Pannexin 1 regulates ventricular zone neuronal development

by

Leigh Wicki-Stordeur
B.Sc., University of Victoria, 2011

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of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Division of Medical Sciences

Supervisory Committee

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Dr. Leigh Anne Swayne (Division of Medical Sciences)
Supervisor

Dr. Craig Brown (Division of Medical Sciences)
Departmental Member

Dr. Stephanie Willerth (Division of Medical Sciences)
Departmental Member

Dr. Chris Nelson (Department of Biochemistry and Microbiology)
Outside Member

Dr. Tuan Trang (Department of Comparative Biology and Experimental Medicine and Department of Physiology and Pharmacology, University of Calgary)
External Member

Abstract

Supervisory Committee
Dr. Leigh Anne Swayne (Division of Medical Sciences)
Supervisor
Dr. Craig Brown (Division of Medical Sciences)
Departmental Member
Dr. Stephanie Willerth (Division of Medical Sciences)
Departmental Member
Dr. Chris Nelson (Department of Biochemistry and Microbiology)
Outside Member
Dr. Tuan Trang (Department of Comparative Biology and Experimental Medicine and Department of Physiology and Pharmacology, University of Calgary)
External Member

Neurons are generated from unspecialized neural precursor cells (NPCs) in a process termed neurogenesis. This neuronal development continues throughout life in the ventricular zone (VZ) of the lateral ventricles, and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. NPCs undergo a complex and highly regulated set of behaviours in order to ultimately integrate into the existing brain circuitry as fully functional neurons. Recently the pannexin (Panx) large-pore channel proteins were discovered. One family member, Panx1 is expressed in the nervous system in mature neurons, and acts as an ATP release channel in various cell types throughout the body. Post-natal NPCs are responsive to ATP via activation of purinergic receptors, which modulate a variety of NPC behaviours. I therefore investigated the hypothesis that Panx1 was expressed in post-natal VZ NPCs, where it functioned as an ATP release channel and regulated neuronal development. In the course of my studies, I found that Panx1 positively regulated NPC proliferation and migration, and negatively regulated neurite outgrowth *in vitro*. Using an NPC-specific Panx1 knock-out strategy, I showed that Panx1 expression was required for maintenance of a consistent population of VZ NPCs *in vivo* in both healthy and injured brain. Together these data indicated that Panx1 directed NPC behaviours associated with neuronal development both *in vitro* and *in vivo*. To further understand the molecular underpinnings of this regulation, I examined the Panx1 interactome, and uncovered a novel association with collapsin response mediator protein 2 (Crmp2). Functional studies suggested that this interaction likely was at least in part responsible for Panx1's negative impact on neurite outgrowth. Overall, my results represent important novel findings that contribute to

our understanding of post-natal neuronal development and the molecular function of Panx1 within the brain.

Publications

Original Research

1. Wicki-Stordeur LE, Sanchez-Arias J, Dhaliwal J, Carmona-Wagner EO, Shestopalov VI, Lagace DC and Swayne LA (2015) Pannexin 1 differentially impacts neural precursor cell maintenance in the ventricular zone and peri-infarct cortex. The Journal of Neuroscience. In revision (manuscript # JN-RM-0436-15).
2. Boyce AKJ, Prager RT, Kim MS, Wicki-Stordeur LE and Swayne LA (2015) ATP stimulates Pannexin 1 internalization by a mechanism involving an ATP sensitive residue and P2X receptors. Biochemical Journal 470(3);319-330.
3. Wicki-Stordeur LE and Swayne LA (2013) Panx1 regulates neural stem and progenitor cell behaviours associated with cytoskeletal dynamics and interacts with multiple cytoskeletal elements. Cell Communication and Signaling 11(1):62.
4. Wicki-Stordeur LE, Boyce AK and Swayne LA (2013) Analysis of a pannexin 2-pannexin 1 chimeric protein supports divergent roles for pannexin C-termini in cellular localization and function. Cell Communication and Adhesion 20(3-4):73-79.
5. Wicki-Stordeur LE, Dzugalo AD, Swansburg RM, Suits JM and Swayne LA (2012) Pannexin 1 regulates postnatal neural stem and progenitor cell proliferation. Neural Development 7:11.

Reviews

1. Boyce AK, Wicki-Stordeur LE, Swayne LA (2014) Powerful partnership: crosstalk between pannexin 1 and the cytoskeleton. Frontiers in Physiology 5:27.
2. Wicki-Stordeur LE and Swayne LA (2014) The emerging Pannexin 1 signalome: a new nexus revealed? Frontiers in Cellular Neuroscience 7:287.
3. Boyce AK, Prager RT, Wicki-Stordeur LE, Swayne LA (2013) Pore positioning: current concepts in Pannexin channel trafficking. Channels (Austin) 8(2).
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1. Wicki-Stordeur LE, Carmona-Wagner EO and Swayne LA (2015) A novel interaction between Pannexin 1 and Collapsin Response Mediator Protein 2 regulates neuronal development. International Gap Junctions Conference. Valparaiso, Chile. Oral Presentation. Awarded **1st prize amongst trainees.**
2. Wicki-Stordeur LE and Swayne LA. Identification of a novel interaction between Pannexin 1 and Collapsin response mediator protein 2 that regulates neuronal

development (2015) Canadian Association for Neuroscience. Vancouver BC, Canada. Poster Presentation.

3. Wicki-Stordeur LE, Boyce AKJ, Kim MS and Swayne LA. A novel Panx1-Crmp2 interaction impacts Panx1 surface expression in neural cells (2014) American Society for Cell Biology. San Diego, California, USA. Poster Presentation.
4. Wicki-Stordeur LE, Carmona-Wagner EO, Bell SC, Boyce AKJ, Lagace DC, Swayne LA (2014) Preclinical Testing of Probenecid for Stroke Recovery. 5th Canadian Stroke Congress. Vancouver, BC, Canada. Poster Presentation.
5. Wicki-Stordeur LE, Prager RT, Boyce AK, Swayne LA (2013) A novel role for pannexin 1 in the regulation of post-natal neural stem and progenitor cell cycle progression. Society for Neuroscience. San Diego, California, USA. Poster Presentation.
6. Wicki-Stordeur LE, Dzugalo AD, Prager RT, Boyce AK, Swayne LA (2013). Pannexin 1 regulates ventricular zone neural stem and progenitor cell behaviours and forms a novel interaction with collapsin response mediator protein 2. Canadian Association for Neuroscience. Toronto ON, Canada. Poster Presentation.
7. Wicki-Stordeur LE, Dzugalo AD, Swansburg RM, Prager RT, Boyce AK, Swayne LA (2012) Panx1 regulates post-natal neurogenesis. Society for Neuroscience. New Orleans, Louisiana, USA. Poster Presentation.
8. Wicki-Stordeur LE, Dzugalo AD, Swansburg RM, Suits JM and Swayne LA (2012) Pannexin 1 regulates postnatal neural stem and progenitor cell proliferation and forms a novel interaction with phosphoglycerate dehydrogenase. Canadian Association for Neuroscience. Vancouver BC, Canada. Poster Presentation.
9. Wicki-Stordeur LE, Dzugalo AD, Swansburg RM, Suits JM and Swayne LA (2012) Pannexin 1 regulates postnatal neural stem and progenitor cell proliferation. Loon Lake Cell Biology Retreat. Maple Ridge BC, Canada. Oral Presentation, Awarded **2nd prize amongst trainees.** Poster Presentation, Awarded **3rd prize amongst trainees.**
10. Swayne LA, Sorbara CD, Wicki-Stordeur LE, Hou W, Figeys D, Bennett SAL (2011) Pannexin 2 regulation of neural stem cells. International Gap Junction Conference. Ghent, Belgium. Poster Presentation.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

*Casp3	activated caspase 3
ACSF	artificial cerebral spinal fluid
AGC	automatic gain control
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CID	collision induced dissociation
CP	cortical plate
Crmp2	collapsin response mediator protein-2
Cx	connexin
DCX	doublecortin
DIV	days *in vitro*
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence substrate
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EPL	external plexiform layer

FDR	false discovery rate
FTMS	Fourier transform mass spectrometry
FT-ICR	Fourier transform ion cyclotron resonance
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCL	granule cell layer
GFAP	glial fibrillary acidic protein
GL	glomerular layer
GO	gene ontology
GST	glutathione s-transferase
HBSS	Hank's balanced salt solution
HRP	horseradish peroxidase
ICV	intracerebroventricular
IP_3	inositol triphosphate
IPL	internal plexiform layer
ITMS	ion trap mass spectrometry
IZ	intermediate zone
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
LTQ	linear trap quadrupole
MCL	mitral cell layer
MGI	mouse genome informatics
MZ	marginal zone
N2a	Neuro-2a

NeuN	neuronal nuclei
NPC	neural precursor cell
NSP	neurosphere
OB	olfactory bulb
Panx	pannexin
Panx1CT	Pannexin 1 C-terminus
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsufonyl fluoride
PVDF	polyvinylidene fluoride
rcf	relative centrifugal force
RIPA	radio-immunoprecipitation assay
RNA	ribonucleic acid
rpm	rotations per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGZ	subgranular zone
SVZ	subventricular zone
TAT	trans-activator of transcription peptide
TBS	TRIS buffered saline
TUJ1	β-III tubulin
UVIC	University of Victoria
VZ	ventricular zone
WGA	wheat-germ agglutinin

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1. Introduction

1.1. Dissertation Overview

Neurons are generated from pools of undifferentiated neural precursor cells (NPCs) in a dynamic process termed neurogenesis. Recent advances demonstrate that in addition to formation of the brain during embryonic and early post-natal development, neurogenesis continues throughout life in restricted regions of the brain: the ventricular zone (VZ) of the lateral ventricles, and the sub-granular zone (SGZ) of the dentate gyrus in the hippocampus (reviewed in Ming and Song, 2011). NPCs in these regions must integrate a multitude of intrinsic and extrinsic signals to ultimately develop into functional neurons. However, the full complement of regulatory signals underlying this complex process is not yet known.

A growing body of work has linked the activity of large-pore channel proteins to control of neurogenesis. Large-pore channels, such as the connexin (Cx) and pannexin (Panx) proteins of the gap junction group, mediate passage of ions and metabolites across cellular membranes. The diverse actions of Cxs have long been implicated in various aspects of neuronal development. For example, NPCs are highly responsive to extracellular signalling molecules, such as adenosine triphosphate (ATP), thought to be released through Cx large-pore channels. However, recent discovery of the Panx channels raised questions as to whether these functions are, instead, Panx-dependent. The work presented here therefore focused on elucidating the role of Panxs, and in particular Panx1, in neuronal development.

I was the first to demonstrate that Panx1 was expressed in post-natal VZ NPCs both *in vitro* and *in vivo* (Wicki-Stordeur et al., 2012; Wicki-Stordeur and Swayne, 2013). The studies

presented here concentrate on this VZ niche. I moved into cell culture models to address whether Panx1 regulated neuronal development *in vitro*. I found that Panx1 was responsible for ATP release from NPCs, and this promoted their proliferation (Wicki-Stordeur et al., 2012). Moreover, Panx1 positively regulated NPC migration and negatively regulated neurite outgrowth (Wicki-Stordeur and Swayne, 2013). These data are presented in Chapter 3.

I next moved into an NPC-specific Panx1 knock-out mouse model to identify how Panx1 impacted VZ neuronal development *in vivo*. Since pathophysiological stimuli alter both VZ neurogenesis (reviewed in Ohab and Carmichael, 2008; Yu et al., 2014) and Panx1 channel activity (Thompson et al., 2006; Weilinger et al., 2012), I also examined whether focal cortical stroke modulated the role of Panx1 within VZ NPCs. My data suggested that Panx1 was critical for maintenance of the VZ NPC population size in both healthy and stroke brains. These experiments are outlined in Chapter 4.

Finally, I looked to uncover potential molecular mechanisms underlying the Panx1-dependent control of neuronal development. To this end, I performed the first large-scale, unbiased screen for Panx1-interacting proteins and identified 480 putative protein interaction partners. I concentrated on proteins with an establised role in neuronal development. I focused on a novel association with collapsin response mediator protein 2 (Crmp2), a well-known regulator of neurite development (reviewed in Ip et al., 2014; Quach et al., 2015). In Chapter 5, I confirmed this interaction in NPCs, both *in vitro* and *in vivo*. Moreover, I demonstrated that this interaction appeared to underlie the Panx1-dependent inhibition of neurite outgrowth discovered in Chapter 3.

Overall, the data presented in this dissertation identified a role for Panx1 in neuronal development both *in vitro* and *in vivo*. From this data I have outlined a putative molecular

mechanism through which Panx1 may be acting, furthering our understanding of the function of Panx1 in the nervous system. Neuronal development remains an important modulator of brain homeostasis throughout life, and is emerging as a putative target for brain repair following injuries. My dissertation work therefore represents important steps in unraveling the molecular modulators of this process.

1.2. Neuronal development

Neurons originate from pools of undifferentiated NPCs through a complex process known as neurogenesis. This process begins during brain development in the embryonic period (reviewed in Temple, 2001; Erzurumlu and Gaspar, 2012; Martynoga et al., 2012), and continues throughout life in discrete regions of the post-natal brain (reviewed in Ming and Song, 2011). Neurogenesis progresses through multiple NPC types, which undergo a complex and precisely regulated set of behaviours including proliferation, migration, differentiation and survival/death. These behaviours are highly responsive to many physiological and pathophysiological stimuli; therefore neurogenesis represents a potential therapeutic target for several injury and disease models, such as stroke. Because of the highly complex nature of the neurogenic niche within the brain, many of the associated cellular functions are often studied in simplified cell culture systems. In this section I will describe embryonic neurogenesis as it pertains to cortical development, and the continuation of neurogenesis within the post-natal brain under both healthy and pathophysiological conditions with a focus on the VZ niche. I will further discuss the use of *in vitro* models of neuronal development, and outline the specific *in vitro* models used in the data presented in this dissertation.

1.2.1. Cortical development

In the embryonic brain, populations of NPCs undergo neurogenesis to produce neurons of the developing cerebral cortex (Figure 1.1; reviewed in Temple, 2001; Erzurumlu and Gaspar, 2012; Martynoga et al., 2012). Radial glia located in the ventricular zone (VZ) surrounding the lateral ventricles, give rise to highly proliferative NPCs. These divide symmetrically at first, thereby amplifying the NPC pool for a number of divisions, before exiting the cell cycle and differentiating into immature neurons. These young neurons migrate along radial glia processes that extend outwards from the VZ to the pia, moving through the intermediate zone into the cortical plate. Here they become mature neurons while developing complex networks of neurites and synapses. By birth the cerebral cortex undergoes lamination, with the cortical plate forming layers II-VI. The cell-sparse marginal zone becomes layer I, and the intermediate zone forms the internal white matter.

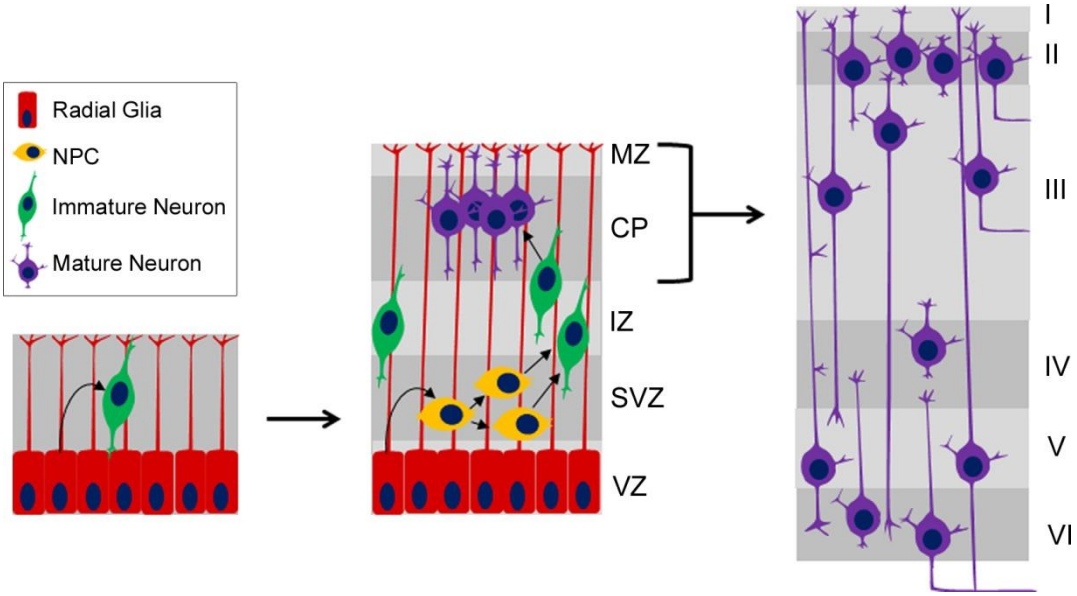


Figure 1.1. NPCs in the embryonic brain form the developing cortex.
Initially the radial glia in the ventricular zone (VZ) form contacts with the pial surface, and divide to form immature neurons. Subsequently, the radial glia give rise to NPCs that in turn differentiate into immature neurons. These cells migrate from the sub-ventricular zone (SVZ), through the intermediate zone (IZ) and into the cortical plate (CP). Here they develop into mature neurons. Shortly after birth, the cortical plate forms into layers II-VI of the cerebral cortex, with the marginal zone (MZ) forming layer I.

1.2.2. Post-natal neuronal development

A historical perspective

Until recent years, the general consensus was that neurogenesis ceased after brain development was complete. Post-natal-derived neurons were first described in various regions of the adult brain in pioneering studies by Altman and colleagues (Altman, 1963; Altman and Das, 1965, 1966; Altman, 1969), yet these studies were largely ignored for 20 years. In the 1980's, the idea of post-natal neurogenesis regained attention when newborn neurons were shown to form

connections with existing circuitry (Kaplan and Bell, 1983; Stanfield and Trice, 1988), and their functional relevance began to be elucidated in songbirds (Paton and Nottebohm, 1984). However, post-natal neurogenesis was not widely accepted until more recently (reviewed in Gross, 2000). The shift was precipitated especially by (1) the isolation and culture of multipotent NPCs from the adult rodent brain (Reynolds and Weiss, 1992; Richards et al., 1992; Reynolds and Weiss, 1996) and (2) the introduction of labelling techniques utilizing nucleotide analogs, such as bromodeoxyuridine (BrdU; Gratzner, 1982), to trace the fate of dividing cells. In the same period of time, a large population of post-natal VZ cells in the rodent brain was found to migrate to the olfactory bulb (OB) and form resident interneurons (Luskin, 1993; Lois and Alvarez-Buylla, 1994). This resulted in a period of intense investigation, in which life-long neurogenesis was identified in multiple species (reviewed in Lindsey and Tropepe, 2006; Bonfanti and Peretto, 2011; Aimone et al., 2014). Considerable interest was generated when this phenomenon was discovered in humans, and even in aged individuals, (Eriksson et al., 1998; Kukekov et al., 1999), indicating that ongoing neurogenesis was potentially important for human cognition. In most mammals, including humans, neurogenesis was found to be exclusively restricted to two regions within the post-natal brain: the SGZ of the dentate gyrus in the hippocampus, and the VZ of the lateral ventricles (Figure 1.2; reviewed in Ming and Song, 2011).

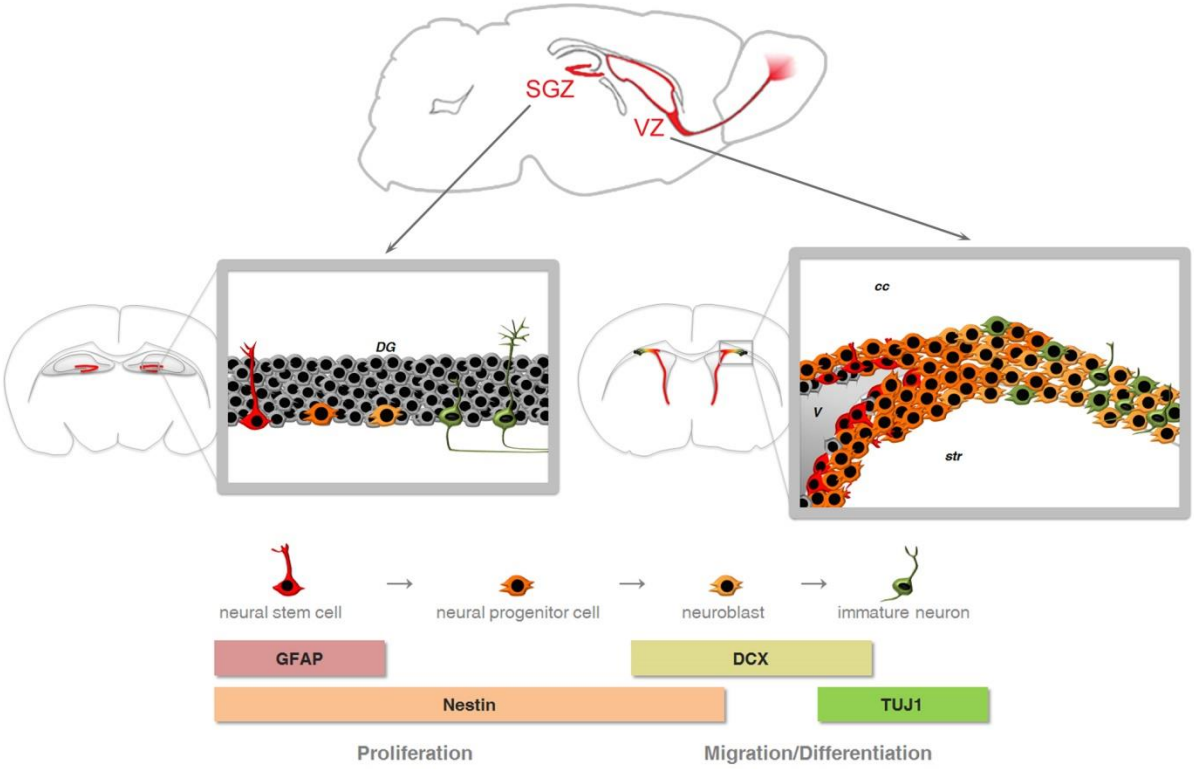


Figure 1.2. Neuronal development continues in discrete regions of the post-natal brain.
Post-natal neurogenesis occurs exclusively in two regions of the mammalian brain: the SGZ of the dentate gyrus in the hippocampus (left) and the VZ of the lateral ventricles (right). In both regions, NPCs undergo a complex and highly regulated process consisting of several distinct cell types identified by specific lineage markers. Relatively quiescent radial glia-like neural stem cells (GFAP+/nestin+) form more rapidly proliferating neural progenitor cells (GFAP-/nestin+) that in turn become migratory neuroblasts (DCX+) upon neuronal commitment. Together these cells are referred to as NPCs. These cells exit the cell cycle as immature neurons (TUJ1+) that eventually become mature hippocampal granule cells (SGZ) or OB interneurons (VZ) and integrate into the existing circuitry.

Post-natal VZ neurogenesis

Within the post-natal brain, in both rodents and humans, neurogenesis continues in the VZ of the lateral ventricles (reviewed in Ming and Song, 2011). In this post-natal niche, NPCs proceed

through a tightly regulated set of behaviours and cell types. Relatively quiescent radial glia-like neural stem cells (type B cells; glial fibrillary acidic protein (GFAP)-positive/nestin-positive) give rise to more rapidly dividing transient amplifying cells (type C cells or intermediate progenitors; GFAP-negative/nestin-positive). These generate neuroblasts (type A cells; doublecortin (DCX)-positive) that move in chains of cells along the rostral migratory stream (RMS). Along this route, neuroblasts become committed to a neuronal lineage and develop into post-mitotic immature neurons (β-III tubulin (TUJ1)-positive). Collectively, these immature neuronal cells are referred to as NPCs.

The VZ niche is a highly specialized microenvironment containing a remarkably diverse population of cells that support and promote the progression of neurogenesis. Here, the type B NPCs lie in close contact to the ventricular wall, with processes and sometimes entire apical surfaces contacting the cerebrospinal fluid (CSF; Doetsch et al., 1999; Conover et al., 2000; Doetsch et al., 2002; Merkle et al., 2007; Mirzadeh et al., 2008; Shen et al., 2008). These cells have many astrocytic qualities (Liu et al., 2006), and reach out long processes into deeper regions of the niche. Many of these processes make contact with endothelial cells of the extensive blood vessel network that runs parallel to the VZ niche (Shen et al., 2008; Tavazoie et al., 2008; Ottone et al., 2014). These are unusually permeable connections, often devoid of astrocytes and pericytes. NPCs therefore integrate signals from the CSF and vasculature, both rich sources of nutrients and soluble factors that modulate NPC behaviours (reviewed in Zappaterra and Lehtinen, 2012; Ottone and Parrinello, 2015; Stolp and Molnar, 2015). There is controversy surrounding the identity of the cells lining the ventricles. They have previously been called ependymal cells, but new evidence suggests they are actually quiescent NPCs (reviewed in Chojnacki et al., 2009). These cells make up a large portion of the ventricular lining and are also

constantly in contact with the CSF. The action of the cilia of these cells creates signalling gradients of CSF-derived growth factors and guidance molecules across the VZ niche (Sawamoto et al., 2006). These in turn regulate NPC migration and fate specification. Vascular cells and circulating factors play a major role in controlling NPC proliferation, and the network of blood vessels provides a structural base for NPC migration along the RMS (Martoncikova et al., 2014; Yuan et al., 2015). Astrocytes also offer structural support along the RMS through formation of 'glial tubes' (reviewed in Sun et al., 2010). Moreover, these cells present soluble and membrane bound signalling factors (Barkho et al., 2006; Platel et al., 2010), as well as important plasma membrane receptors, such as Robo (Kaneko et al., 2010), making astrocytes central contributors to all aspects of neurogenesis. Microglia, the resident immune cells, actively regulate various NPC behaviours; however their role seems to be dependent on the exact balance of pro- and anti-inflammatory molecules released (reviewed in Ekdahl et al., 2009; Su et al., 2014; and supported by Ribeiro Xavier et al., 2015). Neurotransmitter release (dopamine, serotonin), from mature neurons synapsing in the niche, regulates NPC proliferation and differentiation (reviewed in Young et al., 2011). GABA release from late-stage type A NPCs controls behaviour of NPCs during earlier stages of neurogenesis (Liu et al., 2005), providing an important feedback loop that normalizes the number of neurons being born and integrated into existing circuitry.

Throughout neurogenesis, a large proportion of NPCs are lost (Morshead and van der Kooy, 1992). Interestingly, this appears to be due to clearance by phagocytic DCX-positive NPCs, the primary phagocytic cells in the VZ (Lu et al., 2011; Lovelace et al., 2015). There are two critical survival periods: one during the migration of NPCs and immature neurons (Platel et al., 2010), and the other during the maturation of new neurons in the OB (Mouret et al., 2008). The cells

that survive through these critical periods end their journey at the OB core (reviewed in Sakamoto et al., 2014a), where they migrate radially towards the glomeruli and ultimately mature into various subtypes of OB neurons (neuronal nuclei (NeuN)-positive; Figure 1.3). The majority become GABAergic granule neurons, while a minority develop into GABAergic periglomerular neurons. A small subset may also generate glutamatergic juxtaglomerular neurons (Brill et al., 2009).

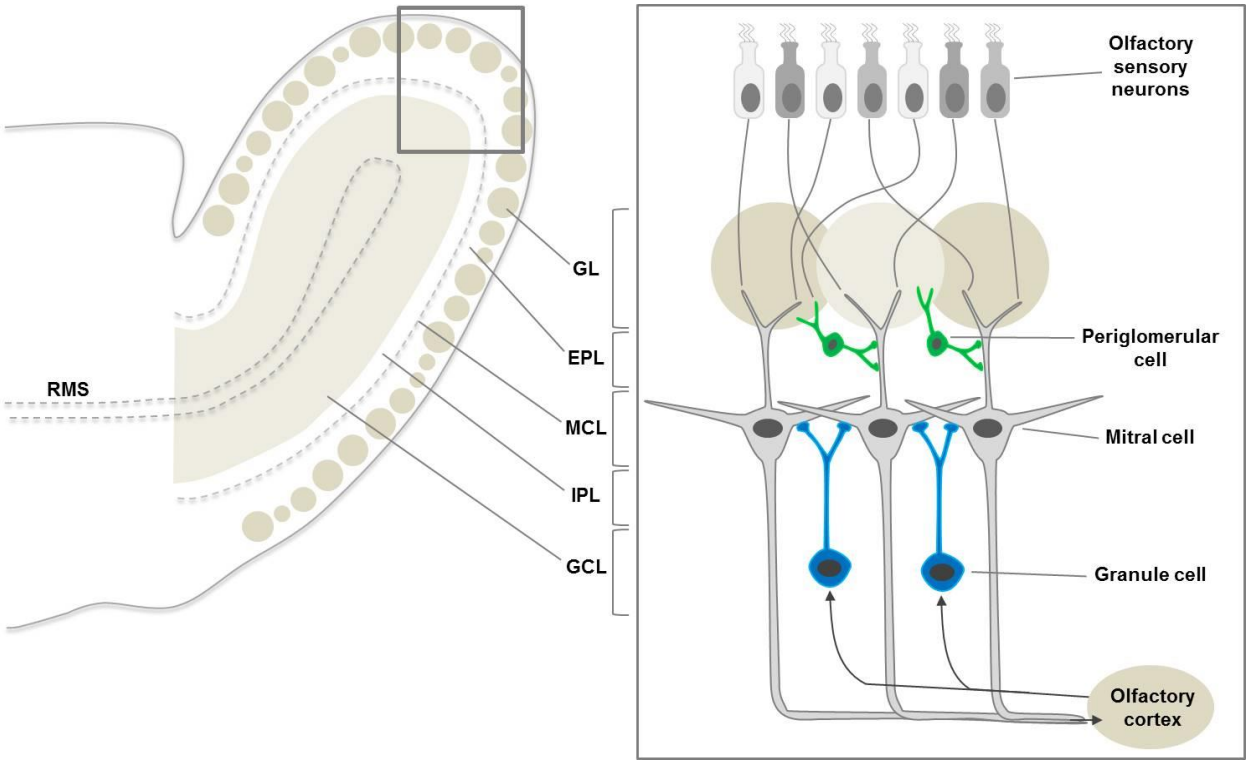


Figure 1.3. Newborn VZ neurons migrate along the RMS into the OB where they integrate with the existing circuitry.

A cartoon sagittal section of a rodent OB is shown on the left. The RMS terminates in the core of the OB. From here, the majority of newborn neurons migrate radially into the granule cell layer where they mature into GABAergic granule cells. A minority of newborn neurons become GABAergic periglomerular cells. A schematic diagram of the OB neuronal circuit is shown on the right. Olfactory sensory neurons provide excitatory input to mitral cells which in turn project to the olfactory cortex. Granule cells form dendro-dendritic synapses with the mitral cells, and receive glutamatergic input from the olfactory cortex. Periglomerular cells receive inputs from olfactory sensory neurons, and also form dendro-dendritic synapses with mitral cells. Granule cells and periglomerular cells mediate lateral inhibition within the OB. GL: glomerular layer, EPL: external plexiform layer, MCL: mitral cell layer, IPL: internal plexiform layer, GCL: granule cell layer.

A massive number of these new neurons enter into the OB each day (reviewed in Sakamoto et al., 2014a), yet the functional correlates of post-natal VZ neurogenesis are only beginning to be unravelled. Many studies have indicated that this process plays a role in the ongoing

maintenance of the OB structure and function (Sakamoto et al., 2014b). The granule and periglomerular cells mediate lateral inhibition within the OB circuit. Adult-born neurons within these populations initially have heightened synaptic plasticity compared to older neurons (Nissant et al., 2009), thereby providing an additional level of plasticity to the OB network. These neurons have been implicated in odor detection and discrimination, as well as short- and long-term olfactory memories, odor-associated learning, and olfactory fear conditioning (reviewed in Sakamoto et al., 2014a). Pheromone-associated behaviours, such as mating and parenting, and many social behaviours in rodents are also dependent on this continual addition of newborn neurons to the OB (reviewed in Peretto and Paredes, 2014). However, the use of a wide range of techniques to modulate VZ neurogenesis has resulted in contradictory studies for each of these putative functions. Therefore, while many important questions have been answered over the past few decades, the full significance of this phenomenon remains to be elucidated.

Post-natal SGZ neurogenesis

Post-natal neurogenesis also occurs in the SGZ of the dentate gyrus in the hippocampus. SGZ NPCs progress through similar cell types and behaviours as seen in the VZ; however some of the nomenclature differs between NPCs of the two regions (reviewed in Ming and Song, 2011). Radial glia-like precursor cells (type I; GFAP-positive/nestin-positive) in the SGZ form non-radial glia-like precursor cells and intermediate progenitor cells (type IIa and IIb, respectively; GFAP-negative/nestin-positive), which give rise to neuronally-committed neuroblasts (type 3; DCX-positive). These generate immature neurons (TUJ1-positive), which migrate to the inner granule cell layer and differentiate into GABAergic hippocampal granule neurons (NeuN-

positive). Shortly thereafter these cells extend processes and form fully functional synaptic connections, with dendrites reaching into the molecular layer and axons projecting through the hilus towards the CA3 region. This process is regulated by the co-ordinated action of astrocytes, vascular cells, mature neurons and microglia (reviewed in Aimone et al., 2014); however unlike in the VZ, SGZ NPCs are not in contact with the CSF or ependymal cells. Similar to VZ neurogenesis, a large portion of NPCs are lost throughout this process (Tashiro et al., 2006b; Sierra et al., 2010).

As in the VZ literature, there are many, often contradictory, studies discussing the role of post-natal SGZ neurogenesis. Newborn neurons in this region also exhibit hyper-excitability and enhanced long-term potentiation compared to their older counterparts, providing an extra layer of plasticity to the dentate gyrus and connected circuitry. Overall, SGZ neurogenesis appears to significantly contribute to a variety of hippocampal-dependent functions (reviewed in Aimone et al., 2014) such as spatial learning, pattern recognition, and memory retention, as well as contextual fear and trace conditioning. Emotional control and affective behaviours also seem to be modified by this phenomenon (reviewed in Deng et al., 2010; Christian et al., 2014; Vadodaria and Jessberger, 2014). Because methodological differences between individual studies have led to inconsistent results throughout the literature, it is not surprising that the involvement of SGZ neurogenesis in some of these behaviours is still under debate.

Injury-induced neurogenesis

Apart from the role post-natal neurogenesis plays in maintaining OB and hippocampal homeostasis, it has also emerged as an exciting putative clinical target in a variety of

pathophysiological situations. NPCs are exquisitely sensitive to changes in their surrounding environment, and their behaviours are therefore dynamically regulated by a wide variety of brain injuries. Early studies noted a phenomenon termed 'injury-induced neurogenesis', in which NPC behaviours were altered following brain insults such as stroke (Takagi et al., 1999; Arvidsson et al., 2001; Jin et al., 2001; Kee et al., 2001), seizure (Bengzon et al., 1997; Parent et al., 1997), and traumatic brain injury (Gould and Tanapat, 1997). Overall, NPC proliferation and differentiation were enhanced, and migration was altered so that NPCs and their progeny were diverted towards the site of injury. In many cases, these diverted NPCs were able to differentiate into the specific cell types lost to the injury, albeit at a low percentage. These studies generated considerable interest in the potential for post-natal neurogenesis to act as an endogenous mechanism for brain repair.

