

Functional insights into novel roles for gap junction protein-protein
interaction networks in liver and brain

Stephanie Fowler

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Department of Biochemistry, Microbiology, and Immunology
Faculty of Medicine
University of Ottawa

Abstract

Gap junctions are highly-conserved communicating junctions composed of the connexin family of proteins. In addition to this channel function, gap junctions mediate adhesive contacts at extracellular domains, and are host to a variety of signalling metabolites at intracellular surfaces. In this thesis, I explore the emerging theme of the connexin interactome. Starting with a non-biased proteomic approach, I identified endogenous protein interactions with the predominant liver and oligodendrocyte connexin, connexin32 (Cx32). Here, I identified novel mitochondrial protein interactions suggesting that Cx32 might localize to mitochondrial membranes, as has been reported for cardiac Cx43. Following proteomic quantitation of WT and Cx32 KO membranes, I determined that loss of Cx32 specifically induces mitochondrial protein expression. Bioenergetic analysis of isolated mitochondria then confirmed that oxygen consumption and rates of reactive oxygen species (ROS) generation were elevated in Cx32 KO mitochondria. In addition to novel intracellular connexin protein interactions, we hypothesized that connexin-mediated glial cell:cell interactions were responsible for mediating fate decisions in the complex hippocampal neurogenic niche environment. We identified that Cx32-mediated glial cell:cell interactions exert significant proliferative and fate specifying pressures on hippocampal progenitor cell types, wherein the loss of Cx32 enables improved histological and functional regeneration following excitotoxic injury. Together, this thesis identifies novel connexin-mediated signalling pathways that provide mechanistic insight into both intracellular and extracellular interactome-dependent functions for Cx32, and outlines a potentially transformative avenue for brain repair.

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Abbreviations

A:A	astrocyte:astrocyte
A:O	astrocyte:oligodendrocyte
ACN	acetonitrile
aCSF	artificial cerebral spinal fluid
ADAM	a disintegrin and metalloprotease
ANT	adenine nucleotide translocase
AU	arbitrary fluorescence units
BDNF	brain derived neurotrophic factor
BME	β -mercaptoethanol
BrdU	bromodeoxyuridine
CA	cornu ammonis
CaM	calmodulin
CBX	carbenoxolone
CMTX	Charcot Marie Tooth disease, X-linked
CNS	central nervous system
CSPG	chondroitin sulphate proteoglycan
Cx26	connexin26
Cx32	connexin32
DAVID	Database for Annotation, Visualization and Integrated Discovery
DCX	doublecortin
DG	dentate gyrus
DIV	days in vitro
Dlg1	discs-large homolog 1
DMEM/F12	Dulbecco's modified Eagle medium
DMP	dimethyl pimelimidate
DTT	dithiothreitol
EGF	epidermal growth factor
ER	endoplasmic reticulum
ERMES	endoplasmic reticulum-mitochondrial encounter structure
ESI	electrospray ionization
FCCP	carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FGF-2	basic fibroblast growth factor
GCL	granule cell layer
GFAP	glial fibrillary acidic protein
GJIC	gap junction intercellular communication
GRA	18-alpha-glycyrrhetic acid
GrDG	granule layer of the dentate gyrus
H&E	hematoxylin and eosin
H₂DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HRP	horseradish peroxidase
IGF-1	insulin-like growth factor-1
IL-1β	interleukin-1 β
IMM	inner mitochondrial membrane
IP	immunoprecipitation

IP-HTMS	immunoprecipitation-high throughput mass spectrometry
IV	importance value
KO	knockout
LC-MS/MS	liquid chromatography-tandem MS
LFQ	label-free quantitation
LGI	leucine-rich glioma inactivated
LNS	laminin- α , neurexin and sex hormone-binding globulin
LTP	long term potentiation
LTQ	linear ion trap quadropole
MAPK	mitogen activated protein kinases
MGPY	Methyl green-pyronin Y
MIB	mitochondrial isolation buffer
mitoK_{ATP}	mitochondrial ATP-sensitive potassium channels
MS	mass spectrometry
MS	Multiple Sclerosis
NL	no lysate
NPC	neural progenitor cell
NPTX2	neuronal pentraxin 2
O:O	oligodendrocyte:oligodendrocyte
OCR	oxygen consumption rate
ODDD	Oculo-dento-digital dysplasia
OMM	outer mitochondrial membrane
OP	Optiprep
OPC	oligodendrocyte progenitor cell
PDGFαR	platelet-derived growth factor α receptor
PKA/C	protein kinase A/C
PLL	poly-L-lysine
PM	plasma membrane
PMSF	phenylmethylsulfonyl fluoride
PNS	post-nuclear supernatant
Prdx	peroxiredoxin
PTGDS	prostaglandin D2 synthase
PVDF	polyvinylidene difluoride
RB	reaction buffer
RCD	regulated cell death
RCR	respiratory control ratio
ROS	reactive oxygen species
SCX	strong cation exchange
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SFXN-1	sideroflexin-1
SGZ	subgranular zone
SHZ	sub-hippocampal zone
SOD	superoxide dismutase
STED-CW	stimulated emission depletion-continuous wave
SVZ	subventricular zone

TAM	tamoxifen
TCA	the citric acid cycle
TEA	tetraethylammonium
TLR	toll-like receptor
TMA	tetramethylammonium
TMRE	tetramethylrhodamine ethyl ester
TNF-α	tumour necrosis factor- α
TOAD-64	turned on after division protein-64 kD
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
Tyr	tyrosine
UCP	uncoupling proteins
WT	wild-type
YFP	yellow fluorescent protein
ZO	zona occludens
$\Delta\Psi$	mitochondrial membrane potential

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Chapter 1: Gap junctions mediate a diverse set of channel-dependent and channel-independent phenotypes

1.1. Gap junctions mediate communication between cells

The development of increasingly complex intercellular communication mechanisms have enabled the evolution of multi-cellularity (Niklas and Newman 2013). Fueled by gene duplications and protein domain shuffling, the development of a diverse series of intercellular contact mechanisms including adherens, septate, tight, and gap junctions is a hallmark of higher-order phyla (Abedin and King 2010). This thesis focuses on elucidating the importance of subcellular localization and functional cell-cell communication of the highly-conserved gap junction family of proteins, structurally composed of connexin proteins in chordates, and innexin proteins in non-chordates (Abascal and Zardoya 2013). The connexin family of proteins consists of 20 and 21 isoforms in mouse and man, respectively (Sohl and Willecke 2004), and are clustered into five phylogenetically divergent subfamilies named α , β , and γ , δ and ζ (Abascal and Zardoya 2013; Hua et al. 2003) (Table 1). Named according to their molecular weights, at least one connexin isoform is expressed in every cell type during development, and in almost every cell type at maturation, excepting terminally differentiated red blood cells, spermatozoa, and skeletal muscle (Mese et al. 2007; Revel et al. 1978). The pannexin family of proteins are the chordate homologs of innexins, and, while they share little sequence similarity with connexins and will not be discussed at length in this thesis, pannexins form single membrane channels, and may also be capable of gap junctional

communication, albeit with some controversy (Chekeni et al. 2010; Penuela et al. 2013; Sosinsky et al. 2011).

The complexity of the connexin proteins permits fine-tuned cellular responses to a variety of extracellular (paracrine) and intracellular (juxtacrine) signaling cues detected in extracellular milieu and coupled cells respectively, yet capacity for connexin-mediated cell-cell communication extends beyond formation of channels in single and junctional membranes. In this thesis, I explore an emerging theme in connexin cellular biology: namely the role of the connexin interactome. Here, connexin interactomes are defined as the complement of extracellular gap junctional coupling partners and their intracellular regulatory/structural proteins associating with specific connexin isoforms at gap junctional plaques. As such, the complete interactome includes connexin:protein complexes formed by two different cells coupled as a result of connexin:connexin docking between cells. I focus on Cx32, the predominant hepatocyte, oligodendrocyte, and Schwann cell connexin, as most of the previous work has described interactions with the widely-expressed Cx43. Cx43 and Cx32 are phylogenetically separated from each other (42% sequence identity, α and β connexins, respectively, see Table 1), and have distinct tissue expression patterns, justifying the need for more connexin-specific knowledge of Cx32 regulation and function, particularly for central nervous system (CNS) conditions related to mutation/loss of Cx32. Indeed, over 400 different mutations in the Cx32 gene underlie the hereditary X-linked Charcot Marie Tooth (CMTX) neuropathy, a demyelinating phenotype that results in reduced motor nerve conduction velocities and distal muscle weakness in humans (Kleopa 2011). In this thesis, I identify

and describe Cx32 interactions with intracellular signaling effectors, organelles, and with other

Table 1.1: Connexin and pannexin proteins in mouse and human.

Family	Mouse	Human	Conductance (pS)	Tissue Expression	% Identity [^]
$\alpha 1$	mCx43	hCx43	90-110	Most widely expressed, present in 34 tissues, and 46 cell types (Laird 2006)	86
$\alpha 3$	mCx46	hCx46	140-152	Lens (Gerido and White 2004); alveolar epithelium (Abraham et al. 2001)	59
$\alpha 4$	mCx37	hCx37	219-300	Vascular smooth muscle (Isakson and Duling 2005); endothelium (Kwak et al. 2002); ovaries (Gershon et al. 2008); skin (Di et al. 2001b)	65
$\alpha 5$	mCx40	hCx40	158-198	Skin (Di et al. 2001b); nervous system (Chang et al. 1999); endothelium (Yeh et al. 1998); heart (Severs et al. 2001)	66
$\alpha 6$	mCx33	----	ND	Testes (Fischer et al. 2005)	
$\alpha 8$	mCx50	hCx50	212	Lens (Gerido and White 2004)	63
$\alpha 9$	----	hCx59	ND	<i>Human genome</i>	50
$\alpha 10$	mCx57	hCx62	57	Mouse oocytes (Ratchford et al. 2008); horizontal cells of the retina (Hombach et al. 2004; Palacios-Prado et al. 2009)	36
$\beta 1$	mCx32	hCx32	58-70	Liver (Paul 1986); skin (Di et al. 2001b); Schwann cells (Bergoffen et al. 1993b); oligodendrocyte lineage cells (Melanson-Drapeau et al. 2003; Orthmann-Murphy et al. 2008); endometrium (Jahn et al. 1995); gland cells (Michon et al. 2005)	69
$\beta 2$	mCx26	hCx26	115-150	Breast (Kalra et al. 2006); skin (Di et al. 2001a); cochlea (Sun et al. 2005); liver (Traub et al. 1989); endometrium (Jahn et al. 1995); astrocytes (Nagy et al. 2011); airway epithelium (Carson et al. 1998); seminiferous tubules (Brehm et al. 2002); pancreas (Serre-Beinier et al. 2002)	73
$\beta 3$	mCx31	hCx31	85/15	Skin (Di et al. 2001b); airway epithelium (Wiszniewski et al. 2007); cochlea (Forge et al. 2003); placenta (Malassine and Cronier 2005)	68
$\beta 4$	mCx30.3	hCx30.3	ND	Skin (Di et al. 2001b)	59
$\beta 5$	mCx31.1	hCx31.1	ND	Skin (Di et al. 2001b)	53
$\beta 6$	mCx30	hCx30	160	Skin (Di et al. 2001b); astrocytes (Nagy and Rash 2000); cochlea (Ahmad et al. 2007); airway epithelium (Wiszniewski et al. 2007); exocrine gland (Michon et al. 2005)	64
$\beta 7$	----	hCx25	ND	<i>Human genome</i>	72
$\gamma 1$	mCx45	hCx45	30	Human pancreatic ductal epithelial cells (Chanson et al. 1999); SA and AV nodes of the heart (Severs et al. 2001); neurons (Maxeiner et al. 2003); vascular system (Figuerola and Duling 2009); skin (Di et al. 2001b); osteoblasts (Steinberg et al. 1994); retina (Massey et al. 2003); uterus (Albrecht et al. 1996)	72
$\gamma 2$	mCx47	hCx47	55	Oligodendrocytes (Orthmann-Murphy et al. 2008; Teubner et al. 2001)	51
$\gamma 3$	mCx29	hCx30.2	ND	Oligodendrocytes (Nagy et al. 2003a)	----
$\delta 2$	mCx36	hCx36	5-15	Retina (Massey et al. 2003); pancreatic beta cells (Serre-Beinier et al. 2009); neurons (Condorelli et al. 2000)	84
$\delta 3$	mCx30.2	hCx31.9	15	Mouse heart (Kreuzberg et al. 2006); mouse brain (Kreuzberg et al. 2008)	50
$\delta 5$	mCx23	hCx23	N/A	<i>Predicted from human and mouse genome</i>	----
ζ	mCx39	hCx40.1	ND	<i>Human genome</i> , developing muscle of mouse (von Maltzahn et al. 2006)	38
Pannexin	mPanx1	hPanx1	70-500 (Bao et al. 2004; Kienitz et al. 2011; Ma et al. 2012; Romanov et al. 2012; Thompson et al. 2008)	Brain; heart; skeletal muscle; skin; testis; ovary; placenta; thymus; prostate; lung; liver; small intestine; pancreas; spleen; colon; blood endothelium; erythrocytes (Baranova et al. 2004)	65
Pannexin	mPanx2	hPanx2	ND	Brain (Baranova et al. 2004); cochlear system (Wang et al. 2009); hippocampal progenitor cells (Swayne et al. 2010)	69
Pannexin	mPanx3	hPanx3	ND	Osteoblasts; synovial fibroblasts, whole joints and cartilage; hippocampus (Baranova et al. 2004); cochlear bone (Wang et al. 2009); Leydig cells; epididymis; efferent ducts (Turmel et al. 2011)	61

G = conductance in picosiemens. ^ = % identity between human and frog.

connexins at heterocellular junctions that suggest novel, pharmacologically-targetable functions for this widely-expressed connexin.

1.1.1 Structure of gap junctions

Hemichannels (connexons) are formed from the oligomerization of six connexin subunits. These hexamers can function as single-membrane channels mediating paracrine signaling with the extracellular environment (Goodenough and Paul 2003; Maes et al. 2014; Saez et al. 2010) or as intercellular channels. Docking of compatible connexons between neighboring cells initiates the formation of gap junctional intercellular channels: contiguous cytoplasmic pores that are permeable to a variety of different signaling metabolites and ions up to 1.5 kDa in size. Specificity of molecular passage depends on the size/charge specificity of the amino acid residues lining the pore. Fine-tuning of metabolic signalling between cells is further enabled by pairing of connexons composed of the same or multiple connexins in either heterotypic or homotypic gap junctions. These channels may be composed of heteromeric or homomeric connexons (Falk 2000b; Falk and Gilula 1998; Segretain and Falk 2004) (Figure 1.1A). Finally, the assembly of multiple coupled connexons at the plasma membrane (PM) of adjacent cells in hexagonal arrays makes up a gap junctional plaque. Each plaque may contain hundreds to thousands of individual channels, and may occupy significant portions of total membrane surface area (Sosinsky and Nicholson 2005).

Figure 1.1: Structure of connexins and gap junctional channels.

(A) Structural organization of homotypic, heteromeric, and heterotypic gap junctions. Hemichannels mediate paracrine signaling with the extracellular environment. **(B)** Amino acid structure of hepatic connexins, Cx32 and Cx26. Dark blue residues indicate conserved cysteine residues that mediate interaction between docked connexons. Six connexins oligomerize to form a single membrane channel called a connexon, or a hemichannels. **(C)** Crystal structure of Cx26 at 3.8 Å resolution.

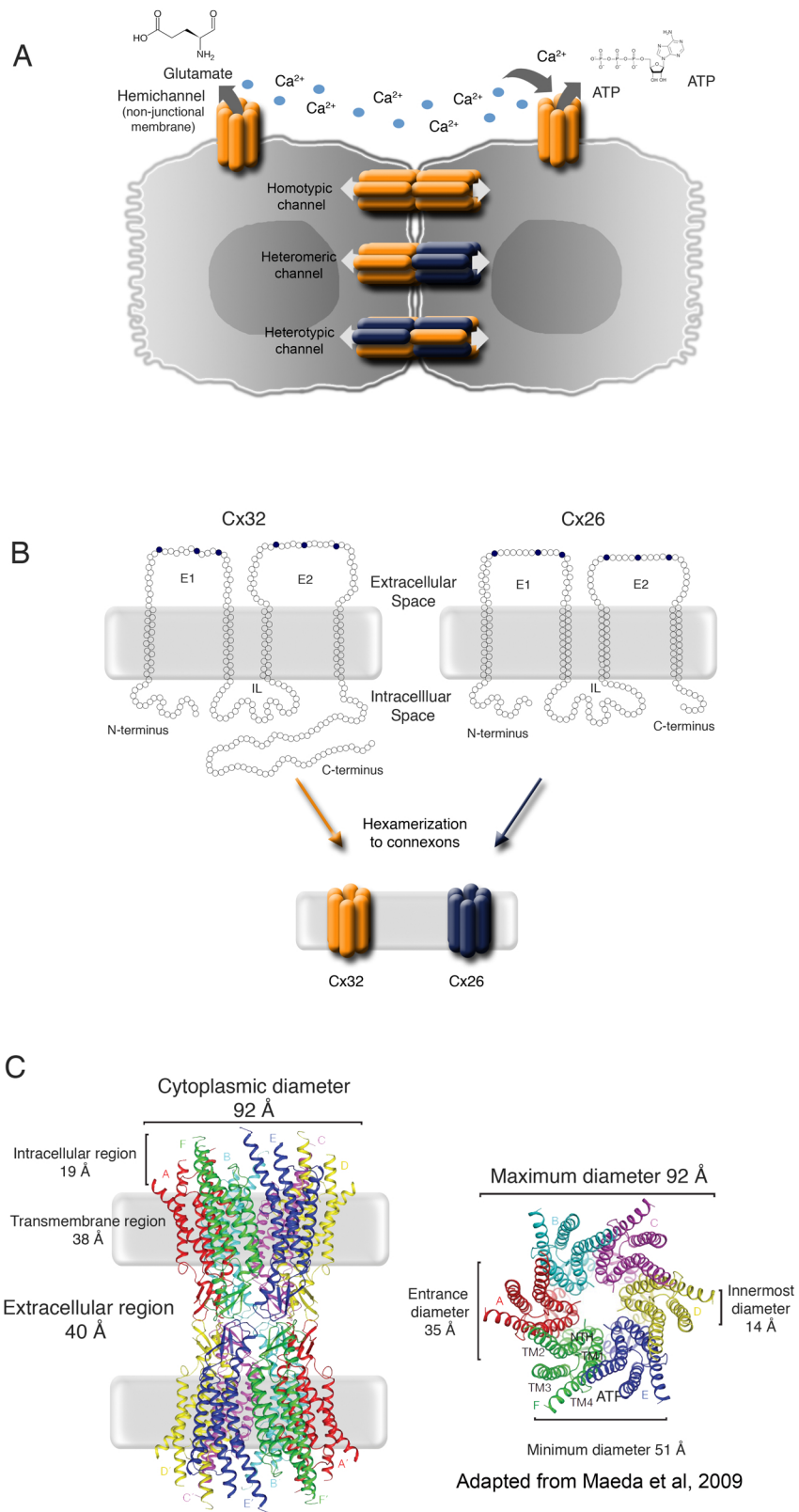


Figure 1.1

Structurally, connexins are four-transmembrane-spanning proteins that possess an intracellular N-terminal intracellular loop, C-terminal domains, and two extracellular loop domains (Martin et al. 2000) (Figure 1.1B). The intracellular regions confer isoform specificity, particularly the intracellular loop and C-terminal tail domains (Manthey et al. 2001), which are shortest for Cx26 (~20 amino acids), and longer for Cx43 and Cx32 (~150 and ~74 amino acids, respectively) (Fiori et al. 2014). Facilitated by six completely conserved cysteine residues, the extracellular loops mediate connexon:connexon docking at apposing membranes (Foote et al. 1998). Currently, the 3.5 Å resolution crystal structure for Cx26 is the best tool available for elucidating the relative importance of specific amino acid residues in gating, intra and inter-molecular interactions, pore properties, and extracellular docking (Maeda et al. 2009). This structure has revealed that Cx26 hemichannels (homotypic Cx26 hexamers inserted into non-junctional membranes) feature 19 Å intracellular regions, a positively charged cytoplasmic entrance (diameter 35 Å), a negatively charged transmembrane pathway (inner diameter 14 Å), and an extracellular cavity that forms a “gap” of 40 Å between coupled cells (Figure 1.1 C). This crystal structure also confirms that the short, N-terminal regions of Cx26 are the most flexible domains, and show that the N-terminus forms hydrogen bonds with the first transmembrane domain of the neighboring protomer, which maintains the open state of the channel. Presumably, voltage-sensitive undocking of the N-termini from the neighboring transmembrane loops also enables the formation of a plug that blocks the pore, thereby regulating channel opening and closing (Maeda et al. 2009; Purnick et al. 2000; Verselis et al. 1994).

1.1.2. Life cycle of gap junctional plaques

Connexin proteins have relatively short half-lives of only a few hours, and as such, gap junction plaques are continually remodeled (Fallon and Goodenough 1981; Laird et al. 1991). Connexins are co-translationally threaded into the endoplasmic reticulum (ER), where they oligomerize to form connexons in the ER or in the trans-Golgi network, depending on the connexin isoform in question (Falk 2000a). Passage through the ER-Golgi intermediate compartment is the next likely path to the cell surface (del Castillo et al. 2010); however, some connexins may reach the cell surface via a Golgi-independent pathway (Thomas et al. 2005). Moreover, there appears to be connexin-specific reliance on microtubules for transport to the membrane (Nielsen et al. 2012). Freeze-fracture electron microscopy studies suggest that connexons are couriered on cytoplasmic vesicles that fuse with the PM to liberate their cargo, which then move laterally in the plane of the PM to reach the outer margins of gap junction plaques (Segretain and Falk 2004). Plaque assembly is facilitated by connexin interaction with membrane scaffolding proteins of the zona occludens (ZO) (Barker et al. 2002), and the cadherin-catenin complex (Wu et al. 2003). Finally, post-translational modifications of connexin proteins take place throughout the secretory pathway, and after final assembly in the gap junctional plaque (Ahmad and Evans 2002; Johnstone et al. 2012; Segretain and Falk 2004) (Figure 1.2).

1.1.3. Cx32 is the predominant gap junctional protein in liver

Hepatocyte Cx32 and Cx26 were isolated from liver in 1974 by Goodenough (Goodenough 1974). Twelve years later, a graduate student in the Goodenough lab (and mentor to our laboratory, Dr David Paul) was successful in cloning the 32-kDa rat

Figure 1.2: Life cycle and channel-independent regulation of connexins.

(1) Connexins are co-translated into the ER, and oligomerize in the ER (2), or in the Golgi (3, 4). Connexins are post-translationally modified as they leave the Golgi, and traffic to the PM on cytoskeletal-guided vesicles (5), passing through lipid raft invaginations (6) on their way to assembly at the outer edge of existing gap junctional plaques (7). Gap junctions are recycled back into the cell in double-membrane structures called annular junctions (8), where connexins are degraded at the proteasome (9) and in lysosomes (10). At gap junctional plaques, connexins interact closely with adherens junctions (11 – cadherin), tight junctions (12 – claudin, 13 - ZO-1 & ZO-2), and with the cytoskeleton (14). Connexins are also subject to phosphorylation by numerous kinases (15) that control almost all aspects of their life cycle.

