutations in the *FLNA* gene. Both Cx43 and FLNA are expressed in the VZ and interact with the actin cytoskeleton and with the binding proteins which have implication in neuronal migration. The mutation in the *FLNA* gene causes the postmitotic neurons to accumulate in the VZ and the IZ, thus causing abnormal cortical lamination, known as periventricular heterotopia (Fox et al., 1998). Therefore, similar to Cx43, FLNA is an important factor that is required for the proper migration of neurons. Taken together, the findings of the research presented in this

thesis may have implications in understanding the mechanisms mediating neurodevelopmental abnormalities.

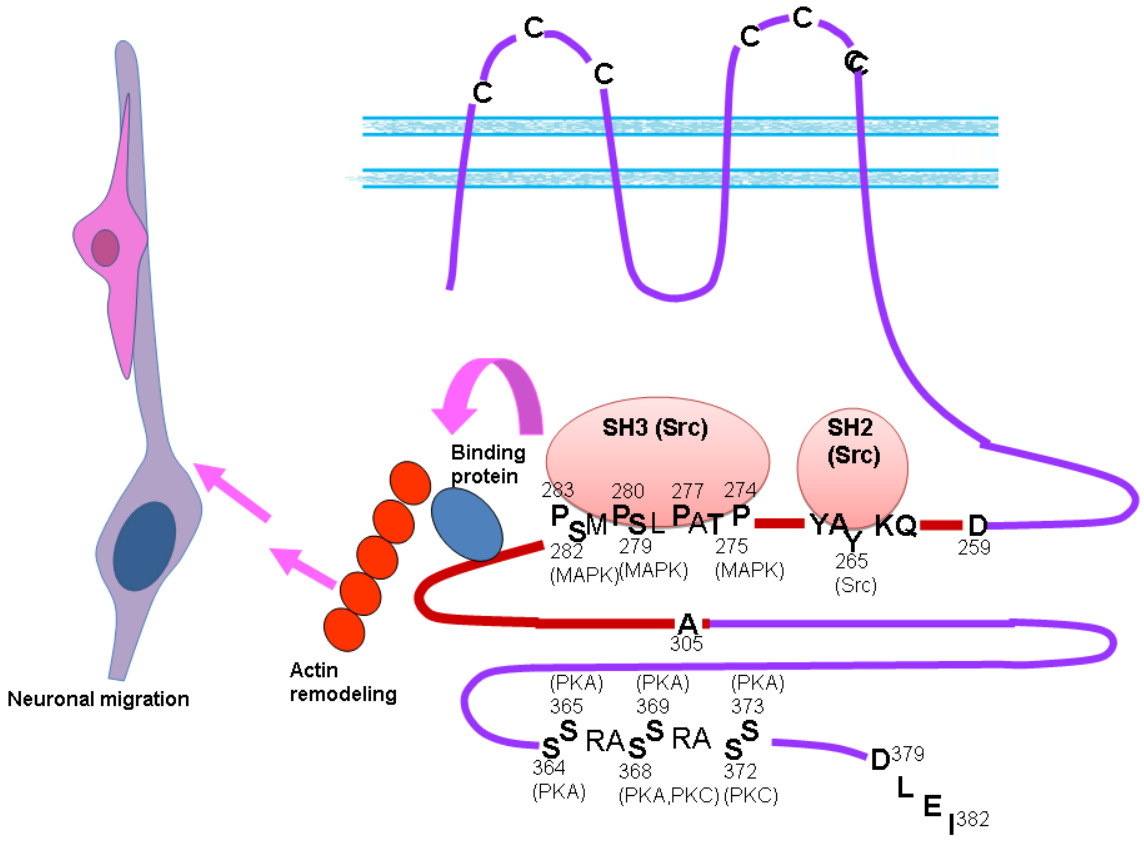


Figure 5.1 Model of Cx43 and signaling molecules in neuronal migration. Src binding domains, SH2 and SH3, facilitate the phosphorylation of Cx43 on Tyr247 and Tyr265. In addition, MAPK phosphorylates Cx43 via amino acid residues 255, 279, and 289. Following the phosphorylation of the C-terminal domain along the amino acid residues 259-305 (red line), Cx43 interacts with the actin cytoskeleton and the binding partners, and allows for the migration of neurons (pink cell) along the radial glial cells (blue cell) through downstream pathways.

5.6 References

Aicardia J (1994) The place of neuronal migration abnormalities in child neurology. Can J Neurol Sci 21:185-193.

Angers-Loustau A, Hering R, Werbowetski TE, Kaplan DR, Del Maestro RF (2004) SRC regulates actin dynamics and invasion of malignant glial cells in three dimensions. Mol Cancer Res 2:595-605.