The most well described occurrence of injury-induced neurogenesis has since been documented in rodent models of stroke (reviewed in Ohab and Carmichael, 2008). Stroke increases NPC proliferation in the VZ and SGZ niches. Within the healthy VZ, 15-21% of NPCs are actively proliferating with a cell cycle length of 18-21 hours (Zhang et al., 2006). At the peak of increased proliferation after stroke, 31% of NPCs are actively proliferating, with a cell cycle length of approximately 11 hours. Moreover, the quiescent NPCs lining the ventricle (previously believed to be ependymal cells) can transform into actively proliferating NPCs upon injury (Li et al., 2002). Stroke therefore increases both the number of actively proliferating NPCs and their rate of proliferation. Stroke also causes a subpopulation of migrating VZ NPCs to re-direct from the RMS towards the site of injury (reviewed in Ohab and Carmichael, 2008). These cells migrate along a network of pre-existing and newly-forming blood vessels with the help of astrocytic processes. Enhanced proliferation and altered migration peak 7-14 days post-injury,

but can persist for weeks and even months (Thored et al., 2006). However, the majority of cells that make it to the injury site do not survive (Arvidsson et al., 2002; Ohab et al., 2006), likely due to the lack of trophic support, the presence of deleterious factors released by the injury, and/or the resulting inflammatory response. The functional significance of injury-induced neurogenesis is therefore not fully understood; yet blocking this response worsens stroke outcomes in rodent models (Raber et al., 2004; Jin et al., 2010; Sun et al., 2012; Wang et al., 2012). Injury-induced neurogenesis also occurs in human stroke patients, with the response largely limited to the VZ niche and RMS. Similar to rodent studies, human VZ NPCs demonstrate both increased proliferation and diverted migration towards the injury (Jin et al., 2006; Macas et al., 2006). This implies that neurogenesis may present a novel approach for treatment strategies following brain injury; however we need a greater understanding of the molecular mechanisms controlling this phenomenon in order to take advantage.

1.2.3. Cell culture models of neuronal development

Neurogenesis is a complex process for which the biological underpinnings are poorly understood. Neuronal development is therefore often studied *in vitro* in simplified culture systems. This allows for precisely defined growth conditions, and thus provides an ideal system to examine the molecular mechanisms regulating various aspects of neurogenesis, including proliferation, migration, survival, fate specification, and neurite outgrowth. *In vitro* systems of neuronal development include cultures of primary NPCs from neurogenic regions of the embryonic or post-natal brain, or of NPC-like cell lines.

In the data presented in this dissertation, I use two different culture models: primary post-natal VZ NPCs (Reynolds and Weiss, 1992), and the Neuro-2a (N2a) cell line. Primary VZ NPCs are isolated by microdissecting and dissociating neonatal VZ tissue, and growing the resulting cells in suspension culture. The non-adherent conditions in combination with application of epidermal and basic fibroblastic growth factors (EGF and FGF-2) favour growth and survival of NPCs over other contaminating cell types. This results in formation of clonal clusters of NPCs termed 'neurospheres' (NSPs), which remain multipotent over multiple passages, and can be differentiated into neurons or glia, depending on culture conditions (Azari et al., 2010). The N2a cell line is derived from a murine neuroblastoma and is a widely used NPC-like model of neural-crest lineage. These cells divide quickly in culture, and are often used to study neuronal development and neurite outgrowth, as they can be easily differentiated into cells that extend neurites and possess many neuronal characteristics (Shea et al., 1985; Huang et al., 2007; Swayne et al., 2010; Wicki-Stordeur and Swayne, 2013).

1.3. Large-pore channels

Large-pore channels are transmembrane proteins that allow passage of ions, small molecules, and metabolites under 1 kDa across the membrane (Wicki-Stordeur and Swayne, 2012). This group of channel proteins consists of connexins (Cx), pannexins (Panx), and aquaporins. Ionotropic purinergic (P2X) receptors and transient receptor potential channels are also occasionally included in this classification. In this dissertation I concentrate on Panxs and Cxs, two large-pore protein families from within the gap junction group of proteins.

While Panxs share no sequence homology with Cxs, the two families are structurally similar (reviewed in Shestopalov and Panchin, 2008). Panx (Panx1, 2, and 3) and Cx family members both consist of four transmembrane domains, two extracellular loops, and three intracellular regions including a short N-terminus, intracellular loop, and highly variable C-terminus. Within the Cx family, six of these subunits oligomerize to form connexon hemichannels which can, in turn, come together on opposing cell membranes to form cell-cell conduits known as gap junctions. Like Cxs, Panx1 forms hexameric channels; however Panx2 channels are formed from eight subunits (Ambrosi et al., 2010). Panx3 is also predicted to hexamerize based on its greater size and sequence similarity to Panx1 than Panx2 (Figure 1.4). There is limited evidence for gap junction formation via these Panx channels (reviewed in Sosinsky et al., 2011), likely due to steric hindrance from glycosylation of the extracellular loops (Figure 1.5). This inability to couple has caused Panx channels to be commonly referred to as 'single-membrane' channels, to distinguish them from Cx hemichannels.

Cxs are among the most well-studied large-pore channels. These proteins were first described in the 1970's as the major structural component of vertebrate intercellular gap junctions (Goodenough, 1974). Since discovery, 21 Cxs have been identified in the human genome, and 20 in the rodent, of which 19 have a human ortholog (reviewed in Sohl and Willecke, 2004). Cxs are expressed in overlapping patterns across almost every cell type within the body, yet individual Cxs exhibit highly variable profiles. For example, Cx43, the most widely expressed, is found in at least 35 distinct tissues, while Cx31.1 is restricted to the skin.

Comparatively, Panxs are a relatively newly discovered family of large-pore channels. They were first identified in 2000 based on their homology to the gap-junction forming proteins in invertebrates, the innexins (Panchin et al., 2000). Of the three Panx family members, Panx1 is

the most ubiquitously expressed, and is found throughout the body in a variety of cell and tissue types (Bruzzone et al., 2003; Baranova et al., 2004; Ray et al., 2005; Vogt et al., 2005). Panx2 and Panx3 exhibit more restricted expression profiles (Baranova et al., 2004; Vogt et al., 2005): Panx2 is limited to the nervous system while Panx3 is expressed in skin, bone, and cartilage. However, these expression profiles are largely based on mRNA analyses. A more recent study found indications of Panx2 protein expression in tissues outside of the nervous system, in which Panx2 transcript could not be detected (Le Vasseur et al., 2014); however, these results have yet to be independently verified. The Panx expression profiles have also not been examined across various stages of development. Therefore the full extent of Panx expression across the body and throughout development is not completely understood.

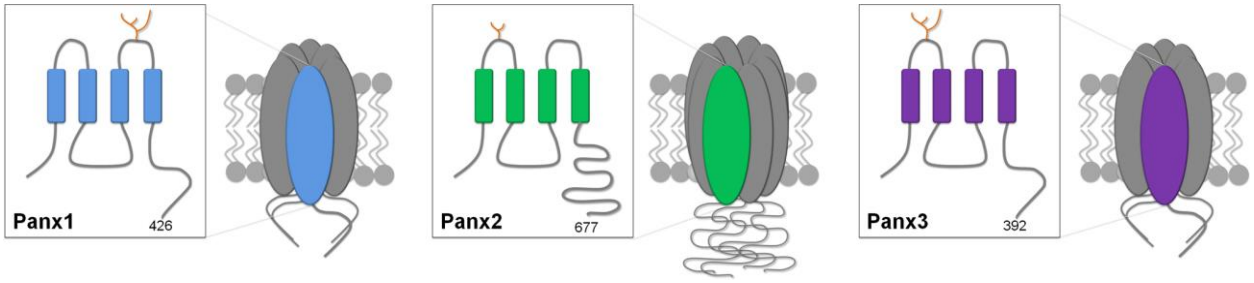


Figure 1.4. Schematic of the Panx family of large-pore channels.

The Panx family consists of three members. Each Panx protein has four transmembrane domains, two extracellular loops, and intracellular N-terminus, C-terminus, and loop region. The C-terminus represents the most variable region between Panxs. Panx1 and Panx3 channels consist of six individual Panx subunits, while Panx2 channels contain eight subunits. These single-membrane channels are unable to form coupled gap junctions due to steric hindrance of glycosyl groups. Panx1 contains a glycosylation site on the second extracellular loop and receives a complex glycosyl group. Panx2 and 3 contain glycosylation sites on the first extracellular loop.

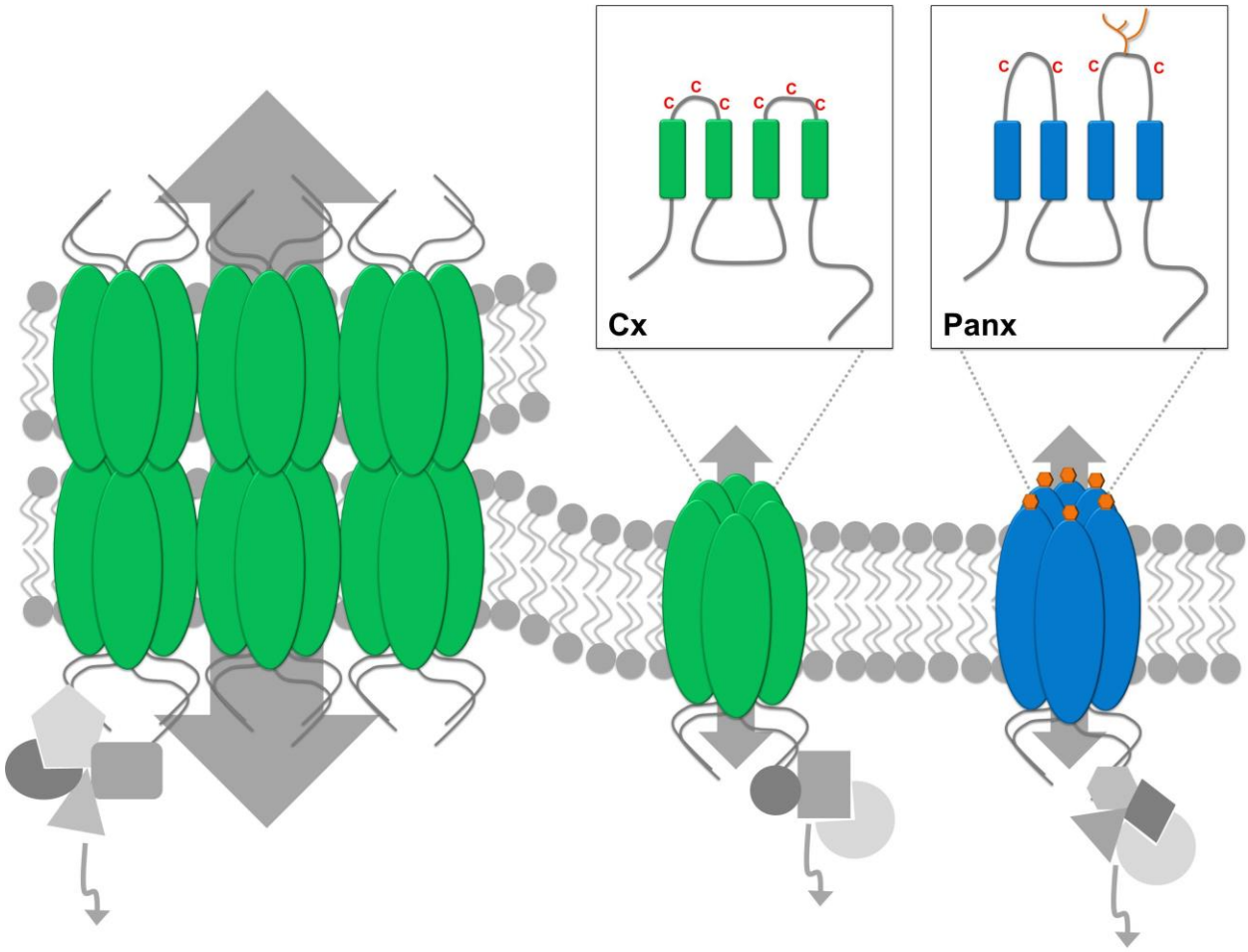


Figure 1.5. Large-pore channels can exert their effects through various mechanisms.

Cxs and Panxs possess similar transmembrane topology. However, Panxs generally have longer extracellular loops with fewer cysteine (C) residues available for disulfide bridge formation. These loops are also glycosylated in Panxs, possibly preventing the formation of coupled gap junction channels through steric inhibition. Cx gap junctions form when Cx hemichannels on adjacent membranes dock with one another. The docked hemichannels are grouped together in gap junction 'plaques' on the plasma membrane, and facilitate intercellular communication by passing ions and small molecules. Cx hemichannels or Panx single-membrane channels are normally closed at the plasma membrane, but open in response to certain stimuli. Once open, these channels pass ions and small molecules between the intra- and extracellular compartments. Cxs and Panxs can also function as signalling nexuses via protein-protein interactions that regulate downstream signalling cascades.

1.3.1. Gap junctions

Large-pore channels have the ability to establish cellular communication networks through the formation of gap junctions. Apart from work in heterologous expression systems and/or overexpression studies (Bruzzone et al., 2003; Vanden Abeele et al., 2006; Ishikawa et al., 2011), limited evidence supports the idea that Panxs are involved in gap junction formation (reviewed in Sosinsky et al., 2011). Instead, these intercellular conduits are established when Cx hemichannels on opposing cell membranes dock with one another (reviewed in Laird, 2006). Each Cx extracellular loop contains three highly conserved cysteine residues which form disulfide bridges that are critically important for hemichannel docking. Upon coupling, the gap junction conduit allows transfer of ions and metabolites, including small molecules and second messengers up to 1 kDa in size, between adjacent cells. This includes important signalling molecules such as ATP, ADP, adenosine, cAMP, glucose, IP_3, and Ca^{2+}. Gap junctions are therefore key regulators of many physiological processes such as cell synchronization, metabolic co-ordination, and differentiation (reviewed in Kar et al., 2012).

1.3.2. Single membrane channels and hemichannels

Until recently, the fundamental function of hemichannels was considered to be formation of gap junctions. It is now widely recognized that Cx hemichannels serve functions distinct from classic gap junctions (reviewed in Dbouk et al., 2009). This idea was further supported by discovery of Panxs, which exclusively form single-membrane channels (reviewed in Sosinsky et

al., 2011). These channels establish a physical conduit between the intracellular and extracellular spaces through which ions and metabolites up to 1 kDa in size can pass. In contrast to gap junctions which are open unless stimulated to close, hemichannels and single-membrane channels are largely believed to exist in a closed state until stimulated to open. Evidence suggests that aberrant opening of these channels can cause cell death via loss of ionic and metabolic gradients, increased cell osmolarity and Ca^{2+} influx (reviewed in Kalvelyte et al., 2003; Chandrasekhar and Bera, 2012; Weilinger et al., 2013). However, controlled opening of these channels contributes to a variety of physiological cellular processes (reviewed in Dbouk et al., 2009; Chandrasekhar and Bera, 2012; Lohman and Isakson, 2014). Cx hemichannels and Panx single membrane channels mediate uptake and release of various molecules between the cytoplasm and the extracellular space, including ATP, adenosine, prostaglandin E2, glutamate, NAD^+, and glucose.

1.3.2.1. ATP release through single membrane channels and hemichannels

A well-documented function shared by Panx single-membrane channels and Cx hemichannels is the release of ATP into the extracellular space (reviewed in Lohman and Isakson, 2014). This phenomenon was originally solely attributed to Cx hemichannels, with Cxs 26, 32, 37, 40 and 43, among others, suggested to release ATP following different stimuli. However, limited evidence existed for hemichannel opening under physiological conditions. Moreover, following discovery of the Panxs, a number of pharmacological agents believed to act as Cx blockers (i.e. carbenoxolone) were found to also inhibit Panx single membrane channels, often with equal or greater efficacy (Bruzzone et al., 2005; reviewed in Dahl et al., 2013 and Lohman and Isakson,

2014). Therefore Panxs, and especially Panx1, have more recently emerged as prominent regulators of ATP release in a variety of tissues (reviewed in Dahl, 2015). Current evidence demonstrates that Cx and Panx1 channels are activated by pathophysiological stimuli such as oxygen/glucose deprivation, hypotonic stress, and strong membrane depolarization. However, Panx1 channels are also activated by weaker depolarization, caspase cleavage, and mechanical stimulation, among other stimuli (reviewed in Lohman and Isakson, 2014). Therefore, Panx1 (or Panx3 in some tissues; Iwamoto et al., 2010; Fu et al., 2015) is believed to be the major ATP release conduit under physiological conditions (yet see Batra et al., 2012; Orellana et al., 2012; Ponsaerts et al., 2012), with both Cxs and Panx1 acting during pathophysiological situations.

ATP and its metabolites (ADP, AMP, adenosine) act as prominent autocrine and paracrine signalling molecules via their activation of plasma membrane receptors (reviewed in Zimmermand, 2011; Cavaliere et al., 2014). This receptor family is composed of metabotropic adenosine receptors (P1), and ATP/ADP-binding ionotropic (P2X) and metabotropic (P2Y) purinergic receptors. P1 and P2Y receptors are G-protein coupled: P1 receptor activity ultimately regulates adenylate cyclase function and therefore cAMP levels, while P2Y receptors influence IP_3 and cAMP signalling pathways to alter cytoplasmic Ca^{2+} levels. The P2X receptors are ionotropic, allowing passage of Na^{+}, K^{+}, and Ca^{2+} across the plasma membrane when activated. Each receptor subtype has multiple isoforms (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11-P2Y14, and P2X1-P2X7) that demonstrate varied sensitivity to ATP and its metabolites. These isoforms are differentially expressed across tissue and cell types, so the purinergic response can be modulated based on which receptor isoforms are present in any given tissue. Moreover, extracellular ATP levels are finely regulated by tissue-specific expression of ectonucleotidases (nucleotide degrading enzymes; reviewed in Zimmermann et al., 2012). It is therefore not surprising that

purinergic signalling impacts various aspects of development and cellular function, including neurotransmission, inflammation, cell growth and differentiation, migration, and cell death (reviewed in Burnstock et al., 2010).

Interestingly, there appears to be a reciprocal regulation between purinergic receptor signalling and single membrane channel and hemichannel function (reviewed in Baroja-Mazo et al., 2013). Several purinergic receptors have been implicated in the activation of Cx and Panx channels, with the P2X7 ionotropic purinergic receptor perhaps the most studied. The P2X7 receptor has long been known to present di-phasic activation kinetics. Sustained stimulation opens a larger pore permeable to molecules up to approximately 900 Da. This large-pore has since been identified as Panx1 in many cell types (Pelegrin and Surprenant, 2006; reviewed in Baroja-Mazo et al., 2013). Moreover, Panx1 (reviewed in Wicki-Stordeur and Swayne, 2014) and Cx43 (Fortes et al., 2004) physically interact with P2X7 receptors in a cell type-specific manner. Panx1 also associates with P2X2 (Li et al., 2011) and P2X4 (Hung et al., 2013) receptors, both of which exhibit large-pore conductance over time; however the physiological relevance of these associations has not yet been determined. P2Y receptor signalling causes Cx hemichannel opening in many cell types (reviewed in Baroja-Mazo et al., 2013), and may also open Panx channels (Locovei et al., 2006a). Many studies demonstrate that P2X and P2Y receptor-based activation of Panx and Cx channels is a result of increased intracellular Ca^{2+} (Suadicani et al., 2004; Locovei et al., 2006a; Pelegrin and Surprenant, 2006; Iglesias et al., 2008), and acts as a positive feedback loop to amplify purinergic signalling. Importantly, Panx1 is also negatively regulated by ATP independent of purinergic receptors, through direct binding of the molecule to Panx1 channels (Qiu and Dahl, 2009). This ATP-dependent inhibition likely acts, at least in part, by signalling for Panx1 channel internalization (Boyce et al., 2015). Together ATP and

purinergic signalling therefore act as important dual regulators of single membrane channel function.

1.3.3. Large pore channels as 'signalling nexuses'

In recent years, the importance of Panx and Cx proteins acting in intracellular signalling has emerged. These actions are largely mediated by protein interactions with Panx (reviewed in Wicki-Stordeur and Swayne, 2014) or Cx proteins (reviewed in Herve et al., 2012; Vinken et al., 2012), which in some cases can influence gene expression. Large-pore channels are thus referred to as cellular 'signalling nexuses'. It has been proposed that Panxs and Cxs act as scaffolds to spatiotemporally confine enzymes, substrates, effectors, and the cytoskeleton. In particular, a large body of literature demonstrates the interaction of various Cxs with such proteins (reviewed in Herve et al., 2012; Vinken et al., 2012). For example, several studies present evidence that interactions between the cytoskeleton and the C-terminus of Cx43 are crucial for cell polarization and migration within the developing brain, independent of channel-function (Elias et al., 2007; Cina et al., 2009; Elias et al., 2010). Moreover, full length Cx43 (Huang et al., 1998) or its C-terminal region (Moorby and Patel, 2001; Dang et al., 2003) localize to the nucleus and inhibit proliferation in several cell types. Signalling nexus functions of Cxs also influence cell differentiation and death pathways (reviewed in Vinken et al., 2012). The corresponding role of Panxs has not been investigated in as great of depth; however recent Panx interaction studies are beginning to unveil the potential molecular players involved (reviewed in Wicki-Stordeur and Swayne, 2014).

1.4. Regulation of neuronal development by Panx and Cx large-pore channels

It has become increasingly evident that the passage of ions and small molecules across cellular membranes is a key effector of neuronal development (reviewed in Yasuda and Adams, 2010; Swayne and Wicki-Stordeur, 2012; Wicki-Stordeur and Swayne, 2012). Since large-pore channels are able to pass higher molecular weight signalling molecules (approximately 1 kDa in size) in addition to ions, they are uniquely placed to influence various cellular pathways underlying neuronal development, such as the purinergic signalling outlined in the previous section. Panx1 and 2 are expressed in the brain in mature neurons (Bruzzone et al., 2003; Baranova et al., 2004; Ray et al., 2005; Vogt et al., 2005). Panx2 is also found in post-natal SGZ NPCs (Swayne et al., 2010). Moreover, a large number of Cxs are expressed in the brain (reviewed in Decrock et al., 2015), in a cell-type specific manner. Cxs 23, 26, 30, 33, 36, 40, 43, and 45 have been identified in various NPCs, with the role of Cx43 being particularly well-described in the context of neuronal development (reviewed in Eugenin et al., 2012; Wicki-Stordeur and Swayne, 2012; Salmina et al., 2014). Gap junction proteins were initially identified as key components of electrical synapses within neural cells; however as outlined in the previous section, Panx and Cx proteins can also act through gap junction-independent mechanisms to influence various NPC behaviours (reviewed in Elias and Kriegstein, 2008).

1.4.1. NPC Proliferation

Several studies have examined the role of Cxs in NPC proliferation. Early work demonstrated that NPCs within the embryonic VZ and developing cortex are coupled via gap junctions in a cell

cycle dependent manner (Bittman et al., 1997). The dynamic switch between gap junction conduits and uncoupled hemichannels across the cell cycle seems to influence NPC division and fate specification. NPCs of the post-natal VZ also show gap junctional coupling (Menezes et al., 2000; Lacar et al., 2011) and express functional hemichannels (Liu et al., 2006). Activity of Cx43channels enhances cell proliferation in cultured NPCs (Duval et al., 2002; Cheng et al., 2004; Malmersjo et al., 2013), likely in a growth factor-dependent manner (Lemcke and Kuznetsov, 2013). Contrastingly, Cx43 negatively regulates proliferation of an NPC-like cell line independently of its channel function (Moorby and Patel, 2001), and analyses in the early post-natal VZ inversely correlate Cx43 expression with levels of DNA synthesis (Miragall et al., 1997).

NPC proliferation is highly responsive to extracellular ATP (reviewed in Cavaliere et al., 2015), which is released through Cx and Panx channels (Figure 1.6; reviewed in Lohman and Isakson, 2014). In embryonic VZ NPCs, Cx hemichannels release ATP, activating P2Y1 receptors and resulting in downstream rises in intracellular Ca^{2+} levels (Weissman et al., 2004). Purinergic receptor-mediated Ca^{2+} waves promote proliferation in both embryonic (Ryu et al., 2003; Weissman et al., 2004) and post-natal NPCs (Mishra et al., 2006; Lin et al., 2007; Suyama et al., 2012; Boccazzi et al., 2014), and are further propagated between NPCs through gap junction conduits (Lacar et al., 2011). Cx-mediated ATP release and Ca^{2+} signalling may also impact VZ NPC proliferation by regulating interkinetic nuclear migration (Liu et al., 2010), a process in which the nucleus moves between apical and basal aspects of the VZ in synchrony with the cell cycle. In the early post-natal VZ, Cx45 facilitates a similar increase in NPC proliferation via ATP release (Khodosevich et al., 2012). Post-natal SGZ NPCs are also stimulated to proliferate in response to extracellular ATP and P2Y1 receptor activation (Cao et

al., 2013). These NPCs express Cx30 and Cx43, which are necessary for their proliferation (Kunze et al., 2009) suggesting NPC Cxs may also contribute to ATP release within this neurogenic niche. Given the known role of Panx1 as an ATP release channel (reviewed in Dahl, 2015), and its expression within the central nervous system (Bruzzone et al., 2003; Baranova et al., 2004; Ray et al., 2005; Vogt et al., 2005), it is reasonable to suggest that Panx1 contributes to NPC proliferation. While Panx2 is also expressed in the brain (Baranova et al., 2004; Vogt et al., 2005; Swayne et al., 2010), it appears to be restricted to intracellular compartments and therefore is not able to act as a plasma membrane conduit for ATP release.

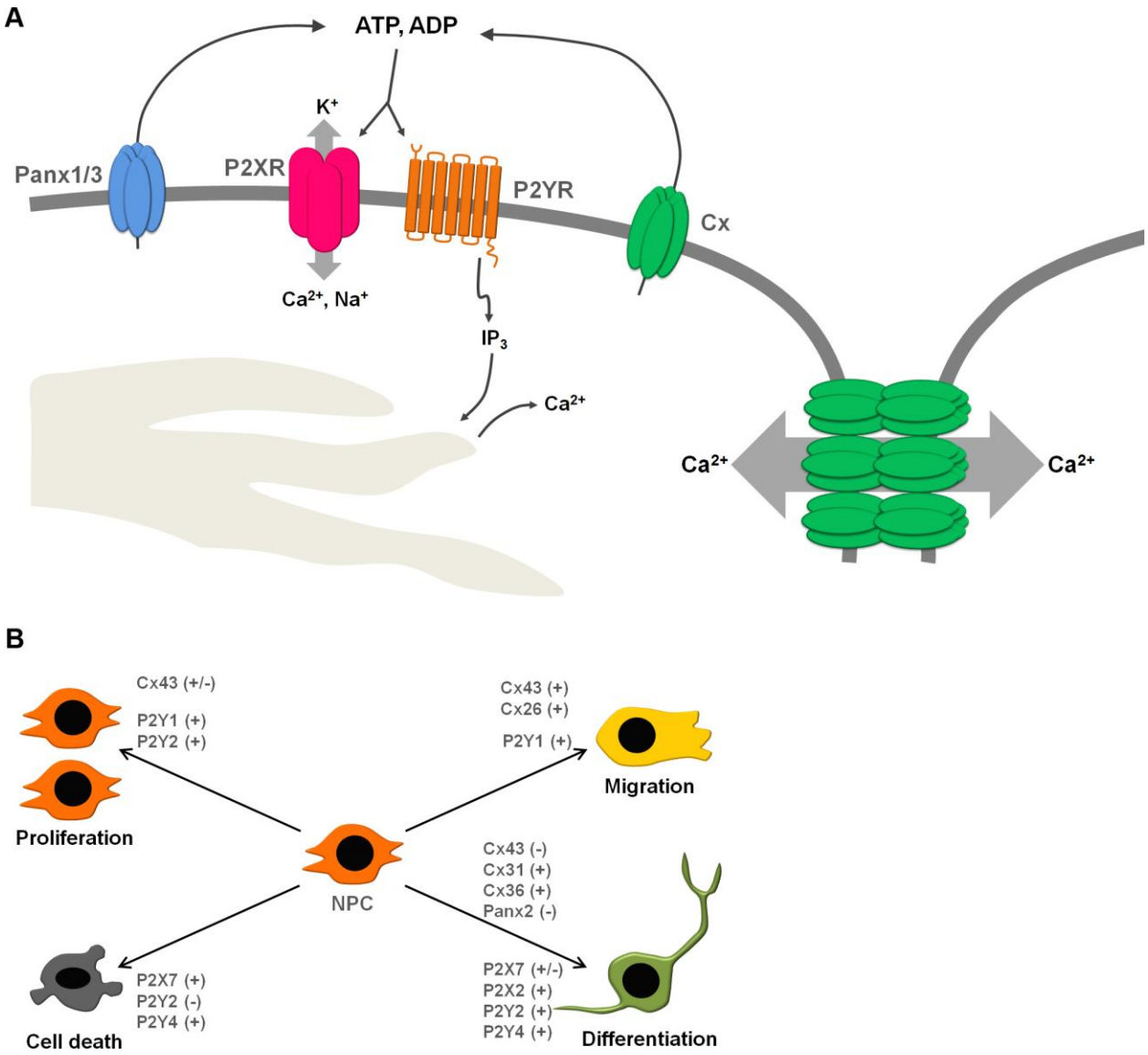


Figure 1.6. Panxs and Cxs regulate neuronal development in part, through nucleotide release and downstream purinergic receptor activation.

(A) Panx single membrane channels and Cx hemichannels release ATP and other nucleotides into the extracellular space. These nucleotides activate ionotropic P2X and metabotropic P2Y purinergic receptors in an autocrine or paracrine manner. P2X receptors open a cationic channel and pass Na^+, K^+ and Ca^{2+}, while G-protein coupled P2Y receptors activate signalling cascades resulting in an IP_3-dependent release of Ca^{2+} from intracellular stores. The resulting Ca^{2+} waves can be propagated via gap junctions between neighbouring cells. (B) Diagram representing the known functions of different Cxs, Panxs, and purinergic

receptors in NPC proliferation, migration, neuronal differentiation, and cell death. (+) positively regulates, (-) negatively regulates.

1.4.2. NPC Migration

NPC migration is dependent on Cx function (reviewed in Matsuuchi and Naus, 2013). Gap junctional coupling promotes migration from early post-natal RMS/VZ explants (Marins et al., 2009) and in the developing cortex, Cx43 and Cx26 are essential for mediating NPC migration along radial glia fibres (Fushiki et al., 2003; Elias et al., 2007; Wiencken-Barger et al., 2007). This action of Cx43 is channel-independent and instead functions through (1) adhesive interactions between Cx extracellular loops on opposing membranes and (2) interactions between the Cx C-termini and underlying cytoskeleton (Elias et al., 2007; Cina et al., 2009; Elias et al., 2010). Cx26 also mediates cell-cell adhesion to promote migration (Valiente et al., 2011).

ATP release through Panxs and Cxs is also expected to play a role in NPC migration. Extracellular ATP promotes NPC migration (Oliveira et al., 2015), likely via activation of P2Y1 receptors (Liu et al., 2008) and downstream cytoskeletal rearrangements (Grimm et al., 2010). Interestingly, an *in vitro* analysis found that Cx43 regulates NPC migration by controlling P2Y1 receptor expression (Scemes et al., 2003); however, further work is required to fully understand the role of Panx and Cx channels in NPC migration.

1.4.3. Neuronal Differentiation

There is emerging evidence implicating multiple Cxs in NPC differentiation. A shift in Cx expression profiles accompanies neuronal differentiation of NPCs (reviewed in Elias and

Kriegstein, 2008), as mature neurons primarily express Cxs 26, 36 and 45 (reviewed in Decrock et al., 2015). Cx43 expression and coupling generally decrease across neuronal differentiation in cultured NPCs (Rozental et al., 1998; Duval et al., 2002; Boucher and Bennett, 2003); however there is evidence of an initial de-coupling at the onset of differentiation, followed by a necessary re-coupling at later stages (Lemcke et al., 2013). Channel-independent effects of Cx43 are also implicated in the inhibition of neuronal differentiation (Santiago et al., 2010; Rinaldi et al., 2014). Other Cxs have opposing effects to that of Cx43: Cx36 promotes neuronal differentiation in cultured NPCs (Hartfield et al., 2011), and channel-independent activity of Cx31 increases neurite outgrowth, a morphological correlate of differentiation, in an NPC-like cell line (Unsworth et al., 2007). One study also demonstrates Panx2 expression in post-natal SGZ NPCs (Swayne et al., 2010) where it controls the timing of neuronal differentiation. These NPCs lose Panx2 during early stages of neuronal commitment, but re-express this protein as mature neurons. Since Panx2 expression is exclusively intracellular in these cells (and others; see Boassa et al., 2015), this function cannot be explained by channel activity at the plasma membrane.

On the other hand, Panx1 and Cx plasma membrane channels likely influence NPC differentiation through release of ATP. Purinergic signalling is a key mediator of neuronal differentiation, especially via ionotropic P2X7 receptors. P2X7 receptors are expressed in the embryonic (Tsao et al., 2013) and post-natal VZ (Messemer et al., 2013), with mRNA also noted in early post-natal SGZ (Tsao et al., 2013). In culture, reduced P2X7 receptor expression coincides with neuronal commitment (Wu et al., 2009; Orellano et al., 2010; Glaser et al., 2014), while receptor antagonists or knock-down stimulate neurite outgrowth (Gomez-Villafuertes et al., 2009; Wu et al., 2009) and axonal elongation and branching (Diaz-Hernandez et al., 2008). Contrasting work from primary embryonic NPC cultures found that P2X7 receptors promote

NPC differentiation (Tsao et al., 2013). Together these studies suggest that P2X7 receptors can have positive or negative effects on neuronal differentiation, likely depending on the experimental conditions. The diverse expression of additional purinergic receptors in specific NPC subpopulations could also contribute to these observations. For example, P2Y2 receptors (Arthur et al., 2005, 2006), P2Y4 receptors (Cavaliere et al., 2005), and P2X2 receptors (Schwindt et al., 2011; Yuahasi et al., 2012) are linked to neuronal differentiation in certain cells. Moreover, there are multiple P2X7 receptor splice variants (Cheewatrakoolpong et al., 2005; Adinolfi et al., 2010) that add further layers of complexity. Preliminary work demonstrates an unidentified purinergic receptor is activated by Cx-mediated ATP release in culture, and promotes neurite outgrowth (Belliveau et al., 2006). Nevertheless, further examination is required to determine whether Panx and Cx channel function are involved in the purinergic control of neuronal differentiation.

1.5. Summary

Neurogenesis continually generates new neurons from VZ NPCs in the post-natal mammalian brain. These NPCs undergo proliferation, migration, and differentiation to ultimately populate the OB as interneurons, contributing to OB structure and associated functions. This neuronal development is exquisitely controlled by both intrinsic and extrinsic factors, yet many of these regulatory processes are poorly understood. Large-pore channel proteins, including Panxs and Cxs, are emerging as key modulators of this process. These channel proteins pass ions and metabolites that act on various signalling pathways, or can function through channel-independent mechanisms as cellular ‘signalling nexuses’. Importantly, Panxs and Cxs are able to release ATP

and other nucleotides, signalling molecules to which NPCs are highly responsive. A large body of work has investigated the effects of Cx proteins in neuronal development; however, since Panxs were only recently discovered, it was not known when I began my graduate work whether they also contributed to VZ NPC behaviours. The following chapters examine the importance of Panx1 in post-natal VZ NPC development, as well as the molecular mechanisms underlying Panx1's function in these cells.

2. Methods

2.1. Experiments in mice

All procedures were carried out in agreement with the guidelines of the Canadian Council for Animal Care, and the University of Victoria and University of Ottawa Animal Care Committees. An equal mix of male and female C57BL/6 mice was used unless otherwise specified.