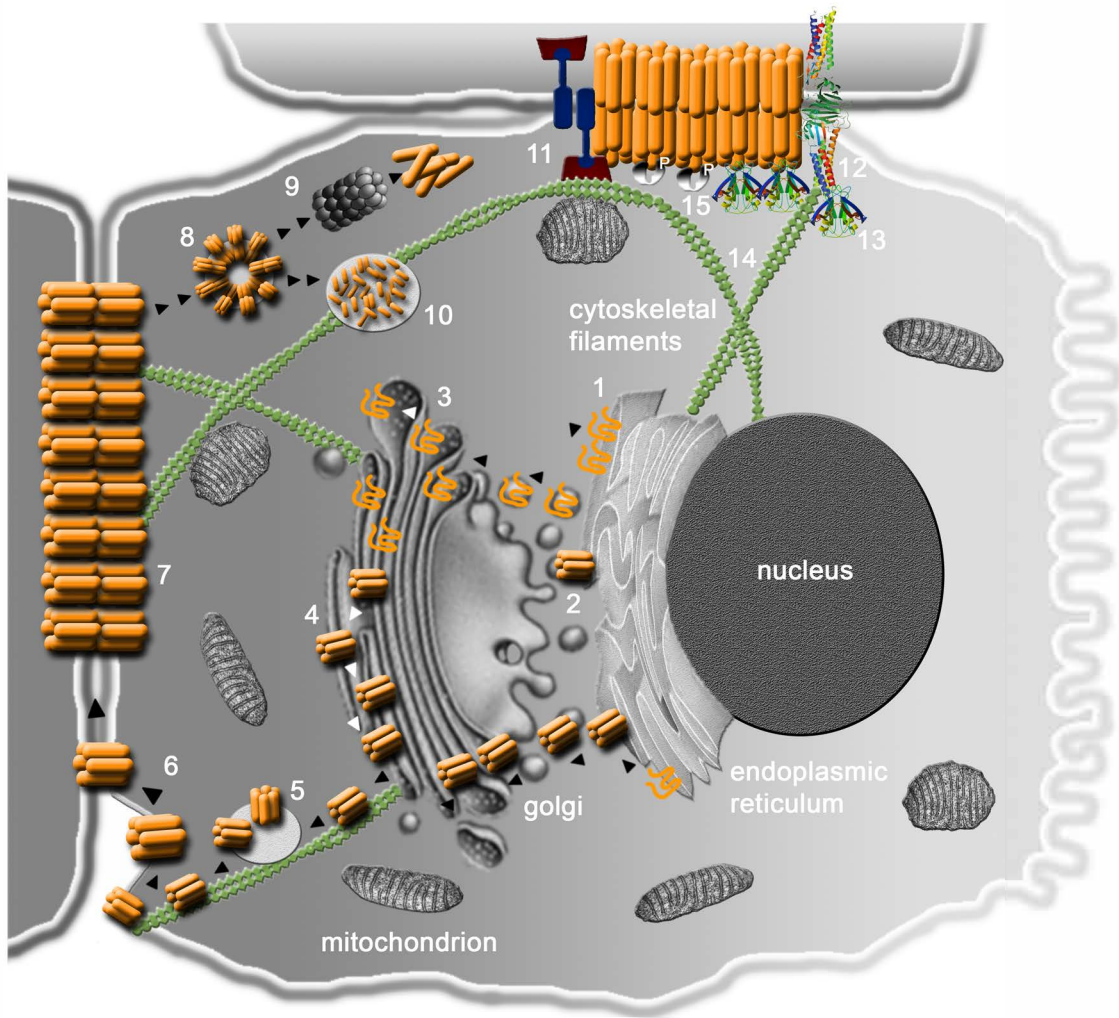


Figure 1.2

liver gap junction protein (Paul 1986), a feat which was also replicated by Gilula's lab that same year (Kumar and Gilula 1986). Gap junctions occupy ~3% of the hepatocyte membrane surface, and Cx32 represents 90% of the total hepatic connexin content. In the liver, gap junctions are required for glycogenolysis (Stumpel et al. 1998), ammonia removal, albumin secretion (Yang et al. 2003), bile secretion (Temme et al. 2001), and xenobiotic biotransformation (Shoda et al. 1999; Shoda et al. 2000). Cx32 gap junctions are also involved in regulating hepatocyte proliferation following hepatectomy (Dermietzel et al. 1987), apoptotic death of hepatocytes (Vinken et al. 2010), and underlie pathophysiological changes related to drug-induced liver failure, hepatitis, cirrhosis, and hepatocellular carcinoma (Vinken et al. 2010). Indeed, intercellular communication via gap junctions was originally described in rat liver cancer cells, which are communication-incompetent (Loewenstein and Kanno 1967), leading to the critical discovery that in most cell types connexin expression acts as a tumor suppressor, and that loss of connexin expression is highly correlated with oncogenesis (Mesnil 2002). This hypothesis was validated in the Cx32-null mouse, which readily develops spontaneous and chemical-induced tumors (Temme et al. 1997).

1.2. Regulation of gap junctional plaques

1.2.1. Regulation by phosphorylation

Hemichannel and gap junction conductance is controlled on several levels by multiple mechanisms mediating both fast and slow channel regulation. Rapid regulation is achieved by trans-junctional voltage-gating, with each connexin isoform displaying multiple voltage-dependent conductance states, and significant differences in voltage sensitivity (Chen and DeHaan 1992; Spray et al. 1979). The phosphorylation state of

the connexin stabilizes junctional conductance over a range of trans-junctional voltages, plays an important role in rapid channel gating, and regulates functional activity of most connexin proteins (Lampe et al. 2000). Cx43 is the most well-studied connexin isoform, and is phosphorylated at 21 serine and two tyrosine residues by a range of kinases including protein kinases A & C (PKA/C), p34/cyclinB kinase, casein kinase 1, mitogen activated protein kinase (MAPK), and Src kinase (Axelsen et al. 2013; Lin et al. 2001; Moreno and Lau 2007; Saez et al. 1998). Phosphorylation of Cx43 on tyrosines 247 and 265 by v-Src kinase (Lin et al. 2001) and/or MAPKs (Cottrell et al. 2003) disrupts gap junctional conductance. Regulatory effects are not, however, universal. For example, phosphorylation of Cx32 by cyclic adenosine monophosphate (cAMP) during PKA activation is correlated with an increase in hepatocyte cell conductance (Saez et al. 1986). Presumably, such heterogeneity in gating dynamics facilitates tissue-specific communication requirements.

1.2.2. Chemical gating by calcium (Ca^{2+}) and pH

In contrast to the rapid voltage-gating of gap junctions, chemical gating is slower, and closes channels more completely than voltage-gating (Peracchia 2004). Chemical gating (mediated by Ca^{2+} , calmodulin (CaM), H^+ and pH) is mainly a protective mechanism of isolating healthy cells from damaged neighbours, yet also occurs at physiological Ca^{2+} and pH levels, supporting a role for this process in general physiology (Lazrak and Peracchia 1993). Chemical gating was uncovered after the observation that cut heart fibers do not heal in the absence of external Ca^{2+} , but heal rapidly when Ca^{2+} is supplied (Deleze 1970). This discovery was then validated by a series of experiments proving that electrical and dye coupling is abolished with

increasing Ca^{2+} concentration (Deleze 1970; Rose and Loewenstein 1975). These findings underlie the large body of work describing connexin involvement in wound healing, which generally follows the hypothesis that early loss of gap junctional communication in the leading wound edge (through channel-inactivation, or by downregulation of connexin expression) compartmentalizes the injured cells to favour wound healing (Becker et al. 2012).

The internal Ca^{2+} concentration required to gate hemichannels and gap junctions is connexin and cell-specific, varying from high nanomolar to low micromolar (insect gland cells, cardiac cells), reaching 40-400 μM in ruptured insect gland cells (Dahl and Isenberg 1980; Oliveira-Castro and Loewenstein 1971; Pfahnl and Dahl 1999). Interestingly, astrocytes are particularly sensitive to low nanomolar intracellular Ca^{2+} concentrations (Enkvist and McCarthy 1994), which has also been observed in Hela cells transfected with Cx43 (Enkvist and McCarthy 1994), and in pancreatic β -cells (Mears et al. 1995). Furthermore, it is well-known that gap junctions and hemichannels can be experimentally forced into the “open” state by reducing extracellular Ca^{2+} in medium, a technique often employed during dye transfer experiments (Pfahnl and Dahl 1999; Verselis et al. 2000).

It is hypothesized that Ca^{2+} regulation is mediated by the intermediate Ca^{2+} -binding protein, CaM, rather than by direct binding with Ca^{2+} (Peracchia 1987; Peracchia 2004; Peracchia et al. 1983). This is consistent with the finding that high Ca^{2+} media does not alter the permeability of Cx32 hemichannels incorporated into liposomes (Bevans and Harris 1999). Indeed, CaM co-localizes with gap junctions from the α , β , and γ subdivisions, and binds directly with two small peptides in the C-terminus of Cx32,

inducing an alpha-helical conformation to the intrinsically disordered protein (Stauch et al. 2012; Torok et al. 1997). Presumably, this change in conformation is associated with channel gating, as has also been reported for other connexin isoforms (Stauch et al. 2012).

Isoform-specific gating by pH has also been demonstrated (Stergiopoulos et al. 1999). For example, decreasing the intracellular pH from 7.6 to 6.5 is sufficient to completely abrogate gap junctional conductance of Cx38 channels, yet reduces conductance of Cx32 channels by only 75% (Wang et al. 1996). Similarly, astrocytic gap junctional communication is relatively insensitive to the intracellular acidification produced during ischemic conditions, which is likely a contributing factor to secondary expansion of ischemic lesions in stroke (Cotrina et al. 1998a). This previous study lends support against conflicting reports that Ca^{2+} may play a role in pH gating by intracellular H^+ ions, as inactivation of astrocyte coupling with a Ca^{2+} ionophore (lasalocid, 40 μM) was not potentiated by exposure to a weak acid, nor did treatment alter the intracellular pH of the astrocytes (Cotrina et al. 1998a).

1.2.3. Critical plaque size

Finally, the number of channels present in the membrane also greatly affects channel function, and is regulated by insertion or removal of channels from gap junctional plaques (Moreno and Lau 2007). Generally operating on a slower time-scale than voltage or gating by phosphorylation, regulation by channel number occurs as groups of gap junctional channels are removed from the plaque as annular rings (Larsen et al. 1979), or added to the plaque as new hemichannels (Laird 1996) (Figure 1.2). Further, there is a critical size dependency on the activity of gap junctional

plaques: plaques smaller than 500 nm across are generally inactive, while the activity of plaques reaching 500 nm is 10-20%, and increases with plaque size (Bukauskas et al. 2000). The isoform-specific half-lives of connexin proteins may also contribute to plaque channel number by regulating complete plaque turnover timescales (Saffitz et al. 2000). For example, cardiac Cx43 has a very short half-life of up to 2 hrs (Laird et al. 1991), hepatocyte Cx32 has a half-life of 5 hrs (Fallon and Goodenough 1981), while epidermal Cx30 was recently shown to have an unusually long half-life of more than 12 hrs (Kelly et al. 2015). Similarly, lens Cx46 protein possesses a half-life greater than 24 hrs (Jiang et al. 1993), likely reflecting the unique metabolic conditions in this avascular organ (Goodenough et al. 1980). In conclusion, under normal physiological conditions, gap junctions have very high open probabilities in the range of 0.6-0.9 (Christ and Brink 1999). This suggests that despite a myriad of complex regulatory influences on connexin function, most gap junction channels are present in the open state, leaving pore selectivity as the main determinant of metabolic coupling (Goldberg et al. 2004).

1.3. Metabolic flux through gap junctions and hemichannels

1.3.1. Channel-dependent communication of gap junctions and hemichannels

Gap junctions are permeable to a variety of ions and polar metabolites less than 1-2 kDa in size. Metabolic transfer amongst coupled cells proceeds down concentration gradients, yet is limited by the specific channel conductance, diameter, charge, and shape selectivity of the pore (Wang and Veenstra 1997). Gap junctions and hemichannels are permeable to inorganic ions, sugars, amino acids, and nucleotides, and impermeable to proteins, nucleic acids, and polysaccharides (Bennett et al. 1991). Selectivity for monovalent cations is very similar across connexin isoforms

(K⁺>Na⁺>Li⁺> tetramethylammonium (TMA)>tetraethylammonium (TEA)), but the same is not true for other molecules. For example, Cx43 channels have 100-300-fold higher selectivity for ATP compared to Cx32 channels, and are also more permeable to AMP, ADP, glucose, glutamate, and glutathione. Conversely, adenosine shows a 12-fold permeability preference through Cx32 channels compared to Cx43 channels; however, phosphorylation of adenosine shifts its permselectivity from Cx32 to Cx43 (Goldberg et al. 2002). Charge alone does not dictate channel selectivity, as IP3-induced Ca²⁺ waves were propagated much more efficiently through Cx32 channels compared to those formed by Cx43 or Cx26 (Niessen et al. 2000). Heterocellularity of junctions also alters channel permeability, as Cx32 homomeric gap junctions are equally permeable to cyclic nucleotides including cGMP and cAMP, while the presence of Cx26 and Cx32 in heteromeric gap junctions introduces selectivity of the channel for cGMP over cAMP (Bevans et al. 1998).

In contrast to gap junctions, hemichannels are mostly inactive under physiological conditions, consistent with the prediction that many open hemichannels would result in cell death (Contreras et al. 2003). Despite this, dye uptake studies indicate that brief hemichannel openings do occur, and that dye transfer is proportional to the total number of hemichannels expressed (Valiunas 2002). Gated by similar mechanisms operating in gap junctional channels, hemichannels are permeable to various signaling metabolites present in the extracellular medium from both healthy and dying cells, and may contribute to both the survival and death of the cells receiving those signals (Contreras et al. 2004). Further, recent reports argue that hemichannels are functional under both physiological and pathological conditions, and have important roles in

regulating cell volume (Quist et al. 2000), release of NAD⁺ (Bruzzone et al. 2001), ATP and glutamate (Orellana et al. 2011), and in the propagation of Ca²⁺ waves (Weissman et al. 2004).

1.3.2. *Pharmacological blockage of gap junctions and hemichannels*

Gap junctions are regulated by a number of chemicals that are known to disrupt gap junctional communication, measured as decreases in electrical coupling, and/or dye transfer amongst coupled cells. The gold standard gap junctional blocker is CBX, a compound derived from glycyrrhetic acid that represents an extremely useful *in vitro* technique to confirm gap junctional involvement in experimental phenotypes (Davidson and Baumgarten 1988; Davidson et al. 1986). Typically active at 100 μ M concentrations, CBX blocks both gap junctions and hemichannels, and does not display any specificity for a particular type of connexin (Juszczak and Swiergiel 2009). Its parent compound, glycyrrhetic acid, is also a non-selective gap junction and hemichannel blocker, and is often used alongside its inactive analog, glycyrrhizic acid, to confirm activity-dependent effects (Davidson and Baumgarten 1988; Davidson et al. 1986). Pannexin 1 (Panx1) channels are also effectively blocked following only 10 μ M CBX/glycyrrhetic acid administration, providing a mechanism of distinguishing effects mediated by pannexins or connexins (Ma et al. 2009). Conversely, the trivalent cations lanthanum and gadolinium preferentially inhibit connexin-mediated hemichannel activity without affecting gap junction or Panx1 channels (Giaume et al. 2013). Additionally, although their relative connexin specificity has not been confirmed, anandamide, long-chain alcohols, fenamates, several anaesthetics, retinoic acid, oleamide, spermine, and

aminosulfonates have all been reported to block at least one member of the connexin family (Juszczak and Swiergiel 2009).

Finally, it should be noted that most chemical gap junction blockers, including CBX and glycyrrhetic acid, are fairly non-specific in their action, and have been known to inactivate a variety of different ion channels (Connors 2012). As such, substantive need for gap junction-specific channel blockers has resulted in the development of a series of connexin-blocking mimetic peptides that show specificity for the connexin family of proteins, with some being more isotype-specific than others (Evans and Boitano 2001).

1.4. Channel-independent communication

1.4.1. Gap junctions are part of multi-adhesion membrane complexes

As has also been reported for many membrane-localized channels and receptors, gap junctions are sites of protein interactions that play essential roles in gap junctional function (Dbouk et al. 2009; Duffy et al. 2002; Giepmans 2006; Herve et al. 2004; Herve et al. 2007; Singh and Lampe 2003). Despite the plethora of proteins confirmed to interact at gap junctions, the functional consequences of many of these proteins remains largely unknown, and have mostly been explored for Cx43. Recent reports indicate that gap junctions are intimately associated with other membrane junctional complexes, and suggest that mammalian junctional proteins require each other to achieve the appropriate conditions for efficient intercellular communication (Figure 1.2).

Evidence of this hypothesis was first confirmed through many reports that connexin proteins interact directly with the tight junction proteins ZO-1 and ZO-2, and are involved in the organization, scaffolding, and trafficking of gap junctions (Barker et al. 2002; Duffy et al. 2002; Kausalya et al. 2001; Kojima et al. 2001; Laing et al. 2005).

As such, ZO-1 is reported to mediate trafficking of connexins from lipid raft domains to gap junctional plaques (Helms and Zurzolo 2004), and act as a scaffold for the recruitment of other regulatory proteins that may be regulated by gap junctions themselves, or by metabolites that pass through the channels (Wu et al. 2003). Further, mammary epithelial cell differentiation relies on the ZO-1/2 interaction with Cx32, Cx30, and Cx43 (Talhouk et al. 2008), and Cx32 has been directly immunoprecipitated with the tight junction proteins occludin and claudin (Kojima et al. 2001; Kojima et al. 1999). Adherens junctions proteins, including the cadherin and catenin proteins also play important roles in the assembly and function of gap junctions (Matsuda et al. 2006; Meyer et al. 1992; Wei et al. 2005). Indeed, gap junctional dye coupling is inhibited in an N-cadherin knockout (KO) mouse model (Xu et al. 2001b), and N-cadherin antibodies prevent both adherens junction formation and functional gap junctional intercellular communication (Meyer et al. 1992). Taken together, these converging reports indicate the importance of both tight and adherens junctions in the assembly, stability, and activity of gap junctional channels, and support the existence of multi-adhesion complexes to provide optimal signaling amongst coupled cells.

1.4.2. Transient interactions of kinases and phosphatases

Connexin-dependent multi-adhesion complexes support a host of other proteins that interact more dynamically at gap junctional plaques. Of particular note is the ability of the C-terminal tail of many connexin isoforms to be phosphorylated at multiple sites by a variety of kinases (Laird 2005; Lampe and Lau 2000; Lampe and Lau 2004; Lau et al. 1996). As previously mentioned, Cx43 is phosphorylated by PKA, PKC, casein kinase 1, MAPK, Src kinase, and p34^{cdc2}/cyclin B kinase at 14 of the serine/tyrosine

residues present in the C-terminal tail region (Lampe and Lau 2004). Accordingly, the phosphatase domain of receptor protein tyrosinase phosphatase μ co-immunoprecipitates with Cx43, and is likely to counteract Src phosphorylation to prevent channel closure (Giepmans et al. 2003). Cx32 is also phosphorylated by cAMP-dependent kinase and PKC (Takeda et al. 1989), and is a moderate substrate for Ca^{2+} -CaM-dependent protein kinase (Saez et al. 1990). Thus, as phosphorylation controls gating and turnover of connexin channels, the ability of gap junctional plaques to support the close interaction with a variety of kinases is likely to explain the rapid and diverse forms of phosphorylative regulation in this protein family.

1.4.3. Cytoskeletal contacts

Gap junctions are also closely connected with the cytoskeleton (Giepmans 2006; Matsuuchi and Naus 2013). In particular, microtubule interactions are essential in the proper trafficking and targeting of newly-synthesized connexons to appropriate domains at the PM (Figure 1.2). Giepmans and colleagues determined that Cx43 possesses a tubulin-binding domain (KGVKDRVKGK), and that a 35 amino acid juxtamembrane region of the C-terminal tail is responsible for mediating this interaction. Interestingly, this sequence is not conserved in Cx31.9, Cx46, or Cx50 sequences, suggesting that direct anchoring or termination of microtubule distal ends is specific to Cx43 (Giepmans et al. 2001). Further, actin is also involved in the delivery of connexons to gap junctional plaques, and co-localizes with connexins (Wall et al. 2007). Indeed, the actin-binding proteins α -spectrin (Toyofuku et al. 1998) and drebrin (Butkevich et al. 2004) are present in Cx43 complexes, and a direct interaction of Cx43 with F-actin and cortactin has been demonstrated in cardiac myocytes (Squecco et al. 2006). Similarly,

Panx1 has also been reported to interact with actin, yet maintains no detectable relationship with microtubules (Bhalla-Gehi et al. 2010).

1.4.4. Trafficking through lipid rafts

Connexin proteins also interact with the lipid-raft-associated caveolin proteins that are responsible for producing membrane invagination at caveolae. These specialized lipid domains are enriched in cholesterol and glycosphingolipids, and are anchor and recruitment sites for membrane proteins and their signaling effectors (Insel et al. 2005; Locke et al. 2005; Patel and Insel 2009). Despite the presence of connexin proteins in lipid raft preparations from detergent-insoluble membranes, lipid rafts are not a terminal assembly point for gap junctions (Locke et al. 2005); rather, they likely act as intermediary targets for certain connexons on their way to the PM (Helms and Zurzolo 2004; Laing et al. 2005). By this mechanism, it is possible that connexins and connexons accumulate protein interactions along the secretory route, or that transient trafficking-specific protein interactions facilitate connexin elaboration at the gap junctional plaque. That being said, interaction with lipid raft proteins implies access to these rich signaling domains, which may have important regulatory implications for connexin function.

1.4.5. Regulation by CaM/Discs-large homolog 1 (Dlgh1)

As mentioned previously, CaM interacts with multiple different connexin family members (Peracchia and Bernardini 1984; Peracchia et al. 1983; Zou et al. 2014). Acting as a Ca^{2+} gate intermediary, CaM interacts at both N- and C-terminal loops of Cx32 (β -connexin), at the intracellular loop region of Cx43, Cx44, and Cx50 (α -connexins), and at the C-terminal region of Cx36 (γ -connexin) (Zou et al. 2014). For

Cx32, the mechanism controlling Ca^{2+} -CaM inhibition of gap junctional communication is thought to be mediated by an alpha helical conformational change in the C-terminal region, which also displaces the known Cx32 binding partner, Dlg1 (Stauch et al. 2012). Dlg1 belongs to the membrane-associated guanylate kinase (MAGUK) family, and is involved with the scaffolding/regulation of ion channels at membrane microdomains. Dlg1 is also a member of the highly-conserved Scribble polarity complex, along with Lethal giant larvae (Lgl), which has roles in cell proliferation, assembly, and maintenance of adherens and tight junctions (Su et al. 2012). Duffy and colleagues report that loss of Cx32 reduces Dlg1 expression at the membrane, and induces translocation of Dlg1 to the nucleus, a phenomenon known to induce cell proliferation (Duffy et al. 2007; Funke et al. 2005; Ishidate et al. 2000). Importantly, the interactions of Cx32 with CaM and Dlg1 may underlie Ca^{2+} -induced increases in Schwann cell proliferation and dysfunction at paranodes, a mechanism with far-ranging implications for myelin sheath regulation (Stauch et al. 2012).

In conclusion, the interaction of connexins with a variety of intracellular connexins is an active area of research, as protein interaction maps provide valuable information about gap junctional function. This field is limited by the technical challenges involved in purifying endogenous gap junctional plaques, and in the validation of protein interaction networks. The current thesis addresses some of these technical concerns, and expands upon our knowledge of Cx32-interacting proteins involved in novel intracellular functions.

1.5. Non-plasmalemmal localization of connexin proteins

1.5.1. Nuclear targeting of connexins

In a seminal publication by Dang et al, convincing evidence was presented that the C-terminal region of Cx43 can target to the nucleus and induce decreased cell proliferation (Dang et al. 2003). The concept that functional gap junctional channels at the PM were not required to induce growth control was excellent support for the importance of examining channel-independent roles in connexin function (Krutovskikh et al. 2000; Moorby and Patel 2001). Presumably, nuclear localization of the C-terminal regions of Cx43 are accomplished through their interaction with known nuclear-localized proteins, including Src, ϵ -PKC (Beyer 1993), and β -catenin (Ai et al. 2000; Dang et al. 2003), yet, a putative nuclear localization sequence has also been identified on the Cx43 C-terminus (Qin et al. 2002). Wild-type (WT) Cx43 can also localize to the nucleus in src and *neu*-transformed liver epithelial cells (de Feijter et al. 1996; Huang et al. 1998b), and Cx32 is also capable of localizing to the nuclear envelope in baby hamster kidney cells (Kumar et al. 1995). It is proposed that nuclear-localized connexins can act as transcription factors; however, this hypothesis has not been extensively tested (Doble et al. 2004; Moorby and Patel 2001).

1.5.2. Mitochondrial targeting of connexins

In addition to nuclear targeting of connexins, the mitochondrial localization of cardiac Cx43 has been described (Boengler et al. 2005; Boengler et al. 2009 (Boengler et al. 2005; Boengler et al. 2009; Dhein 2005). Initially, German scientists discovered that the hearts of Cx43^{+/-} animals could not be preconditioned to an ischemic stress, as has been described for WT animals (Schwanke et al. 2002). Four years later, it was

reported that approximately 5% of total cellular Cx43 is localized to the inner mitochondrial membranes (IMM) of subsarcolemmal but not interfibrillar mitochondria, and that Cx43 localization at the mitochondrion mediates preconditioning to ischemic stress (Boengler et al. 2007; Boengler et al. 2006; Ruiz-Meana et al. 2008; Schulz et al. 2007; Schulz and Heusch 2006). Other groups began reporting that Cx43 was also localized to cardiac mitochondria, (Goubaeva et al. 2007; Penna et al. 2009b) and that its presence was indeed related to pre-and post-conditioning stress (Penna et al. 2009), mitochondrial potassium uptake (Miro-Casas et al. 2009), and apoptosis (Goubaeva et al. 2007). In an unfortunate series of events, a 2012 publication concerning the functional aspects of mitochondrial Cx43 was retracted (Rottlaender et al. 2010; Rottlaender et al. 2012a; Rottlaender et al. 2012b). A second publication from 2010 (Rottlaender et al. 2010) was also retracted, with the primary author citing “intentional, systematic manipulation of the electrophysiological data”, and confirming that the other authors were not involved in the data manipulation (Rottlaender et al. 2012b). These retractions have certainly stymied any recent electrophysiological reports; however, given that multiple independent scientists have confirmed a mitochondrial localization for Cx43, this phenomenon warrants further investigation.