Anthony TE, Klein C, Fishell G, Heintz N (2004) Radial glia serve as neuronal progenitors in all regions of the central nervous system. Neuron 41:881-890.

Bittman K, Owen DF, Kriegstein AR, LoTurco JJ (1997) Cell coupling and uncoupling in the ventricular zone of developing neocortex. J Neurosci 18:7037-7044.

Bittman KS, Becker DL, Cicirata F, Parnavelas JG (2002) Connexin expression in homotypic and heterotypic cell coupling in the developing cerebral cortex. J Comp Neurol 443:201-212.

Butkevich E, Hülsmann S, Wenzel D, Shirao T, Duden R, Majoul I (2004) Drebrin is a novel connexin-43 binding partner that links gap junctions to the submembrane cytoskeleton. Curr Biol 14:650-658.

Cina C, Bechberger JF, Ozog MA, Naus CCG (2007) Expression of connexins in embryonic mouse neocortical development. J Comp Neurol 504:298-313.

Cina C, Maass K, Theis M, Willecke K, Bechberger JF, Naus CC (2009) Involvement of the cytoplasmic C-terminal domain of connexin43 in neuronal migration. J Neurosci 29:2009-2021.

Deans MR, Gibson JR, Sellitto C, Connors BW, Paul DL (2001) Synchronous activity of inhibitory networks in neocortex requires electrical synapses containing connexin36. Neuorn 31:477-485.

Elias LA, Wang DD, Kriegstein AR (2007) Gap junction adhesion is necessary for radial migration in the neocortex. Nature 448:901-907.

Fox JW, Lamperti ED, Ekşioğlu YZ, Hong SE, Feng Y, Graham DA, Scheffer IE, Dobyns WB, Hirsch BA, Radtke RA, Berkovic SF, Huttenlocher PR, Walsh CA (1998) Mutation in filamin 1 prevent migration of cerebral cortical neurons in human periventricular heterotopia. Neuron 21:1315-1325.

Fushiki S, Perez Velazquez JL, Zhang L, Bechberger JF, Carlen PL, Naus CC (2003) Changes in neuronal migration in neocortex of connexin43 null mutant mice. J Neuropathol Exp Neurol 62:304-314.

Giepmans BN, Moolenaar WH (1998) The gap junction protein connexin43 interacts with the second PDZ domain of the zona occludens-1 protein. Curr Biol 8:931–934.

Guerrini R, Filippi T (2005) Neuronal migration disorders, genetics, and epileptogenesis. J Child Neurol 4:287-299.

Elias LA, Wang DD, Kriegstein AR (2007) Gap junction adhesion is necessary for radial migration in the neocortex. Nature 448:901-907.

Kanemitsu MY, Lau AF (1993) Epidermal growth factor stimulates the disruption of gap junctional communication and connexin43 phosphorylation independent of 12-0-tetradecanoylphorbol 13-acetate-sensitive protein kinase C: the possible involvement of mitogen-activated protein kinase. Mol Biol Cell 4:837-848.

Klinghoffer RA, Sachsenmaier C, Cooper JA, Soriano P (1999) Src family kinases are required for integrin but not PDGFR signal transduction. EMBO J 18:2459-71.

Lau AF, Kanemitsu MY, Kurata WE, Danesh S, Boynton AL (1992) Epidermal growth factor disrupts gap junctional–communication and induces phosphorylation of connexin43 on serine. Mol Biol Cell 3:865-874.

Li W, Hertzberg EL, Spray DC (2005) Regulation of connexin43-protein binding in astrocytes in response to chemical ischemia/hypoxia. J Biol Chem 280:7941-7948.

Lin R, Warn-Cramer BJ, Kurata WE, Lau AF (2001) v-Src-mediated phosphorylation of connexin43 on tyrosine disrupts gap junctional communication in mammalian cells. Cell Commun Adhes 8:265-269.

Lin R, Warn-Cramer BJ, Kurata WE, Lau AF (2001) v-Src phosphorylation of connexin43 in Tyr247 and Tyr65 disrupt gap junctional phosphorylation. J Cell Biol 154:815-827.

Lo WK, Shaw AP, Takemoto LJ, Grossniklaus HE, Tigges M (1996) Gap junction structures and distribution patterns of immunoreactive connexins 46 and 50 in lens regrowths of Rhesus monkeys. Exp Eye Res 62:171-180.

Loddenkemper T, Grote K, Evers S, Oelerich M, Stögbauer F (2002) Neurological manifestations of the oculodentodigital dysplasia syndrome. J Neurol 249:584-95.

McLeod TL, Bechberger JF, Naus CC (2001) Determination of a potential role of the CCN family of growth regulators in connexin43 transfected C6 glioma cells. Cell Commun Adhes 8:441-445.