2.1.1. Panx1 expression analyses

For analyses of Panx1 expression in VZ NPCs *in vivo* (Chapter 3), mice were sacrificed by cardiac perfusion at post-natal day (P)15, P30, and P60. Coronal cryosections of these brains (see section 2.4 for methods) were immunostained for Panx1 along with NPC lineage markers.

For Western blot time course analyses of Panx1 and Crmp2 expression in the VZ (Chapter 5), mice were sacrificed at P0, P7, P10, P28, and P60. The VZ was removed by dissection, and made into lysates for SDS-PAGE/Western blot analysis (see section 2.6 for methods)

2.1.2. Virus injections and stroke/sham surgeries

The following procedures were performed at the University of Ottawa as part of a collaboration with Dr. Diane Lagace. Focal cortical ischemia was induced by photothrombosis of the cortical microvasculature (as described in Watson et al., 1985). Briefly, adult (2 – 3 months) "floxed" Panx1-LoxP mice (on a 129 background; confirmed by genotyping; Dvoriantchikova et

al., 2012), or wild-type 129 control mice were anesthetized using isofluorane and maintained at 37°C with a heating pad. A 1% Rose Bengal (Sigma-Aldrich, St. Louis, Missouri, USA) solution (in brain buffer: 0.04 M NaH_2PO_4, 0.16 M Na_2HPO_4) was injected intraperitoneally (i.p.) 2 – 5 minutes before laser illumination. The skull was exposed by a midline incision, and a site 2.25 mm left of the midline and 0.7 mm anterior to bregma was illuminated for 10 minutes by a laser calibrated to 532 nm. Retrovirus was used to target primarily late-stage NPCs (Tashiro et al., 2006b; Tashiro et al., 2006a) in the VZ. CAG-red fluorescent protein (RFP) and CAG-green fluorescent protein (GFP)-Cre viruses were mixed in a 1:1 ratio and injected bilaterally at the time of stroke at coordinates 1.2 mm right and left of the midline, 1.0 mm posterior to bregma and 1.9 mm in depth.

Mice were sacrificed at 2, 5, or 10 days post-injection/photothrombosis (dpi/PT; N = 7 for 2 dpi/PT, and N = 6 for 5 and 10 dpi/PT). Naïve floxed Panx1 mice were given bilateral virus injection without stroke, and sacrificed at 2 or 10 dpi (N = 5 for 2 dpi and N = 6 for 10 dpi). Naïve wild-type 129 control mice were given bilateral virus injection without stroke and sacrificed at 2 dpi (N = 7).

2.1.3. Cell quantifications

For virus quantifications, 600 by 400 μm boxes were drawn around the VZ dorsolateral corner, the ventral boundary of the stroke, and the medial edge of the stroke boundary, aligned with the pial surface as shown in Figure 4.1. In addition, representative coronal slices from each animal were taken for virus quantifications in the rostral migratory stream (RMS; See Figure 1). The area of quantification was equivalent between animals, and was restricted to the circle/oval of

condensed nuclei rostral to the opening of the lateral ventricles. Hoechst 33342 was used as a nuclear counterstain in all images. RFP fluorescence was present in both cytoplasmic and nuclear compartments of the NPCs, while GFP signal was localized to the nucleus. As such, the RMS and VZ counting criteria were that a positive cell must have GFP and/or RFP signal overlapping with a Hoechst-positive nucleus. Panx1-expressing NPCs possessed RFP fluorescence only, and Panx1-null NPCs had nuclear GFP fluorescence with or without RFP fluorescence. Quantification of transduced NPCs in the VZ of wild-type 129 control mice revealed relatively equal expected populations of RFP-positive only and GFP-positive NPCs per VZ (45% vs 55%, each ± 2%; N = 7, 2 dpi). There were no significant differences in NPC labeling between hemispheres in stroke animals and therefore presented pooled contralateral and ipsilateral data for each subsequent analysis. Data are presented as mean number of NPCs per VZ quantification region (outlined above). The data from each individual animal was considered as an independent biological replicate.

For lineage analysis, images of equal area were taken from the dorsolateral corner of the VZ, and overlap between Cre-GFP or RFP, and DCX signal was analyzed. The counting criteria were such that a transduced cell was considered DCX-positive if two thirds of its surface was surrounded by DCX signal in at least one plane of a confocal z-stack.

For proliferation analysis, images of equal area were taken from the dorsolateral corner of the VZ, and overlap between Cre-GFP or RFP, and Ki67 signal was analyzed. The counting criteria were such that a transduced cell was considered Ki67-positive if the corresponding nucleus overlapped with Ki67 signal in at least one plane of a confocal z-stack.

For apoptosis analysis, images of equal area were taken from the peri-infact cortex (as described above) and overlap between Cre-GFP or RFP and activated caspase 3 (*Casp3) was

analyzed. The counting criteria were such that a transduced cell was considered *Casp3-positive if the corresponding nucleus overlapped with *Casp3 signal in at least one plane of a confocal z-stack.

2.2. Primary NPC cultures

Primary NPCs were isolated from P0-P3 C57BL/6 mouse VZ or hippocampus. Briefly, P0-P3 brains were removed and put into artificial cerebrospinal fluid (ACSF; 26 mM $NaHCO_3$, 124 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM D-glucose, 100 U/mL penicillin, 100 µg/mL streptomycin). Coronal sections of 500 µm were made using a vibratome, and VZ tissue or the hippocampus was dissected out. The tissue was incubated at 37°C for 45-60 minutes in dissociation media (26 mM $NaHCO_3$, 124 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$, 3.2 mM $MgCl_2$, 10 mM D-glucose, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mg/mL dispase-1, 10 mg/mL papain, 10 mg/mL DNAse), then triturated into single cells using a fire-polished Pasteur pipette. Cells were plated at $8.8x10^3$ cells/cm^2 in neurosphere (NSP) proliferation media (DMEM/F12, B-27, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 20 ng/mL human EGF, 10 ng/mL FGF-2; Life Technologies, Burlington, Ontario, Canada) on ultra-low attachment cell culture dishes (Corning, Tewksbury, Massachusetts, USA) and expanded as NSPs for 7 days *in vitro* (DIV). EGF and FGF-2 were replenished in the culture medium every 2 days.

For neuronal differentiation, whole VZ NSPs were re-plated at DIV7 onto a poly-d-lysine (PDL; 100 µg/mL) coated surface in neuronal driving differentiation media (Neurobasal-A, B-

27, 0.5 mM Glutamax, 100 U/mL penicillin, 100 μg/mL streptomycin; Life Technologies), and were collected after 5 days by fixing in 3.7% formaldehyde.

2.2.1. Proliferation

For proliferation assays, VZ NSPs were treated at DIV1 with 1 mM probenecid (Panx1 blocker; Silverman et al., 2008), or an equivalent volume of sterile water (vehicle). At DIV7, NSPs were visualized using bright field illumination, and sphere diameters were measured in Photoshop CS5 (Adobe). For proliferation assays using adherent VZ NPCs, NSPs were dissociated at DIV7 in 37°C Versene solution (Life Technologies) with a Pasteur pipette, and plated on a PDL coated surface in proliferation media. Probenecid (1 mM) or vehicle was added the following day, and NPC confluence was tracked in real time using an Incucyte kinetic imaging system (Essen Biosciences, Ann Arbor, Michigan, USA).

2.2.2. Neurite Outgrowth

For neurite outgrowth analysis, VZ NSPs were dissociated at DIV7 in 37°C Versene solution with a Pasteur pipette, and plated on a PDL coated surface in proliferation media. Probenecid (1 mM) or vehicle were added the following day, and cells were collected after 48 hours by fixing in 3.7% formaldehyde, then imaged using bright field microscopy. Images were analyzed in Adobe Photoshop for cell body length, and length of all processes. A process was considered a neurite if it was greater than or equal to the length of the corresponding cell body.

2.3. N2a cell culture

N2a cells were cultured in N2a proliferation media (DMEM/F12, 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin). To differentiate, N2a cells were plated at $1.3x10^4$ cells/cm^2 on a PDL-coated surface in differentiation media (DMEM/F12, 2% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 µM all-trans retinoic acid), and collected after 24 hours.

2.3.1. Transfection

Where indicated, N2a cells were transfected using jetPEI reagent (Polyplus/VWR; Edmonton, Alberta, Canada) according to the manufacturer's protocol, with Panx1-EGFP plasmid (a generous gift from Dr. Dale Laird, University of Western Ontario, Ontario, Canada), EGFP control plasmid, Crmp2-DsRed plasmid (a generous gift from Dr. Rajesh Khanna, University of Arizona), or DsRed control plasmid. For knock-down, cells were transfected using Interferin (Polyplus/VWR) with validated Panx1 (targeting 5'-CCACCUUCGAUGUUCUACAUU-3') or GFP (targeting 5'-AAGCUGACCCUCAAGUUCAUC-3') siRNAs (Dharmacon/Thermo Fisher Scientific, Lafayette, Colorado, USA) according to the manufacturer's protocol.

2.3.2. Proliferation

For proliferation assays in Chapter 3, N2a cells were plated at $3.6x10^4$ cells/cm^2 and treated 4-6 hours later (time 0) with 1 mM probenecid, 30 µM PPADS or vehicle control. Cells were counted at 0, 24, and 48 hours.

For proliferation assays in Chapter 5, N2a cells were plated at $5.3x10^4$ cells/cm^2. Four hours later, cells were treated with 10 µM Panx1 C-terminus (Panx1CT) fragment peptides CT1, CT2, CT3 (Figure 2.1; New England Peptide, Gardner, Massachusetts, USA) or control (TAT; AnaSpec, Fremont, California, USA) and imaged every 2 hours over 5 days using an Incucyte kinetic imaging system. Cell confluence was quantified by the Incucyte software.

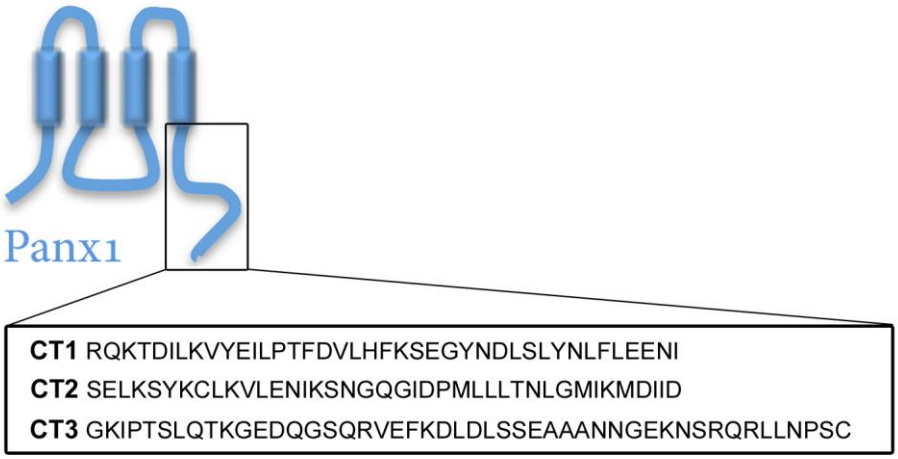


Figure 2.1. Schematic of the Panx1 C-terminal fragment peptides.
Panx1 C-terminal peptides were tagged with a TAT sequence (YGRKKRRQRRR) and used in binding assays to narrow down the interaction region between Panx1 and Crmp2. N2a cells were then treated with the peptides, in a competitive strategy to interfere with the Panx1-Crmp2 interaction, and their proliferation, migration, and neurite outgrowth was analyzed (see Chapter 5 results).

2.3.3. Migration

For migration analyses, N2a cells were grown to confluence before being subject to a scratch wound. The scratch wounds were monitored in real time using an Incucyte kinetic imaging

system, with images taken every 2 hours for 80 hours. The Incucyte software calculated wound width at each time point.

2.3.4. Neurite outgrowth

For neurite outgrowth experiments in Chapter 3, N2a cells ($1.3x10^4$ cells/cm^2) in proliferation media were treated with 1 mM probenecid or vehicle control and collected after 36 hours. N2a cells were also transfected with Panx1 or control siRNAs (see section 2.3.1) and examined 48 hours later. N2a cells overexpressing Panx1EGFP were cultured according to the differentiation protocol outlined above, and collected after 24 hours. Cells were collected by fixing in 3.7% formaldehyde, and imaged on an epifluorescent microscope. Images were analyzed in Adobe Photoshop for cell body length, and number and length of all processes. A process was considered a neurite if it was greater than or equal to the length of the corresponding cell body.

For neurite outgrowth experiments in Chapter 5, N2a cells were plated at $7.9x10^3$ cells/cm^2 16 hours prior to analysis. The media was changed to include 10 µM CT3 or control peptides, and the cells were immediately placed into an Incucyte kinetic imaging system for real-time monitoring of neurite length and branch points, over 24 hours. Quantification was performed in the Incucyte software.

2.3.5. ATP release

For Chapter 3 ATP release assays, N2a cells were plated at $5.3x10^4$ cells/cm^2 16-20 hours prior to analysis. Media was removed and cells were washed once with Hank's balanced salt

solution (HBSS[1]; 137.93 mM NaCl, 5.33 mM KCl, 1.26 mM $CaCl_2$, 0.9 mM $MgCl_2$, 5.56 mM D-glucose, 0.441 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 0.338 mM Na_2HPO_4, pH 7.4) and pre-incubated for 10 minutes in HBSS[1] with or without the addition of 0.5 mM probenecid. Eighty percent of the pre-incubation medium was removed, and replaced with an equal volume of high KCl HBSS[1] (117.93 mM NaCl, 25 mM KCl, 1.26 mM $CaCl_2$, 0.9 mM $MgCl_2$, 5.56 mM D-Glucose, 0.441 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 0.338 mM Na_2HPO_4, pH 7.4) for a final concentration of 20 mM KCl with or without 0.5 mM probenecid, for 10 minutes. Eighty percent of the medium was removed, spun down for 1 minute at 1,500 rcf, and the top portion was analyzed in triplicate for ATP concentration using a commercially available ATP determination kit (Life Technologies). Data were normalized to cell number.

For Chapter 5 ATP release assays, N2a cells were plated at $5.3x10^4$ cells/cm^2 16-20 hours prior to analysis. A subset of cells was transiently-expressing Crmp2-DsRed or DsRed control and another subset was pre-treated for 1 hour with 10 μM CT3 or control peptides prior to start of analysis. The cells were then pre-incubated in HBSS[1] for 10 minutes. Eighty percent of the pre-incubation medium was exchanged for new HBSS[1] and incubated 10 minutes. Eighty percent of the medium was removed, spun down for 1 minute at 1,500 rcf, and the top portion was analyzed in triplicate for ATP concentration. Data were normalized to confluence (quantified by Incucyte scans).

2.3.6. Cell Surface Luminometry

N2a cells stably expressing Panx1EGFP were plated at $5.3x10^4$ cells/cm^2 20 hours prior to analysis. A subset was transiently-expressing Crmp2-DsRed or DsRed, and another subset was

pre-treated with 10 μM CT3 or control peptides 16 hours prior to analysis. Cells were washed once with PBS, fixed for 5 minutes with 4% paraformaldehyde in PBS, and washed 3 times in PBS. Half the wells were then permeabilized in PBS with 0.1% Triton X-100 for 5 minutes, while the other half were incubated in PBS alone. Cells were washed twice with PBS, incubated for 45-60 minutes in blocking buffer (PBS, 2% FBS), and incubated for 60 minutes with Panx1-EL2 primary antibody (1:300; a generous gift from Dr. Dale Laird) in blocking buffer. Samples were washed 4 times in blocking buffer, incubated 45-60 minutes with secondary antibody (1:1000 horseradish peroxidase-conjugated donkey anti-rabbit IgG) in blocking buffer, washed 3 times in blocking buffer and once in PBS. All liquid was removed, Clarity ECL reagent (BioRad, Hercules, California, USA) was added, and the luminescence was recorded on a plate reader. The signal from non-permeabilized cells was normalized to the signal from permeabilized cells to obtain the proportion of total Panx1 signal at the cell surface. The average ratio of surface:total Panx1 signal from each individual biological replicate was plotted.

2.4. Cortical neuronal cultures

The cortical neurons used in Chapter 5 were isolated from P0-P1 C57BL/6 pups. Briefly, P0-P1 cortices were dissected and put into ACSF. The cortical tissue was minced into fine pieces with a scalpel, then incubated at 37°C for 45 minutes in dissociation media, and finally triturated into single cells using a fire-polished Pasteur pipette. Cells were plated at $2.5x10^5$ cells/cm^2 on PDL and laminin-coated coverslips in neuronal media (NeuroCult Neuronal Basal Medium (Stem Cell Technologies), 0.5 mM Glutamax, 2% NeuroCult SM-1 supplement, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μg/mL gentamycin). The media was fully exchanged

the next day to remove dead cells and debris, and the neurons were collected at DIV4 by fixing with 3.7% formaldehyde.

2.5. Microscopy

Mouse brain and NSP cryopreservation and serial cryosectioning were performed as described (Swayne et al., 2010; Wicki-Stordeur et al., 2012). Briefly, brains were cryopreserved in 20% sucrose and NSPs in 15% sucrose for 48 hours before freezing at -80°C overnight. Frozen samples were cut on a cryostat, and 20 µm coronal brain sections (Chapter 3 and 4) and 10 µm NSP sections (Chapter 3) were mounted.

For immunostaining, antibodies were diluted in 10 mM PBS supplemented with 0.3% Triton-X-100 and 3% bovine serum albumin (BSA). To label the N2a cell plasma membrane, live cells were treated with 5 µg/mL TRITC-conjugated wheat germ agglutinin (WGA; Life Technologies) for 5 min at 37°C in HBSS[2] (137 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$), washed 3 times in HBSS, and fixed in 3.7% paraformaldehyde for 15 minutes.

Confocal immunofluorescence imaging was performed as previously described (Wicki-Stordeur et al., 2012; Wicki-Stordeur et al., 2013; Wicki-Stordeur and Swayne, 2013) using a Zeiss LSM 700 (Chapter 3) or a Leica SP8 (Chapters 4, 5) confocal microscope. In general, representative images were produced with Adobe Photoshop CS5 Extended software (Adobe Systems Incorporated, San Jose, California) and uniformly adjusted for brightness/contrast.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from N2a cells and NSPs, and first-strand synthesis and PCR were carried out. Briefly, cells were lysed in 1 mL Trizol (ThermoFisher Scientific, Waltham, Massachusetts, USA), and 200 µL chloroform was added to precipitate protein contaminants. Isopropyl alcohol (500 µL) was added to the aqueous layer to precipitate the RNA, the resulting pellet was washed with 1 mL 75% ethanol, then dissolved in nuclease free water. 4 µg of the resulting RNA solution was cleared of DNA contaminants with DNAse (ThermoFisher Scientific) according to the manufacturer's instructions. Phenol-chloroform solution was added to 50%, then the aqueous phase was removed and incubated overnight in 60 mM sodium acetate and 80% ethanol. The resulting pellet was washed twice with 500 µL 75% ethanol and re-suspended in 10 µL nuclease free water. First strand synthesis was carried out using Superscript II (ThermoFisher Scientific) according to the manufacturer's instructions. PCR was performed on the resulting cDNA using the following cycling parameters: 94^{o}C for 5 min, 35 cycles of 94^{o}C for 30 sec, 57^{o}C for 50 sec, and 72^{o}C for 2 min, and a final step at 72^{o}C for 7 min. Primers were: 5'-CATTGACCCCATGCTACTCC-3', 5'- TCAGCCACAGAAGTCACAGG-3' defining a 248 bp Panx1 amplicon (accession# NM_019482.2) and 5'-TGGTGCTGAGTATGTCGTGGAGT-3', 5'-AGTCTTCTGAGTGGCAGTGATGG-3' defining a 292 bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplicon (accession# NM_008084.2).

2.7. Western blot analysis

Unless otherwise specified, samples were homogenized in RIPA buffer (10 mM PBS [150 mM NaCl, 9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4], 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail at 1 μL/10^6 cells (stock: 0.104 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 0.08 mM aprotinin, 4 mM bestatin hydrochloride, 1.4 mM N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide, 2 mM leupeptin hemisulfate salt, 1.5 mM pepstatin-A; Sigma-Aldrich), PMSF at 2 μL/10^6 cells, 10 μM sodium orthovanadate and 1 mM EDTA for 30 minutes, and centrifuged at 4°C for 20 minutes at 12,000 rpm to remove debris. Samples were boiled (100°C) for 20 minutes in SDS-PAGE loading dye under reducing conditions (dithiothreitol and β-mercaptoethanol) before loading onto gels. Gels were transferred to 0.2 um pore-size polyvinyldene fluoride (PVDF) membrane for 1 hour at 85 V, or 16-18 hours at 22 V. Transfer was confirmed by Ponceau S total protein staining. Blocking and antibody incubations took place in blocking buffer (5% skim milk in PBST; 10 mM Na_2HPO_4, 1.25 mM NaH_2PO_4, 2.7 mM KCl, 137 mM NaCl, 0.1% Tween 20). Blots were quantified by densitometry measurements using ImageJ (http://imagej.nih.gov/ij/).

2.8. GFP Immunoprecipitations and Mass Spectrometry

2.8.1. Immunoprecipitations

Panx1EGFP and EGFP expressing N2a cells were collected 96 hours following transfection. Approximately 4.5x10^7 cells per condition were homogenized on ice in RIPA buffer supplemented with protease inhibitor cocktail and PMSF for 30 minutes, followed by centrifugation at 4°C for 20 minutes at 12,000 rpm to remove debris. Lysates were pre-cleared

for 45-60 minutes with protein-G agarose beads (Roche Scientific, Laval, Quebec, Canada) at 4°C with shaking, then added to 200 µL protein-G bead suspension cross-linked with 5 µg of αGFP monoclonal antibody (Roche Scientific), and incubated overnight at 4°C with shaking. Beads were washed once with RIPA buffer and twice with PBS, and eluted in 2 bead volumes of 0.5 M ammonium hydroxide/0.5 mM EDTA for 30 minutes at room temperature with shaking. The eluent was dried, and one fifth was rehydrated in RIPA, then mixed with SDS-PAGE loading dye under reducing conditions (dithiothreitol and β-mercaptoethanol) to analyze by Western blotting. The remaining sample was analyzed for protein interaction partners at the UVIC-Genome BC Proteomics Centre using high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Resulting data is from three individual biological replicates.

2.8.2. Trypsin Digestion

The following procedures were performed at the UVic-Genome BC Proteomics Centre. Each immunoprecipitation sample was reduced with dithiothreitol at 37°C for 30, and cysteine sulfhydryls were alkylated with iodoacetamide at 37°C for 30 min in the dark. Samples (1 mg each) were digested at 37°C for 16 hours with trypsin (20 µg; Promega).

2.8.3. Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS)

The digested samples were separated by on-line reverse phase chromatography at a flow rate of 300 nL/min using a Thermo Scientific EASY-nLC II system with a reversed-phase pre-

column ReproSil-Pur C18-AQC18 A1 EASY pre-column (100 µm I.D., 2 cm length, 5 µm, 120 Å; Thermo Fisher Scientific, Bremen, Germany) and an in-house prepared reverse phase nano-analytical column Magic C-18AQ (75 µm I.D., 15 cm length, 5 µm, 100 Å; Michrom BioResources Inc, Auburn, California, USA). The chromatography system was coupled to an LTQ Orbitrap Velos mass spectrometer equipped with a Nanospray II source (Thermo Fisher Scientific). Solvents were A: 2% acetonitrile, 0.1% formic acid; B: 90% acetonitrile, 0.1% formic acid. After a 249 bar (~ 5 µL) pre-column equilibration and 249 bar (~ 8 µL) nanocolumn equilibration, samples were separated by a 24 minute gradient: 0 min 5% B, 20 min 40% B, 2 min 80% B, 2 min 80% B.

The LTQ Orbitrap Velos parameters were as follows. Nano-electrospray ion source with spray voltage 2 kV, capillary temperature 225℃. Survey MS1 scan m/z range 400-2000 profile mode, resolution 60,000 @ 400 m/z with AGC target 1E6, and one microscan with maximum inject time 200 ms. Lock mass Siloxane 445.120024 for internal calibration with preview mode for FTMS master scans: on, injection waveforms: on, monoisotopic precursor selection: on, rejection of charge state: 1. The eight most intense ions charge state 2-4 exceeding 5000 counts were selected for CID ion trap MS/MS fragmentation (ITMS scans 2-9) and detection in centroid mode. Dynamic exclusion settings were: repeat count: 2, repeat duration: 15 seconds, exclusion list size: 500, exclusion duration: 60 seconds with a 10 ppm mass window. The CID activation isolation window was: 2 Da, AGC target: 1E4, maximum inject time: 25 ms, activation time: 10 ms, activation Q: 0.250, normalized collision energy: 35%.

2.8.4. Data Analysis

Raw files were analysed with Proteome Discoverer 1.3.0.339 software suite (Thermo Scientific). Parameters for the Spectrum Selection to generate peak lists of the CID spectra were: activation type: CID, s/n cut-off: 1.5, total intensity threshold: 0, minimum peak count: 1, precursor mass: 350-5000 Da. The peak lists were submitted to an in-house Mascot 2.2 server against the Uniprot-Swissprot 20110104 (523151 sequences; 184678199 residues) Allspecies taxonomy database as follows: precursor tolerance: 10 ppm, MS/MS tolerance: 0.6 Da, Trypsin enzyme: 2 missed cleavages, FT-ICR instrument type, fixed modification: carbamidomethylation (C), variable modifications: deamidation (N,Q), oxidation (M), and propionamide (C). Percolator settings: Max delta Cn 0.05, Target FDR strict 0.01, Target FDR relaxed 0.05 with validation based on q-Value. An additional Mus musculus (16381 sequences) only species search was also performed with tolerances as described above. The datasets from three individual biological replicates were combined, and proteins identified in both Panx1EGFP and EGFP control samples were removed from the final Panx1 interaction partners list.

2.8.5. Gene Ontology (GO) Analysis

The curated list of Panx1 protein interaction partners was classified by the Generic GO Term Mapper (http://go.princeton.edu/cgi-bin/GOTermMapper) using the MGI Generic GO Slim databases for (1) Biological Process, (2) Molecular Function and (3) Cellular Component. Data are represented as proportion of total interacting proteins that were amenable to GO term analysis (426/480 interacting proteins) in each category.

2.9. Endogenous immunoprecipitations

N2a cells ($4.5x10^7$ cells/immunoprecipitation), or VZ tissue dissected from pooled P0/P10 or P60 C57BL/6 mice were homogenized in TBS lysis buffer (10 mM Tris base, pH 7.4, 150 mM NaCl, 1% IGEPAL), supplemented with protease inhibitor cocktail, PMSF, and sodium orthovanadate, for 30 minutes on ice, followed by centrifugation at 4°C for 20 minutes at 12,000 rpm to remove debris. The supernatant was pre-cleared for 45-60 minutes with protein-A agarose beads (Roche Scientific) cross-linked to ChromPure rabbit IgG (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) at 4°C with shaking. Pre-cleared lysate (1.5 - 2.5 mg) was added to 200 µL protein-A bead suspension cross-linked with 5 µg of αPanx1-EL2 (generously provided by Dr. Dale Laird, University of Western Ontario, Canada), αCrmp2 polyclonal (Bioss Antibodies, Woburn, Massachusetts, USA), or ChromPure rabbit IgG control, and incubated 1.5 hours at 4°C with shaking. Beads were then washed 2-3 times with TBS/0.5% IGEPAL and 4 times with TBS, and eluted in 2 bead volumes of 0.5 M ammonium hydroxide/0.5 mM EDTA for 30 minutes at room temperature with shaking. The eluent was dried and rehydrated in TBS/1% IGEPAL with SDS-PAGE loading dye under reducing conditions to analyze by Western blotting.

2.10. Protein Purification

Bl21 *Escherichia coli* (*E. coli*; New England Biolabs) were transformed with Panx C-terminus(Panx1CT)-GST (BioBasic Inc., Amherst, New York, USA), Crmp2-GST (a generous gift from Dr. Rajesh Khanna, University of Arizona), or GST control plasmid (GE Healthcare,

Mississauga, Ontario, Canada) according to the manufacturer's instructions, and grown up overnight on LB agar at 37°C. The following day, single colonies were picked and grown in 50 mL LB broth at 37°C overnight. LB broth was added (200 mL) and the cultures were grown 2 hours at 37°C, and then induced with IPTG (1 mM) at 37°C for 4 hours. The bacteria were pelleted and re-suspended in 10 mL cold re-suspension buffer (PBS, 0.05 % Tween20, 2 mM EDTA, 0.1% mercaptoethanol) before being lysed by 2 – 3 passages through a French press at ~1100 psi. GST fusion proteins were recovered by binding with glutathione-agarose beads (Thermo Fisher Scientific) overnight at 4°C, then washed three times with 0.5 mL RIPA, twice with 0.5 mL RIPA without the SDS, three times with 0.5 mL PBS, then re-suspended to 50% with PBS. The final products were analyzed against BSA standards by SDS-PAGE and Colloidal Blue staining (Life Technologies) to estimate concentration.

Purified Panx1CT was removed from the beads by thrombin cleavage. Briefly, the bead-protein complexes were washed twice in thrombin cleavage buffer (50 mM TRIS-HCl pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$, 0.1% mercaptoethanol), and incubated 2 hours in 0.25 mL thrombin cleavage buffer with 10 µL thrombin protease at room temperature. PMSF (15 µL) was added to the beads, incubated for 15 minutes, and repeated once more. The supernatant was removed and analyzed by SDS-PAGE for concentration (against BSA standards).

Purified Crmp2 was removed from the beads by cleavage with Factor Xa (New England Biolabs). The beads were washed twice in Factor Xa cleavage buffer (20 mM TRIS-HCl, pH 8.0, 100 mM NaCl, 2 mM $CaCl_2$) then incubated in 250 µL buffer with 10 µL Factor Xa at room temperature for 4 hours. PMSF (15 µL) was added to the beads, incubated for 15 minutes, and repeated once more. The supernatant was removed and analyzed by SDS-PAGE for concentration (against BSA standards).

2.11. *In vitro* binding assays

Purified Crmp2 (0.03 nmol) or Panx1CT (0.3 nmol) was incubated with 0.034 nmol Panx1CT-GST or Crmp2-GST coupled to glutathione agarose beads at 4°C for 1 hour with shaking. Beads were washed 2-3 times with RIPA buffer, 2 times with TBS/1% IGEPAL and 3 times with TBS, eluted by boiling at 100°C in SDS-PAGE loading dye under reducing conditions, and analyzed by Western blotting.

For peptide binding assays, Crmp2-GST coupled glutathione agarose beads were incubated with 10 μM CT1, CT2, CT3, or control (TAT) peptide for 1 hour at 4°C with shaking. Beads were washed 3 times with RIPA buffer, 2 times with TBS/1% IGEPAL, and 3 times with TBS, then eluted by boiling at 100°C in SDS-PAGE loading dye under reducing conditions, and analyzed by Western blotting.

2.12. Antibodies

Primary antibodies used were anti-Panx1 C-term (1:200; Life Technologies), anti-Panx1 CT395 (1:500 - 1:4000; a generous gift from Dr. Dale Laird, University of Western Ontario, Canada), anti-Panx1 EL2 (1:200; a generous gift from Dr. Dale Laird), anti-GFAP (1:100; Life Technologies), anti-nestin (1:120; Chemicon, Billerica, Massachusetts, USA), anti-DCX (1:1600; Millipore, Billerica, Massachusetts, USA), anti-TUJ1 class III β-tubulin (1:120; RDI), anti-β-actin monoclonal (1:160,000; Sigma-Aldrich), anti-GFP monoclonal (1:1000; Roche Applied Science, Mannheim, Germany), anti-GFP polyclonal (1:10,000; Life Technologies), anti-Ki67 (1:200; BD Pharmingen, BD Biosciences, San Jose USA), anti-cleaved caspase 3

(1:3000; Cell Signalling Technology, Danvers, Massachusetts, USA), anti-Crmp2 monoclonal (1:100 or 1:6,000 – 1:8,000; Novus, Oakville, Ontario, Canada), anti-Crmp2/Toad64 polyclonal (1:100 or 1:4,000-1:10,000; Bioss Antibodies, Woburn, Massachusetts, USA), and anti-HIV1 TAT monoclonal (1:300; Abcam, Cambridge, UK).

Secondary antibodies were Cy3-conjugated anti-rabbit IgG (1:600), DyLight488-conjugated anti-rabbit IgG (1:600), DyLight488-conjugated anti-guinea-pig IgG (1:600), DyLight488-conjugated anti-mouse IgG (1:600), DyLight 405-conjugated AffiniPure donkey anti-rabbit IgG (1:300), DyLight 405-conjugated AffiniPure donkey anti-guinea pig IgG (1:300), and DyLight 649-conjugated AffiniPure donkey anti-mouse IgG (1:300 – 1:400), horseradish peroxidase (HRP)-conjugated AffiniPure donkey anti-rabbit IgG (1:2000 - 1:4000), HRP-conjugated AffiniPure donkey anti-mouse IgG (1:2000 – 1:4000; all from Jackson ImmuoResearch, West Grove Pennsylvania, USA), Alexa Fluor 568-conjugated donkey anti-rabbit IgG (1:600), and Alexa Fluor 568-conjugated donkey anti-mouse IgG (1:600; both from Life Technologies).

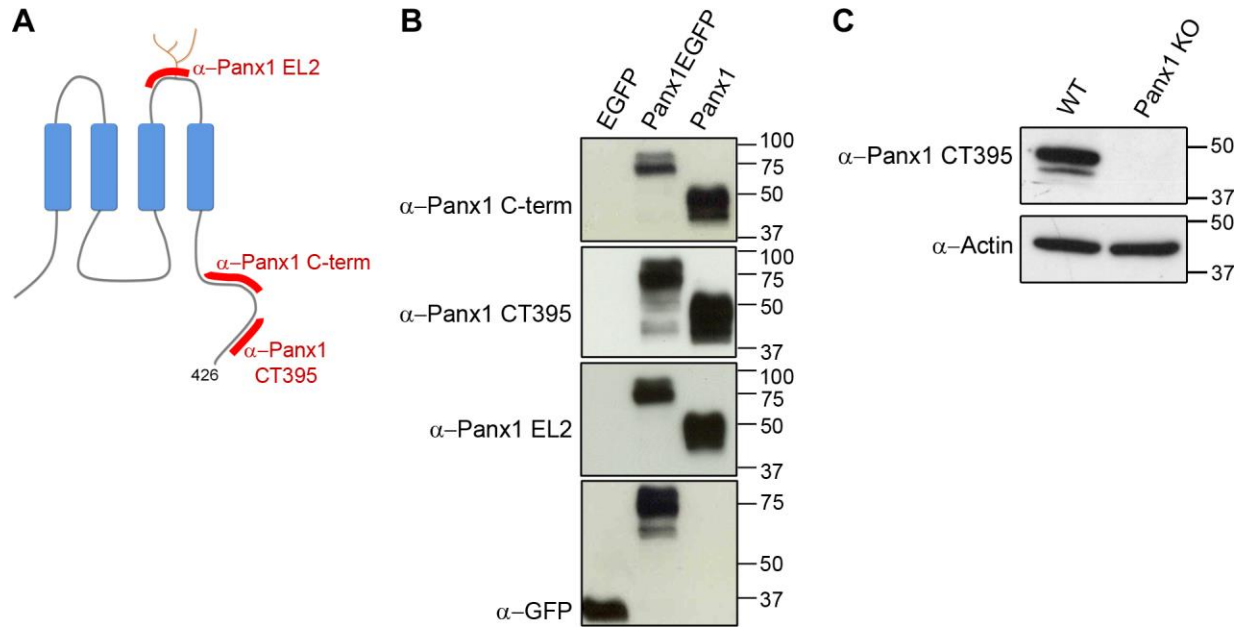


Figure 2.2. Specificity of the various Panx1 antibodies.