Cx43 has also been detected in mitochondria of astrocytes (Kozoriz et al. 2010), endothelial cells (Kiec-Wilk et al. 2012; Li et al. 2002a; Mohammad and Kowluru 2011), stem cells (Lu et al. 2012), hepatocytes (Fowler et al. 2013; Vinken et al. 2013), and pancreatic cancer cells (Sun et al. 2012). Located at the IMM, mitochondrial Cx43 is S-nitrosylated (Soetkamp et al. 2014), and interacts with PKC and K^+ -ATP channels (Waza et al. 2014), nitric oxide synthase (Kirca et al. 2015), apoptosis inducing factor,

and the beta subunit of the electron transfer protein (Denuc et al. 2016). It is hypothesized that mitochondrial Cx43 forms channels in the IMM that pass K^+ (Boengler et al. 2013) and Ca^{2+} (Srisakuldee et al. 2014) ions, and these channels can be blocked with the Gap27 peptide and CBX (Azarashvili et al. 2011; Srisakuldee et al. 2014). Further, loss or selective blockage of cardiac mitochondrial Cx43 reduces complex I activity and ATP generation, without impairing the activity of complex II (Boengler et al. 2012). Interestingly, very low levels of Cx32 are present in mitochondrial extracts from animals wherein cardiac Cx43 was replaced with Cx32, showing that this protein is capable of mitochondrial translocation, yet does not induce the mitochondrial K^+ permeability that is reported for mitochondrial Cx43 (Miro-Casas et al. 2009). Taken together, these data indicate that connexon channels may be located at the IMM, and are important for mitochondrial function in multiple different cell types, raising interesting questions about their possible therapeutic value.

1.6. Glial cells form a gap junction-mediated communication network

1.6.1. The glial cell network

In 1889, the Swiss anatomist Wilhelm His proposed that neuroglial cells formed a multinuclear syncytium of fused cells called the *neurospangium* (His 1889). This theory was highly questioned by many neuroanatomists (Cajal 1911; Lenhossek 1893), yet persisted until the 1950s when electron microscopy demonstrated that glial cells are clearly separated from each other by extracellular space (Beams et al. 1952; Farquhar and Hartmann 1957; Schultz et al. 1957). A new definition for the glial syncytium arose in 1986 when Massa and Mugnaini compiled evidence that glial cells respond as a functional unit to ionic changes in the extracellular space, and attributed this

phenomenon to the gap junction family of proteins (Massa and Mugnaini 1982). This hypothesis was named the “panglial syncytium” by Rash and colleagues in 1997 (Rash et al. 1997), yet has restricted itself over the years, as it has become clear that the glial network is certainly more constrained than the term panglial syncytium suggests (Giaume and Liu 2012). Constraints on glial communication are imposed by a requirement for very specific gap junction pairings amongst oligodendrocytes and astrocytes (Nagy and Rash 2000; Rash et al. 2001a).

1.6.2. Connexins mediate coupling amongst mature glial cells

The main glial cell pairings consist of astrocyte:astrocyte (A:A), astrocyte:oligodendrocyte (A:O), and oligodendrocyte:oligodendrocyte (O:O) channels (Orthmann-Murphy et al. 2008). It has been well established that mature astrocytes express Cx26, Cx30, and Cx43 (Nagy and Rash 2000; Rash et al. 2001a), which participate in Ca^{2+} wave signalling (Cotrina et al. 1998b), trafficking of glutamate and other gliotransmitters (Giaume et al. 1997), and spatial buffering of K^{+} ions (Wallraff et al. 2006). Mature oligodendrocytes express Cx29, Cx32, and Cx47 (Rash et al. 2001a). Both Cx32 and Cx29 are contained within the myelin sheath of oligodendrocytes, where Cx32 forms reflexive junctions between adjacent layers of uncompacted myelin. Additionally, Cx32 is localized to oligodendrocyte somata. Cx29 partially co-localizes with Cx32 along myelinated fibers (Nagy et al. 2003a), while Cx47 forms small gap junctions linking oligodendrocytes with astrocytes, and is not detected in deep myelin layers (Li et al. 2004). Myelin connexins (Cx32, Cx29) are thought to operate as ion-diffusing hemichannels in perinodal myelin sheaths to facilitate absorption and removal

of extracellular K⁺ released from active neurons (Altevogt et al. 2002; Altevogt and Paul 2004; Li et al. 2002b; Nagy et al. 2003a) (Figure 1.3).

The most probable glial connexin cell pairings have been determined by examination of freeze-fracture immuno-electron-microscopy images at junctional membranes, confocal co-immunolocalization, *in vitro* and *in vivo* dye-spreading studies, and by Hela co-expression studies (Magnotti et al. 2011a; Magnotti et al. 2011b; Nagy et al. 2003b). From these indirect assays, functional interaction amongst astrocytes is thought to be mediated by homotypic Cx26, Cx30, and Cx43 channels, and heterotypic Cx26/Cx30 and Cx30/Cx43 channels (Rash et al. 2001b). Oligodendrocyte coupling is likely enabled predominantly by homotypic Cx32 channels, and less so by Cx47 homotypic channels (Dermietzel et al. 1978; Maglione et al. 2010; Wasseff and Scherer 2011), while A:O coupling can be achieved through Cx43/47 and Cx30/Cx47 channels (Maglione et al. 2010), with potential contributions from Cx30/Cx32 and Cx26/Cx32 channels (Altevogt and Paul 2004; Magnotti et al. 2011b; Wasseff and Scherer 2011). Cx29 is thought to form only hemichannels, and does not likely contribute to the glial cell network (Li et al. 2002b) or form heteromeric connexons with Cx32, as the two proteins do not co-immunoprecipitate when expressed in Hela cells (Ahn et al. 2008). Further, IP for oligodendrocyte Cx47 from brain tissue indicates an interaction with astrocyte Cx43, confirming that this connexin pair likely does mediate A:O junctions (Li et al. 2008). To our knowledge, the other proposed glial connexin cell-cell pairings (Cx32/Cx30, Cx32/Cx26, Cx30/Cx47, Cx30/Cx43) have never been tested in whole brain tissue by co-IP.

Figure 1.3: Spatial distribution of Cx32 and Cx29 in oligodendrocyte myelin.

(A) Cx32 is localized to reflexive junctions between adjacent layers of uncompacted myelin. **(B)** Cx29 is present in periaxonal myelin sheaths. Cx32 is also localized at the cell soma, forming junctions with perinodal astrocyte Cx30. Cx47 is localized at the outer-most regions of myelin sheaths, and on the oligodendrocyte cell soma.

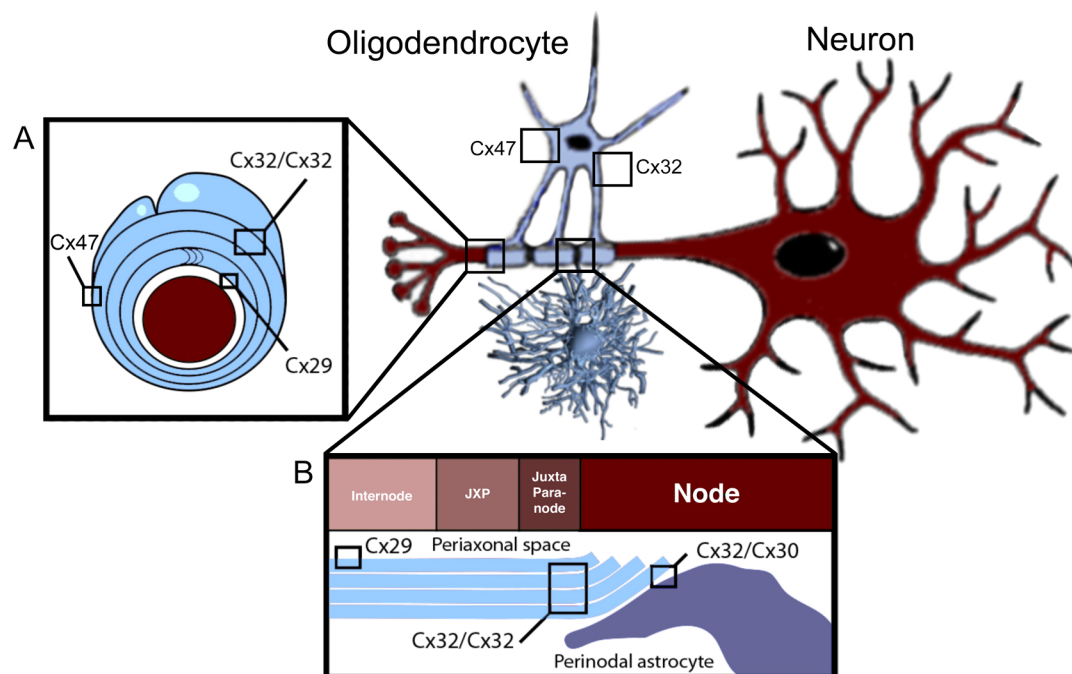


Figure 1.3

1.6.3. Functional redundancy in the glial cell network

A certain degree of functional redundancy is apparent in these glial cell pairings, as evidenced by the relatively mild phenotype of single connexin KO animals, due to compensation by the remaining connexin pairs (Li et al. 2008; Nelles et al. 1996; Odermatt et al. 2003; Scherer et al. 1998). Conversely, deletion of one astrocyte and one oligodendrocyte connexin results in early mortality from severe glial pathology and seizure activity: loss of Cx30 and Cx47 results in death by 13 weeks of age (Tress et al. 2012), while loss of Cx43 and Cx32 results in death by 16 weeks of age (Magnotti et al. 2011a). Interestingly, ablation of two A:A (Cx30 & Cx43) junctions results in a severe dysmyelination phenotype, with sensorimotor and spatial memory impairment, but no premature death (Lutz et al. 2009), while loss of two O:O (Cx32 & Cx47) junctions results in widespread white matter pathology, and death by 6 weeks of age (Menichella et al. 2003). The above findings suggest that O:O and reflexive coupling is more important to survival than A:A coupling, yet it is clear that the complete glial cell network is of tremendous importance for normal physiological brain health.

1.7. Connexins regulate neural and glial progenitor cell fate

1.7.1. The adult neurogenic niche

The adult brain is capable of significant neurogenesis restricted to distinct neurogenic niches in the subventricular zone (SVZ) of the lateral ventricle, and in the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus (Alvarez-Buylla and Lim 2004; Doetsch et al. 1999). In humans, the functional significance of SVZ neurogenesis is highly controversial, as the SVZ contributes significantly to new neurons in the rodent olfactory bulb, but no new neurons are found in the human

olfactory bulb (Wang et al. 2011). Conversely, hippocampal neurogenesis contributes significantly to new granule cell neurons that integrate into the functional hippocampal network. Overall, 33% of the human hippocampus is subject to renewal, leading to the turnover of approximately 2% of hippocampal neurons each year (Spalding et al. 2013). New hippocampal neurons have distinct functions from older neurons: new neurons are required for efficient pattern separation, whereas older neurons contribute more to rapid recall functions (Nakashiba et al. 2012; Sahay et al. 2011). Further, new hippocampal neurons have enhanced synaptic plasticity compared to older neurons, allowing for significant contributions to the neural network for a short period of time after their birth (Ge et al. 2007). This process results in the preservation and maintenance of memory capacity (Kitamura et al. 2009; Sahay et al. 2011; Snyder et al. 2005; Zhao et al. 2008). New neurons are also generated from the SVG following brain injury (Jin et al. 2001) or during a disease state, and are implicated in resistance to stress (Parihar et al. 2011) and psychiatric disorders (Mahar et al. 2014).

Hippocampal neurogenesis is described by a model wherein quiescent Type 1 stem-like cells expressing nestin and glial fibrillary acidic protein (GFAP) are activated to produce rapidly dividing, lineage-determined Type 2a progenitor cells (Kempermann et al. 2004b). These progeny have limited capacity for self-renewal, and have lost GFAP expression. Type 2b cells arise from Type 2a cells, and are characterized by their expression of the microtubule-associated protein doublecortin (DCX). Specification of Type 2b cells to Type 3 neuroblasts signals exit from the cell cycle, and is accompanied by the expression of the immature granule cell markers NeuN and calretinin. Mature hippocampal neurons express calbindin, and are integrated into the

hippocampal circuitry over four-seven weeks of maturation (Aguilar-Arredondo et al. 2015; Ming and Song 2011).

Harnessing the neurogenic or gliogenic potential of progenitor populations holds therapeutic promise for brain repair, however, progress in this field is limited by the inability to direct the fate of NPC populations to appropriate cell types in the damaged microenvironment (Plane et al. 2004; Santhakumar et al. 2000; Yang et al. 2008). This thesis explores connexin-mediated communication in the glial cell network as a means of directing the specification of NPC and glial progenitor populations to regionally-specific phenotypes.

1.7.3. Connexin expression in NPC-glial networks

Postnatal NPCs express multiple connexin isoforms that are compatible with those expressed by mature glial cells (Freitas et al. 2012; Talaveron et al. 2015). Over the course of their specification, NPCs express ten connexin proteins (Cx26, Cx29, Cx30, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, and Cx47); however, early Type 1 and Type 2a hippocampal NPCs express only Cx30, Cx43, and Cx26 (Bittman and LoTurco 1999; Imbeault et al. 2009), while a differentiating population of NG2-glial cells expresses Cx32 (Melanson-Drapeau et al. 2003; Traub et al. 1989). Subsets of NPCs also express Panx1 and Panx2 (Ray et al. 2005; Swayne et al. 2010; Wicki-Stordeur et al. 2012) (Figure 1.4). NPCs are hypothesized to couple to one another and to astrocytes through any Cx30/Cx43/Cx26 channel combination, and NPC contact with mature oligodendrocytes is likely to occur between Cx30/Cx32, Cx26/Cx32 and Cx43/Cx47 channel pairings (Swayne and Bennett 2016). There is also evidence that NPCs communicate with NG2-glia in the SVZ and hippocampal neurogenic niches

Figure 1.4: Connexin and pannexin expression in neuro- and gliogenesis.

Stepwise commitment of Type 1, Type 2a, Type 2b, and Type 3 NPCs toward mature neuronal fates occurs in the SGZ of the hippocampal DG. Type 1 NPCs may also generate astrocytes. Oligodendrogenesis proceeds from embryonic or SVZ/SGZ-derived progenitor populations (El Waly et al. 2014). Connexin and pannexin expression in NPCs and oligodendrocyte progenitor cells (OPCs) was identified by our laboratory and others. Figure adapted from Doetsch 2003, and Pettit et al. 2012.

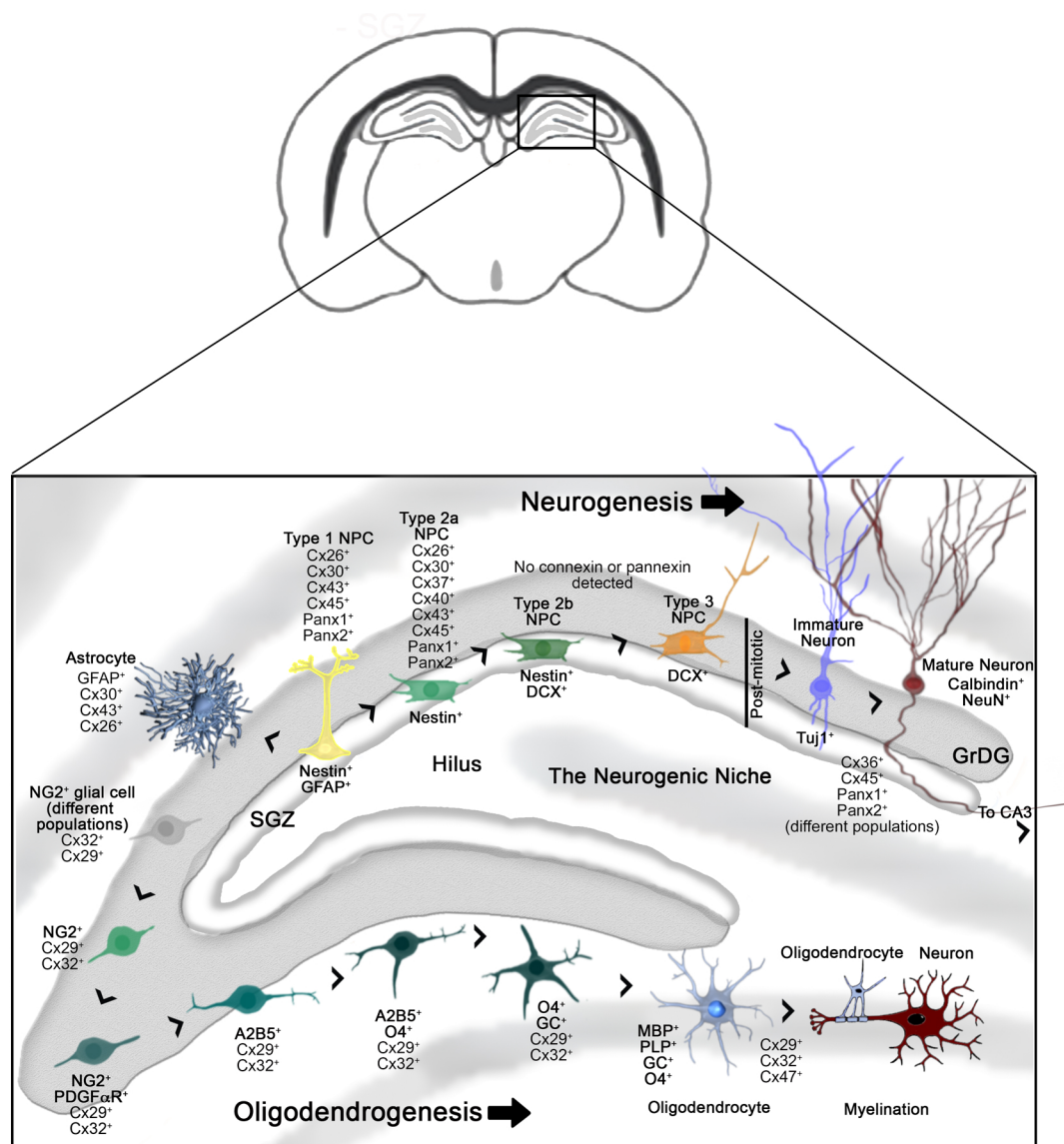


Figure 1.4

(Lacar et al. 2011). Finally, because NG2-glia express Cx32 just prior to terminal oligodendrocyte differentiation (Imbeault et al. 2009; Melanson-Drapeau et al. 2003; Traub et al. 1989) Traub et al. 1989), these cells are likely restricted to communication with mature oligodendrocytes, astrocytes, and NPCs via Cx32/Cx30 and Cx32/Cx26 channels. These modes of communication have yet to be directly tested, and are one focus of this thesis.

1.7.3. Fate regulation by neuronal and glial progenitor cell types

Given that brain development is not impaired in any of the single and double connexin KO animal models, the contribution of cell-cell communication amongst progenitor cell populations, and with mature instructive cell types is unclear. Despite this, our lab and others have provided convincing evidence that gap junction proteins alter the proliferation, specification, and survival of progenitor cell populations (Duval et al. 2002; Freitas et al. 2012; Hartfield et al. 2011; Kunze et al. 2009; Melanson-Drapeau et al. 2003; Ravella et al. 2015; Swayne and Bennett 2016). For example, embryonic NPCs isolated as neurospheres are highly coupled by gap junctions, and experience decreased cell density and altered morphology of differentiated cells when cultures are treated with glycyrrhetic acid (Duval et al. 2002). Further, blockage of gap junctional communication in adult NPCs results in reduction of spontaneous Ca^{2+} activity, and twice as many apoptotic cells. RNA interference knockdown of Cx43 also resulted in increased apoptosis, indicating that NPC connexin networks are required to maintain NPC cell survival (Ravella et al. 2015). Accordingly, there is strong evidence that Cx43 is implicated in maintaining the proliferative state of NPCs (Cheng et al. 2004; Kunze et al. 2009; Miragall et al. 1997), and in mediating proper migration dynamics of migrating

neurons along radial-glial contacts (Elias et al. 2007; Marins et al. 2009) and SVZ neuroblasts (Marins et al. 2009). Data from our laboratory indicates that Cx32 is also implicated in the proliferation, survival, and differentiation of Cx32-expressing NG2-glial cells (Melanson-Drapeau et al. 2003). Here, loss of Cx32 resulted in the hyperproliferation and apoptotic death of nestin⁺/NG2⁺/platelet-derived growth factor alpha receptor⁺ (PDGFR⁺) cells, reflecting a defect in their capacity for terminal oligodendrocyte differentiation in the absence of Cx32.

The contribution of connexin-mediated glial instruction to NPC cell fate has only begun to be described. It is well-understood that astrocytes stimulate neurogenesis from both adult SVZ (Lim and Alvarez-Buylla 1999) and SGZ (Gengatharan et al. 2016; Oh et al. 2009) NPCs, and that these two cell types maintain privileged communication niches that are mediated by gap junctions (Lacar et al. 2011). Presumably, gap junction-permeable instructive cues are exchanged amongst astrocytes and NPCs located within the neurogenic niche environment; however, it is unclear what the singular contributions of Cx43 and Cx30 are to this process, or which connexons might mediate functional mature glial/NPC coupling. Further, it remains to be seen whether mature oligodendrocytes also possess instructive capacity in the niche, or how effective glial instructive cues are in the damaged microenvironment.

1.8. Rationale, hypothesis, and objectives

The objective of this thesis was to identify novel roles for Cx32 protein-protein interaction networks, with specific focus on liver and oligodendroglial cell types. Cx32 is the predominant liver gap junctional protein, mediates reflexive junctions in oligodendrocyte myelin, and is expressed by a population of OPCs (NG2-glia). Loss of Cx32 in mice results in a high incidence of spontaneous liver tumors (Temme et al. 1997), and causes a peripheral demyelinating neuropathy similar to that caused by Cx32 mutation in humans (Scherer et al. 1998). Further, our lab has hypothesized that alteration of connexin-mediated oligodendroglial and astrocytic cell:cell networks is sufficient to alter adult NPC survival and specification (Swayne and Bennett 2016), a hypothesis that is directly tested in this thesis. Involvement of Cx32 in both liver tumorigenicity and instructive NPC microenvironments is not fully understood, particularly in relation to the relatively unexplored channel-independent roles of Cx32. In this thesis, I hypothesized that identification of intracellular and extracellular protein-protein interactomes for liver and oligodendroglial Cx32 would identify unknown roles in novel signaling pathways. To test this hypothesis I proposed three specific aims:

- (1) Identify endogenous intercellular protein-protein interactions of Cx32 from liver tissue (Chapter 2)
- (2) Investigate Cx32 involvement in novel intracellular phenotypes (Chapter 3)
- (3) Determine how connexin-mediated extracellular cell-cell contacts regulate NPC and glial fate specification in uninjured and injured brain microenvironments (Chapter 4)

Chapter 2: Identification of endogenous Cx32 interacting proteins in liver

2.1. Objective of study

The objective of this chapter was to investigate endogenous protein interactions of the liver gap junction protein, Cx32. Protein interactions were identified and validated using tissue subcellular fractionation, mass spectrometry (MS) analysis of interacting proteins, co-IP, and Ingenuity network analyses.

2.2. Author contributions

SLF and SALB conceived and designed the experiments. SLF performed all of the experimental work. MA aided in the microscopy and animal husbandry. HZ ran the MS samples in the laboratory, and under the supervision of DF.

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A detailed protocol for the subcellular fractionation performed in this study is published as an invited chapter in a Springer Press book: **Fowler S**, Akins M, Bennett SAL (2016) Preparation of gap junctions in membrane microdomains for IP and MS interactome analysis. Gap Junction Protocols, Methods in Molecular Biology, Vol 1437: 113-132. New York, NY: Springer Press.