Nakase T, Fushiki S, Söhl G, Theis M, Willecke K, Naus CC (2003) Neuroprotective role of astrocytic gap junctions in ischemic stroke. Cell Commun Adhes 10:413-7.

Nadarajah B, Jones AM, Evans WH, Parnavelas JG (1997) Differential expression of connexins during neocortical development and neuronal circuit formation. J Neurosci 17:3096-3111.

Nadarajah B, Brunstrom JE, Grutzendler J, Wong RO, Pearlman AL (2001) Two modes of radial migration in early development of the cerebral cortex. Nat Neurosci 4:143-150.

Nagy JI, Li W, Hertzberg EL, Marotta CA (1996) Elevated connexin43 inmmunoreactivity at sites of amyloid plaques in Alzheimer's disease. Brain Res 717:173-178.

Neyton J, Trautmann A (1986) Physiological modulation of gap junction permeability. J Exp Biol 124:93-116.

Noctor SC, Martínez-Cerdeño V, Kriegstein AR (2008) Distinct behaviors of neural stem and progenitor cells underlie cortical neurogenesis. J Comp Neurol 508:28-44.

Paznekas WA, Boyadjiev SA, Shapiro RE, Daniels O, Wollnik B, Keegan CE, Innis JW, Dinulos MB, Christian C, Hannibal MC, Jabs EW (2003) Connexin43 (GJA1) mutations cause pleiotropic phenotypic of oculodentodigital dysplasia. Am J Hum Genet 72:408-18.

Roscoe W, Veitch GI, Gong XQ, Pellegrino E, Bai D, McLachlan E, Shao Q, Kidder GM, Laird DW (2005) Oculodentodigital dysplasia-causing connexin43 mutants are non-functional and exhibit dominant effects on wild-type connexin43. J Biol Chem 280:11458-66.

Rufer M, Wirth SB, Hofer A, Dermietzel R, Pastor A, Kettenmann H, Unsicker K (1996) Regulation of connexin-43, GFAP, and FGF-2 is not accompanied by changes in astroglial coupling in MPTP-lesioned FGF-2 treated parkinsonian mice. J Neurosci Res 46:606-617.

Simon AM, Goodenough DA (1998) Diverse functions of vertebrate gap junctions. Trends Cell Biol 8:477-483.

Small JV, Stradal T, Vignal E, Rottner K(2000) The lamellipodium: where motility begins. Trends Cell Biol 12:112-20.

Squecco R, Sassoli C, Nuti F, Martinesi M, Chellini F, Nosi D, Zecchi-Orlandini S, Francini F, Formigli L, Meacci E (2006) Sphingosine 1-phosphate induces myoblast differentiation through Cx43 protein expression: A role for a gap junction-dependent and -independent function. Mol Biol Cell 17:4896-4910.

Weed SA, Karginov AV, Schafer DA, Weaver AM, Kinley AW, Cooper JA, Parsons JT (2000) Cortactin localization to sites of actin assembly in lamellipodia requires i nteractions with F-actin and the Arp2/3 complex. J Cell Biol 151:29-40.

Wiencken-Barger AE, Djukic B, Casper KB, McCarthy KD (2007) A role for connexin43 during neurodevelopment. Glia 55:675-686.

Weissman TA, Riquelme PA, Ivic L, Flint AC, Kriegstein AR (2004) Calcium waves propagate through radial glial cells and modulate proliferation in the developing neocortex. Neuron 5:647-661.

Xu X, Li WE, Huang GY, Meyer R, Chen T, Luo Y, Thomas MP, Radice GL, Lo CW (2001) Modulation of mouse neural crest cell motility by N-cadherin and connexin43 gap junctions. J Cell Biol 154:217-230.

Appendix A

Animal Care Certificate

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE
BREEDING PROGRAMS

Application Number: A06-0271

Investigator or Course Director: Christian Naus

Department: Medicine, Faculty of

Animals:

Mice C57bl6-Cx26 (fl/fl) 58
Mice C57Bl6-GFAP-Cre: Cx43 (fl/fl) 18
Mice C57Bl6-GFAP-GFP:Cx43 (KO/+) 22
Mice C57Bl6-Nestin-Cre:Cx26 (fl/fl) 18
Mice C57Bl6-Nestin-Cre:Cx43 (fl/fl) 18
Mice C57Bl6- Cx43 (fl/fl) 54
Mice C57Bl6-GFAP-Cre:Cx26 (fl/fl) 18
Mice C57Bl6-Cx36 (KO/+) 38
Mice C57Bl6- Cx43(KO/+) 34

Approval Date: October 11, 2006

Funding Sources:

Funding Agency: Canadian Institutes of Health Research
Funding Title: Gap junctions in differentiation and functions of neurons and astrocytes

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above breeding program.

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093