(A) Schematic of Panx1 with red areas indicating the epitopes for Panx1 CT395, Panx1 EL2 (generous gifts from Dr. Dale Laird) and Panx1 C-term (Life Technologies) antibodies. (B) Western blot of lysates from HEK293T cells (which do not endogenously express Panx1) overexpressing EGFP, Panx1EGFP, or untagged Panx1. Blot was probed with the Panx1 C-term, Panx1 CT395, Panx1 EL2, and GFP antibodies. (C) Western blot of lysates from VZ NSPs derived from wild-type or Panx1 knock-out (Panx1-LoxP/CMV-cre-recombinase) mice. No bands are visible in the knock-out lane.

2.13. Statistical analysis

Statistical analyses were performed using Prism for Mac OS X v5.0d software (http://www.graphpad.com; GraphPad Software, San Diego, California, USA). Statistical tests are reported in each Figure legend. All variances are reported as standard error of the mean. Significance was denoted as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****). Exact p values and sample sizes are provided in the Figure legends.

3. Panx1 regulates neuronal development *in vitro*

The majority of the data presented in this Chapter are published in Wicki-Stordeur et al., 2012 and Wicki-Stordeur and Swayne, 2013.

3.1. Overview

New neurons are continuously formed within the post-natal brain from NPCs located in the VZ niche (reviewed in Ming and Song, 2011). These NPCs proceed through a set of behaviours including proliferation, migration along the RMS, and neuronal differentiation, to finally mature into OB interneurons. This process, termed neurogenesis, is highly regulated by a variety of signalling molecules. In particular, purinergic nucleotides (e.g. ATP, ADP, UTP) and their receptors (P2 receptors) act as key modulators of all NPC behaviours (Hogg et al., 2004; Mishra et al., 2006; Lin et al., 2007; Wu et al., 2009). NPCs release episodic bursts of ATP, which acts in an autocrine and paracrine manner on ionotropic P2X and metabotropic P2Y receptors (reviewed in Cavaliere et al., 2015). NPCs highly express P2Y1 and P2Y2 receptors, which when activated, induce proliferation and negatively regulate differentiation (Weissman et al., 2004; Mishra et al., 2006; Lin et al., 2007; Suyama et al., 2012). Together, the expression or activation of different cohorts of P2 receptors helps drive neuronal development from the post-natal VZ (reviewed in Cavaliere et al., 2015).

Large-pore channels release ATP and other nucleotides into the extracellular space. Cx hemichannels were classically believed to act as the ATP release conduits in NPCs; however, recently the Panx large-pore channel proteins were discovered (reviewed in Lohman and

Isakson, 2014). Panxs show similar membrane topology to Cxs, yet function only as unpaired channels and cannot form classic gap junctions (reviewed in Sosinsky et al., 2011). Importantly, Panx channels exhibit similar pharmacology to Cx hemichannels (reviewed in Dahl et al., 2013), bringing into question the functions originally attributed to Cxs.

Within the Panx family, Panx1 and Panx2 are expressed within the brain. Panx2 is found in post-natal NPCs of the SGZ, and it regulates the timing of their neuronal differentiation (Swayne et al., 2010). Panx1 is expressed within mature neurons (Ray et al., 2005; Vogt et al., 2005), as well as in many other cell and tissue types throughout the body where it is known to act as an ATP release channel (reviewed in Dahl, 2015). Because NPC behaviours are highly regulated by purinergic signalling, and Panx1 represents a novel ATP release conduit, **this led to my overall hypothesis that Panx1 regulates post-natal VZ NPC behaviours *in vitro*.** In this chapter I investigated whether Panx1 is expressed in post-natal VZ NPCs. Moreover, I combined studies in the N2a murine neuroblastoma-derived cell line as model NPCs, and in primary VZ NPCs *in vitro*, to elucidate the role of Panx1 in post-natal neuronal development.

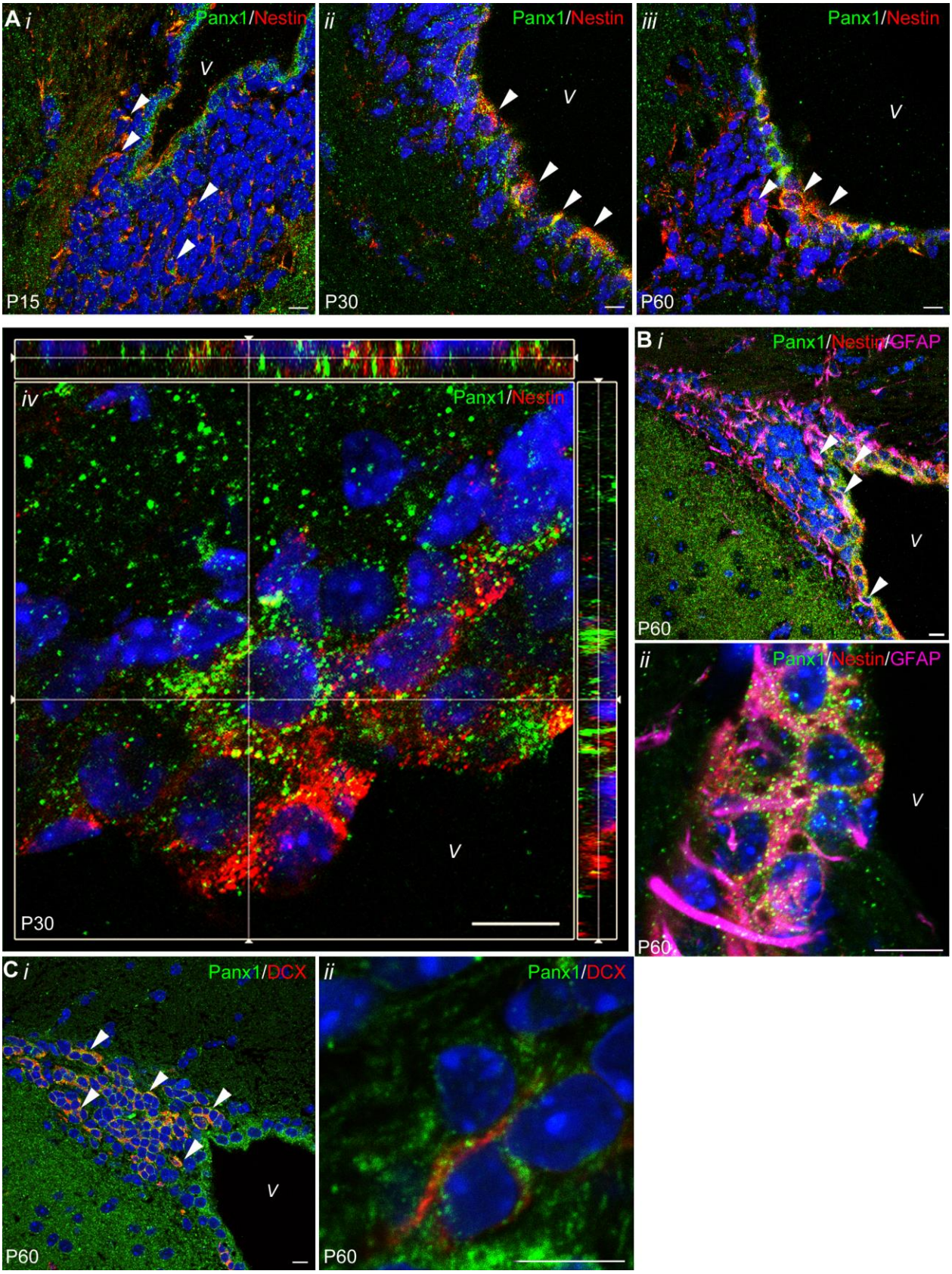
I detected Panx1 in all NPC types (nestin-positive/GFAP-positive type B NPCs, nestin-positive/GFAP-negative type C NPCs, and DCX-positive type A NPCs) *in vivo* and *in vitro*, and in TUJ1-positive immature neurons *in vitro*. Inhibition of Panx1 decreased ATP release, and blocking P2 purinergic receptors reduced NPC proliferation, suggesting a possible role for Panx1 in regulating NPC proliferation. Finally, by modulating Panx1 expression and activity in N2a cells and VZ NPC cultures, I demonstrated that Panx1 positively regulated NPC proliferation and migration, and inhibited neurite outgrowth.

3.2. Results

3.2.1. Panx1 is expressed in post-natal VZ NPCs

To determine if Panx1 was expressed in VZ NPCs, I examined coronal cryosections of postnatal day 15 (P15), 30 and 60 mouse brains immunostained for Panx1 and lineage markers. Panx1 signal was detected in nestin-positive cells at each time point (Figure 3.1A). Subsequent staining with GFAP determined that Panx1 was expressed in both GFAP-positive/nestin-positive (type B) and GFAP-negative/nestin-positive (type C) populations of NPCs at P60 (Figure 3.1B). Additionally, Panx1 signal was present in DCX-positive (type A) NPCs at P60 (Figure 3.1C).

To examine the importance of Panx1 in VZ NPCs, I moved into *in vitro* NPC culture. I dissociated VZ (or SGZ) tissue from P0-P3 mice and grew the resulting NPCs in suspension culture as NSPs (Figure 3.2A). Panx1 mRNA and protein were expressed in VZ (and SGZ) derived NSPs maintained for seven days *in vitro* (DIV), as assessed by reverse-transcriptase polymerase chain reaction (RT-PCR) and western blotting (Figure 3.2B). Using confocal microscopy and immunostaining, I determined that Panx1 signal was again found in both GFAP-positive/nestin-positive and GFAP-negative/nestin-positive populations of VZ NPCs *in vitro* (Figure 3.2C). Furthermore, upon differentiation of the VZ NSPs by culturing in adherent conditions with removal of mitogenic growth factors, Panx1 expression was low in DCX-positive NPCs, but was found in TUJ1-positive immature neurons (Figure 3.2D).


A i
Panx1/Nestin
V
P15
ii
Panx1/Nestin
V
P30
iii
Panx1/Nestin
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P60
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Panx1/Nestin
V
P30
B i
Panx1/Nestin/GFAP
V
P60
ii
Panx1/Nestin/GFAP
V
P60
C i
Panx1/DCX
V
P60
ii
Panx1/DCX
P60

Figure 3.1. Panx1 is expressed in VZ NPCs *in vivo*.

(A) Confocal images of mouse brain slices at the ventricular region. Nestin-positive NPCs exhibit high levels of Panx1 co-expression in postnatal day 15 (P15; i), P30 (ii) and P60 (iii) brains. Arrows indicate areas of co-expression. (iv) Confocal image with orthogonal side-views of P30 VZ demonstrates punctate Panx1 expression in NPCs that form or are adjacent to the ventricular wall. (B) Wide (i) and zoomed (ii) confocal images from a P60 ventricular region demonstrates Panx1 expression in GFAP+/nestin+ and GFAP-/nestin+ NPCs. (C) Wide (i) and zoomed (ii) confocal images from a P60 ventricular region show Panx1 signal in DCX+ NPCs. Hoechst 33342 was used as a nuclear counterstain. All scalebars 10 μm. V: ventricle. *This figure is modified from those published in Wicki-Stordeur et al., 2012 and Wicki-Stordeur and Swayne, 2013.*

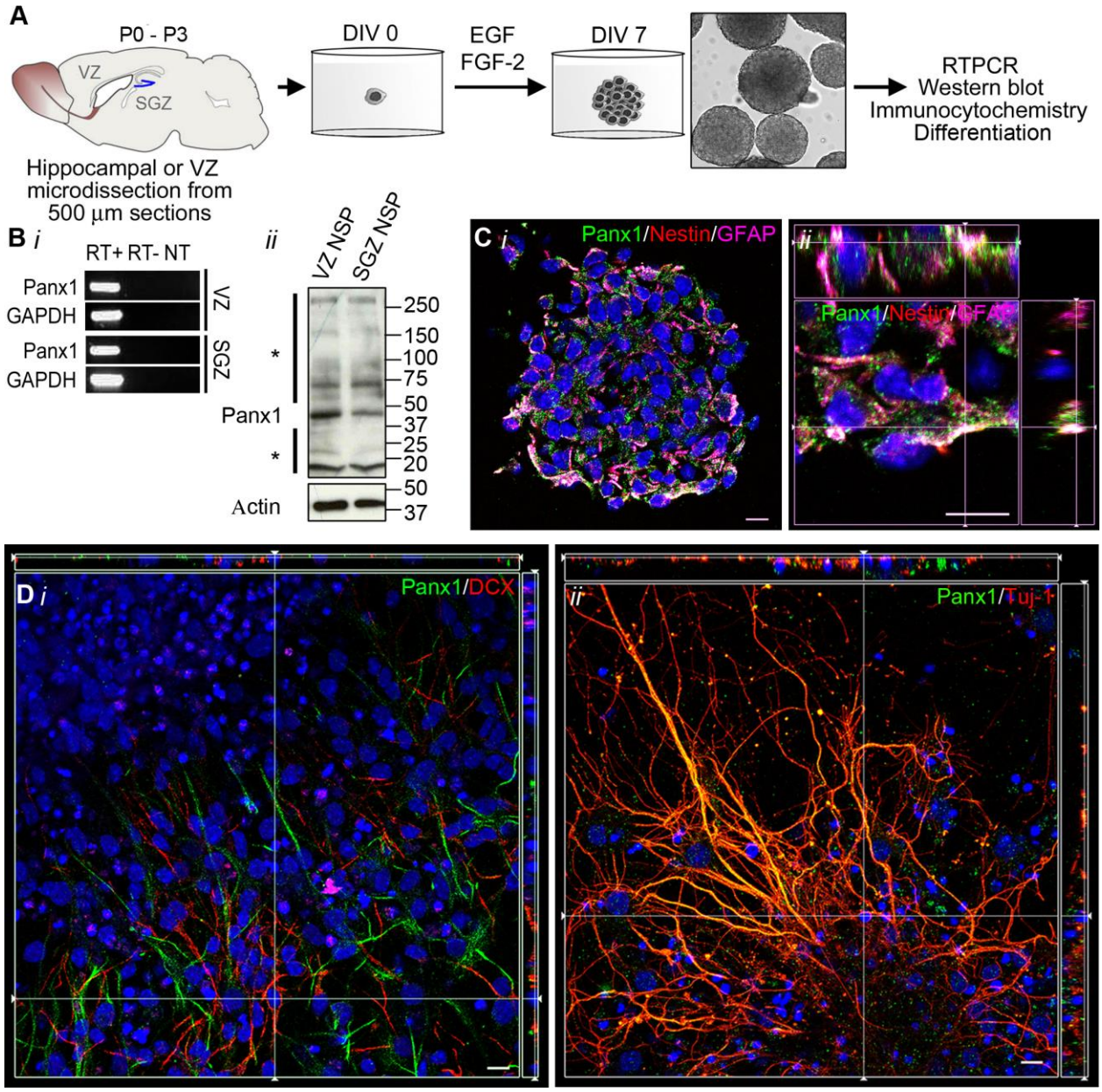

Figure 3.2. Panx1 expression in NPCs is re-capitulated *in vitro* in neurosphere cultures.

(A) Outline of neurosphere (NSP) culture generation from P0-P3 hippocampus or microdissected VZ. NSPs are cultured for seven days *in vitro* (DIV) with addition of growth factors (EGF and FGF-2) every two DIV, before harvesting for subsequent analyses, or differentiation by removal of growth factors and plating on a PDL-coated surface. (B) Panx1 mRNA (i) and protein (ii) are expressed in VZ and SGZ NSPs. To the left of the western blot, the asterisk (*) above 50 kDa denotes high-molecular weight species likely representing various Panx1 oligomeric forms, while the asterisk below 50 kDa denotes low molecular weight Panx1 cleavage products consistent in size with those reported in the literature (Chekeni

et al., 2010). (C) Confocal image showing Panx1 expression in a cryosectioned undifferentiated VZ NSP (i) and a digitally zoomed z-stack with orthogonal side-views (ii) in nestin+ /GFAP+ and nestin+ /GFAP- NPCs. (D) Images from confocal z-stacks with orthogonal side-views show limited Panx1 expression in DCX+ cells in a differentiated VZ NSP, with higher expression in DCX- cells (i). However, Panx1 signal is co-distributed with TUJ-1 in immature neurons from a differentiated VZ NSP. Hoechst 33342 was used as a nuclear counterstain in C-E. Scalebars 10 μm. *This figure is published in Wicki-Stordeur et al., 2012.*

3.2.2. Panx1 mediates ATP release from an NPC-like cell line in vitro

To further study the properties and cell biology of Panx1 in neuronal development, I moved into cell culture models. I first employed the N2a cell line, which endogenously expressed both Panx1 mRNA and protein (Figure 3.3A). Since Panx1 is a well-known ATP release channel in other cell types (reviewed in MacVicar and Thompson, 2009), and ATP is a potent effector of NPC proliferation (Hogg et al., 2004; Mishra et al., 2006; Lin et al., 2007; Wu et al., 2009), I examined the impact of Panx1 on ATP release from N2as. Moreover, I studied the effects of blocking P2 receptors on N2a cell proliferation.

I first treated N2a cells with varying concentrations of KCl, and found that maximal ATP release was stimulated with increased KCl (20 mM) compared to control (5.33 mM) and 0 mM KCl (Figure 3.3B). Treating N2a cells with the Panx1 blocker probenecid (Silverman et al., 2008), significantly decreased ATP release (Figure 3.3C). Furthermore, N2a cell proliferation was significantly reduced upon Panx1 block with probenecid, or treatment with pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS; Lambrecht et al., 1992; Figure 3.3D), which blocks several P2X and P2Y isoforms (Boyer et al., 1994; Communi et al., 1996; Communi et al., 1999; Troadec et al., 1999; Khakh, 2001; Khakh et al., 2001; North, 2002).

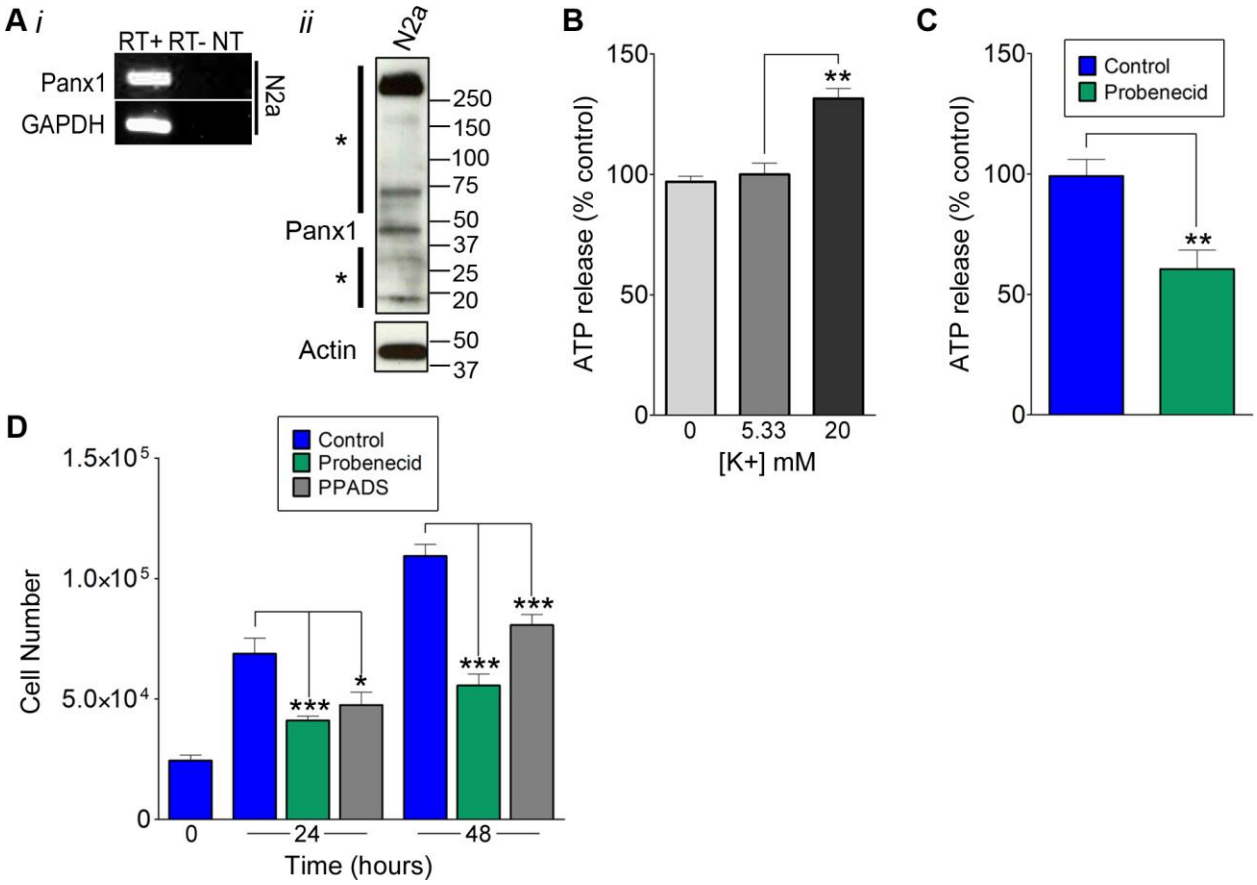

Figure 3.3. Panx1 blockage decreases ATP release from N2a cells and blocking P2 receptors reduces cell proliferation.

(A) Panx1 is endogenously expressed in N2a cells, as demonstrated by RT-PCR (i) and western blot analysis (ii). To the left of the western blot, the asterisk (*) above 50 kDa denotes high-molecular weight species likely representing various Panx1 oligomeric forms, while the asterisk below 50 kDa denotes low molecular weight Panx1 cleavage products consistent in size with those reported in the literature (Chekeni et al., 2010). (B) ATP release from N2a cells is stimulated upon treatment with 20 mM KCl buffer compared to control (5.33 mM) and 0 mM KCl conditions ($p = 0.0013$ by one-way ANOVA, (**) $p < 0.01$ by post-hoc Tukey's multiple comparison test; $N = 3$). (C) Blockage of Panx1 channels with 0.5 mM probenecid significantly decreases ATP release from N2a cells compared to controls ($60.50 \pm 7.913\%$ and $99.17 \pm 6.831\%$, respectively; (**) $p = 0.0061$ by Student's t test; $N = 5$). (D) N2a cell numbers were quantified at 0, 24 and 48 hours for cells treated with 30 µM PPADS, 1 mM probenecid, or vehicle control. PPADS and probenecid treatments significantly reduced N2a numbers at 24 and 48 hours ($p < 0.0001$ by one-way ANOVA, (*) $p < 0.05$ and (***) $p < 0.001$ by post-hoc Tukey's

multiple comparison test; N = 5). *This figure is modified from one published in Wicki-Stordeur et al., 2012.*

3.2.3. Panx1 promotes VZ NPC proliferation in vitro

Given that Panx1 block reduced ATP release from N2a cells, and that Panx1 or P2 receptor block inhibited proliferation, I next tested the hypothesis that Panx1 regulates VZ NPC proliferation. I overexpressed Panx1 tagged with EGFP in the N2a cell model to examine this hypothesis. Similar to previous reported expression in HEK293T cells (Bhalla-Gehi et al., 2010), Panx1EGFP mainly localized to the plasma membrane in N2a cells, with some intracellular aggregates (Figure 3.4A). Panx1 overexpression increased N2a cell proliferation so that the doubling time was reduced from 26.2 hours (with control EGFP expression) to 14.7 hours (Figure 3.4B). Furthermore, treatment of N2a cells with probenecid significantly reduced proliferation of both Panx1EGFP and control EGFP expressing N2a cells (Figure 3.4C).

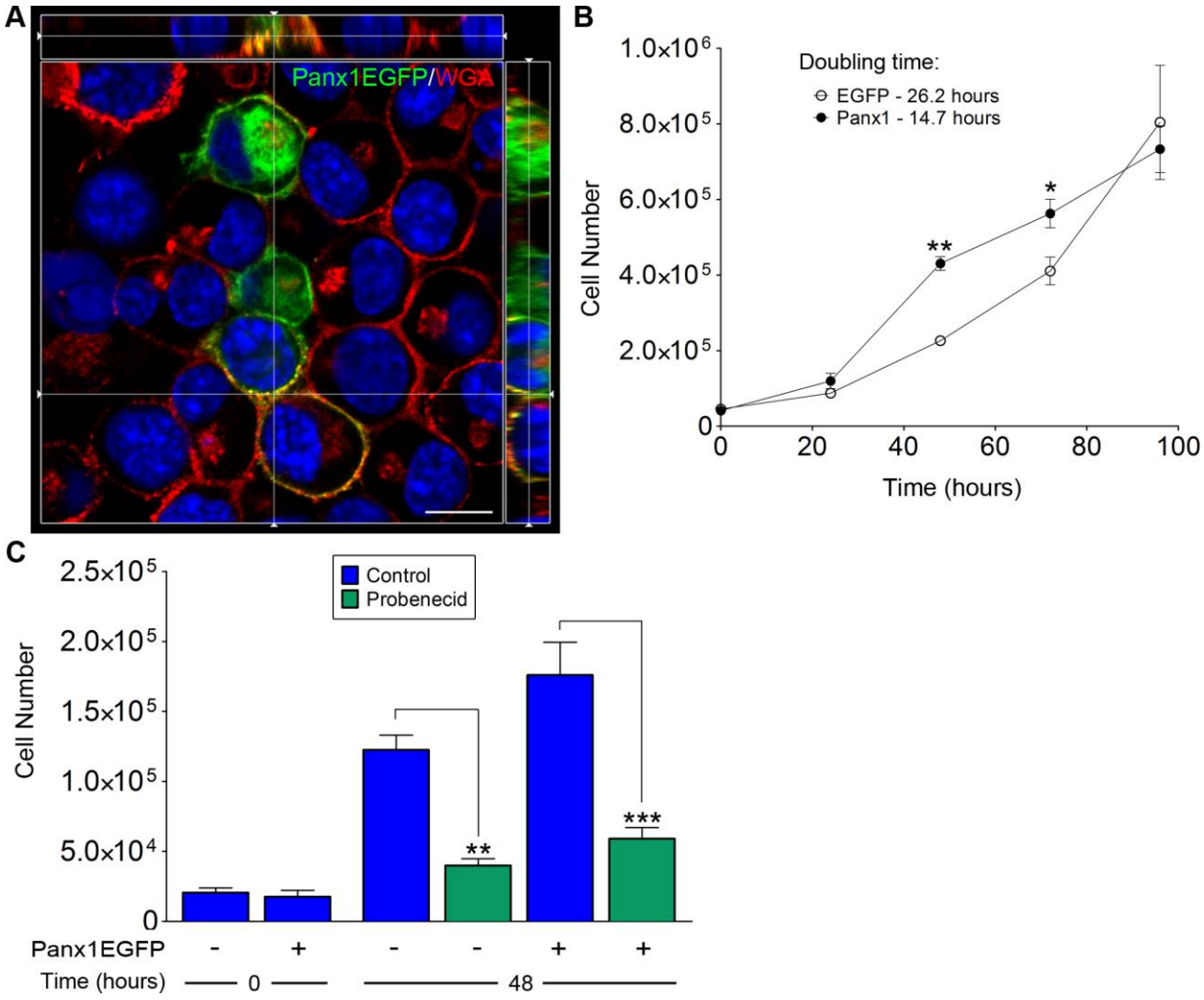

Figure 3.4. Panx1 regulates N2a cell proliferation.

(A) Image from a confocal z-stack with orthogonal side-views of N2a cells overexpressing Panx1EGFP. Panx1 is highly localized to the plasma membrane, stained with wheat germ agglutinin (WGA), as well as to intracellular membranes. (B) N2a cells overexpressing Panx1 exhibited a reduced doubling time (14.7 hours) compared to control cells overexpressing EGFP (26.2 hours) ((*) $P < 0.05$ and (**) $P < 0.01$ by one-way ANOVA with post-hoc Tukey's multiple comparison test, $N = 3$). (C) Treatment of transfected N2a cells with the Panx1 channel blocker, probenecid (1 mM), significantly reduced cell proliferation at 48 hours in both Panx1 and EGFP overexpressing cells ($p < 0.0001$ by one-way ANOVA, (**) $p < 0.01$ and (***) $p < 0.001$ by post-hoc Tukey's multiple comparison test; $N = 3$). *This figure is modified from one published in Wicki-Stordeur et al., 2012.*

To determine whether Panx1 also regulated proliferation of primary NPCs, I created NSP cultures from the VZ, and examined the effect of Panx1 block on their proliferation. NSPs were treated with 1 mM probenecid from DIV1 onwards, and their diameter was measured on DIV7. Probenecid treatment significantly reduced the average NSP diameter (Figure 3.5A). Primary VZ NPCs grown as an adherent monolayer were also treated with probenecid or vehicle from DIV1 onwards for 5 days, and their confluence was monitored in real time. Probenecid treatment significantly reduced NPC confluence compared to control (Figure 3.5B).

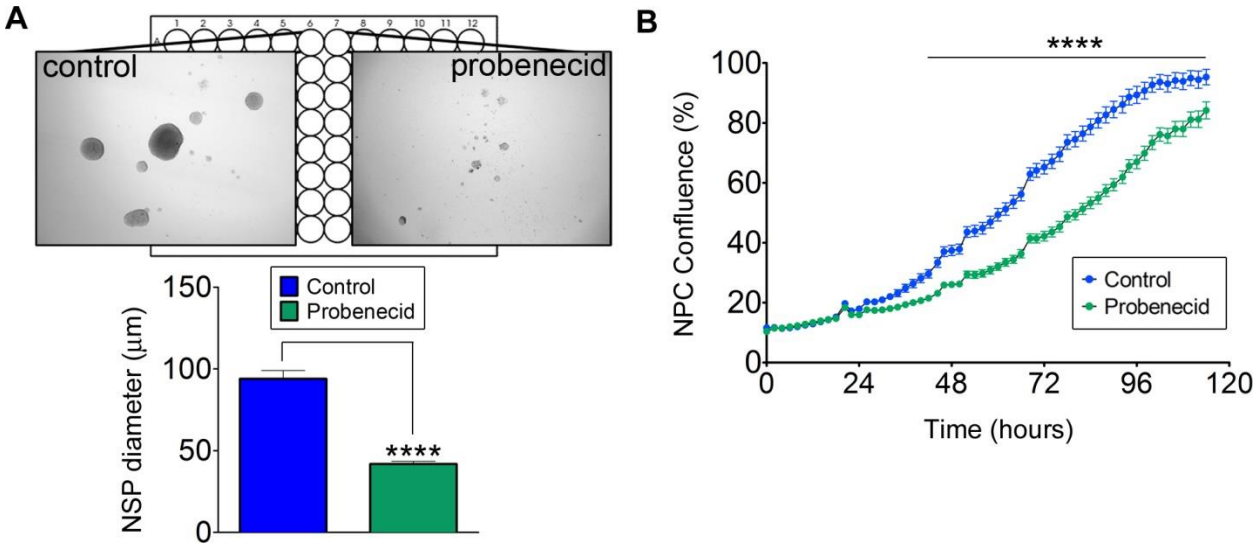

Figure 3.5. Panx1 regulates primary VZ NPC proliferation.

(A) Primary VZ NSPs were treated with probenecid at DIV1, and NSP diameter was measured at DIV7. Probenecid treatment significantly reduced NSP diameter compared to vehicle control (41.85 ± 1.649 µm and 93.97 ± 5.089 µm respectively, (****) $p < 0.0001$ by Student's t test, N = 12). (B) Primary VZ NPCs grown in monolayer culture were treated at DIV1 (time: 0 hours) with probenecid or vehicle control, and their confluence was monitored in real time over 5 days. Probenecid treated NPCs showed significantly reduced confluence compared to control (Time: F = 1454, $p < 0.0001$; Drug: F = 36.36, $p < 0.0001$; Subject (matching): F = 131.7, $p < 0.0001$; Interaction: F = 44.08, $p < 0.0001$ by two factor ANOVA; Bonferroni posthoc $p < 0.0001$ (****) for Drug beginning at 44 hours; N = 6). *This figure is modified from one published in Wicki-Stordeur et al., 2012.*

3.2.4. Panx1 promotes NPC migration in vitro

Since the structurally similar Cx hemichannels (reviewed in Matsuuchi and Naus, 2013), as well as purinergic signalling (Scemes et al., 2003; Liu et al., 2008; Grimm et al., 2010; Oliveira et al., 2015), are known to affect NPC migration I next wanted to investigate whether Panx1 regulated this NPC behaviour. To determine this, I employed a scratch wound closure assay (Liang et al., 2007) using parallel sets of Panx1 siRNA and control siRNA treated N2a cells. In

this assay, migration of cells into the scratch wound leads to a decreased width (wound closure) over time. Panx1 siRNA treated cells exhibited a significant impairment in wound closure compared to control cells (Figure 3.6A, B). Western blot quantifications showed that Panx1 siRNA treatment resulted in approximately 60% knock-down of Panx1 protein expression compared to control siRNA treated cells (Figure 3.6C). Moreover, treatment of N2a cells with probenecid dramatically impaired wound closure (Figure 3.6D, E). Taken together, these results indicate that Panx1 promoted NPC migration *in vitro*.

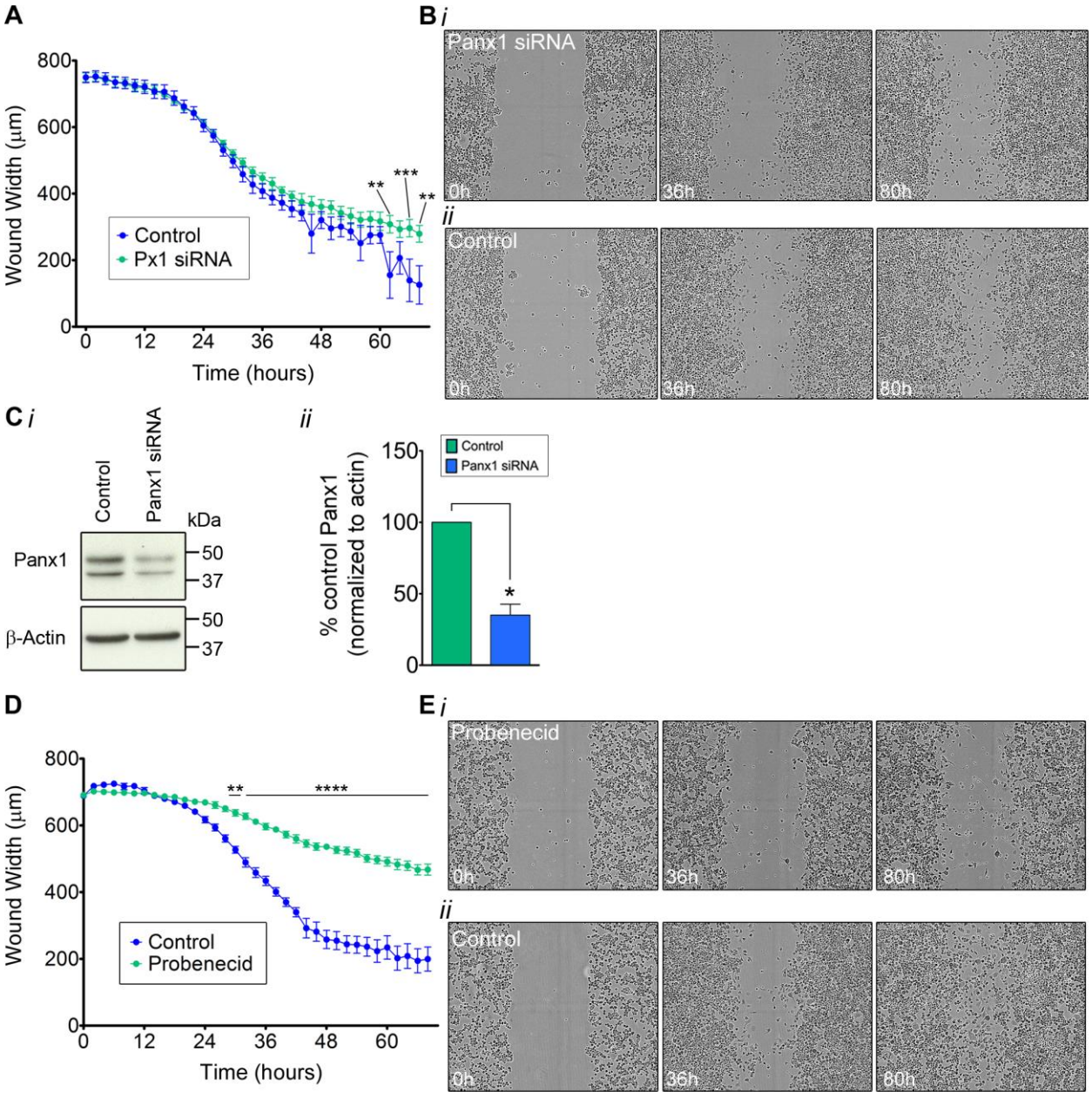

Figure 3.6. Panx1 knock-down or block inhibits N2a cell migration.