The liver connexin32 interactome is a novel plasma membrane-mitochondrial signaling nexus

Stephanie L. Fowler^{1,2}, Mark Akins^{1,2}, Hu Zhou^{2,3}, Daniel Figeys^{2*}, Steffany A.L. Bennett^{1,2*}

¹Neural Regeneration Laboratory, ²Ottawa Institute of Systems Biology, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON, Canada; ³Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China

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Corresponding authors:

Dr Daniel Figeys, dfigeys@uottawa.ca

Tel.: (613) 562-5800 x 8674, Fax: (613) 562-5452

Dr Steffany A.L. Bennett, sbennet@uottawa.ca

Tel.: (613) 562-5800 x 8372

Fax: (613) 562-5452

2.3. Abstract

Connexins are the structural subunits of gap junctions and act as protein platforms for signaling complexes. Little is known about tissue-specific connexin signaling nexuses, given significant challenges associated with affinity-purifying endogenous channel complexes to the level required for interaction analyses. Here, we used multiple subcellular fractionation techniques to isolate Cx32-enriched membrane microdomains from murine liver. We show, for the first time, that Cx32 localizes to the PM and IMM of hepatocytes. Using a combination of immunoprecipitation-high throughput mass spectrometry (IP-HTMS), reciprocal co-IP, and subcellular fractionation methodologies, we report a novel interactome validated using null-mutant controls. Eighteen Cx32 interacting proteins were identified. The majority represent resident mitochondrial proteins, a minority PM, ER, or cytoplasmic partners. In particular, Cx32 interacts with connexin26 and with the mitochondrial protein, Sideroflexin-1 (SFXN-1) at the PM. Cx32 interaction enhances Cx26 stability. Converging bioinformatics, biochemical, and confocal analyses support a role for Cx32 in transiently tethering mitochondria to Cx32-enriched membrane microdomains through interaction with proteins in the outer mitochondrial membrane (OMM), including SFXN-1. Complex formation increases the pool of SFXN-1 that IPs with Cx32 at the PM. Together, these data identify a novel PM/mitochondrial signaling nexus in the Cx32 interactome.

2.4. Introduction

The formation of gap junction channels between two adjacent cells allows for direct cell-cell communication (Simon and Goodenough 1998). Functional diversity is dictated by their structural subunits, a family of 21 connexin proteins in humans (Willecke et al. 2002). Oligomerization of six connexins forms an aqueous pore when inserted into target membranes (Stout et al. 2004). Each pore, known as a connexon or hemichannel, makes up one half of a gap junction channel. In non-junctional PMs, connexons allow for regulated passage of ions and small molecules up to 1.8 kDa to and from the extracellular space (Neijssen et al. 2005; Stout et al. 2004). In junctional PMs, end-to-end docking of two apposing hemichannels enables gap junction intercellular communication (GJIC). Between adjacent membranes of the same cell, connexon alignments create reflexive junctions acting as conduits between membrane compartments (e.g., allowing for molecular traffic from perinuclear to adaxonal cytoplasm between processes of axon-ensheathing oligodendrocytes) (Nualart-Marti et al. 2012). Signaling specificity is achieved by 'mixing and matching' connexin subunits. Assemblies of identical (homotypic channels) or diverse pairings (heteromeric channels) define membrane channels with different permeabilities, gating properties, and docking compatibilities (Manthey et al. 2001).

Only recently have connexin-specific interacting partners been considered part of the gap junction protein signaling nexus. Cx43, the most abundant and ubiquitously expressed connexin, has received the most attention with approximately 25 intracellular interactors identified (reviewed in (Giepmans 2006; Herve et al. 2012)). Transient extracellular Cx43-Cx43 (and Cx26-Cx26) interactions along extracellular docking

domains also act as adhesive contacts, directing the migration of NPCs along radial glia (Elias and Kriegstein 2008; Elias et al. 2007). Finally, connexin complexes can also form functional protein complexes in intracellular organelles. Using selected MS/MS ion monitoring validated by immunofluorescence, Cx43 has been identified in the IMM of cardiac myocytes (Miro-Casas et al. 2009). Using classic biochemical methods, Cx32 has also been reported in hepatic mitochondria but without further sub-organelle localization (Miro-Casas et al. 2009). MS methodologies, however, have fallen short of the level of sensitivity required to explore these mitochondrial interactomes, given difficulties associated with purifying sufficient amounts of membrane-integrated connexin protein.

Mutations in Cx32 are the genetic determinant of CMTX, a debilitating demyelinating peripheral neuropathy (Bergoffen et al. 1993a). Loss of Cx32 (or depletion from the PM) enhances tumourigenesis in liver (Krutovskikh et al. 1994; Moennikes et al. 1999; Omori et al. 2007). Here, to identify potential Cx32 interacting partners that may prevent hepatic disorders when complexed with Cx32, we enriched for connexin-specific membrane microdomains by subcellular fractionation (Miro-Casas et al. 2009), and adapted our IP-HTMS methodology (Abu-Farha et al. 2008; Abu-Farha et al. 2011; Ewing et al. 2007) to study endogenous integral membrane protein complexes. We show that Cx32 can be detected at both the PM and the IMM of healthy hepatocytes and interacts with PM, cytosolic, ER, and mitochondrial resident proteins. Bioinformatic analysis, supported by confocal microscopy, provides further support for an intriguing role for Cx32 in transiently tethering mitochondria to PM microdomains.

2.5. Materials and methods

2.5.1. Generation of N15 C57BL/6 Cx32 KO and congenic WT mice.

Cx32 null-mutant breeding pairs, kindly provided by Dr. Klaus Willecke (Universitat Bonn, Germany) (Nelles et al. 1996), were backcrossed for 15 generations onto a C57Bl/6 background in the Bennett laboratory. Congenic WT mice were derived from heterozygote matings. Male mice used in this study were between 3 and 4 months of age. Genotyping was confirmed at time of weaning and again at time of sacrifice. PCR amplification was performed on a Whatman Biometra TGradient96 system. DNA, isolated from tail snips, was amplified using Primers A, B, and C (A: 5' – TCA TTC TGC TTG TAT TCA GGT GAG AGG CGG – 3', B: 5' – ATA CAC CTT GCT CAG TGG CGT GAA TCG GCA – 3', C: 5' – TCT TAC TCC ACA CAG GCA TAG AGT GTC TGC – 3'). Primer A (forward) and B (reverse) generate a 750 bp WT amplicon. Primer A (forward) and C (reverse) generate a 1300 bp KO amplicon. Primers were synthesized at Integrated DNA Technologies Inc. Cycling parameters were: 95°C for 10 minutes, followed by 30 cycles of 95°C for 60 seconds, 67°C for 60 seconds, and 72°C for 60 seconds. For all studies, liver was dissected and gall bladders removed from both WT and KO mice. For fractionation studies, tissue was processed fresh. For validation IP and Western analysis, tissue was frozen in liquid nitrogen and stored at -80°C until required.

For measurements of ferritin, transferrin, iron, and total iron binding capacity, blood was collected by cardiac puncture from WT and KO mice (7 animals per genotype) between 1-8 months of age. Samples were clotted at RT for 1 h and centrifuged for 10 mins at 1500 x g. Recovered serum supernatant was sent on ice to the

Charles River Research Animal Diagnostic Services Laboratory (Wilmington, MA). Transferrin saturation was calculated as the percentage of iron to total iron binding capacity.

2.5.2. Sucrose gradient fractionation

For each experiment, one liver lobe was homogenized in 1 mL PTN buffer (50 mM sodium phosphate, 1% Triton X-100 [T-8787, Sigma-Aldrich], 50 mM NaCl, pH 7.4, fresh protease inhibitor cocktail (10 mM dithiothreitol (DTT) [Promega #V3151], 1 mM phenylmethylsulfonyl fluoride (PMSF) [OmniPure #7110], 5 µg leupeptin, [Pierce 78435], and 1 µg pepstatin A [Bioshop # PEP 605]) using a Teflon Potter-Elvehjem homogenizer fitted to a 30 mL glass tube. Homogenates were incubated on ice for 30 minutes and centrifuged at 12,000 rpm for 3 minutes at 4°C to remove nuclei. The Triton X-100 supernatant (containing the insoluble membrane fraction) was reserved on ice and diluted with an equal volume of 80% sucrose. Resulting homogenates (40% sucrose) were overlaid with equal volumes of 30% and 5% sucrose in Beckman polyallomer centrifuge tubes, (14x89 mm, #331372) and centrifuged at 32,085 rpm (130,000 x g_{ave}) in an SW40 Ti swinging-bucket rotor overnight (18 hours) at 4°C. Gradients were carefully aliquoted (10 fractions of 500 µL) with fraction 1 being the uppermost, lightest fraction. Protein quantification was performed using the Bio-Rad DC protein assay kit (Bio-Rad, #500-0112).

2.5.3. Optiprep (OP) purification of liver PM and mitochondria

Four livers per genotype (WT and KO) were dissected in ice-cold PBS (10 mM phosphate, 154 mM NaCl). PBS was decanted following collection and tissue weighed prior to being minced with scissors until homogenous. Tissue pieces were washed with

three changes of ice-cold Solution B (0.25 M sucrose, 1 mM EDTA, 2 mM MgCl_2 , 20 mM HEPES-NaOH, pH 7.4). Five mL of solution B with fresh protease inhibitors (1 mM sodium fluoride [Sigma #S-1504], 50 $\mu\text{g/mL}$ aprotinin [Sigma #A-6279], 1 mM sodium orthovanadate [Sigma #S-6508], 1 mg/mL PMSF) was added to the crude lysate at twice the volume per wet weight (g) of liver tissue (e.g., 5 mL buffer A per 2.5 g liver tissue). Lysates were homogenized using a tight-fitting dounce homogenizer and Teflon pestle (approximately 30 strokes). Lysates were transferred to 15 mL Falcon tubes and Solution B was added to fill the tubes prior to centrifugation at 3100 rpm ($1,561 \times g_{ave}$) in a GH-3.8A rotor (Beckman-Coulter) at 4°C for 10 minutes. The supernatant was collected with a Pasteur pipette, transferred to 1.5 mL polyallomer centrifuge tubes (Beckman #357448, with adaptors #355919), and centrifuged at 48,537 rpm ($100,000 \times g_{ave}$) in a TLA100.3 rotor for 50 minutes. The supernatant (containing cytosolic proteins) was discarded, and the pellets were combined and re-suspended in in a 35% OP solution (Sigma #D15556). The 35% OP solution was prepared by diluting a 50% working solution of OP in Solution B (50% working solution of OP: 5 parts OP with 1 part diluent [0.25 M sucrose, 6 mM EDTA, 12 mM MgCl_2 , 120 mM HEPES-NaOH, pH 7.4]). Subsequent dilutions of OP were also prepared by diluting the 50% working solution with Solution B as above.

A discontinuous OP gradient was prepared on ice by slowly overlaying 2 mL of the resuspended pellets with 1 mL 30% OP, 2.5 mL each of 25% and 17.5% OP, and 2 mL each of 10%, and 2.5% OP solutions into 13.2 mL (14 x 89 mm) polyallomer centrifuge tubes. Solution B was added to a point one mm below the surface of the tube (~1 mL). Tubes were carefully balanced and adjusted with Solution B, if

necessary, before assembly into an SW41 Ti rotor (Beckman Coulter). Gradients were centrifuged at 36,555 rpm ($165,000 \times g_{ave}$) at 4°C for 3.5 hours (acceleration = 1, deceleration = 0). The interfaces between the gradients were collected in 0.5 mL volumes by tube puncture. The collected interfaces were diluted with two volumes of Solution B and centrifuged in a TLA100.3 rotor for 50 minutes at 48,500 rpm to collect the pellet. Pellets were re-suspended in RIPA buffer (10 mM PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)) with fresh protease inhibitors (1 mM sodium fluoride, 50 µg/mL aprotinin, 1 mM sodium orthovanadate, 1 mg/mL PMSF) and assayed for protein concentration using the Bio-Rad DC protein assay kit.

2.5.4. Crude mitochondrial isolation

One liver per genotype was placed in ice-cold PBS. Tissue was minced with scissors and washed in PBS. Two volumes of buffer A (20 mM HEPES, 220 mM mannitol, 68 mM sucrose, 80 mM KCl, 0.5 mM EGTA, 2 mM magnesium acetate, 1 mM DTT, pH 7.4, and fresh protease inhibitors (1 mM NaF, 50 µg/mL aprotinin, 1 mM sodium orthovanadate, 1 mg/mL PMSF) were added and tissue homogenized in a dounce homogenizer with Teflon pestle until very smooth (about 30 strokes). The homogenate was centrifuged at 3000 rpm ($738 \times g_{ave}$) in a JA25.50 rotor (50 mL tube) at 4°C for 20 minutes. The supernatant was collected with a Pasteur pipette and transferred to a new tube for centrifugation at 8000 rpm ($5,251 \times g_{ave}$) at 4°C for 15 minutes. This supernatant was discarded and the pellet was washed with 30 mL of buffer A at 8000 rpm at 4°C for 15 minutes as before. The mitochondrial pellet was resuspended in 1 mL buffer A.

2.5.5. Subfractionation of mitochondria following sucrose fractionation

Crude mitochondria were subjected to sucrose gradient fractionation with the following modifications: Mitochondrial samples were mixed with 80% sucrose to achieve a final concentration of 60% sucrose (4 mL total), and were overlaid with 50%, 40%, and 30% sucrose layers (2 mL each), followed by 2 mL homogenization buffer (minus DTT), and centrifuged at 35,000 rpm ($151,263 \times g_{ave}$) for 16 hours at 4°C. Twenty-four fractions of 500 μ L each were collected. WT and KO fractions (WT: fractions 18-22; KO: fractions 17-21) were pooled and protein concentrations assayed using the Bio-Rad DC protein assay kit. Each set of pooled mitochondrial fractions were diluted 2:1 with cold buffer A. Mitochondria were pelleted by centrifugation at 8000 rpm for 15 minutes. Pellets were re-suspended in cold buffer A and 0.6% digitonin (#D141, Sigma-Aldrich) and incubated on ice for 10 minutes to selectively permeabilize the OMM. Mitochondria were again collected by centrifugation at 10,000 g for 10 minutes at 4°C. The supernatant containing outer membrane proteins was reserved. Mitoplasts were re-suspended in 150 mM KCl with fresh protease inhibitors and incubated on ice for 10 minutes prior to centrifugation at 10,000 g for 10 minutes at 4°C. The supernatant containing IMM proteins was reserved.

2.5.6. Endogenous immunoaffinity purification for HTMS

Anti-Cx32-coupled protein G agarose beads were prepared as follows: One mL of a protein G agarose bead slurry (1:1 PBS; Roche #20399) was incubated with 30 μ g monoclonal anti-Cx32 antibody (CX32C13-M, Alpha Diagnostics) overnight at 4°C. Beads were washed with 10 mL of 0.1 M borate buffer (pH 9.0) and re-suspended in 10 mL of the same buffer. Anti-Cx32 antibody was chemically coupled to the protein G

beads by the addition of solid dimethyl pimelimidate (DMP) (Pierce # D8388) to a final concentration of 20 mM. Beads were incubated for 30 minutes at room temperature. The cross-linking reaction was stopped by washing twice with 0.2 M ethanolamine (Sigma #E-9508, pH 8.2). Beads were re-suspended in 10 mL of 0.2 M ethanolamine and incubated at room temperature for 2 h, followed by two washes with 10 mL of PBS. Beads were re-suspended in PBS to a final volume of 1 mL and stored at 4°C. One mg total protein of pooled sucrose fractions 4, 5, and 6 was used as input material for the 1X reaction. Five mg total protein was used for the 5X reaction. The pooled fractions were diluted 1:2 in fresh RIPA buffer with protease inhibitors (1 mM sodium fluoride, 50 µg/mL aprotinin, 1 mM sodium orthovanadate, 1 mg/mL PMSF) and precleared with 100 µL of uncoupled protein G agarose beads per 1 mL of protein lysate for 1 hour at 4°C. Precleared lysates (or an equal volume of RIPA buffer for the no lysate (NL) control) were added to either 400 µL (1X reaction) or 2 mL (5X reaction) anti-Cx32-coupled beads and incubated overnight at 4°C. Beads were washed once in RIPA buffer with 250 mM NaCl, once in RIPA buffer with 125 mM NaCl, and three times in PBS (10 mM) for two minutes with gentle inversion. Proteins were eluted at room temperature for 30 minutes with inversion in one bead volume of ammonium hydroxide elution buffer (0.5 M NH₄OH, 0.5 mM EDTA). Samples were lyophilized in a SpeedVac and solubilized in 45 µL 2X SDS sample buffer (6X buffer diluted to 2X with 0.35M Tris-HCl/0.28% SDS, pH 6.8 buffer [6X buffer: [0.35M Tris-HCl/0.28% SDS, pH 6.8 buffer], and 5% glycerol, 10% SDS, 100 mM DTT, 0.002% bromophenol blue]) with 10% β-mercaptoethanol (BME) at room temperature for 30 minutes. The SDS-solubilized proteins were resolved on NuPAGE Novex 4-12% Bis-Tris gels (NP0335BOX, Invitrogen) at 100 V for 20 minutes,

followed by 150 V. Gels were fixed for 30 minutes in a 5% acetic acid:methanol solution and rinsed twice with double distilled water (ddH₂O) for two minutes each. To minimize background, gels were washed in ddH₂O overnight with shaking at 4°C, sensitized for 2 minutes in a 0.02% sodium thiosulfate solution, and rinsed twice for 30 seconds each with ddH₂O. Fresh silver nitrate (0.1%) solution was incubated for 30 minutes, followed by two 30-second washes in ddH₂O. Gels were developed with two changes of freshly prepared 0.01% formaldehyde in 2% sodium carbonate solution, rinsed with 1% acetic acid, and stored in the same solution at 4°C.

2.5.7. HTMS analysis

HTMS was performed as described (Abu-Farha et al. 2008; Abu-Farha et al. 2011; Ewing et al. 2007) with the following modifications. Briefly, all surfaces in contact with the gel were wiped with 70% ethanol and precautions were taken to minimize contamination of gel and gel pieces with keratin and dust in air. Protein bands were excised on a clean glass plate with a #11 scalpel blade (blade was wiped with 70% ethanol before use, and changed between each band). Gel bands were cut into smaller pieces approximately 3 mm², transferred to 0.6 mL Eppendorf tubes, and washed by vortexing in 50 µL Solution A (50 mM ammonium bicarbonate [EMD #00113164]). The liquid was discarded following a brief centrifugation. Gel pieces were partially dehydrated in 50 µL Solution B (50% acetonitrile [ACN; J.T.Baker #9829-03], 25 mM ammonium bicarbonate) for 15 minutes at room temperature, centrifuged briefly, and the liquid was discarded. Samples were dried in a SpeedVac for 5 minutes without heating, followed by an incubation in 30 µL Solution C (50 mM ammonium bicarbonate, 10 mM DTT) for 15 minutes at 56°C to re-hydrate the gel pieces and reduce disulfide

bonds. Tubes were cooled at room temperature for 15 minutes, centrifuged briefly, and the liquid discarded. To inhibit re-formation of disulfide bonds, thiols were quenched by adding 30 μ L Solution D (100 mM iodoacetamide [SIGMA #I1149] in 50 mM ammonium bicarbonate) in a 15 minute incubation in the dark. Samples were centrifuged, washed in Solution A for 15 minutes, partially dehydrated in Solution B for 15 minutes, centrifuged briefly, and dried in a SpeedVac for 5 minutes without heating. Trypsin enzyme solution was prepared by adding 100 μ L re-suspension buffer to 20 μ g porcine trypsin (Promega, #V5111) and mixing the re-suspended enzyme with 310 μ L of Solution A (sufficient for 10 samples). The enzyme solution was added to each sample at 28 μ L/tube, incubated on ice for 10 minutes, excess solution removed and 10 μ L of Solution E (25 mM ammonium bicarbonate) added to each tube incubated at 37°C overnight. Samples were centrifuged briefly and the solution was transferred to a clean tube. Fifty μ L of Solution E was added to the gel pieces and incubated at room temperature for 20 minutes with occasional vortexing. Analytes were pooled with the other 10 μ L of Solution E from the previous step. Peptides were extracted by incubating each sample in 50 μ L Solution F (5% formic acid [J.T. Baker #9832-03] and 50% ACN) for 20 minutes. The tubes were centrifuged and the peptide solution was pooled into the Solution E washes. Samples were lyophilized and stored at -20°C.

LC-MS/MS was performed by dissolving the peptide samples in 5% formic acid and loading into a 200 μ m x 5 cm precolumn packed in-house with 5 μ m YMC ODS-A C18 beads (Waters, MA, #YMC086529) using a micro Agilent 1100 HPLC system (Agilent Technologies). Peptides were desalted on-line with 95% water (J.T. Baker #9831-03), 5% ACN, and 0.1% formic acid (v/v) for 10 minutes at 10 μ L/min. The flow

rate was split before the precolumn to produce approximately 200 μ l/minutes flow at the column. Following elution, peptides were directed to a 75 μ m x 5 cm analytical column packed with 5 μ m YMC ODS-A C18 beads and eluted using a 1 h gradient (15 to 80% ACN with 0.1% formic acid) into a LTQ linear ion-trap mass spectrometer (Thermo-Electron, USA). MS/MS spectra were acquired in a data-dependent acquisition mode automatically selecting and fragmenting the five most intense peaks from each spectrum generated. Peak lists were generated from the MS/MS .raw file using Mascot Distiller 2.0.0.0 (Matrix Science) to produce .mgf files under default parameters with the exception that for each MS/MS peak, lists were generated assuming a 2+ and a 3+ charge. All .mgf files were analyzed and matched to the IPI database using the Mascot 2.1.04 database search engine (Matrix Science) with trypsin as digestion enzyme, carbamidomethyl of cysteine as a fixed modification, and methionine oxidation as a variable modification. Peptide and MS/MS mass tolerances were set at $\pm$ 2 Da and $\pm$ 0.8 Da, respectively, with 1 miss-cleavage tolerance and the significance threshold set to 0.01 ($p < 0.01$). Finally, an ion score cut-off of 30 was chosen to produce a false-positive rate of less than 1% in the MS data. The most logical assignment of the peptide in the database was reported, and when peptides matched to more than one database entry, only the highest scoring protein was considered. The network of proteins associating with Cx32 was arranged according to subcellular localization and visualized in an interaction network generated using the Osprey Network Visualization System (Osprey version 1.2.0; Samuel Lunenfeld Research Institute). Molecular functions and protein networks were analyzed using the Database for Annotation,

Visualization and Integrated Discovery (DAVID) v6.7 (Huang da et al. 2009) and Ingenuity Systems Pathway Analysis software (IPA version 8.8, Ingenuity Systems Inc.).

2.5.8. Validation of Cx32 interacting partners by co-IP

Validation of protein-protein interaction by co-IP was performed as described above with the following minor modifications: Protein G beads were coupled with monoclonal Cx32 or polyclonal antibodies (Table 1) directed against proteins identified by HTMS analysis. Input proteins for co-IPs were RIPA lysates of homogenized liver tissue. Livers were placed in borosilicate glass culture tubes (VWR, #47729-570) in 2 mL ice-cold RIPA buffer with fresh protease inhibitors. Tissue was homogenized using a variable speed Tissue Tearor (Biospec Products #985370-395) and passed through a 26-gauge needle 8-10 times to break up nuclei. Samples were incubated on ice for 20 minutes, and centrifuged at 16,000 rcf for 30 minutes. Post-nuclear supernatants were adjusted to 5 mg/mL protein with fresh RIPA buffer, and 1 mg of protein was used in each co-IP. Protein G agarose bead volumes were scaled down to 50 μ L (antibody:bead ratio was maintained). In Figure 2C, 50 μ g of input protein and 10 μ L of Protein G agarose beads were used for the PM and mitochondrial IPs. NL controls were also performed substituting an equal volume of RIPA buffer. Eluted, dried, and re-suspended IP product was run on 12.5% Tris-HCl polyacrylamide gels (prepared in-house) and transferred to Immobilon PSQ polyvinylidene difluoride (PVDF) membranes (Millipore, MA, # IPVH00010) for 60 minutes at 100 V or overnight at 22 V.

2.5.9. Immunoblotting

Liver isolated from WT and KO mice was homogenized in RIPA buffer containing protease inhibitors (1 mM NaF, 50 µg/ml aprotinin, 1 mM sodium orthovanadate, 1 mg/mL PMSF, 10 mM PBS). Alternatively, fractions and organelle separations described above were analyzed. Protein concentrations are indicated in each figure caption. Samples processed for Western analysis were diluted to a 1X final concentration using 6X SDS sample buffer (0.35 M Tris-HCl/0.28% SDS, pH 6.8, and 5% glycerol, 10% SDS, 100 mM DTT, 0.002% bromophenol blue) with 10% BME and solubilized at room temperature for 30 minutes. Samples subjected to IP and lyophilization were re-suspended in 2X final SDS sample buffer. Proteins were resolved under reducing/denaturing conditions on 10% or 12.5% Tris-HCl polyacrylamide gels (prepared in-house) and transferred to Immobilon PSQ PVDF membrane at 100 V for 60 minutes. Membranes were blocked in 5% (w/v) skim milk powder-PBS (10 mM phosphate, 154 mM NaCl) with 0.1% Tween-20 (blocking buffer) for 1 h and incubated in primary antibody diluted in the same buffer overnight at 4°C (Table 1). Membranes were rinsed twice in 0.1% PBST (10 mM phosphate, 154 mM NaCl with 0.1% Tween-20) and twice in blocking buffer for 10 minutes prior to a 1 h incubation in horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit (Jackson ImmunoResearch Laboratories, Inc., PA; [anti-mouse #115-035-146; 1:2000] and [anti-rabbit #711-096-152; 1:5000]) secondary antibody diluted in blocking buffer. Signal was detected on X-ray film using SuperSignal West Pico Chemiluminescent Substrate (Pierce, #34808). Densitometry was performed using Image J v1.47b software (NIH). Data were expressed as the percentage of SFXN-1 normalized to Na⁺K⁺-ATPase localizing to either PM or mitochondrial membranes in KO samples relative to WT.

2.5.10. HEK293T culture and transfection of human Cx32 gene

HEK293T cells were maintained in Dulbecco's modified Eagle medium/F-12 (DMEM/F-12; Invitrogen #12400-024) with 10% fetal bovine serum (Invitrogen, #12483-020) and 1% penicillin/streptomycin antibiotic cocktail. The full cDNA coding sequence of the human Cx32 gene (Accession #: NC_000023) was cloned into the pcDNA6TM/V5-His B backbone (Invitrogen, #V220-01), and the empty vector was used as a negative control. HEK293T cells were plated in 6-well tissue culture plates on #1.5 glass coverslips (Zeiss #474030-9000-000). 4 µg of plasmid DNA (human Cx32 or empty vector) and 10 µL of Lipofectamine 2000 reagent (Invitrogen, #11668-019) were each diluted in 250 µL warmed Opti-Mem reduced serum media (Invitrogen, #31985-088) and incubated for 5 minutes. The diluted plasmid and Lipofectamine solutions were gently mixed together and incubated for 20 mins. The medium in each well was replaced with 1.5 mL warmed DMEM with serum (no antibiotic), and 500 µL of DNA:Lipofectamine complexes. Cells were incubated with transfection reagents overnight, and protein expression was allowed to proceed for 24 hrs.

2.5.11. Confocal Microscopy

Cells were washed with 10 mM cold tissue culture PBS, fixed with 3.7% formaldehyde/PBS for 10 mins, and washed again in PBS. Fixed cells were incubated with monoclonal anti-Cx32 (1 µg/mL, #13-8200) and polyclonal anti-SFXN-1 (2.5 µg/mL) antibodies diluted in antibody buffer (10 mM PBS [10 mM phosphate, 154 mM NaCl], 3% BSA, 0.3% Triton-X, pH 7.2) overnight at 4°C. Cells were washed 3X in 10 mM PBS for 5 mins at room temperature, and incubated with Cy3 anti-rabbit IgG secondary antibody (1:800, Jackson ImmunoResearch Labs, Inc. # 711-166-152), and Atto488

anti-mouse IgG secondary antibody (1:1000, Alexis Biochem, #211-205TD-C100) in antibody buffer for 1 h at room temperature. Coverslips were washed 3X in 10 mM PBS for 5 mins at room temperature, and mounted onto glass microscope slides using ProLong® Gold antifade reagent (Invitrogen, #P36930). Colocalization was assessed by confocal laser scanning microscopy on a Leica TCS Sp5 (Leica Microsystems) in z-stacks captured with Leica LAS AF software (version 2.41) using an HCX PL APO CS 63X 1.4 oil objective with a pinhole size of 1 Airy unit (or equal optical slices for multiple fluorophores).

2.6. Results

2.6.1. Isolation of Cx32-enriched membrane microdomains by subcellular fractionation.

To enrich for membrane-integrated Cx32 microdomains, we used a modified lipid raft fractionation protocol to float triton-insoluble membranes in a sucrose gradient (Figure 2.1A). Given connexin antibody cross-reactivity (Fowler et al. 2009), KO fractionations were performed in parallel to ensure specificity (Supplemental Figure 2.1). In WT lysates, Cx32 was detected in fractions 4, 5, and 6 (Figure 2.1A,B). No labelling was observed in any of the KO fractions using the same monoclonal Cx32 antibody (Supplemental Figure 2.1, Table 2.1). Fractions 4, 5, and 6 were located at the interface of 5% and 30% sucrose layers, and in the lightest of the 30% sucrose gradients (Figure 2.1A). Fractions 4-6 were enriched for lipid raft markers caveolin-1 (Cav-1) and flotillin-1 (Flot-1) (Figure 2.1B, Table 2.1). Fractions 4 and 5 were also positive for IMM markers (cytochrome c oxidase subunit IV [CoxIV]) (Figure 2.1B and Table 2.1). The ER marker (heat shock 70kDa protein 5 (glucose-regulated protein,

Figure 2.1: Cx32 is enriched in lipid raft-containing membranes and mitochondrial membranes.

(A) Sucrose gradient fractionation schematic for detergent-resistant membrane flotation. **(B)** Cx32 was detected in fractions 4, 5, and 6 using monoclonal anti-Cx32 (Table 1). Five μg of protein was loaded in each lane. Triplicate fractionations were performed. One representative analysis is shown.

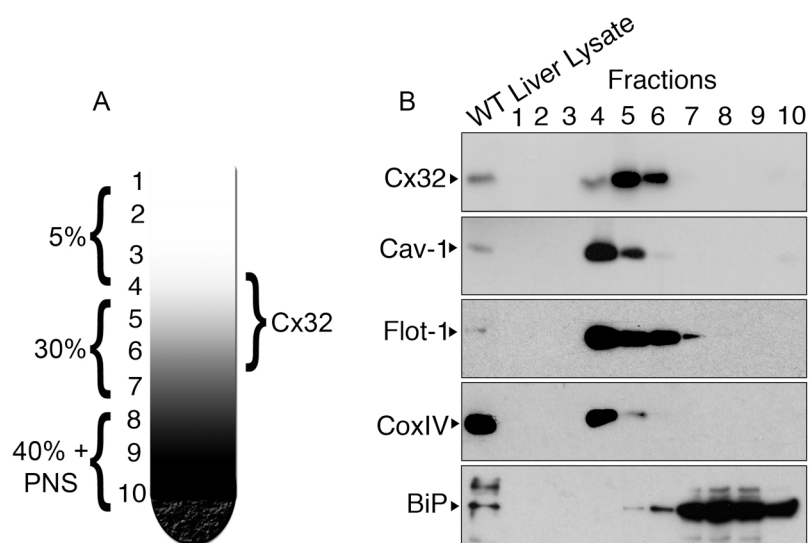


Figure 2.1

78kDa) [BiP]) was detected at low levels in fraction 5 and 6 (Figure 2.1B and Table 2.1). Fractions 7-10 were devoid of Cx32 and enriched for BiP (Figure 2.1B and Table 2.1).

2.6.2. Identification and validation of a Cx32 interactome in liver

Sucrose fractions 4-6 were pooled and incubated with monoclonal anti-Cx32 chemically coupled to protein G agarose beads (Table 2.1). WT Cx32 IP produced several bands that were visually absent in the KO purification in silver-stained gels, notably in the lower molecular weight range (Figure. 2.2A, boxes, WT vs KO). A lysate-free reaction was also prepared by incubating Cx32-coupled beads in RIPA buffer only (Figure 2.2A, NL). Immunoblotting liver lysates or 10% of the Cx32 WT and KO IP input with polyclonal anti-Cx32 antibody (Table 2.1) demonstrated strong, specific enrichment of Cx32 in pooled immunopurified fractions (Figure 2.2A', bottom panel compare WT F4-6 to WT IP F4-6 and KO, NL controls). Following excision of the unique silver-stained bands in WT and corresponding regions in KO lanes (Figure 2.2A, boxes), proteins were eluted from the gel pieces, digested with trypsin, and analyzed by LC-MS/MS. Table 2.1 presents Mascot scores, number of peptides, and the percent coverage for each protein identified from 5 mg of input protein (pooled liver fractions 4-6). Only proteins (a) with Mascot scores of 100 or higher in one or both paired analyses (b) for which at least two peptides were identified that were (c) absent from KO IP-HTMS analyses or (d) for which the same peptide was identified in IPs of both 1 mg and 5 mg WT protein fractions absent from KO both 1 mg and 5 mg analyses met criteria to be included in the reported network (Table 2.2).

Table 2.1: Primary antibodies employed.

Antibody ¹	Catalogue Number	Source	Type ²	[Western]	[IP] ³	[ICC]
ATP5A1	14676-1-AP	Proteintech Group	Polyclonal	0.2 µg/mL		
BiP (GRP78)	610979	BD Transduction	Monoclonal	2.0 µg/mL		
Cav-1	SC-894	Santa Cruz	Polyclonal	1.6 µg/mL		
Cx32	13-8200	Zymed, Invitrogen	Monoclonal			1 µg/mL
Cx32	CX32C13-M	Alpha Diagnostics	Monoclonal	0.5 µg/mL	1.25 µg/50 µL bead slurry	
Cx32	C3470	Sigma	Polyclonal	5.0 µg/mL		
Cx26	51-2800	Zymed, Invitrogen	Polyclonal	4.0 µg/mL	1.5 µg/50 µL bead slurry	
CoxIV	457325	Molecular Probes	Monoclonal	0.4 µg/mL		
Flot-1	610820	BD Transduction	Monoclonal	0.5 µg /mL		
Na ⁺ K ⁺ ATPae	05-369	Millipore	Monoclonal	0.5 µg/mL		
SFXN-1	12296-1-AP	Proteintech Group	Polyclonal	1.5 µg/mL	3.0 µg/50 µL bead slurry	2.5 µg/mL
VDAC1/Porin	ab15895	Abcam	Polyclonal	0.5 µg/mL		

¹Abbreviations: ATP5A1: ATP synthase, H⁺ transporting, mitochondrial F1 complex, alpha subunit 1, BiP: heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa), Cav-1: Caveolin-1, Cx32: Connexin32, Cx26: Connexin26, CoxIV: Cytochrome C Oxidase (Complex IV), Flot-1: Flotillin-1, Na⁺K⁺ATPase: Sodium-potassium adenosine triphosphatase, SFXN-1: Sideroflexin-1, VDAC1: Voltage-dependent anion-selective channel protein 1, IP: Immunoprecipitation, ICC: immunocytochemistry. ²All monoclonal antibodies were raised in mouse. All polyclonal antibodies were raised in rabbit. ³Bead slurry refers to a 1:1 ratio of protein G agarose beads and PBS.

Figure 2.2: Identification of novel Cx32 interactors by IP-HTMS of Cx32-enriched liver membrane fractions and validation by co-IP of tissue lysates.

(A) Silver stain of proteins found in sucrose gradient fractions 4-6 (Figure 2.1B) following IP with monoclonal anti-Cx32 (Table 2.1). Negative controls included IP reactions from KO fractions 4-6 (Supplemental Figure 2.1) and a NL mock IP reaction. Black boxes indicate gel regions excised for tryptic digest. Mobility of precipitating IgG heavy and light chains are indicated by arrowheads. **(A')** Western blot of Cx32-enriched fractions 4-6 (F4-6, 5 μ g) and 1 μ l (10%) of the corresponding IP products from these same fractions analyzed in (A). Analysis confirmed presence of bait (Cx32) in WT samples and lack of cross-reactivity in KO samples. Cx32-M indicates use of monoclonal anti-Cx32; Cx32-P indicates use of polyclonal anti-Cx32 antibody (Table 2.1). **(B)** IP-HTMS identified one known (Cx26) and 17 novel Cx32 interacting partners in replicate screens of 1 mg and 5 mg input protein. Interaction network generated by Osprey version 1.2.0 of proteins present in WT but not KO IP-HTMS. Proteins identified in both the 1 mg and 5 mg screens are represented with larger icons and thicker interaction lines than proteins identified only in the 5 mg screen. **(C)** Reciprocal co-IPs of liver lysates for Cx32 and Cx26. **(D)** Reciprocal co-IPs for Cx32 and SFXN-1. Specificity was also verified in independent IPs using an isotype IgG control (Supplemental Figure 2.2). **(E)** IP for Cx32 and blotting for ATP5A1 did not validate a Cx32-ATP5A1 interaction.

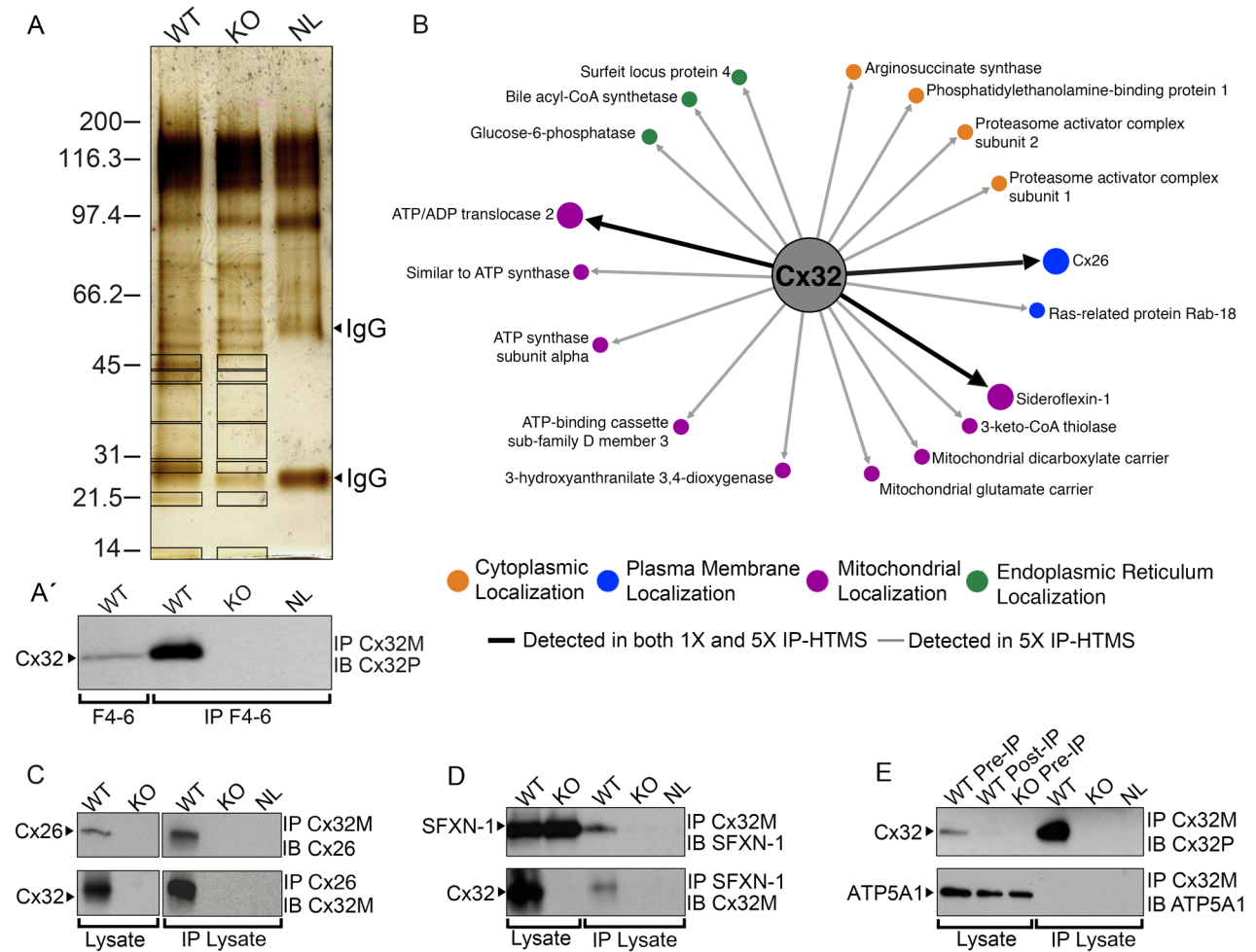


Figure 2.2

Table 2.2: LC-MS/MS identification of Cx32 and binding partners from liver tissue.

			Mascot score	# of peptides	% Coverage
IPI00118459	Gap junction beta-1 protein (GJB1, Cx32)	Cell junction; Cell membrane; Gap Junction	160/370*	12/40*	10/21.2*
IPI00125162	Gap junction beta-2 protein (GJB2, Cx26)	Cell junction; Cell membrane; Gap Junction	179/208*	4/15*	15/19.5*
IPI00115454	Sideroflexin-1 (SFXN-1)	Mitochondrion membrane; Ion transport	102/97*	1/1*	4/4*
IPI00127841	ADP/ATP translocase 2 (ANT-2, SLC25A5)	Membrane; Mitochondrion Transport;	57/241*	1/5*	2.7/21.1*
IPI00313236	Bile acyl-CoA synthetase (SLC27A5)	Membrane; ER	441	8	14.5
IPI00226430	acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase) (ACAA2)	Membrane; Mitochondrion; Lipid metabolism; Fatty acid metabolism	396	7	30.2
IPI00133249	Surfeit locus protein 4 (SURF4)	Membrane; ER; Golgi Apparatus	301	4	20.8
IPI00130280	ATP synthase subunit α , (ATP5A1)	Mitochondrion inner membrane; ATP synthesis; Ion transport	228	4	7.6
IPI00135646	ATP-binding cassette sub-family D member 3 (ABCD3)	Membrane; Peroxisome Mitochondrion inner membrane; Transport;	183	4	7.3
IPI00408961	3-hydroxyanthranilate 3,4-dioxygenase (HAD)	Cytoplasm; Pyridine nucleotide biosynthesis;	176	3	11.9
IPI00124223	Proteasome activator complex subunit 1 (PSME1)	Proteasome	165	3	15.6
IPI00317074	Mitochondrial dicarboxylate carrier (SLC25A10)	Mitochondrion inner membrane; Transport;	161	4	15.0
IPI00461407	similar to ATP synthase, H ⁺ transporting, mitochondrial F1 complex, O subunit	Mitochondrion inner membrane;	153	3	21.6
IPI00109275	Mitochondrial glutamate carrier 1 (GC-1, SLC25A22)	Mitochondrion inner membrane; Symport; Transport;	145	3	11.1
IPI00134746	Argininosuccinate synthase (ASS1)	Amino acid biosynthesis; Arginine biosynthesis; Ligase	118	2	6.3
IPI00119645	Glucose-6-phosphatase (G6pc)	ER; Gluconeogenesis; Carbohydrate Biosynthesis	111	2	8.4
IPI00137730	Phosphatidylethanolamine-binding protein 1 (PEBP1)	Cytoplasm; ATP/Lipid/Nucleotide Binding; Protease inhibitor	111	3	21.4
IPI00116770	Ras-related protein Rab-18 (RAB18)	Cell Membrane; Protein transport;	110	2	10.7
IPI00124225	Proteasome activator complex subunit 2 (PSME2)	Proteasome	108	2	9.6

*Asterisks indicate peptides detected in WT and not KO in two independent screens of 1 mg input and 5 mg input. Mascot scores and peptide coverage are reported only for both the 1 mg and 5 mg input screen separated by /. All other proteins were identified using 5 mg input only.

Eighteen interactors were identified (Figure 2.2B), including the known Cx32 binding partner, Cx26 (Diez et al. 1999). In total, 72% of the proteins identified by endogenous Cx32 IP-HTMS were intrinsic membrane proteins; 50% were mitochondrial resident proteins (Figure 2B). We validated by co-IP one PM (Cx26) and two IMM proteins, one novel (SFXN-1) and one predicted to be an artifact of detergent-insoluble sucrose fractionation (ATP5A1) (Zheng et al. 2009) (Figure 2.2C-E). Tissue lysates were used instead of fractionated membranes to identify robust interactions. Both Cx26 and SFXN-1 reciprocally co-immunoprecipitated with Cx32 (Figure 2.2C,D). KO controls validated specificity of the interaction, which was further confirmed by immunoprecipitating WT protein using an isotype control rabbit IgG antibody (Chemicon (Millipore), #PP64). Only the SFXN-1 antibody specifically immunoprecipitated Cx32 (Supplemental Figure 2). As expected (Zheng et al. 2009), we did not confirm a protein-protein interaction between Cx32 and ATP5A1 (Figure 2E) despite detection of Cx32 in the crude liver homogenates (pre-IP), effective IP, and clearing of target protein from lysate post-IP. ATP5A1 was detectable in both pre and post Cx32 IP as well as in the KO control (Figure 2.2E).

In addition to confirming antibody specificity and screening for non-specific interactions, we also used Cx32 null-mutant liver lysates to assess effect of complex formation on the stability of validated Cx32 binding partners. Loss of Cx32 effectively reduced total Cx26 but not SFXN-1 protein levels in liver. Where immunoblotting for Cx26 produced a band at approximately 24 kDa in WT liver lysates, Cx26 was barely detected in Cx32 KO lysates (Figure 2.2C, top panel, lysate, Supplemental Figure 2.3).

Loss of Cx32 had no effect on overall levels of SFXN-1, detected as a single band at approximately 35 kDa (Figure 2.2D, top panel, lysate).

2.6.3. Cx32 interacts with SFXN-1 at both the PM and the IMM

To explore the Cx32-SFXN-1 interaction, we sought to localize the Cx32-SFXN-1 complex with increasingly rigorous sub-cellular membrane fractionation techniques. Our initial IP-HTMS screen was performed targeting Cx32 primarily integrated into PM lipid rafts. In this separation, however, lipid rafts partially co-fractionate with mitochondrial membranes (Figure 2.1A). To separate plasma from mitochondrial membranes, we performed detergent-free fractionation of liver tissue using a discontinuous OP gradient (Figure 2.3A). In both WT and KO samples, PM markers (Na⁺K⁺-ATPase) were enriched at the 2%-10% interface; the VDAC1/porin marker for OMMs was detected at the 17.5%-25% interface (Figure 2.3B, WT M, KO M). In two separate gradients, mitochondrial membrane contamination was never detected in PM fractions; low levels of Na⁺K⁺-ATPase, however, were consistently detected in mitochondrial fractions (Figure 3B, compare Na⁺K⁺-ATPase with porin-positive fractions). SFXN-1 was enriched in mitochondrial (M) but was also detected at lower levels in PM fractions (PM) (Figure 2.3B). This finding was further supported by the distribution of SFXN-1 across fractions 4-6 of our original detergent-resistant membrane sucrose gradient representing both PM and mitochondrial membranes (Supplemental Figure 2.4).

Figure 2.3: Cx32 interacts with SFXN-1 at PM. Loss of Cx32 reduces SFXN-1 levels at the PM.

(A) Schematic of the OP gradient used to generate PM and mitochondrial membrane (M) fractions from WT and KO post-nuclear supernatants cleared of cytosolic proteins. The PM fraction floated at the interface between the 2.5% and 10% OP gradient, while the mitochondrial fraction was found at the interface between the 17.5% and 25% OP gradient. (B) SFXN-1 localizes primarily to the porin-positive mitochondrial fractions with some SFXN-1 detected in Na⁺K⁺ATPase-positive PM fractions. SFXN-1 levels at the PM were reduced in KO compared to WT. A replicate purification is presented in the right panel with less mitochondrial protein loaded to highlight the observation that, in KO liver tissue, SFXN-1 is reduced only in the PM and not from the mitochondrial fraction. Quantitation is presented in Results. (C) A Cx32 interaction with SFXN-1 is primarily detected at the PM but is also present in mitochondrial membranes following biochemical fractionation. (D) Confocal colocalization of ectopically expressed Cx32 (green) and endogenous SFXN-1 (red) in HEK cells transfected with human Cx32 suggests that *in situ* interaction is only detected at the PM at both junctional (insets and orthogonal views, arrow) and non junctional (arrow) membranes. (E) To further purify mitochondrial membranes from PMs and subfractionate inner (IM) and outer (OM) mitochondrial membranes, sucrose gradient flotation was performed on crude mitochondria purified under detergent-free conditions. Twenty-four fractions were collected from both WT and KO samples. Fractions were assayed for expression of Cx32 and cellular fractionation markers (Supplemental Figure 2.5). (F) In purified mitochondrial preparations, Cx32 localizes to IM; SFXN-1 localizes to both IM and OM.