(A) Panx1 siRNA knockdown in the N2a cell line caused a reduction in cell migration in a scratch wound closure assay (Time: $F = 1454$, $p < 0.0001$; Drug: $F = 36.36$, $p < 0.0001$; Subject (matching): $F = 131.7$, $p < 0.0001$; Interaction: $F = 44.08$, $p < 0.0001$ by two factor ANOVA; Bonferroni posthoc (**) $p < 0.01$ at 62 and 68 hours, (***) $p < 0.001$ at 66 hours; $N = 12$). Wounds were monitored in real time. (B) Representative shots of scratch wound closure for (i) Panx1 knock-down and (ii) control N2a cells. (C) Western blot of N2a cell lysates 48 hours post-transfection shows successful Panx1 knockdown (i). The

percent knockdown at 48 hours was ~60% less than control Panx1 levels (ii; (*) $p = 0.0135$ by one-sample t test against a hypothetical value of 100; N = 3). (D) N2a cell treatment with probenecid significantly impaired wound closure in a scratch wound closure assay monitored in real time (Time: $F = 314.2$, $p < 0.0001$; Drug: $F = 68.76$, $p < 0.0001$; Subject (matching): $F = 37.02$, $p < 0.0001$; Interaction: $F = 55.50$, $p < 0.0001$ by two factor ANOVA; Bonferroni posthoc (**) $p < 0.01$ at 28 hours, (****) $p < 0.0001$ at 30 hours onwards; N = 12). (E) Representative shots of scratch wound closure for (i) probenecid and (ii) control treated cells. *This figure is modified from one published in Wicki-Stordeur and Swayne, 2013.*

3.2.5. Panx1 inhibits neurite outgrowth in vitro

In initial immunostaining for Panx1 in NPCs, both *in vitro* and *in vivo*, the DCX-positive NPCs seemed to show reduced Panx1 signal compared to the nestin-positive NPCs. Since DCX labels the late-stage, neuronally-committed NPC population, this led me to hypothesize that Panx1 expression decreases with NPC differentiation. Using *in vitro* culture models, I found that Panx1 expression was dramatically reduced in N2a cell culture upon their differentiation with retinoic acid and low serum (Figure 3.7A). A significant decrease in Panx1 levels was also noted in primary VZ NPC culture upon differentiation by removal of mitogenic growth factors (Figure 3.7B).

Interestingly, immunostaining and confocal microscopy in differentiated N2a cells and primary VZ NPCs revealed highly clustered endogenous Panx1 signal within the developing neurites (Figure 3.7A, B). Such neurite outgrowth is a morphological characteristic of NPC differentiation *in vitro*. Taken together with the observed reduction in Panx1 levels upon differentiation, I examined whether artificially reducing Panx1 expression or activity (in the absence of other differentiation signals) could induce neurite extension. Indeed, probenecid treatment induced marked neurite outgrowth in both N2a cells and primary VZ NPCs (Figure

3.7C-I). Panx1 siRNA knockdown in N2a cells similarly increased neurite outgrowth in the absence of other signals (Figure 3.7J). Moreover, Panx1 overexpression inhibited neurite outgrowth in retinoic acid/low serum differentiating N2a cells (Figure 3.7K).

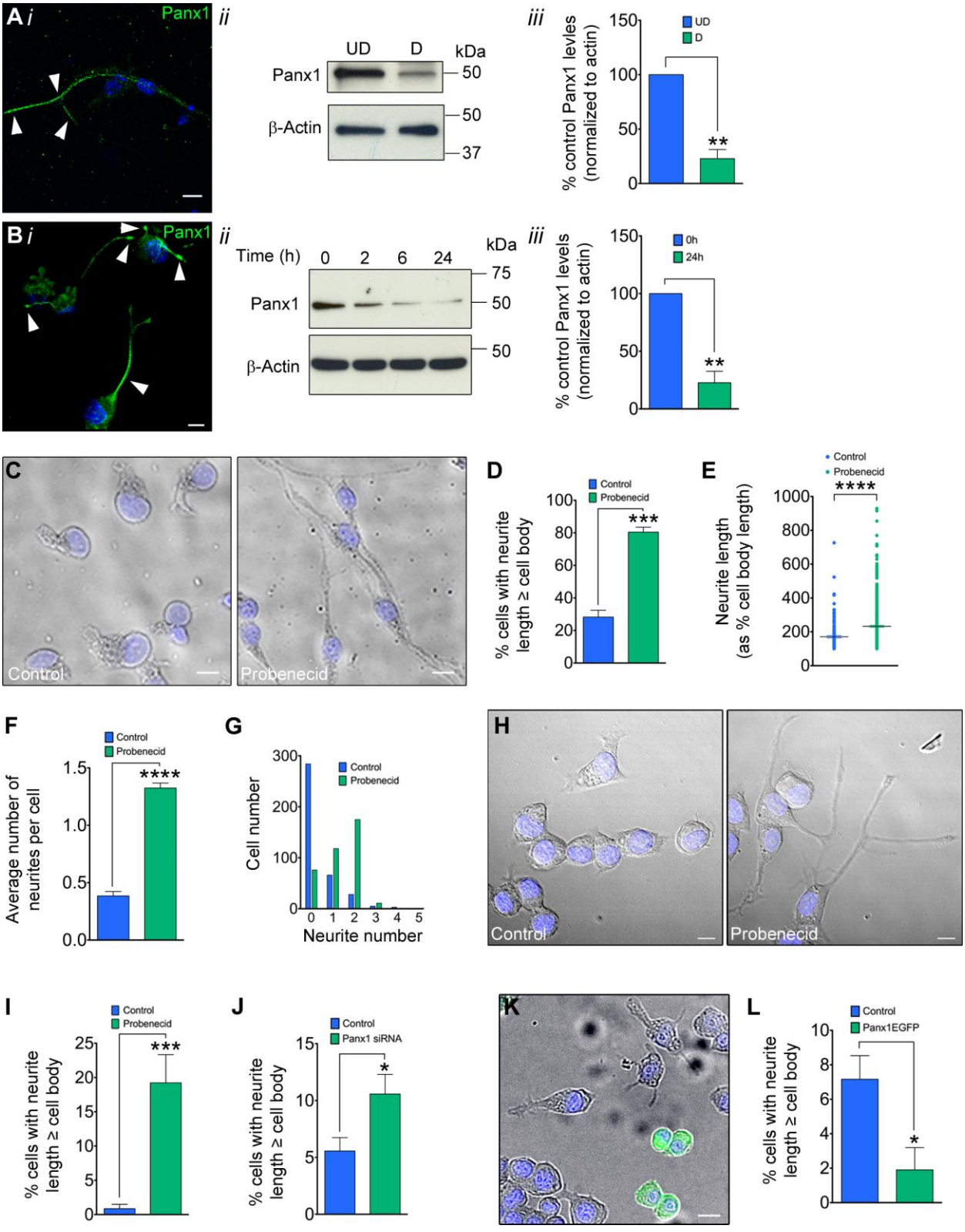

Figure 3.7. Panx1 decreases across neuronal differentiation and is important for neurite outgrowth.

(A) Confocal image of VZ-derived NPCs under neuronal driving conditions immunolabelled for endogenous Panx1 (i). Arrowheads indicate Panx1 in the neurite. VZ NSPs were re-plated and maintained in proliferative conditions (UD; un-differentiated) or neuronal driving conditions (D; differentiated) for 5 days. Panx1 expression assessed by Western blotting (ii) was significantly lower in differentiated compared to undifferentiated NSPs (iii; (**) $p = 0.0027$ by one-sample t test against a hypothetical value of 100; N = 4). (B) N2a cells were differentiated for 24 hours in low-serum media with 10 μM retinoic acid. Samples were collected at 0, 2, 6, and 24 hours. (i) Confocal image of endogenous Panx1 staining in N2a cells 24 hours after induction of differentiation. Arrowheads indicate Panx1 in neurites. Western blotting (ii) revealed significantly reduced Panx1 expression in 24 hour differentiated samples compared to 0 hour controls (iii; (**) $p = 0.0015$ by one-sample t test against a hypothetical value of 100; N = 5). (C) Representative images of VZ NPCs (dissociated NSPs) treated with 1 mM probenecid or vehicle control for 48 hours. A process with length greater than or equal to the corresponding cell body length was considered a neurite. (D) The percent of VZ NPCs possessing a neurite increased with probenecid treatment ((***) $p = 0.0005$ by Student's t test; N = 3). (E) The length of VZ NPC neurites increased with probenecid treatment compared to control ((****) $p < 0.0001$ by Student's t test; N = 3). (F) Probenecid treatment significantly increased the average number of neurites per NPC compared to control ((****) $p < 0.0001$ by Student's t test; N = 3), and (G) dramatically altered the neurite number distribution. (H) Representative images of N2a cells treated for 36 hours with 1 mM probenecid or vehicle control. (I) Probenecid treatment increased the percent of N2a cells possessing a neurite ((***) $p = 0.0009$ by Student's t test; N = 10). (J) Panx1 siRNA knockdown increased the proportion of N2a cells possessing a neurite ((*) $p = 0.0378$ by Student's t test; N = 5). (K) Representative image of Panx1EGFP-expressing N2a cells 24 hours after induction of differentiation. (L) Significantly fewer N2a cells overexpressing Panx1EGFP possessed one or more neurites compared to un-transfected same-plate controls ((*) $p = 0.0438$ by Student's t test; N = 8). Hoechst 33342 was used as a nuclear counterstain. All scalebars are 10 μm. *This figure is published in Wicki-Stordeur and Swayne, 2013.*

3.3. Discussion

Here I report the discovery of Panx1 expression in NPCs and immature neurons both *in vivo* and *in vitro*. *In vivo*, Panx1 expression was high in type B and type C NPCs, and was also found in migrating type A NPCs. This expression was recapitulated *in vitro* in primary VZ NPC

cultures (and was recently confirmed by Talaveron et al., 2015); however Panx1 expression was very low in type A NPCs *in vitro*. Using *in vitro* NPC and N2a cell cultures, I demonstrated that Panx1 block with probenecid reduced proliferation, while Panx1 overexpression enhanced proliferation. Although probenecid has also been shown to target organic anion transporters (Enomoto et al., 2002; May et al., 2008), it is specific for Panx1 within the gap junction group of proteins (Cxs and Panxs; Silverman et al., 2008). Moreover, the increase in cell proliferation resulting from Panx1 overexpression was completely abolished by probenecid treatment, suggesting this is a Panx1-specific effect.

As large-pore ion- and metabolite-permeable channels, Panxs make ideal signal integrators within the neurogenic microenvironment of the VZ niche. Panx1 is known to act as a nucleotide release channel in many tissue and cell types throughout the body (reviewed in Lohman and Isakson, 2014). NPCs basally release ATP, which acts in an autocrine and paracrine manner on P2Y1 and P2Y2 receptors to enhance proliferation (Weissman et al., 2004; Mishra et al., 2006; Lin et al., 2007; Suyama et al., 2012). My current data indicate that Panx1 is likely responsible, at least in part, for this nucleotide release from NPCs, and promotes NPC proliferation through downstream activation of purinergic receptors. NPC migration and differentiation are also tightly controlled by purinergic signalling (reviewed in Cavaliere et al., 2015). Interestingly, in other cell types a positive feedback loop is created by ATP activating Panx1 in a P2 receptor-dependent manner (Locovei et al., 2006a; Pelegrin and Surprenant, 2009); however at higher ATP concentrations, Panx1 activity is inhibited via direct binding of the molecule to residues within its extracellular loops (Qiu and Dahl, 2009) leading to channel internalization (Boyce et al., 2015). Together these mechanisms likely function to maintain homeostatic nucleotide

concentrations within the VZ niche, and properly regulate purinergic control of NPC proliferation.

Panx1 knock-down, or block with probenecid also significantly impaired wound closure in a scratch wound assay. Interestingly, probenecid treatment was much more effective in preventing this wound closure than Panx1 siRNA-mediated knock-down. The maximal knock-down obtained by Panx1 siRNA was 60%, leaving a significant proportion of functional Panx1 protein within the cells. I was also unable to track Panx1 knock-down to specific cells using this method, therefore the wound closure could have been completed by cells expressing normal levels of Panx1. In contrast, probenecid globally blocks Panx1 channel activity in all cells (Silverman et al., 2008), although its exact mechanism of action is unknown. Given that extracellular ATP promotes migration (Oliveira et al., 2015), it is likely that Panx1's role as a nucleotide release channel also underlies its effect on NPC migration.

Expression of Panx1 *in vivo* seemed lower in the migratory type A (DCX-positive) NPCs compared to the luminal type B (GFAP-positive/nestin-positive) NPCs. Moreover, Panx1 expression was almost non-existent in DCX-positive NPCs *in vitro*, and showed a dramatic reduction upon neuronal differentiation of cultured NPCs. This decreased expression likely reduced ATP release and purinergic signalling, which corresponds with a number of reports indicating decreased P2 receptor expression and function upon neuronal differentiation (Wu et al., 2009; Orellano et al., 2010; Glaser et al., 2014). This led me to examine whether Panx1 played a role in neurite outgrowth, a physical correlate of N2a cell and NPC neuronal differentiation. Panx1 block or knock-down induced neurite outgrowth in the absence of any other differentiation stimuli (e.g. retinoic acid, low serum), while Panx1 overexpression blocked neurite outgrowth in the presence of differentiating stimuli.

How else might Panx1 influence NPC behaviours? Recently an interaction between Panx1 and the actin cytoskeleton was described (Bhalla-Gehi et al., 2010; Wicki-Stordeur and Swayne, 2013), and Panx1 channels are activated by membrane deformation (Bao et al., 2004; Seminario-Vidal et al., 2011). Multiple cytoskeletal rearrangements occur during cell division, migration, and neurite outgrowth, which would likely activate the mechanosensitive Panx1 channel, perpetuating ATP release and downstream purinergic signalling. Our recent work demonstrated that Panx1 interacts with both actin and Arp3 (see Chapter 5 - Panx1 interactome analysis; Wicki-Stordeur and Swayne, 2013), a component of the Arp2/3 complex involved in the nucleation of new actin filaments. Actin and the Arp2/3 complex are integral in migration (Sawa et al., 2003; Schaefer et al., 2008), neurite extension (Schaefer et al., 2008; Gupton and Gertler, 2010; Firat-Karalar et al., 2011), cell adhesion (Korobova and Svitkina, 2008), and even cell division (reviewed in Firat-Karalar and Welch, 2011). A similar interaction between Cx43 and the actin cytoskeleton was previously described as crucial for NPC migration during cortical development, and functioned through channel-independent mechanisms (Elias et al., 2007; Cina et al., 2009; Elias et al., 2010). The interaction between Panx1 and the actin cytoskeleton may assume this role in N2a cells as they do not express endogenous Cx43 (Lin et al., 2014) and exhibit extremely low junctional coupling (Huang et al., 2007), indicating limited expression of other Cxs.

Interestingly, I also detected cleavage fragments of Panx1 in both N2a cells and primary NPCs (See Figures 3.2 and 3.3) consistent with recently reported caspase 3 cleavage activity that causes constitutive channel opening during apoptosis (Chekeni et al., 2010). In recent years, novel non-apoptotic roles for caspase activation have emerged, including involvement in proliferation, migration, and differentiation in various cell types (reviewed in Lamkanfi et al.,

2007; Kuranaga, 2012). In NPCs, transient caspase activation was associated with neuronal differentiation (Fernando et al., 2005; Pistritto et al., 2012) and proliferation (Yan et al., 2001; Yoneyama et al., 2014). Perhaps constitutive Panx1 activity generated by non-apoptotic caspase activation might also be relevant to, or necessary for its role in NPC behaviours.

Another Panx family member, Panx2, was also recently demonstrated to regulate differentiation of post-natal SGZ NPCs (Swayne et al., 2010). Similar to previous heterologous expression studies (Penuela et al., 2009), my current data demonstrate that Panx1 has a very different subcellular distribution profile than that reported for Panx2 in NPCs (Swayne et al., 2010; Wicki-Stordeur et al., 2013). In N2a cells, Panx2 was mainly located in late endosomes (Wicki-Stordeur et al., 2013), while my current results show that Panx1 is more widely distributed, and often found at the plasma membrane. Moreover, Panx1 appears to be abundantly expressed within VZ NPCs, while Panx2 is limited to a subset of SGZ NPCs (Swayne et al., 2010) and no evidence exists for its expression in the VZ. Therefore, although Panx1 and Panx2 both influence NPC differentiation, it is unlikely that a physical or functional interaction exists in VZ NPCs.

During embryonic development, Cx43 hemichannels were reported to act as ATP release conduits in radial glial cells of the VZ (based on experiments using pharmacological inhibitors that we now know also act on Panx single membrane channels; Weissman et al., 2004); however corresponding studies in the post-natal VZ have not been performed. Panx1 channels were discovered only recently (Panchin et al., 2000; Bruzzone et al., 2003), and are expressed at relatively high levels within the embryonic and early post-natal brain (Vogt et al., 2005). Given the data presented here indicating Panx1 expression and ATP release function in post-natal VZ NPCs, it would be interesting to examine potential crosstalk or overlap between Cx43 and Panx1

signalling in NPCs during both embryonic and post-natal neurogenesis. Perhaps there are subpopulations expressing either Cx43 or Panx1, or if co-expressed, are coupled to or regulated by different signalling paradigms. For example, Panx single membrane channels are activated under more physiologically relevant stimuli than Cx hemichannels, which are generally thought to open in pathological situations (reviewed in Lohman and Isakson, 2014; but see Batra et al., 2012; Orellana et al., 2012; Ponsaerts et al., 2012).

Recent studies have generated considerable interest in the reparative potential of post-natal VZ neurogenesis following brain injury or disease (reviewed in Burns et al., 2009; Leker et al., 2009; Liu et al., 2009). To be able to harness this potential, it is essential to understand the regulatory factors underlying NPC behaviours, including the role of Panx1. Interestingly, Panx1 channels are activated under several pathological circumstances, including elevated extracellular K^+ (Silverman et al., 2009), ischemia (Thompson et al., 2006), caspase cleavage during apoptosis (Sandilos et al., 2012), and elevated amyloid beta peptide exposure (Orellana et al., 2011), a pathological feature of Alzheimer's disease. NPCs are also highly responsive to changes in the surrounding environment, and are sensitive to injury and disease (reviewed in Lazarov and Marr, 2010; Lazarov et al., 2010). Given my current data demonstrating a role for Panx1 in regulating NPC behaviours under healthy conditions *in vitro*, injury-induced NPC responses could conceivably involve pathological activation of Panx1 channels. Therefore understanding the fundamental role of Panx1 channels in NPCs is important for cellular plasticity in the healthy brain, and potentially in injury-induced responses (explored in Chapter 4).

4. Panx1 differentially impacts NPC maintenance in the VZ and peri-infarct cortex

4.1. Overview

In the post-natal brain, VZ NPCs continuously undergo neurogenesis, a process consisting of proliferation, and differentiation that takes place as NPCs migrate along the RMS (reviewed in Ming and Song, 2011). A large proportion of these NPCs and immature neurons are cleared at different stages of migration (Morshead and van der Kooy, 1992; Platel et al., 2010; Lu et al., 2011), while the remaining cells populate the OB as mature interneurons and integrate into the existing circuitry. This ongoing process is involved in maintaining OB structure and function, and is important for olfactory-related learning and memory, as well as various social behaviours (e.g. Mak and Weiss, 2010; Sakamoto et al., 2014). Importantly, VZ neurogenesis is highly responsive to pathological states, such as stroke. The injury-induced response consists of increased NPC activation and proliferation, and diverted migration towards the area of injury (reviewed in Ohab and Carmichael, 2008). This process positively contributes to stroke outcomes (Raber et al., 2004; Jin et al., 2010; Zhang et al., 2010; Wang et al., 2012), but remains poorly understood.

In the previous chapter, I discovered Panx1 expression in post-natal VZ NPCs (Wicki-Stordeur et al., 2012; Wicki-Stordeur and Swayne, 2013). I demonstrated that Panx1 expression in NPCs *in vitro* positively regulated their proliferation (Wicki-Stordeur et al., 2012) and migration, and negatively regulated their neurite outgrowth (Wicki-Stordeur and Swayne, 2013), a morphological aspect of differentiation. In the present chapter, **I investigated the hypothesis**

that Panx1 regulates NPC behaviours *in vivo* in the post-natal brain. Since NPC behaviours are modulated by stroke (reviewed in Ohab and Carmichael, 2008), **I also examined whether stroke altered the effect of Panx1 expression in VZ NPCs.**

Panx1 is expressed in the brain within mature neurons and VZ NPCs, as well as in many other tissue and cell types throughout the body (Baranova et al., 2004; Ray et al., 2005; Vogt et al., 2005; Wicki-Stordeur et al., 2012). Here, in collaboration with Dr. Diane Lagace's group at the University of Ottawa, I selectively ablated Panx1 expression in VZ NPCs by intracerebroventricular (ICV) injection of control and cre-recombinase retroviruses in a 1:1 mixture (Tashiro et al., 2006b; Tashiro et al., 2006a) in floxed Panx1 mice. These viruses expressed different fluorescent markers in order to track Panx1-null and Panx1-expressing NPC numbers over time, essentially a measure of NPC 'maintenance'. I examined the impact of Panx1 deletion on NPC maintenance in the healthy and stroke VZ, as well as the peri-infarct cortex.

Overall, my results suggested that Panx1 was differentially important for the maintenance of NPCs, based on their location. Panx1 was required to maintain a consistent population of NPCs within the VZ niche. In the context of stroke, which stimulates NPC proliferation, the effects of Panx1 knockout were similar, but significantly delayed. In contrast, maintenance of NPCs within the peri-infarct (that originated in the VZ) was enhanced by Panx1 knockout. Together these results represent the first *in vivo* examination of the NPC-specific role of Panx1 in the healthy brain, as well as in the context of stroke.

4.2. Results

4.2.1. Panx1 is required for maintenance of VZ NPCs in vivo

I previously detected Panx1 expression in VZ NPCs *in vitro* and *in vivo* (Wicki-Stordeur et al., 2012; Wicki-Stordeur and Swayne, 2013), and demonstrated that Panx1 promoted NPC proliferation and migration, while inhibiting neurite outgrowth *in vitro.* I therefore hypothesized that Panx1 was important in the regulation of VZ NPCs *in vivo*. To investigate this prediction, I deleted Panx1 selectively in VZ NPCs using a retrovirus strategy (Tashiro et al., 2006b; Tashiro et al., 2006a). In collaboration with Dr. Diane Lagace's group at the University of Ottawa, I performed ICV injections with a combination of Cre-GFP and RFP-control retroviruses (1:1 ratio) into floxed-Panx1 mice (Figure 4.1A). Any NPC labeled with Cre-GFP (with or without RFP) was Panx1-null, while RFP-only NPCs were Panx1-expressing. As outlined in the cartoon in Figure 4.1B, I investigated both naïve and stroke conditions, quantifying the number of fluorescently labelled NPCs in the dorsolateral corner of the VZ, the RMS, and the peri-infarct cortex. The number of labelled NPCs over time gave a measure of NPC maintenance (i.e. the preservation of a consistent population size). Since naïve animals underwent a similar surgical procedure for virus injection (without dye injection/laser illumination), they were considered as sham controls for stroke surgery (henceforth referred to as 'naïve/sham').

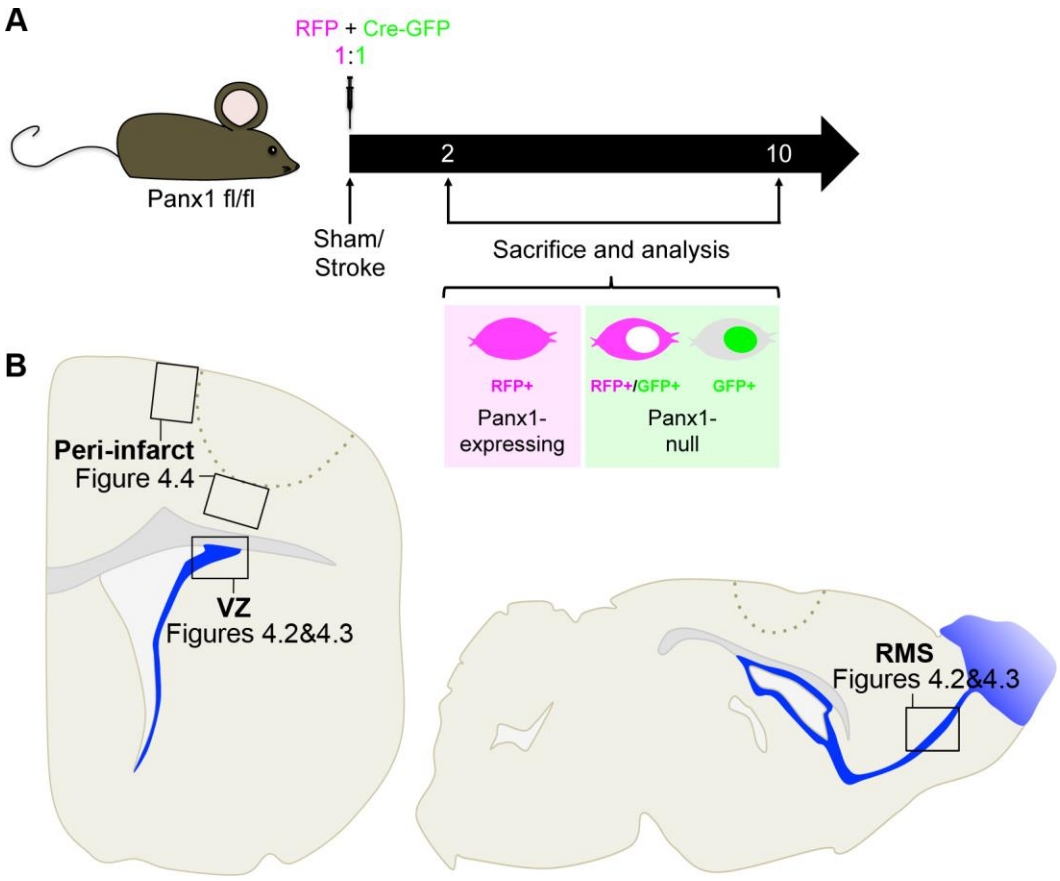

Figure 4.1. Experimental outline for retrovirus-mediated Panx1 deletion in VZ NPCs.
(A) Panx1-LoxP mice were given ICV injections of retroviral particles to introduce Cre-GFP or RFP-control vectors (1:1 ratio). Naïve/sham and photothrombotic stroke conditions were examined 2 and 10 days following surgery. Panx1-expressing (RFP+ only) and Panx1-null (Cre-GFP+ and Cre-GFP+/RFP+) NPCs were counted. RFP signal was present throughout the cell, while Cre-GFP signal was localized to the nucleus. (B) Cartoon representing areas of quantification in the VZ, RMS, and peri-infarct cortex. Labels refer to the Figures in which the corresponding data can be found.

I initially introduced the retroviruses into floxed Panx1 mice without stroke (naïve/sham animals) and counted the number of Panx1-null and Panx1-expressing NPCs in the VZ (Figure 4.2A). Previous quantification in wildtype mice (see Chapter 2, section 2.1.2) established that equal proportions of GFP-positive and RFP-positive only NPCs were expected in the VZ if

Panx1 deletion had no effect. However, initially (2 days post-injection; dpi) there were ~70% fewer Panx1-null NPCs (GFP-positive) than Panx1-expressing NPCs (RFP-positive only) in the VZ. Over time the number of Panx1-expressing NPCs decreased (and there was no statistically-significant change in the number of Panx1-null NPCs) such that by 10 dpi, there was no significant difference between the two NPC populations. I confirmed that virtually all infected NPCs (both Panx1-null and Panx1-expressing) were DCX-positive at 2 dpi (Figure 4.2B).

Since my previously published data demonstrated that Panx1 regulated NPC proliferation *in vitro* (Wicki-Stordeur et al., 2012), I reasoned that a defect in NPC proliferation resulting from Panx1 deletion could have caused the lower abundance of Panx1-null NPCs observed here. In order to examine the proliferation status of infected NPCs, I immunostained for Ki67, a marker of actively cycling cells (reviewed in Scholzen and Gerdes, 2000). The percentage of Ki67-positive infected NPCs was independent of Panx1 expression status (Figure 4.2C). Another potential explanation for the low abundance of Panx1-null NPCs in the VZ was increased migration of Panx1-null NPCs out of the VZ and into the RMS. However, the number of Panx1-null NPCs was also low in the RMS (Figure 4.2D), ruling out this possibility. I immunostained for activated caspase 3, a marker for cells undergoing apoptosis (reviewed in Thornberry and Lazebnik, 1998), but did not detect any activated caspase 3-positive cells in the VZ (data not shown). Together these results suggest that Panx1 is essential for maintenance of VZ NPCs, but does not affect proliferation, migration, or caspase 3-dependent apoptotic mechanisms *in vivo*.

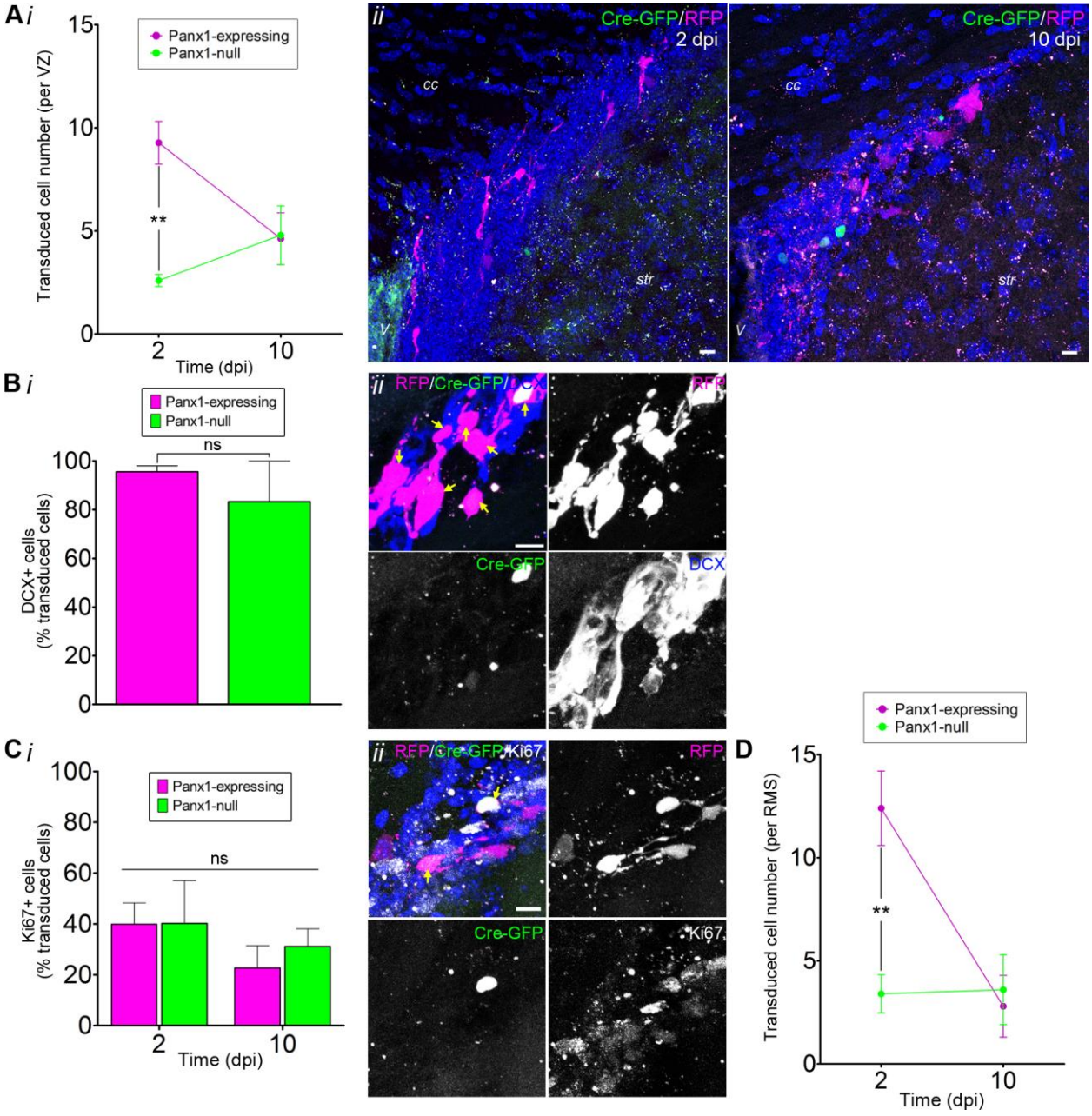

Figure 4.2. Panx1 deletion is associated with a loss of VZ NPCs.

(A) i. Panx1-null (GFP+) and Panx1-expressing (RFP+ only) NPC numbers per VZ at 2 and 10 dpi in naïve/sham animals. The number of Panx1-null NPCs was lower than Panx1-expressing NPCs at 2 dpi (2 dpi, N = 5; 10 dpi, N = 6; Expression: $F(1,18) = 7.898$, $p = 0.0116$; Time: $F(1,18) = 1.123$, $p = 0.3033$; Interaction: $F(1,18) = 8.764$, $p = 0.0084$; by two factor ANOVA; Bonferroni posthoc $p < 0.01$ (**) for Expression at 2 dpi). ii. Maximum intensity projections of representative confocal Z-stacks of the VZ at 2

(left) and 10 dpi (right; scalebars, 10 µm). V: ventricle, cc: corpus callosum, str: striatum. Hoechst 33342 was used as a nuclear counterstain. (B) i. The vast majority of Panx1-expressing and Panx1-null VZ NPCs were immunoreactive for DCX (2 dpi, N = 6, p = 0.3930 by unpaired t test). ii. Maximum intensity projection of a representative confocal Z-stack demonstrating DCX-immunoreactivity of transduced VZ NPCs (scalebar, 10 µm). Arrows indicate DCX+ transduced cells. (C) i. The percentage of transduced VZ NPCs immunoreactive for Ki67 was not affected by time post-injection or Panx1 expression status (2 dpi, N = 4; 10 dpi, N = 6; Expression: $F(1,16) = 0.1885$, $p = 0.6699$; Time: $F(1,16) = 1.633$, $p = 0.2195$; Interaction: $F(1,16) = 0.1582$, $p = 0.6961$ by two factor ANOVA). ii. Maximum intensity projection of a representative confocal Z-stack from the VZ showing Ki67-immunoreactivity of transduced VZ NPCs (scalebar, 10 µm). Arrows indicate Ki67+ transduced NPCs. Hoechst 33342 was used as a nuclear counterstain. (D) Quantification of transduced NPC numbers in the RMS at 2 and 10 dpi (2 dpi, N = 5; 10 dpi, N = 6; Expression: $F(1,16) = 7.293$, $p = 0.0158$; Time: $F(1,16) = 9.584$, $p = 0.0069$; Interaction: $F(1,16) = 10.42$, $p = 0.0053$ by two factor ANOVA; Bonferroni posthoc $p<0.01$ (**) for Expression at 2 dpi).

4.2.2. Panx1 is required to maintain elevated VZ NPC numbers after stroke

Cortical stroke causes VZ NPCs to hyper-proliferate for several weeks despite their distance from the focal injury site (reviewed in Ohab and Carmichael, 2008). I therefore used the photothrombotic (PT) model of stroke, in combination with the retrovirus knock-out strategy, to investigate whether focal cortical stroke modifies the effects of Panx1 deletion on VZ NPC maintenance. Note that virus injection and photothrombosis were performed during the same surgical procedure.

As expected, I observed a similar increase in the total numbers of infected NPCs (GFP-positive and RFP-positive populations combined) at 2 dpi/PT compared to naïve/sham controls (naïve/sham: 11.9 ± 1.3, stroke: 41.5 ± 10.1 transduced cells/VZ, $p = 0.0264$ by unpaired t test). At 2 dpi/PT, Panx1-null and Panx1-expressing NPCs were equally abundant (no significant

difference by two-factor ANOVA) in the VZ (Figure 4.3A), and virtually all infected NPCs were DCX-positive (Figure 4.3B). However, by 10 dpi/PT, the number of Panx1-null NPCs was significantly reduced to naïve/sham levels while the number of Panx1-expressing NPCs remained elevated. Analysis of an intermediate time point demonstrated that the Panx1-null NPC numbers were already largely (albeit not significantly) reduced by 5 dpi/PT.

The percentage of Ki67-positive NPCs was independent of Panx1 expression status (Figure 4.3C), suggesting the compromised Panx1-null NPC maintenance was not due to impaired proliferation. Moreover, the number of infected NPCs in the RMS following stroke was independent of Panx1-expression status (Figure 4.3D), indicating there was no effect of Panx1 deletion on NPC migration. I also immunostained for activated caspase 3 and again did not detect any activated caspase 3-positive cells in the VZ (data not shown). Taken together, these results suggest that Panx1 is also essential for maintaining elevated VZ NPC numbers after stroke, and does not affect proliferation, migration, or caspase-3-dependent apoptotic mechanisms.

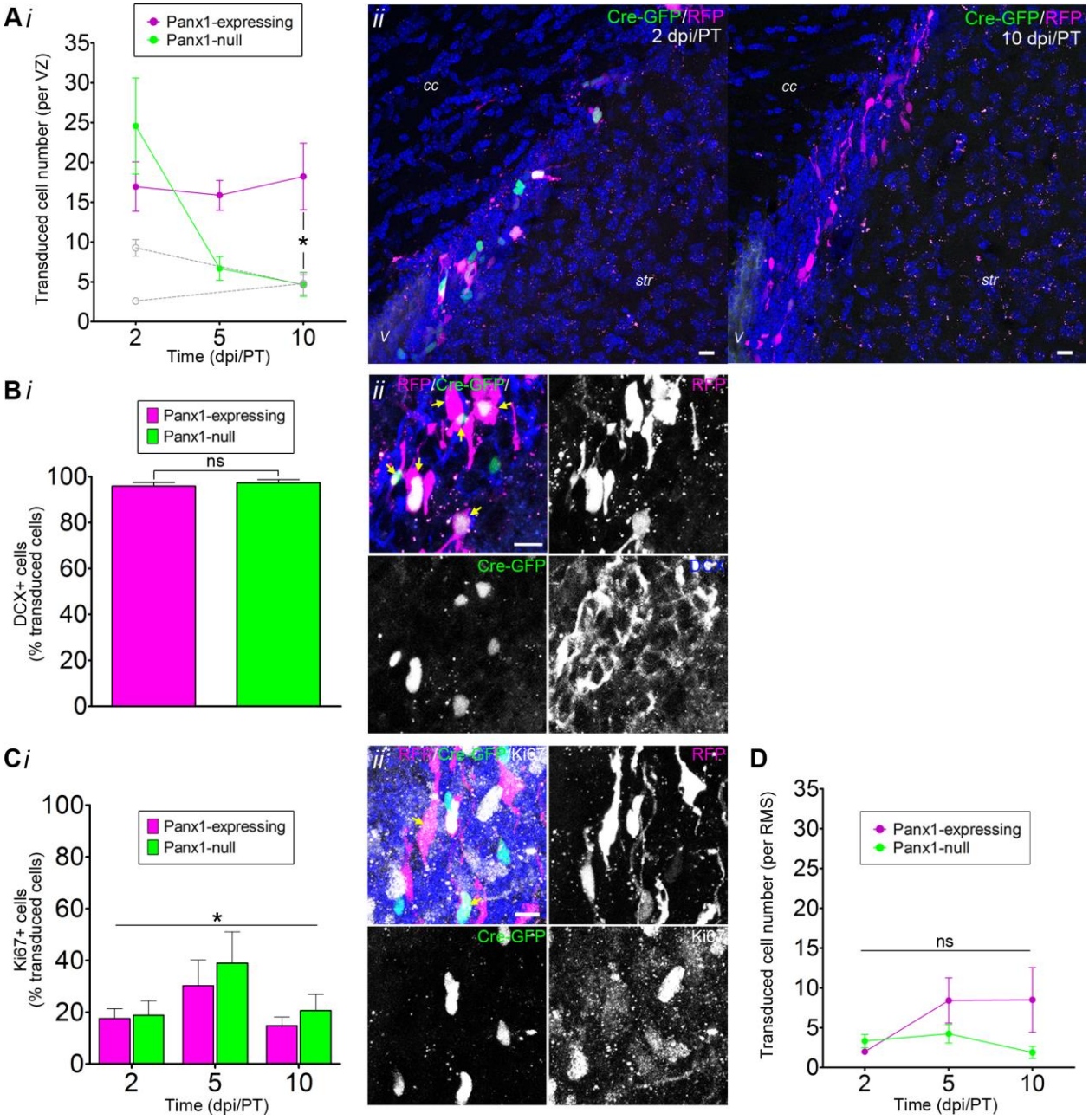

Figure 4.3. Panx1 is essential for maintaining elevated VZ NPC numbers after stroke.

(A) i. Panx1-null and Panx1-expressing NPC numbers per VZ at 2, 5, and 10 dpi/PT. Naïve/sham data from Figure 2A is overlaid in light grey. The number of Panx1-null NPCs significantly decreased over time (2 dpi/PT, N = 7; 5 and 10 dpi/PT, N = 6, Expression: $F(1, 32) = 2.854$, $p = 0.1008$; Time: $F(2,32) = 4.644$, $p = 0.0169$; Interaction: $F(2,32) = 4.902$, $p = 0.0139$ by two factor ANOVA; Bonferroni posthoc $p<0.05$ (*) for Expression at 10 dpi/PT). ii. Maximum intensity projections of representative confocal Z-

stacks of the VZ show transduced NPCs at 2 (left) and 10 dpi/PT (right; scalebars, 10 µm). V: ventricle, cc: corpus callosum, str: striatum. Hoechst 33342 was used as a nuclear counterstain. (B) i. The vast majority of transduced VZ NPCs were immunoreactive for DCX (2 dpi/PT, N = 6, p = 0.5202 by unpaired t test). ii. Maximum intensity projection of a representative confocal Z-stack from the VZ demonstrating DCX-immunoreactivity of transduced VZ NPCs (scalebar, 10 µm). Arrows indicate DCX+ transduced NPCs. (C) i. The percentage of transduced VZ NPCs immunoreactive for Ki67 was not affected by Panx1 expression status, but was affected by time post-stroke (2 dpi/PT, N=7, 5 and 10 dpi/PT, N = 5; Expression: $F(1,28) = 0.8049$, $p = 0.3760$; Time: $F(2,28) = 3.461$, $p = 0.0454$; Interaction: $F(2,28) = 0.1508$, $p = 0.8607$ by two factor ANOVA). ii. Maximum intensity projection of a representative confocal Z-stack demonstrating Ki67-immunoreactivity of transduced VZ NPCs (scalebar, 10 µm). Arrows indicate Ki67+ transduced NPCs. Hoechst 33342 was used as a nuclear counterstain. (D) Quantification of Panx1-null and Panx1-expressing NPC numbers in the RMS at 2, 5, and 10 dpi/PT. The number of transduced NPCs was not affected by Panx1 expression status and did not significantly change over time (2 and 5 dpi/PT, N=6; 10 dpi/PT, N=5; Expression: $F(1,28) = 3.593$, $p = 0.0684$; Time: $F(2,28) = 1.812$, $p = 0.1820$; Interaction: $F(2,28) = 1.998$, $p = 0.1545$ by two factor ANOVA).

4.2.3. Panx1-null NPCs persist within the peri-infarct cortex

Following stroke, a population of VZ NPCs are diverted from their normal migration path and directed towards the site of injury (reviewed in Ohab and Carmichael, 2008). Panx1 has been implicated in death of mature neurons (reviewed in Weilinger et al., 2013), and in inflammatory signalling (reviewed in Makarenkova and Shestopalov, 2014) that persists in the peri-infarct cortex for days after the acute ischemic event (reviewed in Brouns and De Deyn, 2009). I therefore hypothesized that deletion of Panx1 could influence the maintenance/survival of VZ NPCs that migrated into the peri-infarct cortex. There was a significantly greater abundance of Panx1-null NPCs in the peri-infarct cortex at 5 dpi/PT that persisted at 10 dpi/PT (Figure 4.4A, B). Given that I did not observe a surge of Panx1-null NPCs into the peri-infarct cortex (nor the RMS) at 2 dpi/PT, this increase in Panx1-null NPCs was not likely due to accelerated migration

of Panx1-null NPCs from the VZ. Analysis of the expression of activated caspase 3 within the transduced peri-infarct NPCs suggested a low percentage of these NPCs (<10%) were apoptotic (Figure 4C). These results suggest that Panx1-null NPCs were able to persist for longer in the peri-infarct cortex compared to their Panx1-expressing counterparts.

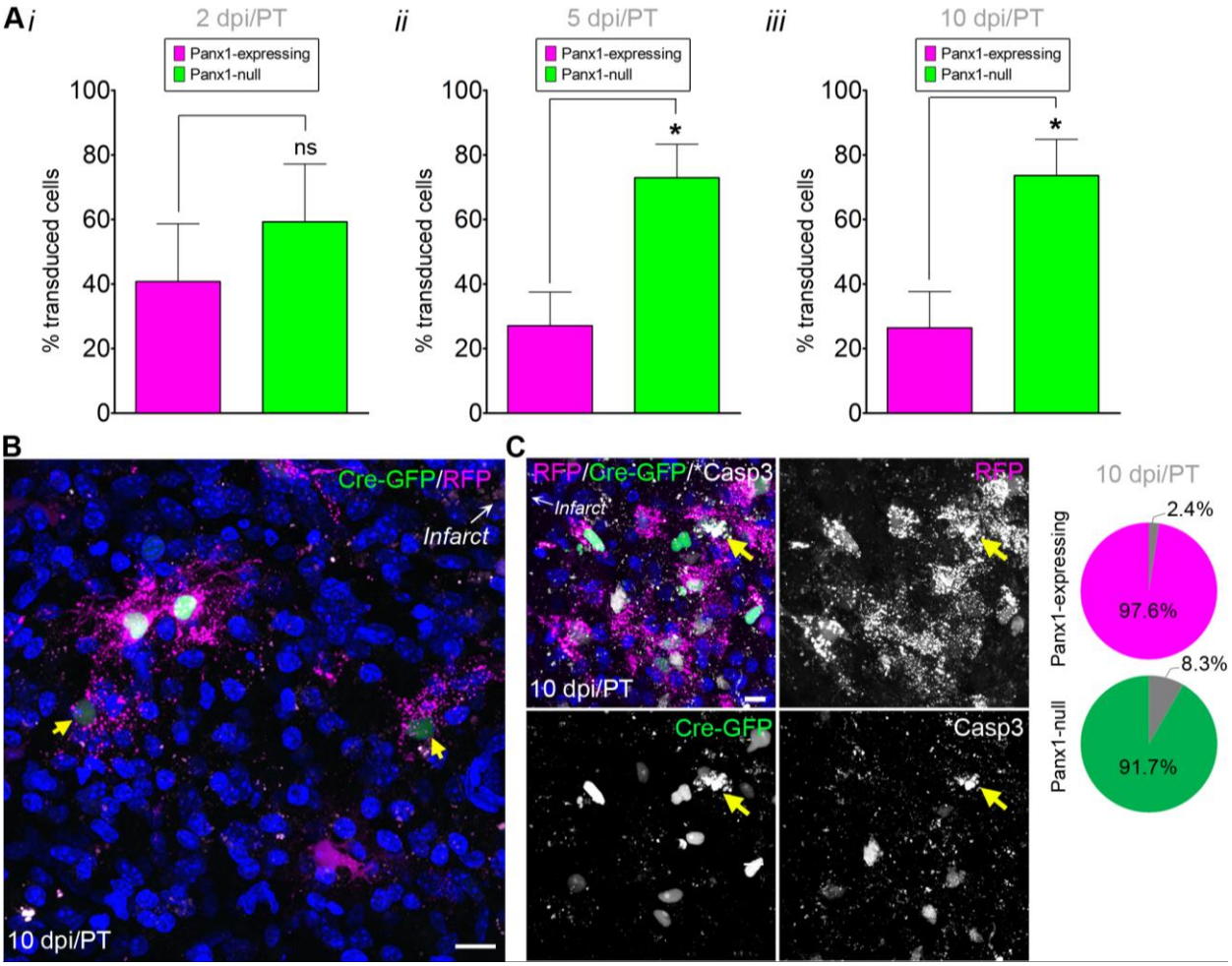

Figure 4.4. Panx1-null cells persist in the peri-infarct cortex.

(A) Percentages of Panx1-null and Panx1-expressing NPCs in the peri-infarct cortex. i. Panx1-null and Panx1-expressing NPCs were equally abundant 2 dpi/PT (N = 6, p = 0.4812 (not significant; ns) by unpaired t test). Note that 1 of the 7 brains did not have a single transduced NPC in the peri-infarct at 2 dpi/PT; data are represented as percent Panx1-null NPCs of total transduced NPCs due to a large

variability in NPC number reaching the peri-infarct cortex between mice. ii. Panx1-null and Panx1-expressing NPC percentages at 5 dpi/PT (N = 4, p = 0.0208 (*) by unpaired t test). Note that 2 of the 6 brains did not have a single transduced NPC in the peri-infarct at 5 dpi/PT. iii. Panx1-null NPCs were more abundant than Panx1-expressing NPCs at 10 dpi/PT (N = 6, p = 0.0180 (*) by unpaired t test). (B) Maximum intensity projection of a representative confocal Z-stack from the peri-infarct tissue 10 dpi/PT. Arrows indicate faint GFP+ nuclei. (C) Maximum intensity projection of a representative confocal Z-stack from the peri-infarct tissue 10 dpi/PT with arrows indicating activated caspase (*Casp3)+ cells. Pie charts indicate the percentage of total RFP+ (Panx1-expressing; upper pie chart) or GFP+ (Panx1-null; lower pie chart) cells that were *Casp3+ in the peri-infact at 10 dpi/PT across all animals. Hoechst 33342 was used as a nuclear counterstain (scalebars, 10 μm).

4.3. Discussion

In this chapter, I built on my previous discovery of Panx1 expression in GFAP-positive/nestin-positive, GFAP-negative/nestin-positive, and DCX-positive post-natal VZ NPCs (Wicki-Stordeur et al., 2012; Wicki-Stordeur and Swayne, 2013) by examining the effect of an NPC-specific Panx1 knock-out in healthy (naïve/sham) and stroke-injured brain. My Chapter 3 data demonstrated that blocking Panx1 reduced VZ NPC numbers *in vitro* (Wicki-Stordeur et al., 2012), indicating that Panx1 was involved in the regulation of NPC proliferation and/or maintenance. The current experiments demonstrated that *in vivo*, Panx1 was required for maintenance of VZ NPC numbers independent of a proliferative effect.

To initially test the hypothesis that Panx1 impacted VZ NPCs behaviours *in vivo*, I used a retrovirus strategy (Tashiro et al., 2006b; Tashiro et al., 2006a) to selectively knock-out Panx1 in late-stage (DCX-positive) VZ NPCs in healthy floxed Panx1 mice. At an early time point following Panx1 ablation (2 dpi), there were 70% fewer Panx1-null than Panx1-expressing NPCs in the VZ. By 10 dpi the number of Panx1-expressing NPCs had also decreased so that there was

no longer a significant difference between the two populations. Since it is well known that a large proportion of NPCs are lost over the course of neuronal development (Morshead and van der Kooy, 1992), this could be the underlying cause for the apparent loss of Panx1-expressing NPCs over time.

I next examined the impact of focal cortical stroke on Panx1-null and Panx1-expressing VZ NPC populations. Stroke-enhanced VZ NPC proliferation can persist for weeks, and even months following injury (reviewed in Ohab and Carmichael, 2008). As expected, I observed an expansion of the overall population of transduced/labeled NPCs following stroke. Interestingly, this increase was both ipsilateral and contralateral to stroke, similar to a previous report by Jin and colleagues where they found a bilateral response to focal stroke (Jin et al., 2001). This is perhaps not surprising given that the unknown mechanisms underlying stroke-induced neurogenesis likely include trans-synaptic connections, long-distance diffusion of chemical modulators, and/or delivery of signalling molecules via the vasculature and CSF. Both Panx1-null and Panx1-expressing NPC populations were increased shortly following stroke (2 dpi/PT). This was in contrast with my naïve/sham results, where the Panx1-null population was smaller than the Panx1-expressing population at 2 dpi. However, while the Panx1-expressing NPC numbers remained elevated for over a week following stroke, there was a robust decline in the Panx1-null NPC population. Together these data suggest that while stroke stimulation initially overrode the effect of Panx1 knock-out, Panx1 expression was necessary for maintaining the heightened NPC response. It should be noted that since stroke was induced within the same time period as retrovirus injection, the stroke stimuli preceded actual decreases in Panx1 protein levels. Therefore, I was not able to address whether Panx1 was required for the initial stroke-

induced NPC activation. Future studies where Panx1 deletion occurs prior to stroke will be needed to answer this question.

NPCs release ATP in episodic bursts events that promote NPC proliferation and/or maintenance (Lin et al., 2007). In particular, P2Y1 receptor activity regulates NPC proliferation in the VZ (Suyama et al., 2012; Boccazzi et al., 2014). Panx1 acts as an ATP release channel in several tissue and cell types (reviewed in Dahl, 2015). My previous data demonstrated that Panx1 also mediated ATP release from an NPC-like cell line *in vitro*, and positively regulated proliferation and/or maintenance of cultured NPCs (Wicki-Stordeur et al., 2012). It was therefore reasonable to hypothesize that deletion of Panx1 would impair NPC proliferation *in vivo*. However, I observed no differences in proliferation between Panx1-null and Panx1-expressing VZ NPC populations in naïve or stroke animals, as assayed by immunoreactivity for the proliferation marker Ki67. It should be noted that Ki67 levels are variable across the cell cycle (reviewed in Scholzen and Gerdes, 2000), raising the possibility that Panx1 deletion altered the cell cycle in such a way as to obscure detection of differences. Moreover, it is also possible that the remaining Panx1-null NPCs were resistant to the effects of Panx1 deletion (through perhaps normal heterogeneity in gene expression levels within the population; Johnson et al., 2015), or had recovered from proliferative defects associated with Panx1 deletion at the time points analyzed (through compensatory mechanisms; see Lohman and Isakson, 2014). Still, the simplest explanation for these results was that Panx1 deletion did not significantly affect VZ NPC proliferation *in vivo*.

The loss of Panx1-null NPCs from the VZ might also have been explained by their increased migration out of the VZ, or elevated apoptosis. My Chapter 3 data indicated that Panx1 positively regulated NPC migration *in vitro* (Wicki-Stordeur and Swayne, 2013). However, my

current analysis of labelled NPCs within the RMS revealed no significant difference in migration between Panx1-null and Panx1-expressing NPC populations *in vivo*. There was also no obvious expression of activated caspase-3 in Panx1-null or Panx1-expressing VZ NPCs, indicating that Panx1 expression status had no impact on caspase-3 dependent cell death in the VZ. Together, my data suggested that deletion of Panx1 impaired NPC maintenance within the VZ, independent of decreased proliferation, increased migration, or increased apoptosis.

In the VZ, a large proportion of NPCs are normally lost (Morshead and van der Kooy, 1992). This phenomenon was previously assumed to be a result of apoptotic cell death. However, recent evidence found that these NPCs are actually cleared by neighboring phagocytic NPCs (DCX-positive neuroblasts), the primary phagocytic cells in the VZ (Lu et al., 2011), in a process termed 'phagoptosis' (reviewed in Brown and Neher, 2012; Brown et al., 2015). Further work demonstrated that this phagoptosis is regulated by non-canonical functions of the P2X7 receptor (Lovelace et al., 2015). In this mechanism, the P2X7 receptor interacts with non-muscle myosin to mediate phagocytosis; however, in the presence of ATP this interaction is disrupted, abolishing further phagocytosis (Gu et al., 2009; Gu et al., 2010; Gu et al., 2011; Gu et al., 2012). This suggests that extracellular ATP acts as a 'don't-eat-me' survival signal for VZ NPCs, in addition to its well-defined role in purinergic receptor-mediated regulation of NPC maintenance and differentiation (reviewed in Cavaliere et al,. 2015). Since my Chapter 3 data demonstrated that Panx1 mediates ATP release from NPC-like cells *in vitro* (Wicki-Stordeur et al., 2012), it is feasible that Panx1 deletion rendered VZ NPCs susceptible to clearance by resident phagocytic NPCs, resulting in the reduced Panx1-null NPC numbers observed here.

Stroke induces a subset of VZ NPCs to deviate from their normal migration route and instead move towards the site of injury (reviewed in Ohab and Carmichael, 2008). It is therefore not

surprising that I initially observed comparatively fewer labelled NPCs in the stroke RMS than under naïve/sham conditions. Improved stroke outcomes are associated with survival of these diverted NPCs (reviewed in Xiong et al., 2010). At 5 dpi/PT and 10 dpi/PT I detected a larger proportion of Panx1-null than Panx1-expressing NPCs in the peri-infarct cortex. A low percentage of both Panx1-null and Panx1-expressing NPC populations showed signs of apoptosis (< 10% of labelled cells). This suggests that loss of Panx1 improved NPC maintenance in the peri-infarct cortex, as the Panx1-null NPCs persisted in this region longer than their Panx1-expressing counterparts.

In the peri-infarct cortex, 'find-me/eat-me' signals are expressed by surviving neurons exposed to sub-lethal stimuli. These signals attract phagocytic microglia, rendering these otherwise viable neurons vulnerable to phagoptosis (Neher et al., 2011; Neniskyte et al., 2011; Geiger-Maor et al., 2012; Neher et al., 2013). 'Find-me/eat-me' signals include phosphatidylserine exposure and release of ATP and other nucleotides, among other things (reviewed in Patel et al., 2013). Supporting this idea, Panx1 was previously identified as the release conduit for ATP and UTP 'find-me' signals for phagocytic macrophage (Chekeni et al., 2010). Microglial P2Y12 receptors are also activated by ATP, and stimulate microglial chemotaxis and phagocytosis of the target cell (Honda et al., 2001; Irino et al., 2008; Ohsawa et al., 2010). Therefore, it would be reasonable to speculate that Panx1 deletion protects cells from phagoptosis in the peri-infarct cortex by reducing release of nucleotide 'find-me/eat-me' signals (in stark contrast to the ATP-mediated *inhibition* of phagoptosis in the VZ; Lovelace et al., 2015).

In addition, the peri-infarct cortex contains low levels of oxygen and nutrients, released factors from dead and dying cells, cytokines, and activated immune cells. Over-activation of Panx1 channels by any of these pathological stimuli could lead to loss of ionic and metabolic gradients,

and result in NPC death (similar to its proposed function as a ‘death-pore’ in mature neurons following injury; reviewed in Thompson, 2015). Taken together with recent studies implicating Panx1 activity in neuron death under pathological circumstances (Thompson et al., 2006; Bargiotas et al., 2011; Bargiotas et al., 2012; Dvoriantchikova et al., 2012; Xiong et al., 2014; Cisneros-Mejorado et al., 2015b), the current results add to a growing body of literature (reviewed in Dahl and Keane, 2012) indicating that targeting Panx1 within the peri-infarct (in combination with other interventions) may improve cell survival. However, tracking Panx1-null peri-infarct NPCs over a longer time-course will be required to fully address the effects of Panx1 on peri-infarct NPC survival and its impact on stroke outcomes.

Overall these data contribute to our understanding of the importance of Panx1 within the brain, under both healthy conditions and in the context of stroke. Moreover, these results suggest that the role of this channel in NPCs is site-dependent. Specifically, Panx1 plays an important role in promoting the maintenance of NPC numbers in the VZ niche, yet targeting Panx1 within the peri-infarct cortex could improve cell survival around the injury.

5. Panx1 regulates NPC neurite outgrowth through a novel interaction with Crmp2

5.1. Overview

My previous data demonstrated that Panx1 regulated NPC behaviours both *in vitro* (Chapter 3; Wicki-Stordeur et al., 2012; Wicki-Stordeur and Swayne, 2013) and *in vivo* (Chapter 4). However, limited data existed to explain the molecular underpinnings of this Panx1-dependent regulation. Therefore, in the present chapter I investigated the Panx1 interactome to gain insight into its involvement in established signalling pathways and protein networks in NPCs. Since the discovery of Panxs, a handful of studies have obtained limited information on the Panx1 interactome in different cell types (Figure 5.1; reviewed in Wicki-Stordeur and Swayne, 2014). Here I performed the first large-scale, unbiased proteomics screen for Panx1 protein interaction partners in NPCs. Using immunoprecipitations coupled to high performance liquid chromatography and tandem mass spectrometry (LC-MS/MS), I identified a novel protein interaction partner, collapsin response mediator protein 2 (Crmp2), as well as other Crmp family members (that can form heterotetramers; Wang and Strittmatter, 1997) in multiple replicates. This cytoskeletal-associated protein is highly expressed in the nervous system (recently reviewed Hensley et al., 2011; Ip et al., 2014), and has a well-defined role in neurite outgrowth, one of the NPC behaviours regulated by Panx1 (Wicki-Stordeur and Swayne, 2013). **This led to my hypothesis that Panx1 regulates NPC behaviours in part through its interaction with Crmp2.**

In this chapter, I first present a large-scale Gene Ontology (GO) analysis of the identified Panx1 interacting proteins. Many of these proteins were associated with cell proliferation, motility and differentiation. I then focused on validating the novel association between Panx1 and Crmp2 in NPCs, and examined its functional impact on NPC proliferation, migration, and neurite outgrowth. Blocking this interaction promoted NPC neurite outgrowth, without altering proliferation or migration. Moreover, Panx1 protein localization and ATP release were unaffected. Together these data suggest that the Panx1-Crmp2 interaction underlies my previously reported modulation of NPC neurite outgrowth by Panx1.

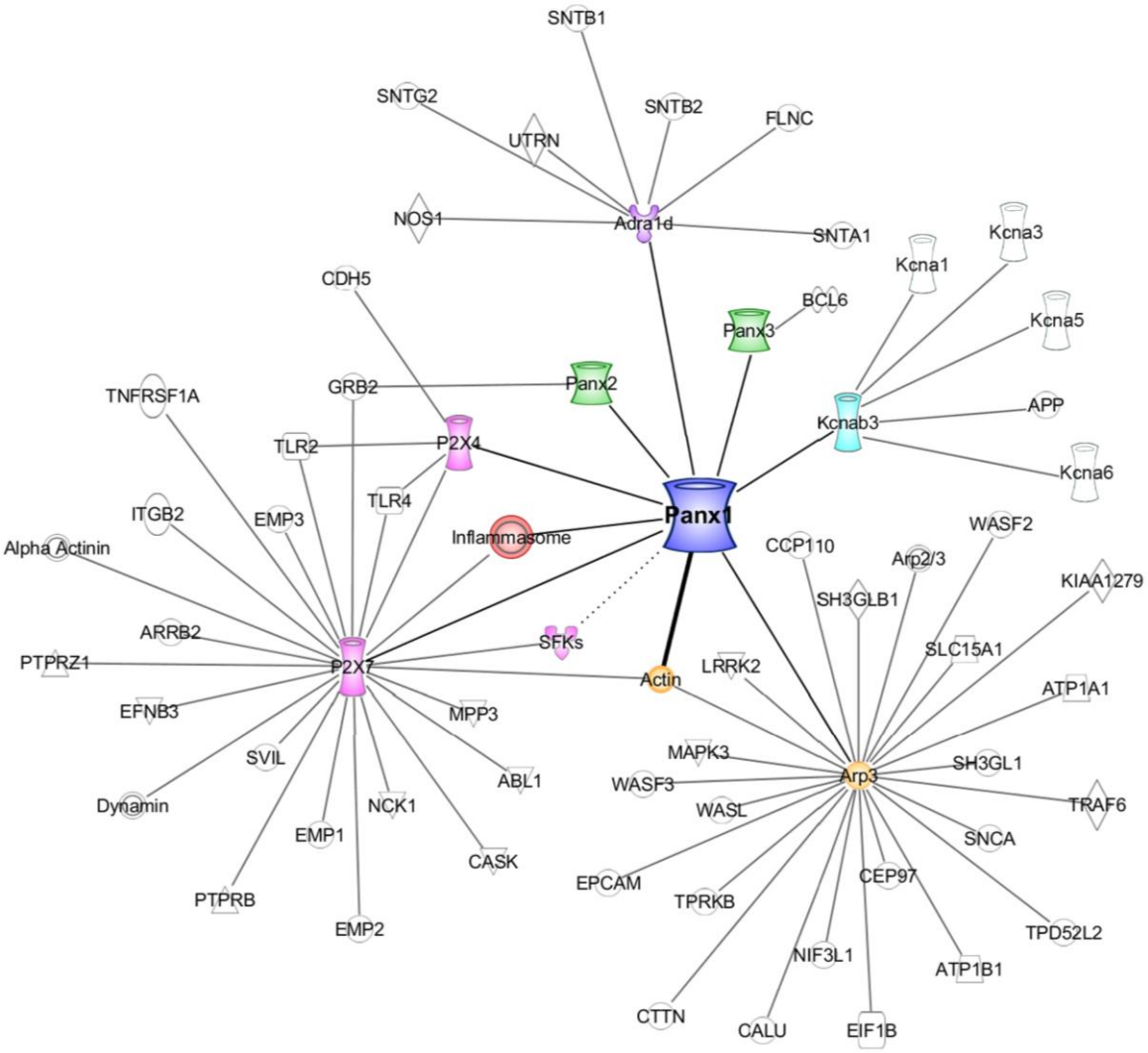

Figure 5.1. The reported Panx1 interactome.

A diagram generated using Ingenuity Pathways Analysis [IPA; Ingenuity Systems (Qiagen), Redwood City, CA, USA] representing a curated list of the Panx1 protein interaction partners, and the subsequent IPA-derived interaction networks of these proteins (excluding actin). The thick line between Panx1 and actin represents a direct interaction. The dotted line between Panx1 and SFKs indicates a likely link between the two proteins; however, no physical interaction data is yet available.

5.2. Results

5.2.1. Identification of novel Panx1 interacting proteins

I immunoprecipitated Panx1 from N2a cells expressing Panx1EGFP or EGFP, and identified co-precipitated proteins by high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The EGFP tag does not affect the trafficking or functioning of the Panx1 (Lai et al., 2007; Bhalla-Gehi et al., 2010; Wicki-Stordeur et al., 2013) and therefore was deemed suitable for use in identification of interacting proteins. All proteins co-precipitating with the EGFP control were excluded from further analysis. The resulting Panx1-specific list contained 480 unique proteins identified as putative Panx1 interaction partners. The corresponding genes were analyzed using GO Slim term classifications (http://go.princeton.edu/cgi-bin/GOTermMapper) for Biological Process (Figure 5.2), Molecular Function (Figure 5.3) and Cellular Component (Figure 5.4). Of the proteins/genes amenable to GO analysis (426/480), those classified under Biological Process terms relating to cell proliferation, migration, and differentiation are listed in Table 1. A large proportion of the analyzed interacting proteins (93 genes; 20%) were classified under the Cellular Component GO term ‘Cytoskeleton’ (GO:0005856; Table 2). This GO term encompasses components of the intermediate filament, actin, and microtubule cytoskeleton, as well as any other polyfilamentous structures.

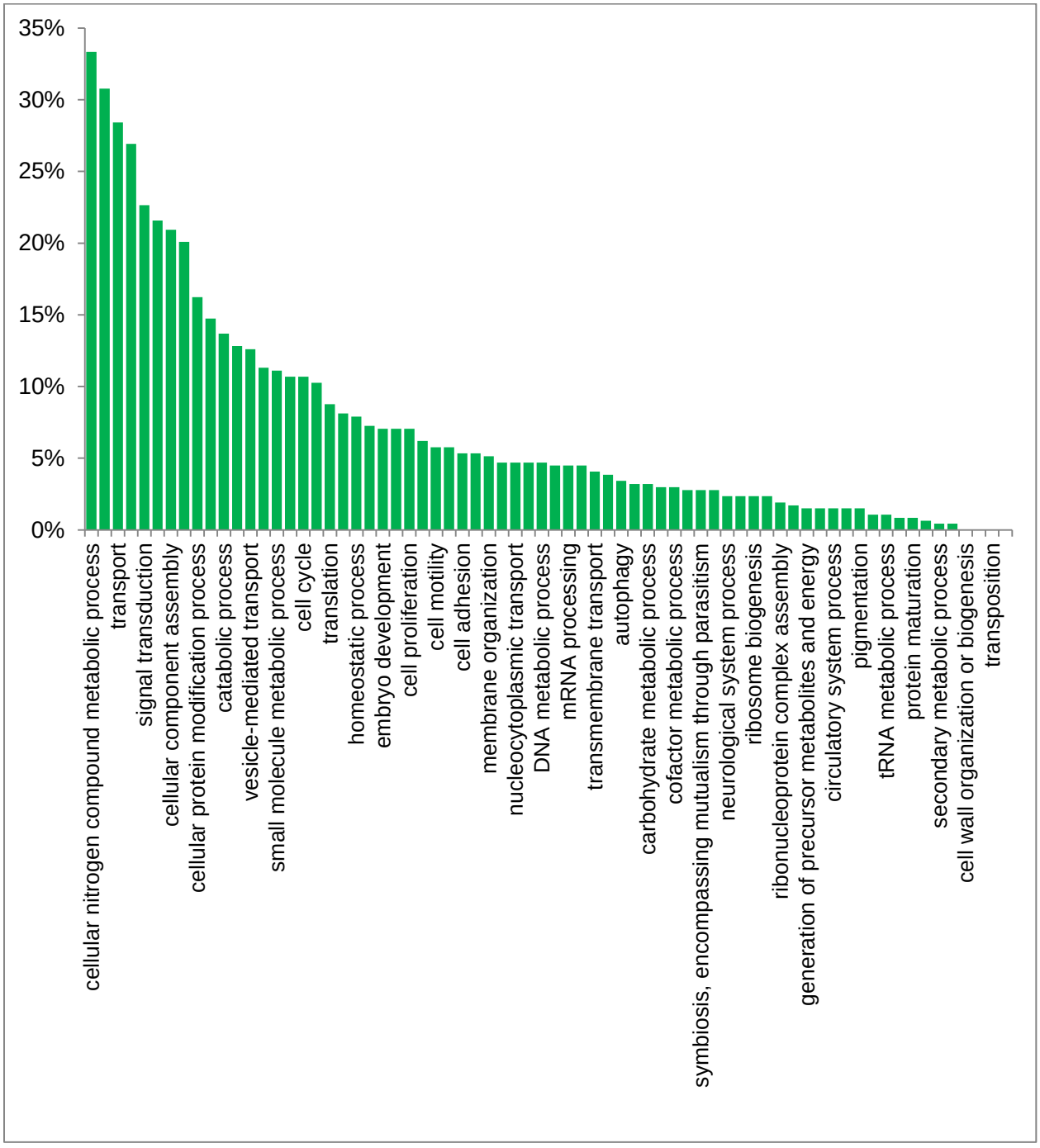

Figure 5.2. Biological Process GO Slim term analysis of Panx1 interacting proteins.