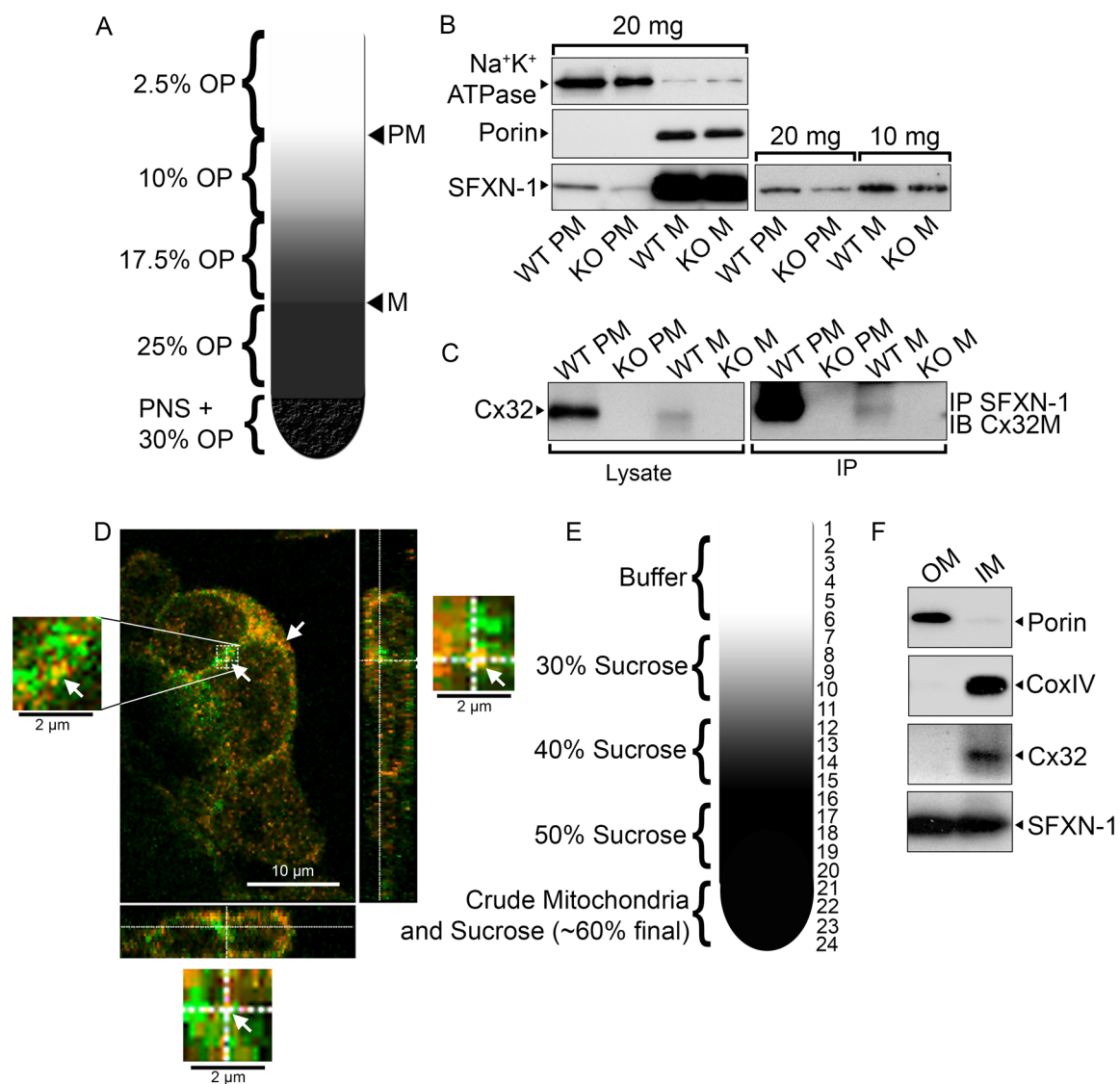


Figure 2.3

Next, pooled PM-specific or mitochondrial (M)-specific fractions isolated from the OP gradient (Figure 2.3A) were immunoblotted for Cx32 (Figure 2.3C, left panel). As expected, Cx32 was enriched in PM fractions of WT samples (Figure 2.3C, left panel, WT PM). Low levels of Cx32, however, were also present in mitochondrial fractions (Figure 2.3C, left panel, WT M). Finally, we immunoprecipitated SFXN-1 from both PM and mitochondrial membrane fractions, and immunoblotted for Cx32 (Figure 2.3C, right panel). The interaction between these two proteins was strongest at the PM, but was also detectable in mitochondrial membranes (Figure 2.3C, right panel). KO controls confirmed specificity of Cx32 detection in both compartments (Figure 2.3C, left and right panels).

To rule out the possibility that this mitochondrial pool of Cx32 was not carry over from contaminating PM present in mitochondrial fractions, we purified crude mitochondrial preparations under detergent-free conditions, isolated contaminating PMs from mitochondria by sucrose gradient flotation (Figure 2.3E, Supplemental Figure 2.5), and separated the inner and OMMs prior to western blotting. As shown in Supplemental Figure 2.5, contaminating PM in our mitochondrial preparations were found in fractions 8-15 overlapping with fractions 13-16 enriched for CoxIV (Supplemental Figure 2.5). Fractions 17-23 represented pure mitochondria devoid of PM contamination in both WT and KO flotations (Supplemental Figure 2.5). Consistent with the OP gradients (Figure 2.3A-C), Cx32 was enriched at PM but was also clearly detectable in the mitochondria-only fractions (Supplemental Figure 2.5). A biologically relevant interaction between Cx32 and SFXN-1 would require that both interacting partners be present in the same mitochondrial membrane compartments. OMMs were

further isolated from pooled fractions 17-23 using a 0.6% digitonin solubilization protocol. IMM in remaining mitoplasts were osmotically disrupted in a 150 mM KCl solution and collected by centrifugation. The OMM sample was enriched in VDAC1/porin and devoid of CoxIV (Figure 2.3F). The IM sample was enriched in CoxIV and devoid of VDAC1/porin (Figure 2.3F). Mitochondrial Cx32 localized exclusively to the IMM. SFXN-1 was found equally distributed across both OMM and IMM compartments (Figure 2.3F).

While these data clearly show that Cx32 and SFXN-1 are detected at both PM and IM, biochemical fractionation cannot rule out an exchange of interacting proteins from different compartments post-lysis. To address this, we localized *in situ* Cx32-SFXN-1 interactions by confocal microscopy in human Cx32-transfected HEK cells (Figure 2.3D). Cx32 (green) puncta overlap with the SFXN-1 (red) signal at both junctional membranes (arrows), and non-junctional membranes (arrows). Colocalization was observed at the cytoplasmic face of the PMs. This staining pattern is consistent with the hypothesis that Cx32 in gap junctional plaques at PM can interact with SFXN-1 at the PM and/or between apposed plasma and OMMs, but not with the hypothesis that the interactions can occur at the IMM (Figure 2.3D).

To explore consequences of this PM-OMM interaction, we asked whether SFXN-1 localization was influenced by its interaction with Cx32. We compared SFXN-1 levels in both mitochondrial and PM compartments of WT and KO liver. We found that localization of SFXN-1 at the PM, but not at IMM/OMMs, was reduced by $54\% \pm 17\%$ in KO liver as determined by densitometry of replicate independent fractionations compared to WT (100%) (Figure 2.3B). This change represented a shift in subcellular

localization (Figure 2.3B) without altering total SFXN-1 levels (Figure 2.2D) suggesting that PM Cx32 likely interacts with SFXN-1 resident in OMM and that detection of SFXN-1 at PM may represent a sub-pool of SFXN-1 removed from apposing mitochondrial membranes during co-IP without affecting total cellular levels.

2.6.4. Network analysis of dual PM/mitochondrial Cx32 interactomes

Taken together, the interaction of Cx32-SFXN-1 at the PM and reciprocal localization at the IMM suggested the presence of overlapping organelle-specific Cx32 protein networks in liver. To explore the nature of these networks, we performed functional annotation clustering using DAVID (Huang et al. 2009) and protein interaction network analysis using Ingenuity Systems Pathway Analysis software (Figure 2.4). Cx32 interactors clustered into two main functional classifications: (1) drug metabolism, lipid metabolism, molecular transport and (2) endocrine system development and function, molecular transport, cellular development. Pathway analysis identified three overlapping pathways implicated in (1) liver metabolic response to adipose-derived leptin, (2) liver response to insulin/insulin receptor signaling, and (3) interferon gamma responsiveness/iron metabolism impaired in hepatic carcinoma (Figure 2.4A). Dysfunction in leptin and insulin pathways, potentially mediated through disruptions in Cx32 interactions with SLC27A5, SLC25A10, ASS1, ABCD3, SLC25A5, and GP6 (Figure 2.4A) is consistent with previous reports that C57BL/6 X 129/SV Cx32 null-mutant mice exhibit a small reduction (~17%) in body weight (Nelles et al. 1996) also observed in our colony backbred to a pure C57BL/6 lineage (data not shown). Proposed regulation of interferon gamma responsiveness/iron metabolism by a Cx32 interactome with SFXN-1, PMSE1, PMSE2, and PEBP-1 (Figure 2.4A) is, however,

novel. To test directly whether Cx32 KO mice show any dysfunction in these pathways, we measured circulating ferritin (Figure 2.4B), transferrin (Figure 2.4C), iron (Figure 2.4D), total iron binding capacity (Figure 2.4E), and percent transferrin saturation (Figure 2.4F). Cx32-null mutant mice exhibited a mild, significant hyperferritinemia (Figure 2.4B) without any indication of systemic iron overload (Figure 2.4C-E) or changes in transferrin saturation (Figure 2.4F). Taken together, this phenotype is consistent with hyperferritinemia observed following liver injury and as a result of systemic inflammatory conditions (Naz et al. 2013).

Figure 2.4: Ingenuity network analysis of the Cx32 protein interactome.

(A) Proteins (listed by gene name) are represented as nodes (shapes indicate functional annotation). A documented biological relationship between two nodes is represented as a grey or orange edge (line). Grey edges represent interactions within a single network; orange edges cross-link nodes from multiple interacting networks. Proteins identified in our screen are represented as grey nodes with black edges (lines). White nodes represent gene products that participate in these networks, but were not identified in this study. Interactors that are not implicated in these primary networks are not included. The direct Cx26-Cx32 interaction validated in our screen is a known interaction and thus appears as a grey edge. All grey and orange edges are supported by references maintained by the Ingenuity Pathways Knowledge Base. Solid grey edges represent direct interactions; indirect interactions are represented by dashed lines. Circular lines describe a feed-back loop wherein a node has been shown to self-regulate. (B) Serum analysis of blood collected from WT and KO animals reveals that KO animals exhibit mild hyperferritinemia, without concurrent changes in serum transferrin (C) iron (D), total iron binding capacity (E), or % transferrin saturation (F).

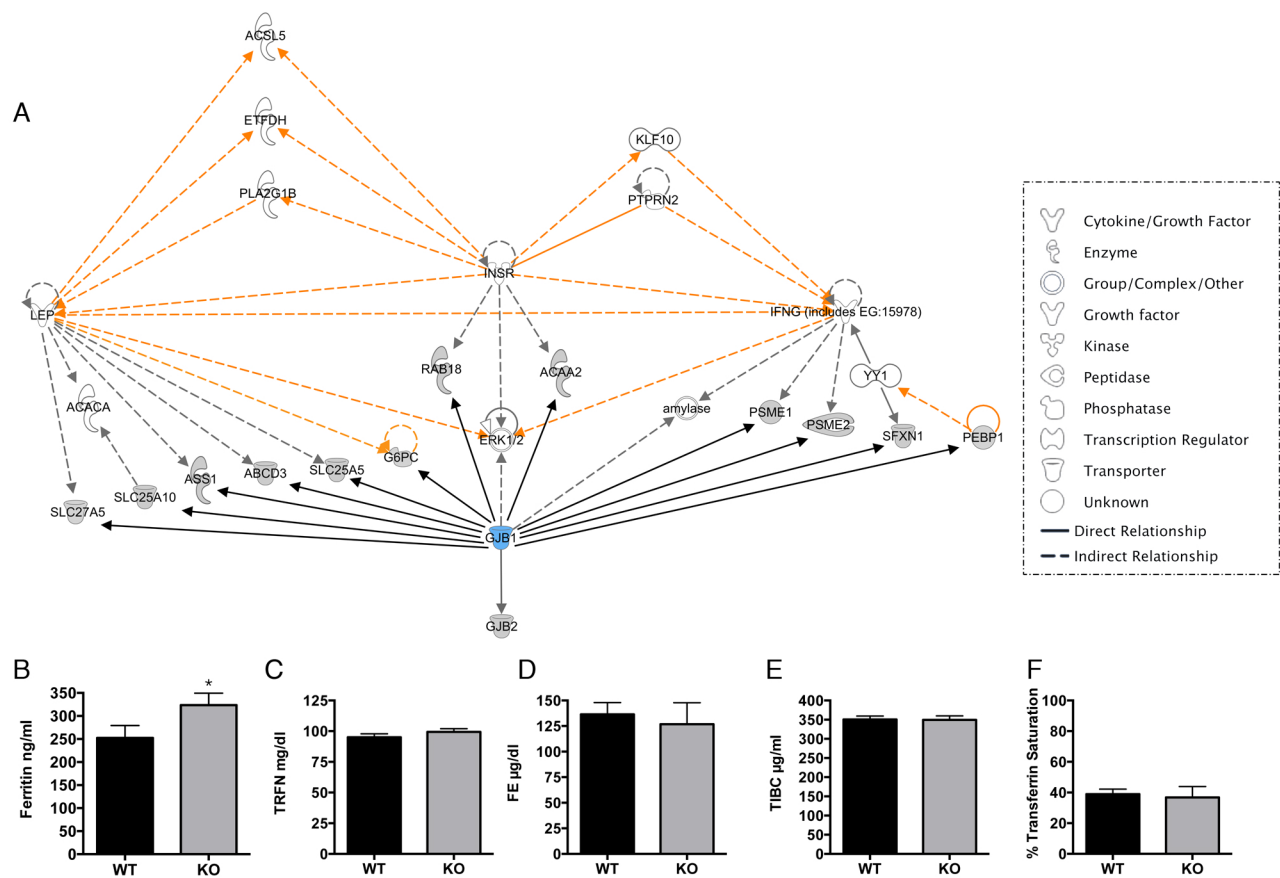


Figure 2.4

2.7. Discussion

Here, we report a novel endogenous Cx32-interacting network in mouse liver identified by immunoaffinity purification of Cx32-enriched subcellular membrane microdomains. Using unbiased IP-HTMS, we mapped 18 interacting partners. Fifty percent were resident mitochondrial proteins; 22% were cytoplasmic proteins; 17% localize to the ER, and 11% were predicted to be resident in PM. By reciprocal co-IP, subcellular fractionation, and confocal microscopy, we found that Cx32 forms protein-protein complexes at the PM (i.e., with Cx26) and either sequesters SFXN-1 at the PM or forms protein-protein complexes between Cx32 and SFXN-1 resident in apposing PMs and OMMs. Bioinformatic analysis places these interactions into three overlapping networks: (1) liver metabolic response to adipose-derived leptin, (2) liver response to insulin/insulin receptor signaling, and (3) interferon gamma responsiveness/iron metabolism. The observation that Cx32, integrated into PM, can interact with resident OMM proteins further suggests a novel role for connexins in possibly transiently tethering mitochondria to connexin-enriched PM domains (Figure 2.5). Together, these data highlight a novel PM/mitochondrial signaling nexus mediated by the Cx32 interactome.

This study represents the first endogenous connexin network mapped by unbiased MS approaches. The majority of reported connexin interactors have been identified using ectopically-expressed tagged proteins, or exogenous peptides as “bait” (Giepmans 2006; Herve et al. 2012; Palatinus et al. 2012). Endogenous partners have been inferred primarily from careful immunofluorescence co-localization studies (Giepmans 2006; Herve et al. 2012; Palatinus et al. 2012). These data have provided

essential insight into channel-independent connexin function, notably for Cx43 (Giepmans 2006; Herve et al. 2012; Palatinus et al. 2012), however, tissue-specific network analyses are lacking. To our knowledge, only two MS-based proteomic screens have been performed both using a C-terminal fragment of Cx43 as bait (Singh and Lampe 2003; Singh et al. 2005). The primary obstacle has been the challenge associated with affinity purifying endogenous membrane channel complexes in sufficient amounts for MS detection. To address this, we enriched for Cx32-positive membrane microdomains by subcellular fractionation prior to IP-HTMS. Cx32 null-mutant controls were used to identify and eliminate background interactors. Success of this strategy was validated by the fact that our top 'hit' was the known Cx32 binding partner, Cx26 (Diez et al. 1999). Cx26 and Cx32 have been shown to colocalize in heterotypic (and presumably heteromeric) channels in liver hepatocytes (Diez et al. 1999). Moreover, we also find that Cx26 protein levels are reduced in liver of Cx32 KO mice, consistent with earlier reports (Diez et al. 1999). This reduction is likely due to a loss of Cx26 protein stability in absence of Cx32/Cx26 complexes as steady-state Cx26 mRNA levels in WT and KO liver are comparable (Nelles et al. 1996).

Certainly, further improvements can be made to increase identification of connexin-interacting proteins by IP-HTMS. We have recently developed a gel-free centrifugal proteomic reactor to analyze membrane proteins (Zhou et al. 2011) and have begun to adapt this technology to analyze fractionated membrane microdomains enriched in Cx32. Our centrifugal proteomic reactor is a simplified, user-friendly, adaptation of gel-free digestion. We exploit the fact that most proteins are positively charged at pH <3 and thus a protein/trypsin mixture will bind tightly to a small

volume of strong cation exchange (SCX) beads while nonionic detergents and contaminants are readily washed away (Zhou et al. 2011). We then use DTT and iodoacetamide for protein reduction and alkylation, activate the trypsin by increasing pH to 8, and elute the resulting peptides in alkaline buffer for LC-MS/MS analysis (Zhou et al. 2011). Using this gel-free methodology, we have been able to detect 945 PM proteins and 955 microsomal membrane proteins in hepatic cells (Zhou et al. 2011). More than 800 out of these 945 and 955 proteins were not identified by the conventional in-gel digestion approach (Zhou et al. 2011). We anticipate that these improvements will further expand upon this study's identification of a mitochondrial protein-connexin interactome. Application will require further optimization as we have found that the phospholipid-Cx32 interactions (Locke and Harris 2009) in our fractionated microdomains complicate the required pH-dependent binding to cationic beads. Despite clear presence of Cx32 in IP samples, detection of bait connexins by HTMS has not been consistent using the centrifugal reactor (Zhou et al. 2011). We are currently exploring the efficacy of mild cross-linking followed by delipidation to circumvent this problem and increase the number of connexin binding proteins we can identify.

Despite the fact that we may be able to identify more interacting partners in future, we report here that the majority of novel Cx32 interactors detected were resident mitochondrial proteins. In previous targeted approaches, PM and cytoplasmic proteins predominate in connexin complexes. In rat hepatocytes, Cx32 has been shown to co-localize with the tight junction proteins occludin and claudin-1, and the scaffolding protein ZO-1 (Kojima et al. 2001; Penes et al. 2005). In the brain, oligodendrocytic Cx32 co-localizes with ZO-1 and the ZO-1-associated nucleic acid-binding protein

(Penes et al. 2005). In lipid rafts, Cx32 interacts with caveolin-1, the ubiquitously expressed calcium binding protein, CaM, and the membrane-associated guanylate kinase family member, Dlg-1 (Duffy et al. 2007; Schubert et al. 2002; Torok et al. 1997). Cx32 presumably sequesters Dlg-1 at the PM as acute removal enables its translocation to the nucleus (Duffy et al. 2007). Here, we show that the Cx32-SFXN-1 interaction may have a similar effect with Cx32 sequestering a subpool of the resident mitochondrial protein SFXN-1 to the PM. Comparison of our quantitative biochemical analyses and confocal localization studies, however, suggests that the primary function of the Cx32-SFXN-1 interaction is likely not to regulate localization of binding partners. First, *in situ* interaction with SFXN-1 at the PM assessed by confocal microscopy is not ubiquitous in Cx32-overexpressing HEKs. Second, unlike the effect of Cx32 null-mutation on Cx26 stability, overall SFXN-1 protein levels are not significantly altered, nor is mitochondrial subcellular localization determined by biochemical assessment in Cx32 KO hepatocytes. Rather, by confocal assessment, small, regionally distinct, pools of SFXN-1, apparently at the OMM, can be seen to co-localize with PM-resident Cx32 in direct apposition in HEKs ectopically expressing human Cx32. In null-mutant hepatocytes, SFXN-1 levels at PM are quantitatively reduced compared to WT as determined by direct biochemical assay and MS. Taken together, these observations provide tantalizing support for the emerging hypothesis that connexins may transiently tether mitochondria to PM when binding partners come into apposition (Delmar 2011) further supported by evidence that the complex association with Cx43 at the PM is transient yet strong enough to be co-immunoprecipitated (Sato et al. 2011).

Despite this evidence, we were mindful that mitochondrial contaminants routinely co-purify with detergent-resistant lipid raft preparations and are often misinterpreted as *bona fide* interactors (Zheng et al. 2009). Spurious interactions with F(1)/F(0) ATPase subunits and voltage-dependent anion selective channels are common (Zheng et al. 2009). Here, we used co-IP and bioinformatic analysis to rule out Cx32 interactions with the ATPase subunit ATP5A1 and the unidentified protein similar to ATP synthase, H⁺ transporting, mitochondrial F1 complex, O subunit identified in our IP-HTMS screen. We show definitively that ATP5A1 does not co-IP with Cx32. Moreover, F(1)/F(0) ATPase subunits are not predicted to participate in the three main pathways identified by Ingenuity Pathway analysis. Our validation, however, of SFXN-1-Cx32 complexes at both plasma and mitochondrial membranes is consistent with a previous report that Cx32 is present in isolated liver mitochondria (Miro-Casas et al. 2009). Indeed, Cx43 is certainly capable of translocating to the IMM of cardiomyocytes (Miro-Casas et al. 2009), where Cx43 channels protect cells from excitotoxic insult (Boengler et al. 2009; Rottlaender et al. 2010; Rottlaender et al. 2012a; Ruiz-Meana et al. 2008). It is, however, important to note that in this study, while we have biochemical evidence indicating that Cx32 and SFXN-1 are both present in IMM, we do not have evidence to suggest that an interaction occurs at this location. *In situ* imaging indicates that the primary interaction between these two proteins occurs between PM-localized Cx32 and OMM SFXN-1. Thus, while our data is the first demonstration that Cx32 is present at IMM, we must conclude that the low level Cx32-SFXN-1 interaction detected by IP in isolated mitochondrial membranes is likely due to contamination of these fractions with trace amounts of PMs as shown in our data. Given that intracellular interactions

between Cx32 and SFXN-1 were rarely detected in our HEK cell transfectants, we favour the hypothesis that the interaction seen in tissue lysates is due to transient interactions of OMM SFXN-1 with Cx32-containing gap junction plaques (Lebiedzinska et al. 2009).

SFXNs are an evolutionarily conserved family of mitochondrial tricarboxylate carrier proteins (Li et al. 2010; Miotto et al. 2007; Ye et al. 2003). SFXN-1 is a five-membrane spanning integral membrane protein predicted to be either a channel or carrier protein. Mutations in SFXN-1 are the genetic determinant of transient embryonic and neonatal anemia presented in the *flexed-tail (f/f)* mouse model, (Fleming et al. 2001) wherein defects in iron metabolism lead to a transient iron overload and intramitochondrial iron deposits in erythrocytes (Fleming et al. 2001). Interestingly, presentation of iron defects coincides with the onset of hepatic erythropoiesis, and persists through postnatal day 7 subsiding between the second and third week postnatal (Fleming et al. 2001). This time course corresponds closely with the developmental increase in hepatic Cx32 levels that do not reach adult levels until postnatal day 21 (Berthoud et al. 1992). Iron overload has been shown to reduce the presentation of Cx32 at PM in favour of intracellular distribution (Bilello et al. 2003). Thus, iron deficits in SFXN-1-deficient mice may promote the mitochondrial localization of Cx32, although this hypothesis remains to be tested. Here, we asked the reverse question: What effect does Cx32 null mutation have on ferritin status and iron metabolism? Based on the predicted involvement of Cx32 protein-protein interactions with the interferon gamma response in hepatocytes, we highlight a novel regulation of isolated hyperferritinemia observed in the absence of iron overload that is characteristic

of dysregulated chronic inflammatory responses (Naz et al. 2013). Ferritin elevations are seen in a number of liver disorders including alcoholic and non-alcoholic steatohepatitis, viral hepatitis, and acute liver injury (Beaton and Adams 2012). Moreover, elevated ferritin levels are associated with insulin resistance and purported to be the hepatic manifestation of metabolic syndrome (Beaton and Adams 2012). Our data implicate the connexin-interacting nexus identified here in leptin, insulin, and interferon gamma signaling pathways. Interestingly, these pathways also link the Cx32 interactome identified in this study to the regulation of liver tumorigenesis (Krutovskikh et al. 1994; Krutovskikh et al. 1995; Liu et al. 2009; Moennikes et al. 1999). For example, the interactions between Cx32 and the mitochondrial solute carrier proteins SLC25A10 and SLC25A5 as well as one of rate-limiting synthases in the arginine biosynthesis pathway (Ass1) are predicted to underlie the metabolic response to leptin in hepatocytes, notably in the exchange of citrate for malate between cytosol and mitochondria required for fatty acid synthesis, lipogenesis, and the regulation of protein catabolism, ammonia levels, and nitric oxide generation (Leung et al. 2012; Mizuarai et al. 2005). Cx32 interaction may act to stabilize membrane presentation, as shown here and by others for Cx26 (Nelles et al. 1996), given that both Cx32 and SLC25A5 are downregulated in human hepatocellular carcinomas (Krutovskikh et al. 1994; Lee et al. 2009). Certainly, mutations in ASS1 that impair protein stability are known causes of citrullinemia type I leading to liver failure, as well ethanol-induced liver injury (Faghfoury et al. 2011; Leung et al. 2012). This pathway analysis is consistent with previous reports that Cx32 null-mutant mice are mildly underweight compared to controls (Nelles et al. 1996).