Graphical representation of the proportion of LC-MS/MS identified Panx1 interacting proteins that are classified by each GO Slim term in the 'Biological Process' category.

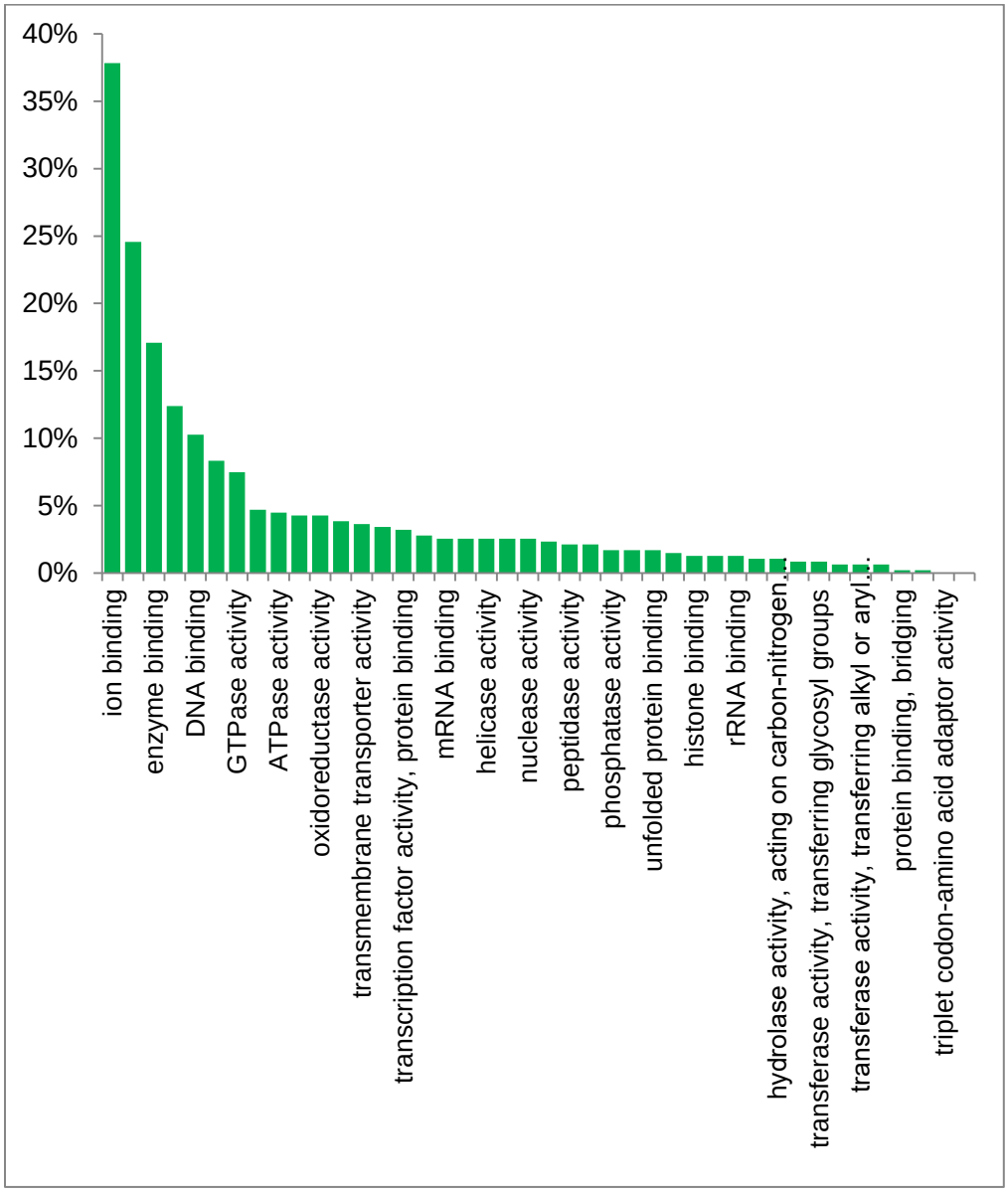

Figure 5.3. Molecular Function GO Slim term analysis of Panx1 interacting proteins.

Graphical representation of the proportion of LC-MS/MS identified Panx1 interacting proteins that are classified by each GO Slim term in the 'Molecular Function' category.

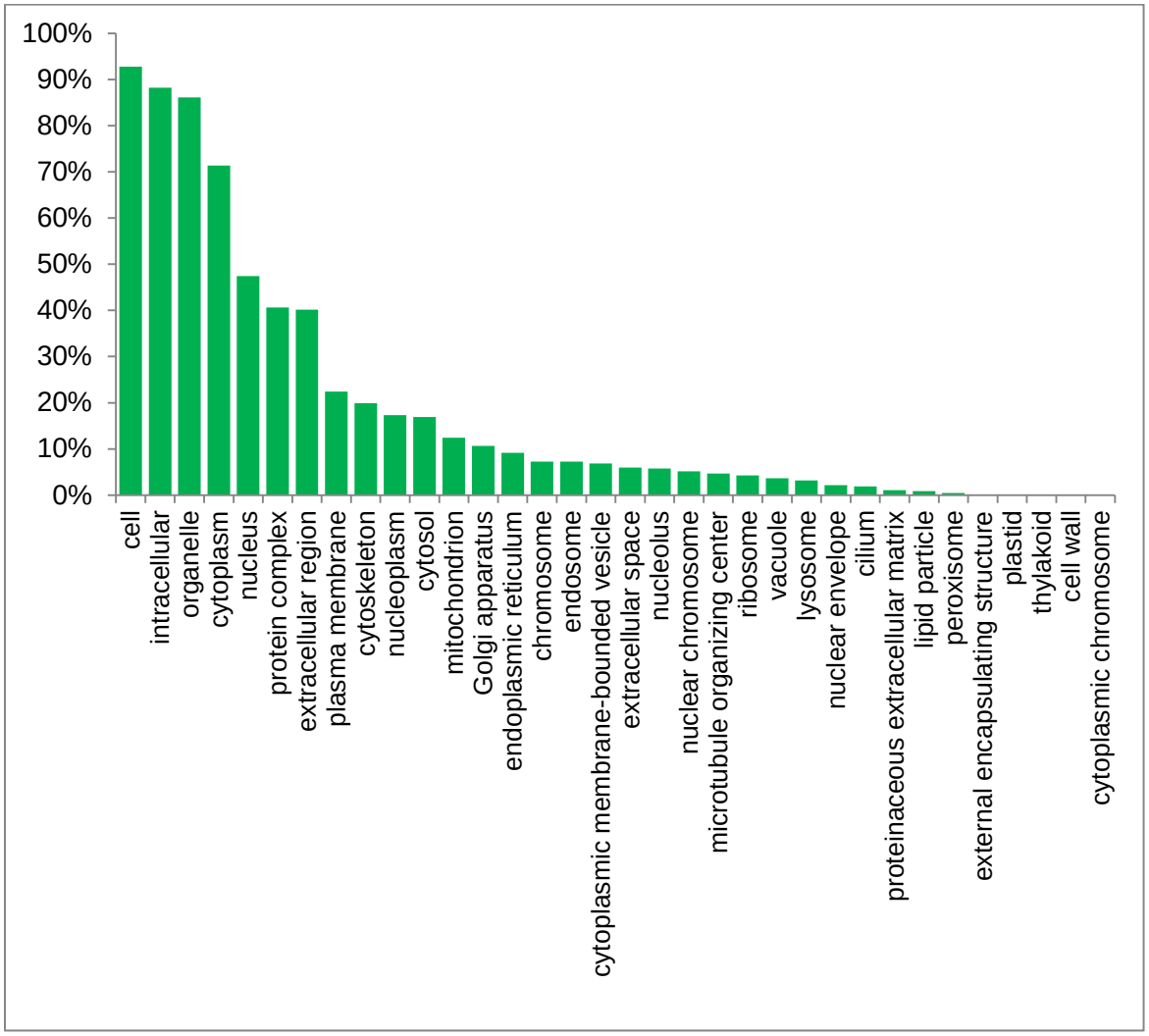

Figure 5.4. Cellular Component GO Slim term analysis of Panx1 interacting proteins.

Graphical representation of the proportion of LC-MS/MS identified Panx1 interacting proteins that are classified by each GO Slim term in the 'Cellular Component' category.

Table 1. Panx1 interacting proteins involved in proliferation, migration, differentiation.

List of Panx1 interacting proteins classified by Biological Process GO Slim terms relating to cell proliferation, migration, and differentiation. The proportion of the annotated interacting protein list (426 genes) and the annotated mouse genome (24187 genes) falling under each GO Slim term is listed.

GO Slim term (GO ID)	Gene List	Proportion of interacting proteins	Proportion of mouse genome
Proliferation			
cell cycle (GO:0007049)	Actr3, Anp32b, Arl8b, Bat1, Camk2b, Chmp5, Clasp2, Clock, Crocc, Ddb1, Dlgap5, Dst, Dync1h1, Ensa, Gnb2l1, H2afx, Hspa2, Ino80, Khdrbs1, Map4, Map4k4, Mapk1, Mapk3, Mapre1, Mcm5, Nasp, Ncapd2, Nras, Nudc, Pdcd6ip, Phb2, Phgdh, Piwil2, Poc5, Ptprk, Rab11a, Rab35, Rps15a, Rps6, Rps6ka3, Sept1, Sept2, Sept9, Smc2, Tnik, Trim21, Trim35, Tubgcp3, Usp9x, Xrn1	10.68%	5.74%
cell proliferation (GO:0008283)	Anp32b, Cckbr, Cd47, Cend1, Dhcr7, Eif5a, Enpep, Fkbp1a, Gfap, Grn, Hdac2, Hspd1, Kdm1a, Maged1, Mapk1, Mll3, Mllt4, Nasp, Nf1, Nras, Phb2, Pik3cb, Pkn1, Ptges3, Ptprk, Rps15a, Rps6, Rps9, Setdb1, Slc25a5, Slit3, Trim35, Twist2	7.05%	7.21%
cell division (GO:0051301)	Actr3, Arl8b, Camk2b, Chmp5, Clasp2, Ensa, Ino80, Map4, Mapre1, Ncapd2, Nudc, Pdcd6ip, Phb2, Pik3cb, Piwil2, Rab11a, Rab35, Sept1, Sept2, Sept9, Smc2, Usp9x	4.70%	2.45%
Migration			
cell motility (GO:0048870)	Arpc5, Cd47, Cend1, Clasp2, Dnaja1, Dock10, Dpysl3, Enpep, Gna12, Gna13, Gnb2l1, Gsn, Has1, Hist1h1t, Lama1, Lgals9, Myo9b, Nf1, Pik3cb, Pik3cd, Pkn1, Ptprk, Rab11a, Rab1A, Srgap2, Usp9x, Vcl	5.77%	4.94%
Differentiation			
cell differentiation (GO:0030154)	Actc1, Actn1, Actr3, Agrn, Bdh2, Camk2b, Cend1, Cfl2, Chsy1, Clasp2, Clock, Cnot1, Crmp1, Crtac1, Csrp2, D1Pas1, Dbnl, Ddx5, Dhcr7, **Dpysl2**, Dpysl3, Dst, Eef2, Eif2b1, Eif2c2, Eif4g1, Eif5a, Elavl1, Epas1, G6pdx, Gdi1, Gfap, Gna11, Gna12, Gna13, Gnb2l1, Golga4, Grn, H3f3a, Hdac2, Hils1, Hist1h1t, Hist1h4a, Hmgb3, Hnrnpab, Hnrnpk, Hspa1l, Hspa2, Hspe1, Kdm1a, Krt75, Krt8, Krt84, Lama1, Lgals9, Lrrk1, Lsm1, Map4, Map4k4, Mapk1, Mapk3, Mllt4, Muc4, Mypn, Naa15, Nf1, Phgdh, Pik3cd, Piwil2, Ppp1cc, Ppp2r1b, Ptprk, Rab11a, Rab1A, Rab35, Rab8a, Rap1a, Rpl22, Rps11, Rps3a, Rps6, Sept2, Slc7a5, Slit3, Snd1, Spen, Srgap2, Stmn2, Tmod1, Tnik, Tnpo2, Tpt1, Tsnax, Twist2, Uchl3, Usp9x, Vasp, Vcl, Vps33a, Vps33b, Zfhx3	21.58%	15.10%

Table 2. Cytoskeletal Panx1 interacting proteins.

List of Panx1 interacting proteins classified under the Cellular Component GO Slim term 'cytoskeleton'. The proportion of the annotated interacting protein list (426 genes) and the annotated mouse genome (24187 genes) falling under each GO Slim term is listed.

GO Slim term (GO ID)	Gene List	Proportion of interacting proteins	Proportion of mouse genome
cytoskeleton (GO:0005856)	Actc1, Actn1, Actr1a, Actr1b, Actr3, Actr3b, Alkbh8, Arl8b, Arpc5, Arpc5l, Camk2b, Camsap1l1, Capza1, Ccdc114, Cfl2, Clasp2, Clip2, Coro2b, Coro6, Crmp1, Crocc, Csrp1, Dbnl, Dis3l, Dlgap5, **Dpysl2**, Dpysl3, Dst, Dync1h1, Eppk1, Filip1, Fkbp4, G6pdx, Gfap, Gnb2l1, Gsn, H1f0, Ino80, Krt15, Krt25, Krt7, Krt73, Krt75, Krt8, Krt84, Lasp1, Lrrc49, Map1lc3b, Map4, Mapk1, Mapk3, Mapre1, Marcks, Mical3, Mms19, Mtpn, Myh7, Myl1, Myl6, Myo1b, Myo5b, Myo9b, Nudc, Nudt21, Pdcd6ip, Plec, Poc5, Ptges3, Ptpn12, Pxk, Rab11a, Rab8a, Rbm39, Sept1, Sept2, Sept9, Sfi1, Slc16a1, Slc16a3, Slc25a5, Tchp, Tmod1, Tnik, Tpt1, Tuba1a, Tuba1c, Tuba3a, Tubgcp3, Twf1, Ubr4, Vasp, Vcl, Xrn1	19.87%	7.46%

5.2.2. Panx1 and Crmp2 are co-expressed in VZ NPCs and cortical neurons

From the putative list of Panx1 interaction partners, I concentrated on protein hits that had a previously defined role in NPC behaviours and neuronal development. I chose to focus specifically on Crmp2 (*Dpysl2*), which is a well-known modulator of neurite development (reviewed in Ip et al., 2014). This Panx1 interaction partner was classified by GO Slim terms 'Cell Differentiation' and 'Cytoskeleton' (See Tables 1&2). Crmp2 was identified in all three immunoprecipitation/LC-MS/MS replicates. Moreover, Crmp1 and Crmp3 (*Dpysl3*), other Crmp family members that can form heterotetramers with Crmp2 (Wang and Strittmatter, 1997), were also identified.

I first looked to see whether Panx1 and Crmp2 were expressed within the VZ *in vivo*. Western blots of VZ tissue from P0, P7, P10, P28, and P60 mice showed Panx1 and Crmp2 expression at all time points, with Panx1 expression significantly decreasing after P10 (Figure 5.5A). Crmp2 expression was not significantly altered over this time course. Immunostaining for Panx1 and Crmp2 revealed co-expression and partial co-distribution of the two proteins in primary post-natal VZ NPCs (Figure 5.5B) and in DIV4 post-natal cortical neurons (Figure 5.5C).

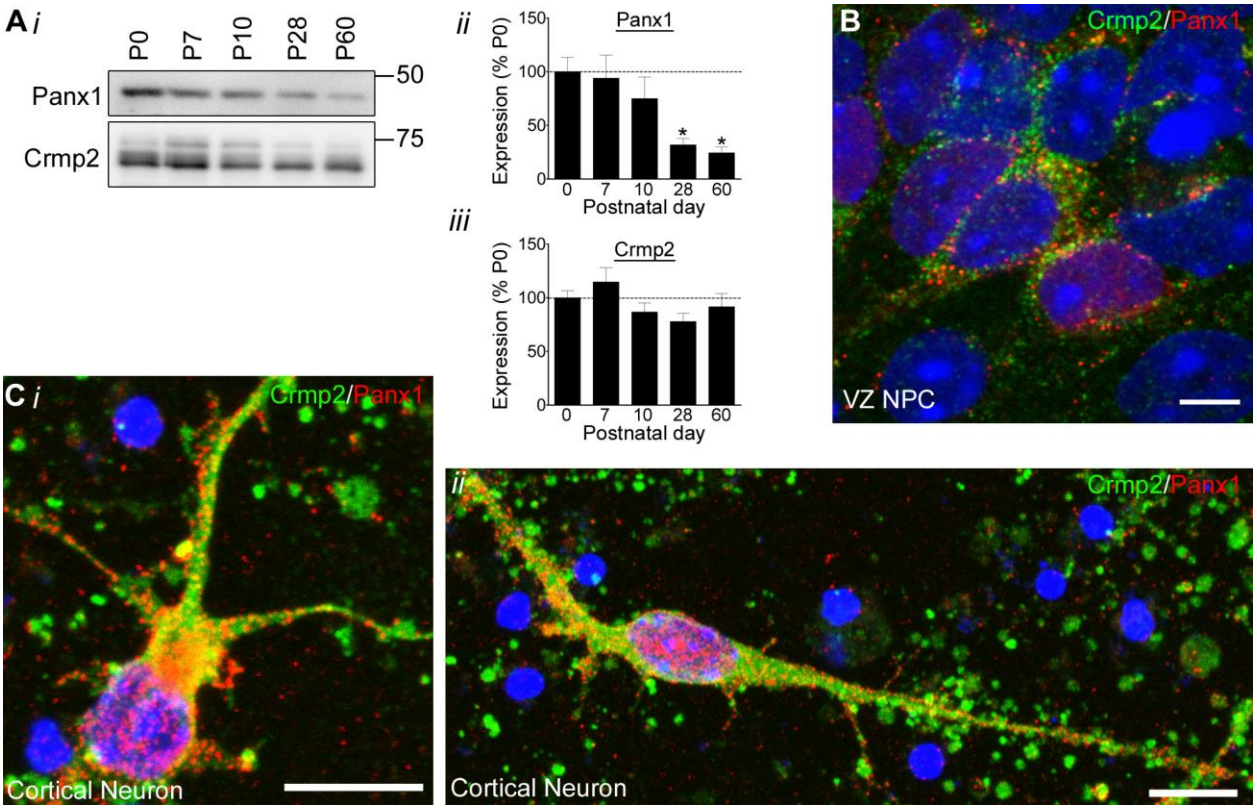

Figure 5.5. Panx1 and Crmp2 are co-expressed in NPCs.

(A) Western blot of microdissected VZ lysates from P0-P60 mice, probed for Panx1 and Crmp2 (i). Quantification of Panx1 (ii) and Crmp2 (iii) signal normalized to P0 demonstrates a significant decrease in Panx1 expression within the VZ by P28 (N = 3; $p = 0.0134$ by one way ANOVA; Dunnett's multiple comparison test post-hoc P28 and P60 $p < 0.05$ (*)), but no change in Crmp2 levels. (B) Confocal image of primary VZ NPCs in adherent culture, immunostained for Panx1 and Crmp2. (C) (i and ii) Maximum intensity projections of confocal z-stacks from DIV4 cortical neurons immunostained for Panx1 and Crmp2. Hoechst 33342 was used as a nuclear counterstain in all images. Scalebars are 10 µm.

5.2.3. Panx1 interacts with Crmp2 in NPCs

I next performed *in vitro* protein interaction assays to confirm my mass spectrometry results. Panx1 immunoprecipitation from N2a cell lysates demonstrated co-precipitation of Crmp2 compared to control IgG immunoprecipitation (Figure 5.6A). Similarly, Crmp2

immunoprecipitation from N2a cell lysates demonstrated co-precipitation of Panx1 (Figure 5.6B). Panx1 immunoprecipitations from P0/P10 VZ tissue (Figure 5.6C) and P60 VZ tissue (Figure 5.6D) also showed co-precipitation of Crmp2.

To examine whether the Panx1-Crmp2 interaction was direct, or required accessory proteins, I performed *in vitro* binding assays using purified proteins. Because the Panx1 C-terminus (Panx1CT) is the largest intracellular region, I first purified this fragment. Panx1CT was enriched when incubated with purified Crmp2-GST compared to GST control (Figure 5.6E). Similarly, Crmp2 was enriched when incubated with Panx1CT-GST compared to GST control (Figure 5.6F). Finally, I developed Panx1CT fragment peptides to further narrow down the interaction region (Figure 5.7A). An *in vitro* binding assay using the different Panx1CT fragment peptides demonstrated enrichment of the most distal portion of the Panx1CT (CT3; aa 379 – 426) with Crmp2-GST compared to GST control (Figure 5.7B). Taken together, these data confirm that Panx1 and Crmp2 interact in NPCs, and that this interaction occurs directly through the distal Panx1CT.

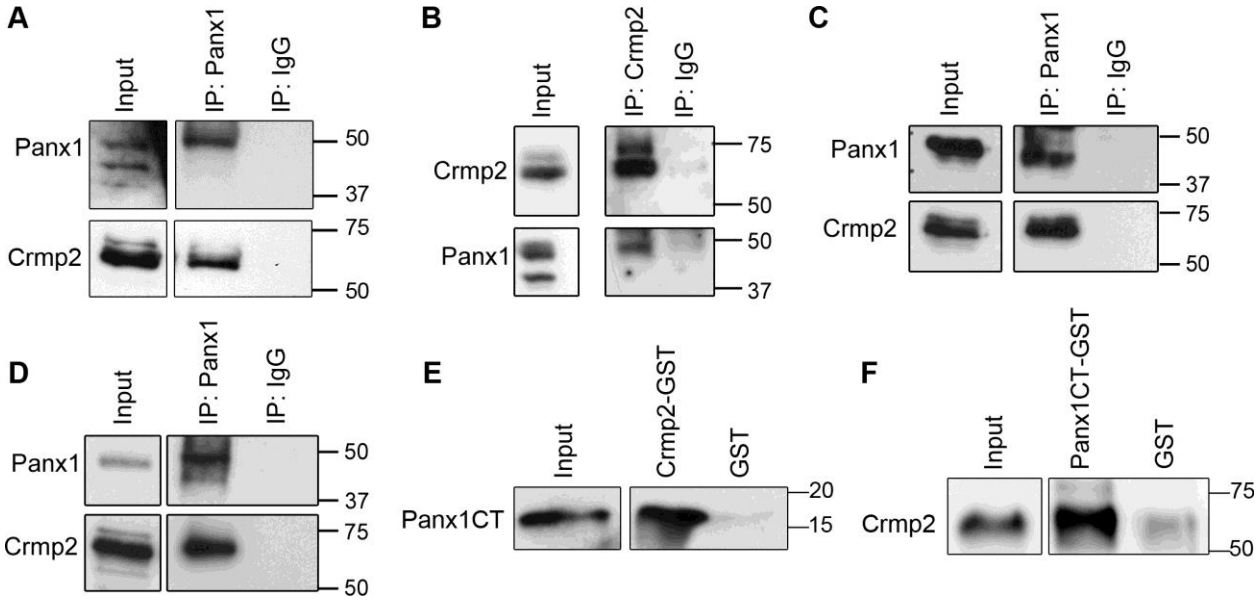

Figure 5.6. Panx1 and Crmp2 interact in NPCs via the Panx1 C-terminus.

(A) Panx1 immunoprecipitation from N2a cells demonstrates enriched co-precipitation of Crmp2 compared to IgG control. (B) Crmp2 immunoprecipitation from N2a cells shows enriched co-precipitation of Panx1 compared to IgG control. Panx1 immunoprecipitations from (C) P0/P10 VZ tissue and (D) P60 VZ tissue show enriched co-precipitation of Crmp2 compared to IgG controls. (E) *In vitro* binding assay using purified proteins demonstrates enrichment of Panx1 C-terminus (Panx1CT) with Crmp2-GST compared to GST control. (F) *In vitro* binding assay demonstrates enrichment of Crmp2 with Panx1CT-GST compared to GST control.

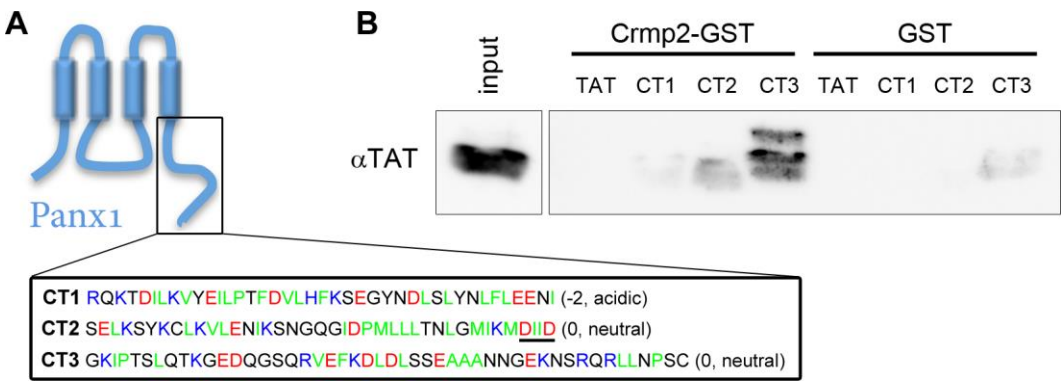

Figure 5.7. Crmp2 interacts with the distal portion of the Panx1CT.
(A) Schematic of Panx1 CT fragment peptides. Acidic amino acids are in red, basic amino acids are in blue, hydrophobic uncharged amino acids are in green, and all other amino acids are in black. A caspase cleavage site is underlined in the CT2 sequence. The overall charge state of each peptide is indicated in the brackets. (B) An *in vitro* binding assay between purified Crmp2 and Panx1CT fragment peptides demonstrates enrichment of the distal portion of Panx1CT (CT3) with Crmp2 compared to GST control (N = 3).

5.2.4. *Blocking the Panx1/Crmp2 interaction enhanced neurite outgrowth* in vitro

I next used the CT3 interacting peptide in a blocking strategy to interfere with the Panx1-Crmp2 interaction in NPCs. I treated N2a cells with CT3 (10 µM) and examined the effect on proliferation, migration, and neurite outgrowth. CT3 treatment significantly increased neurite length and branch points in the absence of any other differentiation stimuli (Figure 5.8A). Preliminary experiments showed this CT3 effect was specific, as no alterations to neurite length or branch points occurred upon treatment with CT1 or CT2 (data not shown). CT3 treatment did not impact N2a cell proliferation (Figure 5.8B), but slightly impaired wound closure in a scratch wound assay (Figure 5.8C).

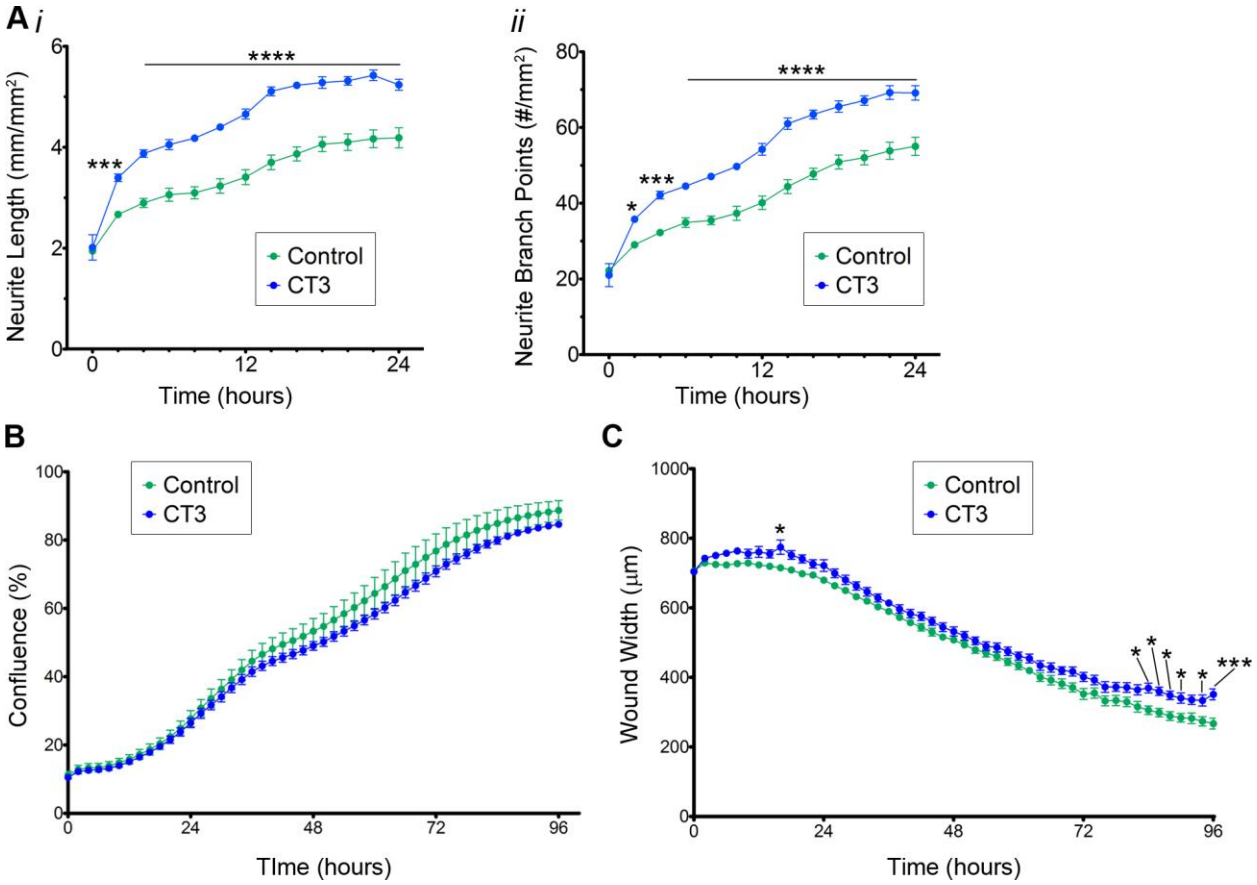

Figure 5.8. Treatment of N2a cells with the CT3 peptide dramatically increases neurite outgrowth. (A) N2a cells treated with CT3 peptide (10 µM) in the absence of other differentiating stimuli developed significantly longer neurites (i; N = 12, Peptide: F = 59.84, p < 0.0001; Time: F = 204.4, p < 0.0001; Interaction: F = 8.953, p < 0.0001; Subjects (matching): F = 18.09, p < 0.0001 by two factor ANOVA; Bonferroni posthoc p < 0.001 (***), p < 0.0001 (****)) with more branch points (ii; N = 12, Peptide: F = 52.82, p < 0.0001; Time: F = 251.0, p < 0.0001; Interaction: F = 9.451, p < 0.0001; Subjects (matching): F = 13.94, p < 0.0001 by two factor ANOVA; Bonferroni posthoc p < 0.05 (*), p < 0.001 (***), p < 0.0001 (****)) than control TAT treated cells. These data are representative of three independent biological replicates. (B) CT3 treatment did not alter N2a cell proliferation (N = 3, Peptide: F = 1.065, p = 0.3604; Time: F = 1421, p < 0.0001; Interaction: F = 2.266, p < 0.0001; Subjects (matching): F = 322.3, p < 0.0001 by two factor ANOVA). (C) Wound closure was slightly impaired with CT3 treatment in a scratch wound assay (N = 12, Peptide: F = 6.951, p = 0.0158; Time: F = 975.4, p < 0.0001; Interaction: F = 2.125, p < 0.0001; Subjects (matching): F = 92.97, p < 0.0001 by two factor ANOVA; Bonferroni posthoc p < 0.05 (*), p < 0.001 (***)).

5.3.5. The Panx1-Crmp2 interaction does not affect Panx1 localization or ATP release

Since Crmp2 has previously been shown to regulate the trafficking of plasma membrane proteins (Arimura et al., 2009; Brittain et al., 2011), I also examined the impact of this novel Panx1-Crmp2 interaction on Panx1 channel localization in NPCs. I used a cell-surface lumino-immunodetection assay to detect the relative proportions of cell surface and intracellular Panx1EGFP in a stably-expressing N2a cell line. Treatment of N2a cells stably expressing Panx1EGFP with CT3 (10 μM) peptide did not alter the proportion of cell-surface Panx1 (Figure 5.9A). Overexpression of Crmp2 also had no effect on Panx1 surface expression (Figure 5.9B).

Panx1 regulates ATP release from N2a cells (Wicki-Stordeur et al., 2012). I therefore investigated whether the Panx1-Crmp2 interaction affected ATP release from N2a cells. Neither CT3 treatment (Figure 5.9C), nor Crmp2 overexpression (Figure 5.9D), significantly altered the amount of ATP released from N2a cells. Together these data suggest that the Panx1-Crmp2 interaction does not significantly influence Panx1 localization or ATP release-functions.

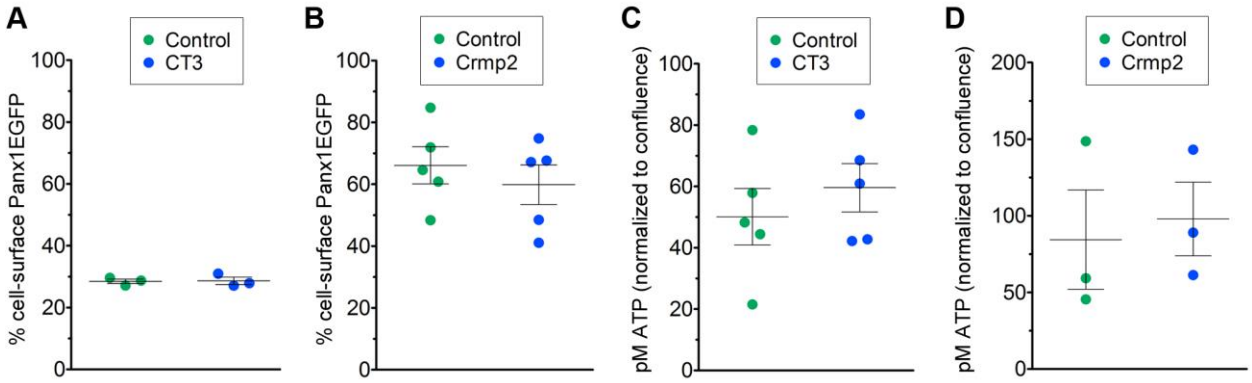

Figure 5.9. The Panx1-Crmp2 interaction does not significantly influence Panx1 localization or ATP release.