Further, given that SFXN-1 likely facilitates transport of components, notably pyridoxine, required for iron utilization into and out of the mitochondria (Fleming et al. 2001), its function may depend upon a network of channel transporters moving required components through PM to mitochondria. The interaction of Cx32 at the PM and SFXN at the OMM could serve to tether a pool of subplasmalemmal mitochondria to the PM and potentially facilitate transport of pyridoxine into the mitochondria. In support of this hypothesis, another Cx32 interactor identified in this study, ANT-2, has been shown to function as both a mitochondrial and PM translocase (Detke and Elsabrouty 2008; Loers et al. 2012; Radichev et al. 2009; Sigal and Resh 1993). Moreover, the known Cx32 interactor caveolin-1 also binds to the ABCD transporters (Woudenberg et al. 2010), and our identification of a potential Cx32-ABCD3 interaction, possibly including caveolin-1 in complex, provides further support for the idea that Cx32 participates in the tethering of mitochondria to lipid raft domains. As Cx32 localizes to the basolateral membrane domains of hepatocytes wherein the majority of transporters are located (Fort et al. 2011), these interactions would presumably enable transient mitochondrial docking at site of metabolic import (Figure 2.5). Similar functional interactions have been described in developing muscle cells between the L-type calcium channel $\alpha 2/\delta 1$ subunit and the ATP5B subunit of the mitochondrial ATP synthase complex (Garcia 2011). This interaction is most pronounced during muscle cell differentiation, and, as observed here for the Cx32-SFXN-1 complex, occurs both in intracellular membranes and at the level of the PM (Garcia 2011).

Figure 2.5. Schematic of hepatic gap junctions showing possible sites of Cx32-SFXN-1 interaction.

Cx32 (orange) forms heterotypic and heteromeric gap junction channels with Cx26 (blue). Cx32 is also present on IMMs. SFXN-1 (red) is present on both outer and IMMs, and possibly at PM. Sites of Cx32-SFXN-1 interaction include 1) PM gap junctional plaques either as (i) transient interactions, (ii) novel PM resident proteins, (iii) part of the docking mechanism tethering subplasmalemmal mitochondria to the PM, or (iv) to promote the transport of small molecules into mitochondria, and 2) the IMM (unknown role).

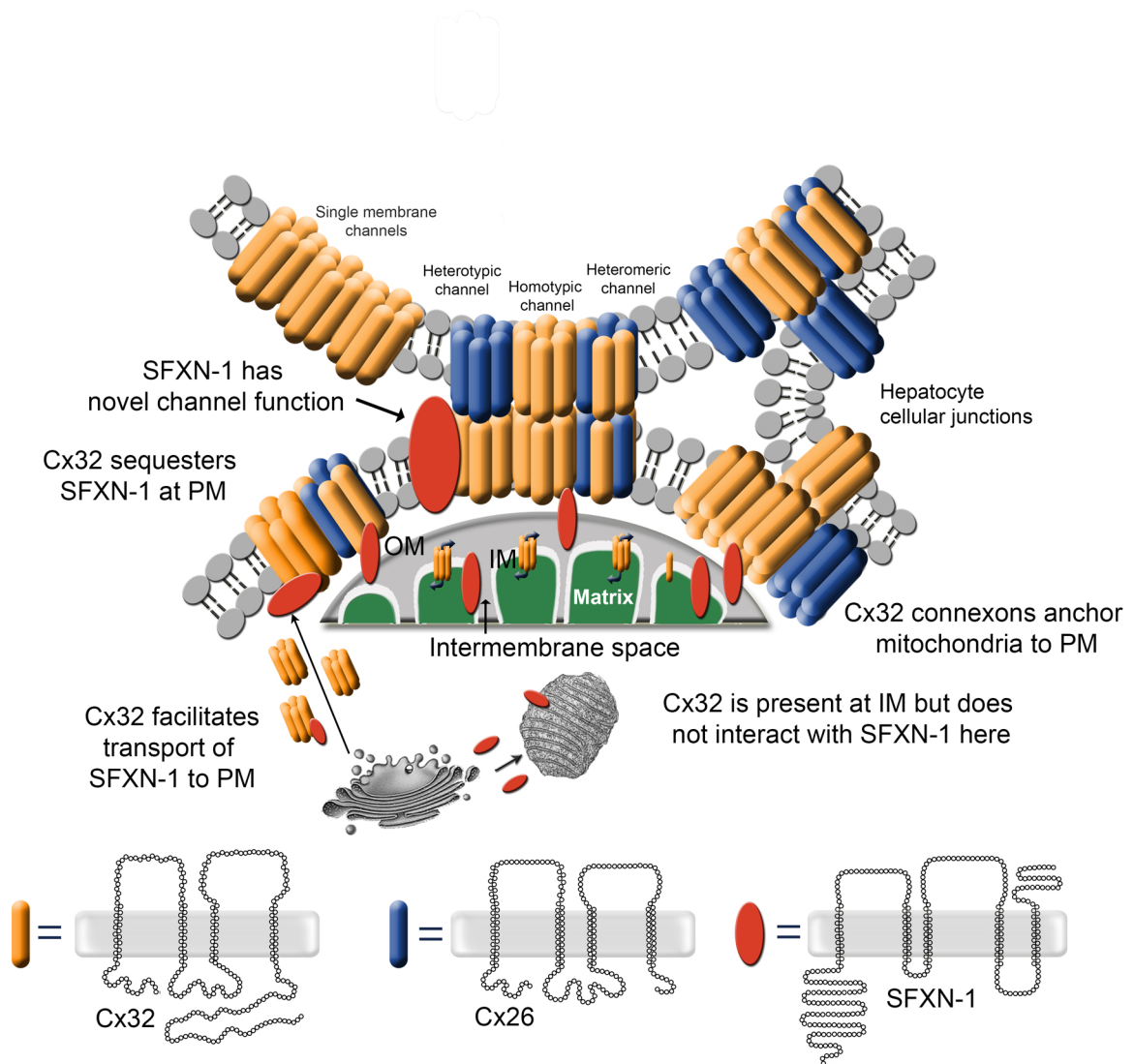
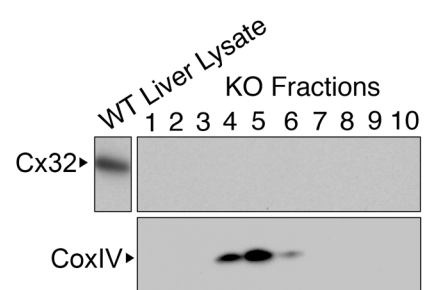


Figure 2.5

In summary, this work describes the identification of a network of liver-specific Cx32 interacting proteins enriched in mitochondrial targets. These interactions were validated by co-IP and detergent-free subcellular fractionation, and provide evidence for a novel signaling nexus made possible by Cx32 interactions with resident proteins at plasma and mitochondrial membranes that are implicated in liver function and hepatocyte growth, differentiation, and tumour progression.

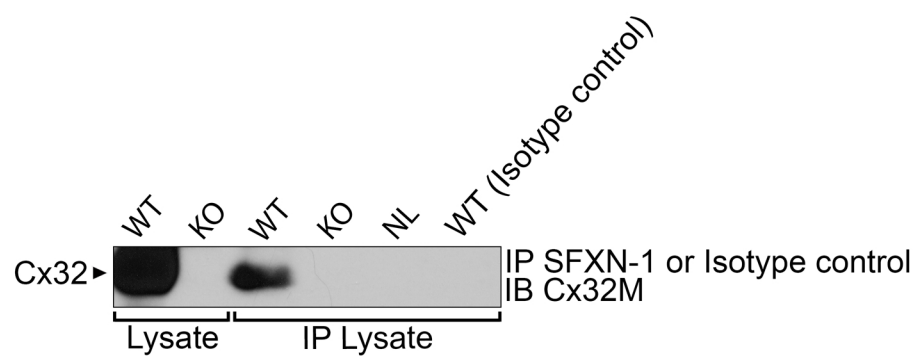
Supplemental Figure 2.1: Cx32 is not detected in KO sucrose gradient fractions.

To exclude non-specific interactors and confirm monoclonal antibody specificity (Table 1), KO liver tissue was fractionated as in Figure 1. CoxIV was used to confirm protein integrity in fractions 4-6. Note that the Cx32 lysate presented was immunoblotted at the same time and analyzed at the same exposure as the Cx32 fractions.



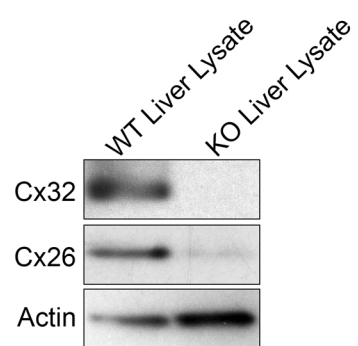
Supplemental Figure 2.2: Independent validation of SFXN-1 and Cx32 co-IP with isotype IgG control.

IP for SFXN-1 and blotting for Cx32 in WT and KO liver lysate confirmed interaction not present following IP of WT liver lysate using a rabbit IgG isotype control.



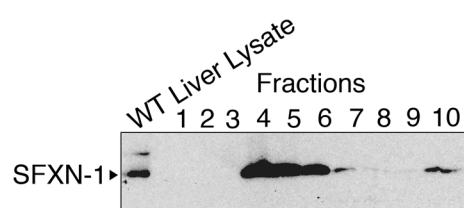
Supplemental Figure 2.3: Cx26 protein levels are reduced in Cx32 KO liver.

Cx26 levels are lower in KO compared to WT liver (see also Figure 2.2C). Actin was used as a loading control to confirm protein integrity.



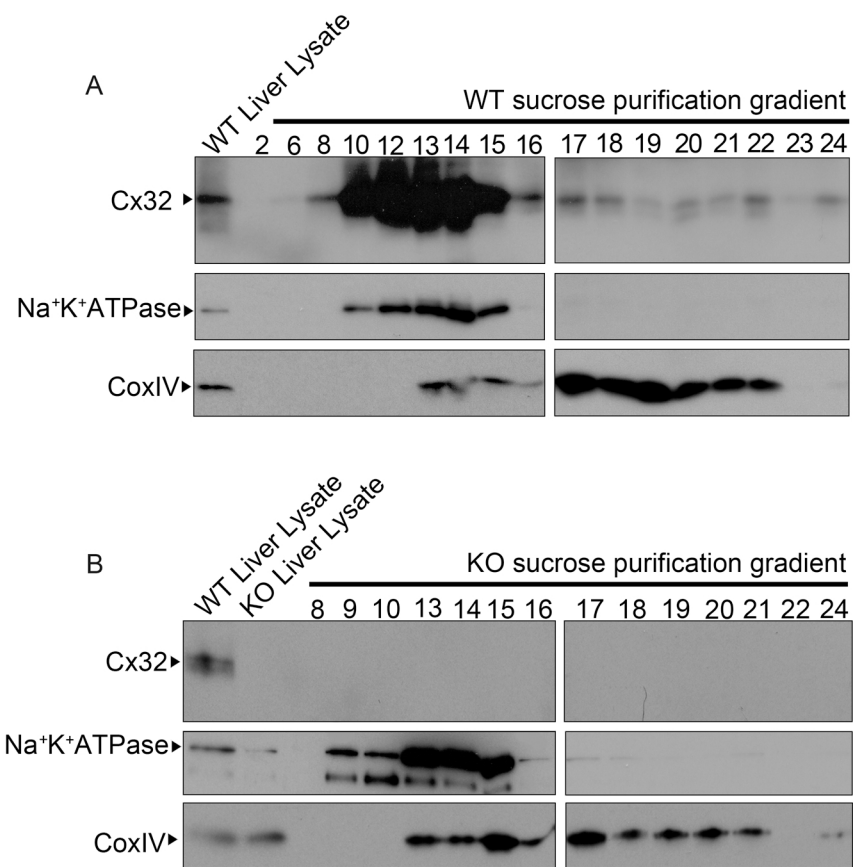
Supplemental Figure 2.4: SFXN-1 co-fractionates with Cx32.

Detergent-resistant membranes from the sucrose gradient depicted in Figure 2.1A were immunoblotted for SFXN-1. SFXN-1 overlaps with Cx32 (Figure 2.1A) in fractions 4-6. Antibodies are listed in Table 2.1.



Supplemental Figure 2.5: WT and KO sucrose flotation of crude mitochondrial preparations.

(A) WT. (B) KO. Contaminating Na⁺K⁺ATPase-positive PM is detected in fractions 10-15 (WT) and 9-15 (KO) of crude WT mitochondria separated by sucrose gradient (Figure 2.3D). CoxIV-positive mitochondrial membranes overlap with fractions 13-16. Pure CoxIV-positive mitochondrial membranes are enriched in fractions 17-22 (WT) and 17-21 (KO). Cx32 is primarily detected in PM fractions but clearly present in mitochondrial fractions devoid of any PM contamination. KO controls confirm monoclonal Cx32 antibody specificity (Table 2.1).



2.8. Associated Content

Supporting Information.

Supporting figures and materials. This material is made available free of charge via the Internet at <http://pubs.acs.org>

Author Information

*D.F. email: dfigeys@uottawa.ca; tel: (613) 562-5800 x 8674; fax: (613) 562-5452
or *S.A.L.B. email: sbennet@uottawa.ca; tel: (613) 562-5800 x 8372; fax: (613) 562-5452

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Conflict of interests: The authors declare no competing financial interests.

Chapter 3: Altered respiratory and functional characteristics of hepatic mitochondria lacking Cx32

3.1. Objective of study

In Chapter 2, identification and quantitation of protein-protein interactions in WT and Cx32 KO mice implicated a Cx32 PM-mitochondrial interacting nexus in the regulation of mitochondria by gap junction proteins. Thus, the objective of this chapter was to use a loss of function approach to investigate the phenotypic significance of this interactome.

3.2. Author contributions

SLF and SALB conceived the experiments. SLF performed all of the experimental work. RM assisted in the design and execution of the experiments for mitochondrial bioenergetics, ROS, and membrane potential assays. ME and RM provided interpretive assistance. MA performed the confocal and STED-CW microscopy imaging. HZ performed the MS analysis in the laboratory of DF.

Altered respiratory and functional characteristics of hepatic mitochondria lacking Cx32

Fowler, S^{1,2}, Mailloux, RJ², Akins, M, Zhou, H, Figeys, D, Harper, M-E², Bennett, SAL^{1*}

¹Neural Regeneration Laboratory, Ottawa Institute of Systems Biology, Centre for Catalysis Research and Innovation, Ottawa Brain and Mind Research Institute,

²Department of Biochemistry, Microbiology, and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada.

* Corresponding author

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Corresponding author:

Dr Steffany A.L. Bennett, sbennet@uottawa.ca

Tel.: (613) 562-5800 x 8372

Fax: (613) 562-5452

3.3. Abstract

Proteomic profiling of WT and Cx32 KO membranes indicates that loss of Cx32 induces global upregulation of proteins involved in the citric acid (TCA) cycle, and respiratory electron transport pathways. Given that previous work from our laboratory has shown that Cx32 is capable of interacting with mitochondrial proteins (Chapter 2), the following study was undertaken to determine if isolated hepatic mitochondria possess different functional characteristics from WT mitochondria. Using purified mitochondria, I have shown that Cx32 protein is present at IMMs, and is also present in mitochondrial organelles immobilized on PLL-coated coverslips. Super-resolution STED-CW microscopy revealed that Cx32 protein localizes around the circumference of isolated mitochondria in ~80 nm wide clusters. Bioenergetic assessment of mitochondrial oxygen consumption rates (OCRs) indicated that Cx32 KO mitochondria respire at significantly higher rates than WT mitochondria. Cx32 KO mitochondria also produce more ROS, and possess increased mitochondrial membrane potentials ($\Delta\Psi$ Ms) compared to WT mitochondria. Together, these data suggest that mitochondrial Cx32 is directly involved in the regulation of mitochondrial function, and that loss of Cx32 may result in pathological ROS production.

3.4. Introduction

Gap junctions are highly-conserved channel-forming structures in vertebrate PMs that mediate both juxtacrine and paracrine signaling (Evans and Martin 2002; Goodenough 1974; Goodenough and Paul 2009; Skerrett et al. 2002). Gap junctions form contiguous channels that connect adjacent cells, but also form single membrane channels in non-junctional membranes (Goodenough and Paul 2003; Maes et al. 2014; Saez et al. 2010). The connexin family of proteins form the structural units of PM gap junction channels, yet have recently been localized to mitochondrial membranes in various cell types (Fowler et al. 2013; Kiec-Wilk et al. 2012; Kozoriz et al. 2010; Li et al. 2002a; Lu et al. 2012; Mohammad and Kowluru 2011; Sun et al. 2012; Vinken et al. 2013). We have shown that the main hepatocyte, oligodendrocyte, and Schwann cell connexin, Cx32, specifically localizes to IMMs in hepatocyte mitochondria (Fowler et al. 2013). Further, in addition to interacting with a variety of PM-localized proteins, we have identified that Cx32 is capable of interacting with proteins at the both the OMM and IMM (Fowler et al. 2013). These mitochondrial Cx32 interacting proteins are intimately involved with oxidative phosphorylation and energy production, thus, it is plausible that Cx32 may have intrinsic function in mitochondrial membranes.

We have previously shown that Cx32 is present at IMMs by immunoblotting from OP-purified mitochondrial lysates, and digitonin-solubilized subfractions thereof (Fowler et al. 2013), while others have demonstrated the presence of Cx32 in Percoll-purified mitochondrial preparations by immunoblotting and HPLC-MS/MS analyses (Miro-Casas et al. 2009). Despite this evidence, the presence of Cx32 protein in mitochondrial organelles has yet to be established by direct immunofluorescent

analysis, nor has a role for its mitochondrial localization been elucidated. There is, however, precedent for connexin pore-forming function in mitochondrial membranes. It is well accepted that Cx43 is targeted specifically to subsarcolemmal IMMs in cardiac myocytes (Boengler et al. 2005; Boengler et al. 2009). In cardiac cells, Cx43 may act as an ion-permeable hemichannel (Boengler et al. 2013; Srisakuldee et al. 2014), as its loss impairs complex I activity and ATP generation (Boengler et al. 2012), and prevents the ROS-induced protection afforded by ischemic or pharmacological preconditioning stressors (Heinzel et al. 2005).

As of yet, there have been no direct investigations of Cx32 function at the mitochondrial membrane. It has been shown previously that loss of Cx32 results in uncontrolled cellular growth and tumorigenesis (King and Lampe 2004a; King and Lampe 2004b) through direct activation of the MAP kinases ERK1 and ERK2 (Irani et al. 1997; Ogrunc et al. 2014), which can be activated by increased mitochondrial ROS (Meves et al. 2001). Accordingly, there is evidence that the anti-oxidant superoxide dismutase (SOD) is elevated in Cx32 KO hepatocytes (Stahl et al. 2005). Here, to provide mechanistic insight into possible metabolic/energetic roles of Cx32, we compared proteome of Cx32-enriched membrane microdomains at PM, IMM and OMM, and lysosomes of WT mice with the same fractions isolated from Cx32 KO mice. We show here that most highly upregulated proteins in Cx32 KO membranes were associated with mitochondrial function and energy metabolism. Given this knowledge, we confirmed that Cx32 protein is present in mitochondrial membranes by super-resolution STED-CW microscopy of isolated mitochondria. We then used the high-throughput Seahorse XF Analyzer to show that loss of Cx32 results in significantly

increased basal, leak, and carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP)-induced maximal OCRs in healthy hepatic mitochondria. Increased respiratory rates were also associated with increased ROS production, and increased $\Delta\Psi$ M. These data provide the first direct evidence that Cx32 has bona fide roles in mitochondrial membranes, and suggest that increased ROS production from overactive mitochondria may underlie pathological outcomes in cells lacking functional Cx32.

3.5. Materials and Methods

3.5.1. Mice

Cx32 null-mutant breeding pairs, kindly provided by Dr. Klaus Willecke (Universitat Bonn, Germany) (Nelles et al. 1996), were backcrossed for 15 generations onto a C57Bl/6 background in the Bennett laboratory. Male mice between 3-4 months of age were used in the current study, and genotyping was confirmed at time of weaning, and at sacrifice as previously described (Fowler et al. 2013).

3.5.2. Membrane protein fractionation

Murine cerebrum freshly dissected from WT or Cx32 KO mice (n=3 per genotype) were washed in ice-cold PBS followed by HB (0.25 M sucrose, 1 mM EDTA, 2 mM MgCl_2 , 20 mM HEPES-NaOH, pH 7.4). Brains were minced on an ice-cold Teflon plate using a razor blade, and transferred to a glass homogenizer with 2 mL of HB. Tissue was homogenized with 20 strokes of a tight-fitting Teflon pestle, or until smooth (identical number of strokes used for each brain; each brain was homogenized and processed separately). Smooth homogenates were centrifuged at 2000 x g for 10 mins to pellet nuclei and tissue debris, and the supernatant (1 mL) was collected and centrifuged at 100,000 g for 60 min. Each resultant membrane pellet was mixed with a

30% OP solution (1.2 mL 50% OP working solution (5 volumes of OP and 1 volume of OP diluent [0.25 M sucrose, 6 mM EDTA, 12 mM MgCl₂, 120 mM HEPES-NaOH, pH 7.4]) and 0.8 mL HB), and pipetted into the bottom of separate 13 mL polyallomer centrifuge tubes (331372) for the swinging-bucket rotor (SW41Ti). A discontinuous gradient was formed by carefully overlaying the protein sample with 2 mL each of 25%, 17.5%, 10%, and 2.5% OP solutions (made from 50% OP working solution, and HB). The top of the centrifuge tube was filled and balanced with HB, and tubes were centrifuged at 165,000 x g for 3.5 hours (acceleration = 1, deceleration = 0, or no brake). The gradient was collected identically between samples, as shown in Figure 3.1A, in 1 mL volumes. Fractions 1-5 were assayed for protein concentration, subjected to immunoblot characterization, and pooled to generate the membrane sample subjected to MS-MS profiling. Heavier fractions were not enriched for Cx32, and were not included in the analysis so as to enable the detection of less abundant proteins by preventing mitochondrial membrane over-representation (Tuli and Ransom 2009).

3.5.3. Immunoblotting

Brain membranes or liver mitochondrial subfractions were diluted to a 1X final concentration with 6X SDS sample buffer (0.35 M Tris-HCl/0.28% SDS, pH 6.8, and 5% glycerol, 10% SDS, 100 mM DTT, 0.002% bromophenol blue) with 10% BME and solubilized at room temperature for 30 min. Proteins (20 µg) were resolved under reducing/denaturing conditions on 12.5% Tris-HCl polyacrylamide gels (prepared in-house), and transferred to Immobilon PSQ PVDF membrane at 100 V for 60 min. Membranes were blocked in 5% (w/v) skim milk powder-PBS (10 mM phosphate, 154 mM NaCl) with 0.1% Tween-20 (blocking buffer) for 1 h and incubated in primary

antibody diluted in the same buffer overnight at 4°C (mouse anti-Na⁺K⁺ATPase, [BD Biosciences #610915], mouse anti-Cx32 [Alpha Diagnostics #CX32C13-M, 0.5 µg/mL], mouse anti-CoxIV [Molecular Probes #457325, 0.4 µg/mL], rabbit anti-LAMP-1 [Sigma #SAB3500285, 1/1000] or rabbit anti-VDAC1 [Abcam #ab15895, 0.5 µg/mL]). Membranes were rinsed twice in 0.1% PBST (10 mM phosphate, 154 mM NaCl with 0.1% Tween-20) and twice in blocking buffer for 10 min prior to a 1 h incubation in HRP conjugated anti-mouse or anti-rabbit (Jackson ImmunoResearch Laboratories, Inc., PA; [anti-mouse #115-035-146; 1:2000] and [anti-rabbit #711-096-152; 1:5000]) secondary antibody diluted in blocking buffer. Signal was detected on X-ray film using SuperSignal West Pico Chemiluminescent Substrate (Pierce, #34808).