(A) Panx1 surface localization was examined by cell-surface lumino-immunodetection assays using N2a cells stably overexpressing Panx1EGFP. The proportion of Panx1EGFP found at the plasma membrane in cells treated with CT3 or TAT control peptides (10 µM) was approximately equal ($p = 0.9118$ by Student's t-test; $N = 3$). (B) The proportion of Panx1EGFP found at the plasma membrane did not change with overexpression of Crmp2 in N2a cells ($p = 0.4974$ by Student's t-test; $N = 3$). (C) Panx1 channels regulate ATP release from N2a cells. The concentration of ATP in the extracellular media did not significantly differ between cells pre-treated with CT3 and TAT control peptides ($p = 0.4569$ by Student's t-test; $N = 5$). (D) Overexpression of Crmp2 also did not alter the extracellular ATP concentration ($p = 0.7556$ by Student's t-test; $N = 3$).

5.3. Discussion

Here I expand on my data presented in Chapter 3 and 4 demonstrating that Panx1 influenced NPC behaviours *in vitro* (Wicki-Stordeur et al., 2012; Wicki-Stordeur and Swayne, 2013). Panx1 promoted NPC proliferation and migration, and negatively regulated neurite outgrowth. Moreover, Panx1-dependent ATP release and activation of downstream purinergic receptor signalling cascades was necessary, at least in part, for the Panx1-dependent effect on NPC proliferation (Wicki-Stordeur et al., 2012). In the present chapter I investigated additional

signalling pathways and protein networks that could underlie the role of Panx1 in NPCs, by performing the first unbiased proteomics screen for Panx1 protein interaction partners. I identified several novel Panx1 interacting proteins, and grouped these using GO term analysis. Many of these interacting proteins were classified by terms associated with proliferation ('Cell Cycle', 'Cell Proliferation', and 'Cell Division), migration ('Cell Motility'), and/or differentiation ('Cell Differentiation').

An unexpectedly large number of Panx1 interacting proteins were also classified under the GO Slim term 'Cytoskeleton' (20% of interacting proteins), which built on previous studies linking Panx1 to the actin cytoskeleton (Bhalla-Gehi et al., 2010; Wicki-Stordeur and Swayne, 2013). Crmp2 (*Dpysl2*) was identified under both 'Cytoskeleton' and 'Cell Differentiation'. Crmp2 is a cytoplasmic phospho-protein that binds to and stabilizes tubulin heterodimers, promoting plus-end microtubule growth (Gu and Ihara, 2000; Fukata et al., 2002; Lin et al., 2011). In fact, Crmp2 was first identified as a critical effector in semaphorin-3a signalling (Goshima et al., 1995), which acts as a repulsive cue for growth cone guidance (reviewed in Roth et al., 2009). Recent studies demonstrated Crmp2 involvement in several other cellular behaviours associated with microtubule dynamics, including polarity, migration, neurite outgrowth and retraction, and axonal transport (reviewed in Hensley et al., 2011; Khanna et al., 2012; Ip et al., 2014). Crmp2 levels increased with neuronal maturation (Minturn et al., 1995b; Minturn et al., 1995a; Gaetano et al., 1997; Byk et al., 1998), and in NPCs it promoted aspects of morphological differentiation, such as neurite formation, specification, and elongation (reviewed in Hensley et al., 2011; Khanna et al., 2012;Ip et al., 2014). Importantly, early studies demonstrated that Crmp2 expression was highly restricted to the nervous system (Goshima et al., 1995; reviewed in Charrier et al., 2003); therefore any effect of a Panx1-Crmp2 interaction would be largely limited

to this tissue. Interestingly, I also identified Crmp1 and Crmp3 as Panx1 protein interaction partners. These other Crmp family members likely form heterotetrameric complexes with Crmp2 (Wang and Strittmatter, 1997). Taken together, this led me to focus on the putative Panx1-Crmp2 interaction for further validation and functional analyses in NPC cultures.

Immunoprecipitations demonstrated that Panx1 and Crmp2 co-precipitated from both neonatal and adult mouse VZ, as well as in N2a cells. This is the first evidence linking Panx1 to the microtubule cytoskeleton. Microtubules serve as transport tracks, position organelles, produce cellular forces, and act as signalling cues within cells (reviewed in Kapitein and Hoogenraad, 2015). Together with the actin cytoskeleton, microtubules are crucial to proper neuronal development, and contribute to NPC proliferation, migration, and differentiation (reviewed in Geraldo and Gordon-Weeks, 2009; Hoogenraad and Akhmanova, 2010; Firat-Karalar and Welch, 2011; Kapitein and Hoogenraad, 2015). Interestingly, blocking the Panx1-Crmp2 interaction strongly increased neurite outgrowth and branch development from N2a cells, yet had little to no effect on cell proliferation or migration. Neurite outgrowth generally requires stabilization of bundled microtubules within the lengthening neurite shaft, and a region of dynamic instability in the growth cone (reviewed in Kapitein and Hoogenraad, 2015). Moreover, transport of organelles and other cellular components along microtubules to the tip of the neurite was proposed to drive process elongation. Recent studies demonstrated that Crmp2 function stabilized microtubules (Gu and Ihara, 2000; Fukata et al., 2002; Lin et al., 2011) and regulated axonal transport (Arimura et al., 2009). The increase in neurite outgrowth observed here upon blocking the Panx1-Crmp2 interaction could therefore be explained by an increase in functional Crmp2 levels. This suggests that Panx1 negatively regulated Crmp2 through their physical association.

This hypothesis is supported by the expression profiles of Panx1 and functional Crmp2 across post-natal neurogenesis (Figure 5.10). Panx1 is highly expressed in proliferative nestin-positive/GFAP-positive and nestin-positive/GFAP-negative (type B and C) NPCs within the VZ (Wicki-Stordeur et al., 2012), and as demonstrated in Chapter 3, its levels drop dramatically following induction of neuronal differentiation (Wicki-Stordeur and Swayne, 2013). Crmp2 is highly regulated by phosphorylation (reviewed in Yamashita and Goshima, 2012). Un-phosphorylated Crmp2 binds and stabilize microtubules, whereas phosphorylation by Rho kinase and/or Gsk-3β/Cdk5 forces its dissociation, causing microtubule de-stabilization and catastrophe (Arimura et al., 2000; Brown et al., 2004; Arimura et al., 2005; Uchida et al., 2005). Crmp2 function was previously shown to increase across neurogenesis (Minturn et al., 1995b; Minturn et al., 1995a; Gaetano et al., 1997; Byk et al., 1998), within the same time period that Panx1 down-regulation occurs. Together, this suggests that in undifferentiated NPCs, high levels of Panx1 inhibit Crmp2 function, maintaining NPCs in a proliferative state. However, following induction of neuronal differentiation, significant reductions in Panx1 expression allow release of functional Crmp2 and promote neurite outgrowth. Whether Crmp2 phosphorylation status is important for its interaction with Panx1 is still unclear, and represents an area for future study.

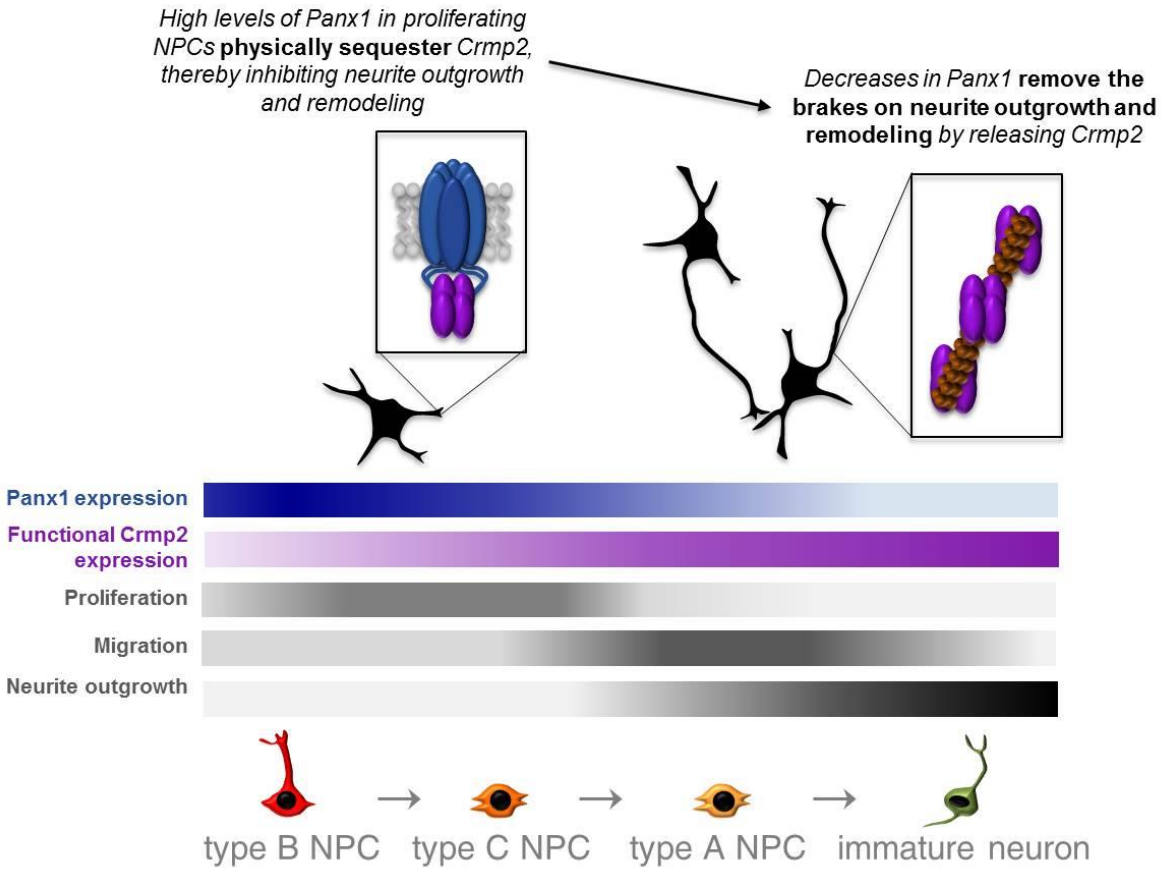

Figure 5.10. Working model: Panx1 negatively regulates Crmp2 function through their physical interaction.

High levels of Panx1 are found in proliferating type B and C NPCs in the post-natal VZ. Decreased Panx1 expression and increased Crmp2 function accompany NPC neuronal specification and neurite outgrowth. Together this suggests that high levels of Panx1 physically sequester and inhibit Crmp2, negatively regulating neurite outgrowth and maintaining NPCs in a proliferative state. However, a decrease in Panx1 expression upon induction of neuronal differentiation releases functional Crmp2, allowing neurite outgrowth to proceed.

It is important to note that Crmp2 is also able to bind actin filaments (Arimura et al., 2005) seemingly independent of its phosphorylation state. Panx1 stability at the plasma membrane was previously shown to be dependent on interactions with the actin cytoskeleton, and independent of

microtubules in a fibroblast-like cell line (Bhalla-Gehi et al., 2010). Since my current results demonstrated the Panx1-Crmp2 interaction did not alter overall Panx1 surface expression or ATP release function, it seems unlikely that this association influences Panx1-actin cytoskeleton dynamics. However, it is possible that Panx1 regulates both Crmp2-microtubule and Crmp2-actin interactions. Actin cytoskeleton remodelling is a major component of dendrite formation in developing neurons, while stabilization of the microtubule cytoskeleton is more heavily involved in axon outgrowth (reviewed in Conde and Caceres, 2009; Kapitein and Hoogenraad, 2015; Takano et al., 2015). It would therefore be interesting to examine whether Panx1 influences the Crmp2-microtubule association in axons, and the Crmp2-actin filament interaction in dendrites.

What other interacting proteins might be involved in Panx1's role in NPCs? While here I chose to focus on Crmp2, the proteomics screen uncovered 479 other potential interacting proteins, some of which have defined roles in neuronal development. For example, another microtubule binding protein called Clasp2 was identified, and is known to influence neuronal process formation, polarity and synaptic function (Beffert et al., 2012). Srgap2, a Rho-GTPase activating protein, is involved in neuronal migration, differentiation (Guerrier et al., 2009), and synaptic development (Charrier et al., 2012). Finally, Phgdh, a critical enzyme in the serine biosynthesis pathway, is necessary for proper brain morphogenesis (Yoshida et al., 2004), and is upregulated in post-natal VZ type B and C NPCs (Kinoshita et al., 2009). Interestingly, a recent publication implicated Panx1 as the d-serine release channel in astrocytes (Pan et al., 2015). Perhaps in this case Panx1 acts both as a channel and a scaffold, confining the d-serine metabolic machinery close to the site of release. The validation and functional significance of these (and other) putative interacting proteins will be the focus of future work in the lab.

The current results contribute a large body of data to the existing Panx1interactome literature, and provide the groundwork for future studies examining the molecular function of Panx1 in NPCs and other cell types. Moreover, these results uncovered novel evidence reinforcing the established link between Panx1 and the cytoskeleton, and suggest that the relationship between Panx1 and Crmp2 underlies, at least in part, the regulation of NPC neurite outgrowth by Panx1. Taken together, these data contribute to our understanding of the fundamental role of Panx1 in NPCs, and the signalling networks through which it acts.

6. General Discussion

The studies conducted in this dissertation investigated the role of the Panx1 ion and metabolite channel in NPC behaviours associated with neuronal development. In this chapter I will discuss the contributions of my present findings to the current understanding of Panx1, and the molecular determinants underlying its cellular role. I will also discuss potential translational implications of my data, and will mention potential future directions that could expand on these results.

6.1. Panx1-mediated ATP release regulates cell behaviours

Panx1 is expressed in multiple cell and tissue types, where it has often been found to function as an ATP release channel (reviewed in Lohman and Isakson, 2014). My *in vitro* results (Chapter 3) indicated that Panx1 also acts as an ATP release conduit in NPCs (Wicki-Stordeur et al., 2012), initiating downstream purinergic receptor signalling in an autocrine and paracrine fashion. I demonstrated that this Panx1 activity positively regulates proliferation of NPCs. Panx1 was previously found to enhance fibroblast proliferation through a similar ATP release and purinergic signalling mechanism (Pinheiro et al., 2013); however, a number of other studies demonstrated it acts as a negative regulator of proliferation (Lai et al., 2007; Penuela et al., 2014), although many did not directly address the involvement of ATP. Similar contrasting studies exist with respect to the importance of Panx1 in cell migration (Penuela et al., 2012; Wicki-Stordeur and Swayne, 2013; Penuela et al., 2014) and differentiation (Celetti et al., 2010; Wicki-Stordeur and Swayne, 2013; Langlois et al., 2014), depending on the cell type examined. This is likely due, in part, to

differences in the cohort of purinergic receptors present within specific cell types (reviewed in Burnstock et al., 2010), so that the nucleotide release function of Panx1 can have varying downstream effects on cellular development.

It should also be noted that Panx1 is also found in tissues in which ATP release and purinergic signalling does not elicit these developmental responses. Within the brain, Panx1 was originally detected in post-mitotic, mature neurons (Ray et al., 2005; Vogt et al., 2005). Many studies have linked its activity in these cells to neuronal death and dysfunction (reviewed in Dahl, 2012; Weilinger et al., 2013); however, additional physiological roles are emerging. Panx1 channels are located at post-synaptic sites on hippocampal and cortical neurons (Zoidl et al., 2007). Given that ATP can act as an excitatory neurotransmitter (reviewed in Cisneros-Mejorado et al., 2015a) , it is not surprising that further study revealed a role for Panx1 in maintaining synaptic strength in this system, via release of ATP (Prochnow et al., 2012).

What about crosstalk with Cxs? Cx hemichannels were originally attributed as the ATP release conduits of cells. Interestingly, the identified functions of Cx43 in NPCs seem to be similar to those reported here for Panx1. For example, Cx43 promotes proliferation in embryonic (Weissman et al., 2004) and cultured NPCs (Duval et al., 2002; Cheng et al., 2004; Malmersjo et al., 2013), mediates migration within the developing cortex (Fushiki et al., 2003; Elias et al., 2007; Wiencken-Barger et al., 2007; Cina et al., 2009; Elias et al., 2010), and shows reduced expression corresponding with neuronal differentiation (Rozental et al., 1998; Duval et al., 2002; Boucher and Bennett, 2003). However, these functions may be altered in the mature brain, as Cx43 expression was found to be inversely correlated with levels of DNA synthesis in the post-natal VZ (Miragall et al., 1997). It would be interesting to examine the crosstalk between Panx1 and Cx43 in both embryonic and post-natal NPCs, to determine if they perhaps function during

separate periods of time, are expressed in distinct NPC sub-populations, or are responsive to different stimuli.

6.2. Panx1 and the cytoskeleton

There is growing evidence of a functional association between Panx1 and the cytoskeleton (reviewed in Boyce et al., 2014). This is perhaps unsurprising, due to the mechnosensitive nature of the channel (Bao et al., 2004; Locovei et al., 2006b; Seminario-Vidal et al., 2011; Richter et al., 2014). Early work demonstrated a role for the microfilament cytoskeleton in cell-surface trafficking and stabilization of Panx1 (Bhalla-Gehi et al., 2010). Ectopically expressed Panx1 was subsequently found to associate with actin, in a mechanism involving direct interaction between actin and the Panx1CT. Recent work from our group confirmed this interaction between endogenous proteins in N2a cells and uncovered a novel interaction with the actin-associated protein Arp3 (Wicki-Stordeur and Swayne, 2013). My proteomics screen for Panx1 interacting partners (Chapter 5) provided further evidence for a cytoskeletal association. A large proportion of the identified interacting proteins were classified as cytoskeletal proteins (20% of interacting proteins) when analyzed by GO analysis. While most of these have yet to be validated, such a large proportion of cytoskeletal interacting proteins implies a significant role for the cytoskeleton in Panx1 trafficking, stability, and/or function.

I validated an interaction between Panx1 and Crmp2, one of the identified cytoskeletal interacting proteins. Interestingly, a recent study examining the Crmp2 interactome in frontal cortical tissue did not identify Panx1 (Martins-de-Souza et al., 2015), perhaps indicating that this interaction is NPC-specific. Crmp2 represents only the second protein interaction partner to

demonstrate a direct interaction with Panx1 (actin is the other). This was also the first indication of a link between Panx1 and the microtubule cytoskeleton, as previous work found that microtubules were not important for Panx1 trafficking or stabilization at the membrane (Bhalla-Gehi et al., 2010). My current experiments demonstrated that the Panx1-Crmp2 interaction did not impact Panx1 plasma membrane localization, supporting these previous data. Instead, my results suggested that perhaps Panx1 negatively regulates microtubule extension, by physically sequestering Crmp2 and inhibiting its function. Future experiments will be required to examine microtubule dynamics following alterations to Panx1 expression or activity, and in the presence of the CT3 blocking peptide to inhibit the Panx1-Crmp2 interaction. These experiments are currently underway in the lab.

6.3. Implications for developmental neurogenesis

The studies presented in this dissertation concentrated on post-natal neurogenesis, but also have implications for developmental neurogenesis. Embryonic neuronal development proceeds through similar cell types and behaviours, sharing many of the signalling pathways with post-natal neurogenesis (reviewed in Temple, 2001; Erzurumlu and Gaspar, 2012; Martynoga et al., 2012). Panx1 is highly expressed in the embryonic and early post-natal brain (Ray et al., 2005; Vogt et al., 2005), a time period of intense neuron birth and migration (Figure 6.1; reviewed in Temple, 2001; Erzurumlu and Gaspar, 2012; Martynoga et al., 2012). My current data, and that of others (Ray et al., 2005; Vogt et al., 2005), have shown that Panx1levels dramatically decline after P14, a critical period of dendritic and synaptic development (reviewed in Heng et al., 2010). Given that my results from Chapter 3 indicate that Panx1 positively regulates NPC proliferation

and migration, and inhibits process formation *in vitro*, it is possible that this developmental decrease in Panx1 expression contributes to the cellular activity within this critical period.

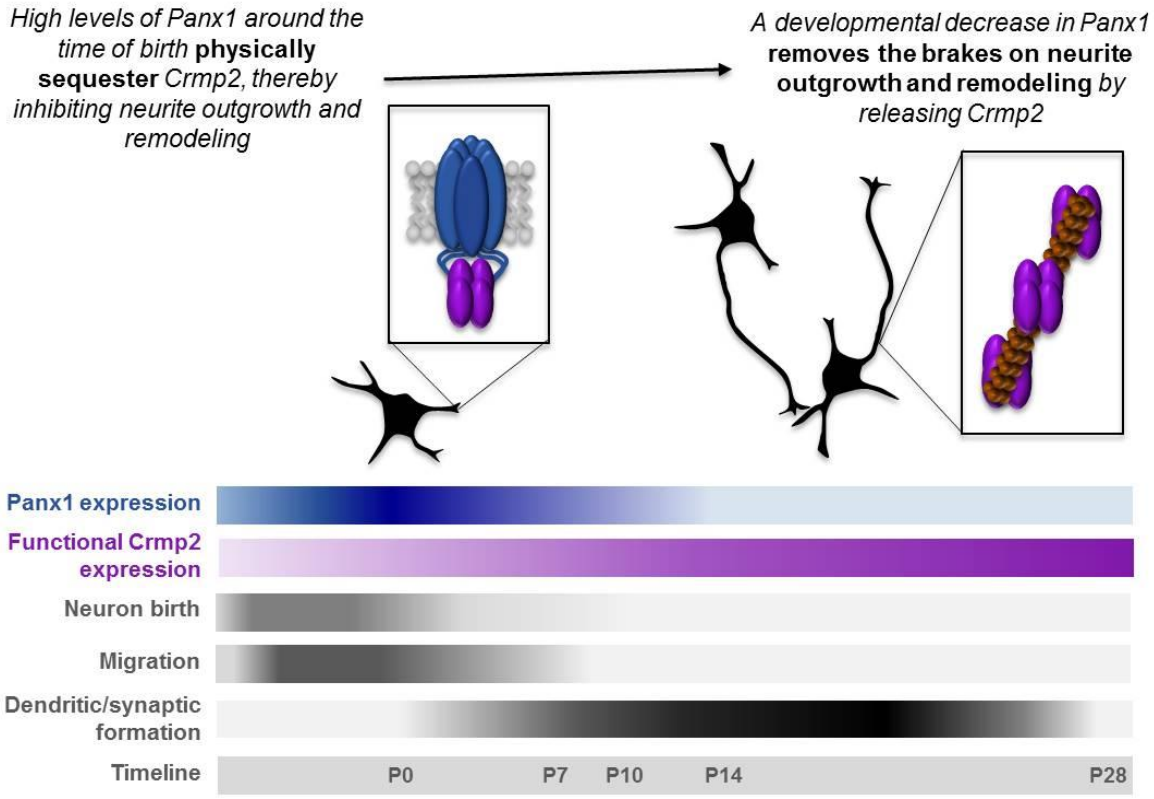

Figure 6.1. Decreases in Panx1 expression within the developing brain could help direct NPC behaviours.

Panx1 is highly expressed in embryonic and early post-natal brain, a time corresponding to intense periods of NPC birth and migration. Panx1 expression significantly declines weeks after birth, corresponding to initiation of a critical period of dendritic and synaptic formation in newborn neurons. Crmp2 function increases within this same time period. It is possible that these co-ordinated alterations to Panx1 and Crmp2 activity help direct neuronal development within the cortex.

This is supported by preliminary results from our lab using young mice treated with the Panx1 blocker probenecid . Early analyses indicate abnormal dendritic spine development on layer V

pyramidal neurons in the cortices of probenecid treated mice (data not shown); however further experiments using both Panx1 block and Panx1 knock-out mice (Figure 6.2) are required to confirm these effects. These studies are currently being undertaken by other students within our lab. Moreover, since Panx1 seems to regulate neurite extension *in vitro* through its interaction with Crmp2, future work will use the CT3 blocking peptide in a similar treatment strategy on developing mouse pups, and examine the impact on dendrite and spine morphology in the young brain.

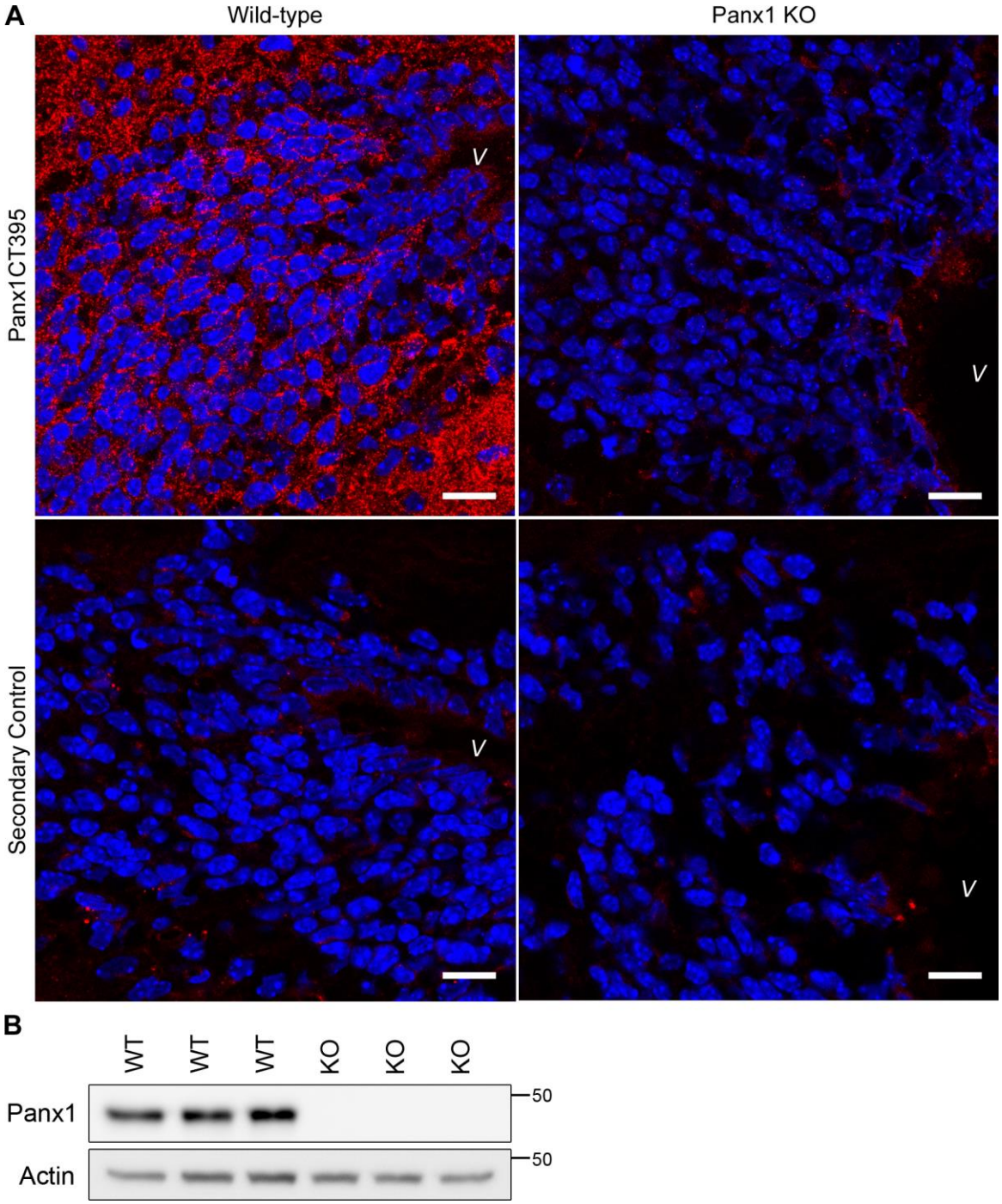

Figure 6.2. Panx1 knock-out mice represent tools for future studies.

(A) Confocal images of P10 wild-type and Panx1 knock-out (KO; Panx1-LoxP/CMV-Cre-recombinase) littermate coronal sections in the ventricular region. Sections were immunostained for Panx1 or using secondary control procedures. Slight background can be seen in Panx1 KO and secondary control brains,

with true Panx1 signal in the wild-type. Hoechst 33342 was used as a nuclear counterstain. Scalebars are 10 µm. (B) Western blot of lysates from P10 wild-type (WT) and Panx1 KO littermate brains, probed for Panx1 and actin loading control. No bands are present in the KO lanes when probed for Panx1.

Abnormalities in neuronal development during these critical early periods can lead to developmental disorders such as autism spectrum disorders (ASD) and schizophrenia (reviewed in Berger-Sweeney and Hohmann, 1997; Pang et al., 2008). Interestingly, a recent genome wide association study revealed a SNP within the Panx1 gene coding region that is connected to ASD (Davis et al., 2012). Perhaps this SNP alters Panx1 expression or function in NPCs, and thereby contributes to the phenotypical abnormalities that classify ASD brains, including brain overgrowth and areas of cortical irregularity (Stoner et al., 2014; Zielinski et al., 2014; Wallace et al., 2015). Studies are currently underway in the lab to elucidate the effect of this SNP on Panx1 function, and NPC behaviours.

Schizophrenia is another brain disorder with links to abnormal dendrite and dendritic spine development (reviewed in Moyer et al., 2015). Although no evidence exists linking Panx1 to this disorder, two SNPs within the Crmp2 gene (*Dpysl2*) were associated with susceptibility to schizophrenia (Lee et al., 2015). A second study found another set of SNPs located within regulatory regions of this gene that were also linked to schizophrenia, and seemed to function by down-regulating Crmp2 expression (Liu et al., 2014). It would be interesting to examine whether these Crmp2 SNPs differentially affect NPC behaviours, and whether Panx1 might also be involved.

6.4. Injury-induced NPC responses

NPCs are activated by pathological stimuli from brain injury or disease (reviewed in Lazarov and Marr, 2010; Lazarov et al., 2010), such as stroke. Stroke-activated NPCs hyperproliferate and exhibit re-directed migration towards the site of injury (reviewed in Ohab and Carmichael, 2008). Panx1 channels are also sensitive to pathological stimuli, opening under conditions associated with stroke such as oxygen-glucose deprivation (Thompson et al., 2006; Weilinger et al., 2012), increased extracellular K^+ (Silverman et al., 2009), and caspase cleavage during apoptosis (Sandilos et al., 2012). Stroke may also increase Panx1 expression in the surrounding tissue (Jiang et al., 2012); however the existing evidence is based on transcript analysis which does not necessarily translate to protein levels. My work presented here (Chapter 4) examining the targeted deletion of Panx1 in VZ NPCs, demonstrated that Panx1 is required to maintain stroke-induced increases in VZ NPC numbers. Perhaps stroke enhanced Panx1 activity or expression in VZ NPCs, and this up-regulated NPC proliferation. Although the exact mechanism is unknown, stroke-elevated NPC activation positively contributes to stroke outcomes (Raber et al., 2004; Jin et al., 2010; Wang et al., 2012). Future experiments should therefore examine the functional and behavioural correlates associated with Panx1 deletion in NPCs following stroke.

Emerging evidence suggests that stroke re-activates developmental pathways within the brain (reviewed in Murphy and Corbett, 2009). These include re-initiation of axon sprouting and dendrite remodeling (reviewed in Carmichael, 2008), in addition to the increases in NPC proliferation and migration (reviewed in Ohab and Carmichael, 2008), and perhaps the potential elevation of Panx1 levels (Jiang et al., 2012). Interestingly, Crmp2 is also altered by stroke, exhibiting hypophosphorylation following ischemia (Zhou et al., 2008). This activation of Crmp2 likely contributes to the re-initiation of morphological plasticity within the injured brain (reviewed in Carmichael, 2008).

Previous studies have largely linked Panx1 activity to cell death following stroke stimuli. Early work demonstrated that Panx1 was responsible for ischemia-induced neuronal depolarizations and resulting cell death (Thompson et al., 2006), while more recent studies have shown that Panx1 activity is detrimental to stroke outcomes (Bargiotas et al., 2011; Bargiotas et al., 2012; Dvoriantchikova et al., 2012; Xiong et al., 2014). My targeted Panx1 knock-out data add to this growing body of evidence, as I found that Panx1-null NPCs were able to persist longer than Panx1-expressing NPCs in the peri-infarct cortex. This suggests that in the peri-infarct, Panx1 is detrimental to cell survival, likely via previously proposed mechanisms linking hyperactive Panx1 function to ionic dysregulation and cellular death (reviewed in Weilinger et al., 2013). Interestingly, Crmp2 overexpression has been associated with neuronal sparing following stroke (Hou et al., 2009). It is feasible to suggest that Panx1 might also negatively impact stroke recovery by inhibiting Crmp2 function, and preventing re-initiation of neuronal morphological plasticity within the peri-infarct cortex. Targeting Panx1 might therefore represent a potential therapeutic treatment, as has been suggested in recent years (reviewed in Dahl and Keane, 2012); however taken in concert with my post-stroke VZ results, the site specific function of Panx1 in NPCs must be first further addressed. Future experiments could also try blocking the Panx1-Crmp2 interaction with the CT3 inhibitory peptide as an alternative intervention following stroke.

6.5. Translation to human neurogenesis

The studies undertaken for this dissertation focused on a rodent model of post-natal VZ neurogenesis; however, this phenomenon also occurs in adult and even aged humans (Eriksson et

al., 1998; Kukekov et al., 1999). Both SGZ (Eriksson et al., 1998) and VZ (Curtis et al., 2007; Ernst et al., 2014) neurogenesis are observed in humans, although NPCs from the VZ do not appear to significantly contribute to the OB (Bergmann et al., 2012). Instead, many VZ NPCs migrate to the striatum where they differentiate into striatal interneurons (Ernst et al., 2014). Similar to rodents, pathological stimuli such as stroke activate human VZ NPCs to migrate towards the site of injury in what is thought to be an endogenous attempt at brain repair (Jin et al., 2006; Macas et al., 2006). Future studies could determine whether the human ortholog of Panx1 is expressed in human NPCs. Given the existence of human NPC-like cell lines, such as SH-SY5Y neuroblastoma cells (reviewed in Kovalevich and Langford, 2013), initial *in vitro* experiments could determine whether Panx1 might regulate human neuronal development as well.

6.6. Summary

Panx1 large-pore channels are expressed in post-natal VZ NPCs and modulate their behaviours both *in vitro* and *in vivo*. Panx1 promotes NPC proliferation and migration, and inhibits neurite outgrowth. This Panx1-dependent regulation is accomplished, in part, by (1) ATP release through the channel and downstream purinergic receptor activation, and (2) crosstalk with cytoskeletal elements, namely Crmp2, to potentially regulate microtubule dynamics. Other putative molecular modulators of Panx1 function in NPCs were discovered by an interactome analysis and will be validated in future studies. The data I presented in this dissertation represent the first demonstration of Panx1 expression and function in NPCs, and contribute to our overall understanding of the role of Panx1 in the brain.

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