3.5.4. *Centrifugal Proteomic Reactor*

Following pooling of fractions 1-5 from the OP membrane fractionation, 20 µg of protein was solubilized using an extraction buffer containing 50% methanol, 2 M urea, and 2% CHAPS, according to protocols described by (Zhou et al. 2011). Briefly, samples were transferred to 1.5 mL Eppendorf tubes, and mixed with 10 µL of SCX slurry and 1.2 mL of 50% methanol/5% formic acid by vigorous vortexing. The samples were centrifuged at 16,100 x g for 2 min, and the pellet was treated with 1.2 mL of 0.5% formic acid. The samples were reduced by mixing with 20 µL of 150 mM NH₄HCO₃, 20 mM DTT (shaking at 800 rpm, 56°C, 15 min). Following reduction, the DTT was diluted with 1.2 mL 0.5% formic acid, and the samples were re-centrifuged. Alkylation was performed by addition of 20 µL of 150 mM NH₄HCO₃/100 mM iodoacetamide for 15 mins at RT in the dark, and the reaction was stopped by adding 1.2 mL of 0.5% formic acid. After centrifugation, the pellets were dissolved in 20 µL of 1M NH₄HCO₃

containing 2 µg trypsin, and were digested on a shaker at 800 rpm at 37 °C for 2 h. Formic acid (5%, 1.2 mL) was added to elute the pH 2.5 fraction by centrifugation, followed by nine additional pH fractions (pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 8.0, 12) with 300 µL of respective pH buffers. The fractionated samples were subjected to liquid chromatography-tandem MS (LC-MS/MS) analysis.

3.5.5 LC-MS/MS

The LC-MS/MS was performed as previous described (Zhou et al. 2011). Briefly, samples were acidified with formic acid to a final concentration of 5% (v/v) and loaded on a 200 µm x 100 mm fused silica precolumn packed in-house with 10 cm of 5 µm ReproSil-Pur C18 beads (200 Å; Dr. Maisch GmbH, Ammerbuch, Germany) using a 1100 micro-HPLC system (Agilent Technologies, Santa Clara, CA). After desalting, the flow was split, and peptides were eluted through a second 75 µm x 150 mm column packed with 3 µm C18 beads at ~150 nL/min. The peptides were eluted using a gradient (5–80% ACN with 0.1% formic acid) over a 2 h period into an electrospray ionization linear trap quadrupole (LTQ)-Orbitrap mass spectrometer (Thermo Electron, Waltham, MA). MS/MS spectra were acquired in a data-dependent acquisition mode that automatically selected and fragmented the 10 most intense peaks from each MS spectrum generated.

3.5.6. MS Data Analysis

Maxquant (<http://maxquant.org/>, version 1.0.13.13) was used to generate peak lists from raw files, and Mascot (version 2.2.02 Matrix Science, London, UK) was used to search the protein sequence database using the following parameters: carbamidomethyl (C) was set as a fixed modification, whereas oxidation (M, +15.99492

Da) was set as a variable modification. Acquired MS/MS spectra were searched against the decoyed rat International Protein Index protein sequence database (version 3.52, 39,906 protein sequences; European Bioinformatics Institute) augmented with the reversed sequence of each entry in the forward database. The precursor and fragment mass tolerances for the searching were set at 7 ppm and 0.5 Da, respectively. Trypsin was selected as the digestive enzyme with two potential missed cleavages. Only fully tryptic peptides ranked first were accepted in our database searching results. The false positive rate was controlled at <1% using the equation $\text{false positive rate} = \frac{\# \text{ of false peptides}}{\# \text{ of true peptides} + \# \text{ of false peptides}} \times 100$. The proteins with at least two unique peptides, and a Mascot score greater than 24 were accepted for subsequent analysis. MaxQuant (version 1.1.1.36) was used for label-free quantitation (LFQ) of the proteins detected in WT and Cx32 KO membrane samples (Cox et al. 2014). REACTOME analysis was used to generate biological pathway annotations (www.reactome.org/). To rank REACTOME pathways identified with high significance (low p -values) and high degrees of representation in our datasets, an importance value (IV) was applied to the data generated by the REACTOME database: $\% \text{ identities} * \log_{10} (1/(p - \text{value}))$, where the % identities is the number of proteins identified in a given pathway from our dataset, divided by the total number of significantly regulated proteins*100.

3.5.7. Mitochondrial isolation for bioenergetic assay

Bioenergetic assays were performed on freshly isolated mitochondria. Briefly, dissected livers (WT and Cx32 KO) were washed in 20 mL mitochondrial isolation buffer (MIB: 70 mM sucrose, 220 mM mannitol, 1 mM EGTA, 2 mM HEPES, 1% BSA, pH 7.2),

and cut into $<1\text{ mm}^2$ pieces using a razor blade on an ice-cold teflon plate. Liver pieces were washed 3X in ice-cold MIB, and carefully homogenized in fresh buffer in a 30 mL glass homogenizer with loose-fitting pestle until a rough homogenate was obtained (~10 strokes). The rough homogenate was further homogenized with a tight-fitting pestle until a smooth homogenate was obtained (~8 strokes). Smooth homogenates were centrifuged at $800 \times g$ for 9 mins at 4°C in 30 mL high-speed centrifuge tubes, and the supernatants cleared of undisrupted tissue were transferred into new tubes. Supernatants were centrifuged at $12,000 \times g$ for 9 mins at 4°C . The supernatants were discarded, and any fat was removed from the side of the tube using a kimwipe. Mitochondrial pellets were carefully resuspended in 2 mL of MIB using a disposable plastic transfer pipette to limit shear stress on the organelles. Resuspended mitochondria were incubated on ice for 10 mins, prior to the addition of ~30 mL MIB (to fill the tubes) and re-centrifuged at $12,000 \times g$. The final supernatants were discarded, and the mitochondrial pellets were re-suspended in ~2 mL BSA-free MIB. If mitochondria were to be used for bioenergetic assays, the suspension was assayed for protein content using the Bio-Rad DC protein assay, and diluted to 0.2 mg/mL in BSA-free MIB, and kept on ice for no longer than 2 h.

3.5.8. Mitochondrial purification for confocal and STED-CW microscopy

If mitochondrial suspensions were to be used for microscopy, they were first purified on discontinuous OP density gradients to deplete the sample of PM contamination. Briefly, the final pellet generated above was not diluted, and was resuspended in 1.5 mL MIB. The crude mitochondrial suspension was brought to a 30% OP final concentration by the addition of 1.8 mL of WS (50% WS: 5 parts OP with

1 part OP diluent [0.25 M sucrose, 6 mM EDTA, 12 mM MgCl₂, 120 mM HEPES-NaOH, pH 7.4] to 1.2 mL of mitochondrial suspension in a new tube. This 3 mL mitochondrial homogenate was thoroughly mixed by pipetting up and down with a disposable transfer pipette, and was transferred to 13.2 mL (14 x 89 mm) polyallomer centrifuge tubes. Mitochondria were carefully overlayed with 2.5 mL each 25% and 17.5% OP, and 2 mL each 10%, and 2.5% OP solutions to form the density gradient. The 25-2.5% OP solutions were prepared by dilution of the 50% WS of OP with MIB. Each centrifuge tube was carefully balanced and adjusted with MIB before assembly into an SW41 Ti rotor (Beckman Coulter). Gradients were centrifuged at 36,555 rpm (165,000 x g_{ave}) at 4°C for 3.5 hours (acceleration = 1, deceleration = 0). The interfaces between the gradients were collected in 1 mL volumes by pipetting from the top of the tube. The interface between the 25% and 17.5% OP layers was previously shown to be enriched for mitochondrial proteins (porin), and devoid of PM proteins (Na⁺K⁺ ATPase) (Fowler et al. 2013). Once collected, these fractions were chilled on ice, and processed immediately for microscopy. If used for immunoblotting, fractions were diluted 1:2 in RIPA buffer (10 mM PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with fresh protease inhibitors (1 mM sodium fluoride, 50 µg/mL aprotinin, 1 mM sodium orthovanadate, 1 mg/mL PMSF), solubilized on ice for 30 mins, assayed for total protein concentration, and stored at -80°C.

3.5.9. Mitochondrial sub-fractionation for immunoblotting

WT and Cx32 KO liver mitochondria were prepared as above for bioenergetic analysis (4.5.2), and 100 µg of each genotype was sedimented by centrifugation at 12,000 x g for 10 mins. Mitochondrial pellets were resuspended in 500 µL hypotonic

solution (5 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.2, with KOH), and incubated on ice for 5 minutes to sensitize and loosen the OMMs. Sensitized mitochondria were centrifuged at 12,000 x g for 5 mins, and the supernatant was discarded. To the pellet, 600 uL of a 2% digitonin solution (20 mg/mL) was added such that the digitonin:protein ratio was 1.2 mg:10 mg. This solution was quickly transferred to a 15 mL centrifuge tube with a tiny (<1 cm diameter) magnetic stir bar. The tube was inverted onto a petri dish filled with ice-water, and the entire apparatus was placed onto a stir-plate set to medium speed for 15 mins. Following digitonin incubation, the mitochondrial solution was diluted with 3 volumes of ice-cold MIB (no BSA), and centrifuged at 8000 x g for 10 mins. The resultant supernatant was reserved on ice as the OMM sample, while the pellet was washed in 2.5 mLs MIB, and re-centrifuged at 8000 x g for another 10 mins. The pellet containing only IMs was resuspended in 1 mL RIPA buffer (10 mM PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with fresh protease inhibitors (1 mM sodium fluoride, 50 µg/mL aprotinin, 1 mM sodium orthovanadate, 1 mg/mL PMSF). The low-speed supernatant was concentrated and buffer replaced with RIPA buffer to 0.5 mL using a 10Kd cut-off centrifugal filter device. All samples were assayed for protein concentration prior to immunoblotting.

3.5.10. Preparation of isolated mitochondria for confocal and STED-CW microscopy

During the 3.5 hr OP gradient ultracentrifugation, 18 mm round #1.5 glass coverslips were coated with 0.1% Poly-L-Lysine (PLL; Sigma-Aldrich) diluted in sterile water for 2 h at RT in 12-well tissue culture plates (250 uL PLL solution/well). All washes, fixing and antibody incubations were performed with the coverslips inside the

tissue culture plate wells. The PLL solution was aspirated from the coverslips, and wells were washed 3X with tissue culture PBS. Following collection of the purified mitochondria at the 25% and 17.5% OP interface, the sample was diluted in MIB to a concentration of 1 mg/mL. 250 μ L of MIB, and 25 μ L of 1 mg/mL mitochondrial suspension were added to each well. Plates were covered and incubated at 4°C for 1 h to allow mitochondria to settle. Attached mitochondria were gently washed 1X with 10 mM PBS, and fixed with 4% (w/v) paraformaldehyde in PBS for 10 min at RT. After three washes with PBS, fixed mitochondria were incubated in 250 μ L primary antibodies (anti-rabbit Tom20 [Santa Cruz # sc-11415, 4 μ g/mL], anti-mouse Cx32 [Invitrogen #13-8200, 1 μ g/mL]) diluted in antibody buffer (10 mM PBS, 0.3% Triton-X100, 3% bovine serum albumin) overnight at 4°C. Coverslips were washed 3X in 10 mM PBS, and incubated for 2 hrs in anti-rabbit (Alexa488) or anti-mouse (Atto488) secondary antibody diluted 1/800 in antibody buffer. Following 3 additional PBS washes, mitochondrial preparations were coverslipped with 10 μ L of ProLong Gold antifade mounting solution (Invitrogen), and allowed to cure overnight at RT in the dark.

3.5.11. Confocal and STED-CW Microscopy and analyses

Confocal images (Tom20 and Cx32) and continuous wave stimulated emission depletion (STED-CW) images (Cx32 only) were acquired using a Leica SP5/STED-CW microscope, a 63x oil immersion objective, and a 100x oil immersion objective (HCX PL APO CS 100x/1.40 OIL STED, Leica Germany), respectively, as described in Zheng et al., 2011 (Zheng et al. 2011), and in Chapter 3. Tom20 was visualized using an anti-rabbit Alexa488 conjugated secondary antibody, excited by an argon-ion laser at 458 nm. Tom20 fluorescence images were overlaid with differential interference contrast

(DIC) images of isolated mitochondrial preparations. Cx32 was visualized using an anti-mouse Atto488 conjugated secondary antibody. For confocal images, the Atto488 fluorophore was excited by an argon-ion laser at 458 nm, while STED depletion was achieved with a 592 nm continuous wave laser line. Scanning at 458 nm was performed at 85% laser power, 800 gain, -1.0 offset, with frame averaging and accumulation of 3, and a detection window of 465 – 485 nm. STED 592 depletion laser power was 100%. STED-CW images were processed using Leica deconvolution software (LAS AF v2.6.3.8173). Briefly, a point spread function (PSF) was generated using a Lorentz transformation of 70 nm, and the image was deconvolved using the generated PSF and signal energy set to regularization parameter 0.05. Images were further processed using the baseline mean function, with background adjustment of 80. The Cx32 KO confocal image was obtained from a population of Cx32 KO mitochondria that were shot and processed at the same time, using the same parameters as the WT preparation.

3.5.12. Mitochondrial bioenergetics (Seahorse XF Analyzer)

Oxygen consumption was investigated using the Seahorse XF24 Extracellular Flux Analyzer (Seahorse Biosciences; North Billerica, MA) as described by Mailloux et al (Mailloux et al. 2013, {Mailloux, 2014 #884}) and Rogers et al (Rogers et al. 2011). Briefly, 50 μ L of freshly-isolated mitochondria (10 μ g of mitochondria from 0.2 mg/mL suspension) was loaded into Seahorse tissue culture plate wells. The plates were centrifuged at 2000 x g for 20 min at 4°C to attach mitochondria, and 350 μ L of reaction buffer (RB) (MIB containing 10 mM KH_2PO_4 , 5 mM MgCl_2 , 0.2% w/v defatted BSA, 10 mM pyruvate, and 2 mM malate) was added to each well, giving a final volume of 400

μL. Following a 10-min incubation at 37°C, plates were loaded into the XF24 Analyzer for bioenergetic analyses.

Prior to the bioenergetic determinations, the OCR was first determined under state 2 conditions (substrate only: 10 mM pyruvate, and 2 mM malate). ADP was titrated into the reactions at 0.5 mM, 0.1 mM, and 0.5 mM to determine the maximal OCR for WT mitochondria, and to ensure that oxidative phosphorylation was active in the preparation. In two separate mitochondrial isolations (total n of 4), WT liver mitochondria respired maximally and without significant decline over the measurement time under our experimental conditions using 0.1 mM ADP (state 3 respiration). For both isolations, a CytC titration (5 μM and 10 μM) was performed using this established ADP concentration (0.1 mM) to ensure OMM integrity. If the OMM was compromised during mitochondrial isolation and/or attachment to the Seahorse microplate, CytC would be released from the organelle, thus, application of exogenous CytC would permeate the OMM, and the OCR would increase. Absence of an increase in OCR is indicative of an intact OMM.

Bioenergetic determinations were then performed for WT and Cx32 KO mitochondria by sequentially injecting ADP (0.1 mM), oligomycin (3.2 μM; ATP synthase inhibitor), FCCP (8 μM; chemical uncoupler), and antimycin A (AntA; 4 μM; complex III inhibitor) to test state 3 (ADP-stimulated respiration), state 4 (oligomycin: proton leak-dependent respiration), maximal respiration (FCCP), and respiration independent of the respiratory chain (antimycin A). For all measurements of OCR, the measurement interval was 3 min, which included 1 min mix, and 2 min measure periods. All respiration values were obtained by averaging 5 technical replicates, and were

corrected for background OCR by the Seahorse XF24 software. OCRs were further corrected by subtracting the AntA-dependent OCR values (respiration not associated with the respiratory chain, when employed).

3.5.13. ROS kinetics of isolated mitochondria

Mitochondria were isolated as for bioenergetic assays, and ROS production kinetics were measured using 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) in a BioTek Synergy MX2 microplate reader (Winooski, VT) as described by Mailloux et al (Mailloux et al. 2013; Mailloux et al. 2014). Upon exposure to oxidants in respiring mitochondrial preparations, the acetate groups of nonfluorescent H₂DCFDA are cleaved to produce the highly fluorescent 2',7'-dichlorofluorescein (DCF) (excitation 480 nm, emission 528 nm). Freshly-isolated mitochondria were diluted to 0.5 mg/mL in RB containing 20 μ M H₂DCFDA, without pyruvate or malate substrates, and 230 μ L was added to each well (115 μ g). For all experiments, mitochondria were prewarmed in RB for 10 min at 37°C. After a 2 min baseline collection, reactions were initiated by the addition of substrates pyruvate (10 mM) and malate (2 mM), and the increase in H₂DCFDA fluorescence was collected for another 2 mins. Reactions devoid of mitochondria served as the control, and were subtracted from all baseline and experimental read values. Data are presented as arbitrary fluorescence units (AU) per mg of protein, and rate data is presented as the fold change in the rate of change in fluorescence/mg of protein following the addition of pyruvate and malate.

3.5.14. TMRE analysis of mitochondrial membrane potential

Mitochondria were prepared as above for ROS production kinetics, except that mitochondria were diluted to 0.5 mg/mL, and added to the plate in RB only (no

substrates). Tetramethylrhodamine ethyl ester (TMRE) was added at 10 nM, and baseline fluorescence was measured for 2 mins to monitor potentiometric dye incorporation into the mitochondrial matrix, and subsequent fluorescent quenching (excitation 544 nm, emission 590 nm). Substrates pyruvate (10 mM) and malate (2 mM) were then injected, and fluorescence was monitored for another 2 mins. Finally, FCCP was injected at 10 nM to depolarize mitochondria, thereby releasing TMRE from the mitochondrial matrix. A spike in fluorescence upon addition of FCCP confirmed operation of the assay in quench mode. As above, mitochondria were prewarmed in RB for 10 min at 37°C, prior to initiation of the assay. Similarly, reactions devoid of mitochondria served as the control, and were subtracted from all experimental read values.

3.6. Results

3.6.1. Proteomic profiling of neural membranes in presence and absence of Cx32

Neural membrane microdomains, enriched in Cx32 from WT cerebra, were compared to fractions simultaneously isolated from Cx32 KO cerebra (n=3/genotype, Figure 3.1A). Cx32 localized to fractions 1-5 enriched in Na⁺K⁺ATPase (plasma), CoxIV (mitochondrial), and LAMP-1 (lysosomal) membranes (Figure 3.1A). Heavier density membranes did not contain Cx32 (data not shown). Fractions 1-5 from each biological replicate were pooled. Following in-tube trypsin digestion on our centrifugal proteome reactor, resulting tryptic peptides were subjected to LC-MS/MS analysis on a linear ion trap Orbitrap mass spectrometer. We used MaxQuant LFQ intensities to compare proteomes. We found that three percent of total proteins identified (30 of 1002) were statistically significantly up-regulated by at least 1.5-fold in Cx32 KO

cerebra; 0.7% were significantly downregulated by at least 1.5 fold (7 of 1002) (Figure 3.1B).

3.6.2. Mitochondrial pathways are increased, while synapse-related proteins are decreased in the absence of Cx32

Pathway analyses were performed on all statistically significantly altered LFQ intensity ratios elevated or depleted in the absence of Cx32 by at least 1.5-fold using the REACTOME database. Pathways were ordered and displayed according to calculated IVs ($\% \text{ identities} * \log_{10}(1/(p - \text{value}))$), taking into account the percent of proteome altered by the loss of Cx32 (Figure 3.2). Of the significantly upregulated proteins in the Cx32 KO, the pathways with highest IVs were associated with mitochondrial function, and include TCA cycle and respiratory electron transport, pyruvate metabolism, respiratory electron transport and heat production by uncoupling proteins, cristae formation, and formation of ATP by chemiosmotic coupling. Pathways most significantly decreased (highest IVs) in the Cx32 KO were associated with a variety of biological processes related to synaptic function including: N-glycan trimming in the ER and the calnexin/calreticulin cycle, leucine-rich glioma inactivated (LGI)-a disintegrin and metalloprotease (ADAM) interactions at synapses, post-translational protein phosphorylation, regulation of insulin-like growth factor (IGF) transport and uptake by IGF binding proteins, and RHO GTPase activation of formins. Table 3.1 includes all proteins with significantly different *p*-values between genotypes.

Figure 3.1: Loss of Cx32 alters the brain membrane proteome.

(A) Schematic of the discontinuous OP gradient used to fractionate total membranes from fresh brain tissue. The top five fractions were enriched in Cx32 and defined by PM, mitochondrial and lysosomal membrane protein markers. Fractions 1-5 were pooled for LC-MS/MS analysis. **(B)** Volcano plot of $-\log P$ -value (Y-axis), assessed by two-tailed Student's t-test of $\log_2$ LFQ intensities with α set to 0.05) and $\log_2$ fold change (Cx32 KO/WT) (X-axis). Proteins statistically significantly upregulated in Cx32 KO cerebra by at least 1.5 fold are indicated in red; proteins statistically significantly downregulated in Cx32 KO cerebra by at least 1.5 fold are indicated in cyan.

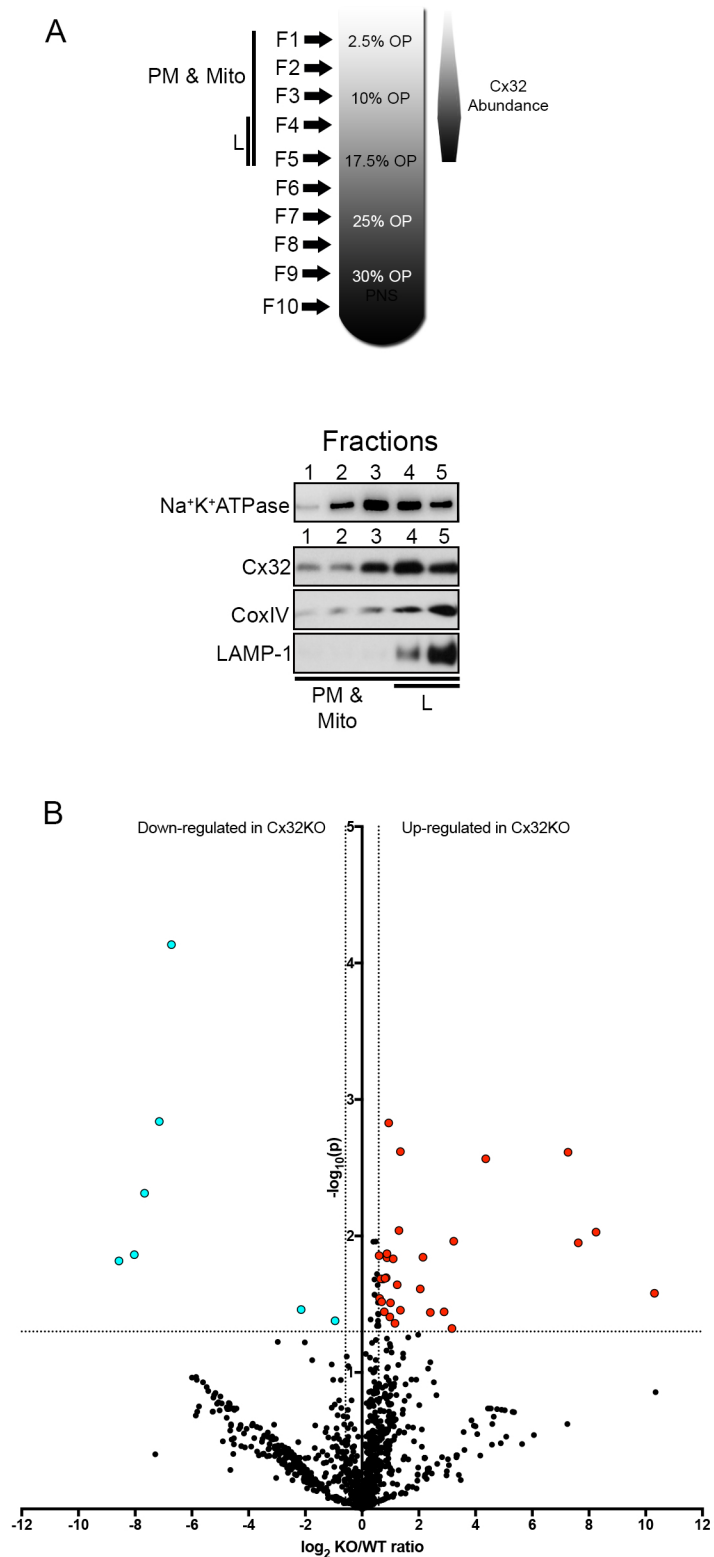


Figure 3.1

3.6.3. *Cx32 is present in purified mitochondrial organelle preparations*

Given that the most significantly upregulated proteins in Cx32 KO neural membranes were related to mitochondrial function, we next assessed whether mitochondrial energetics were altered in mitochondria isolated from Cx32 KO mice. To maximize yield, we used hepatic mitochondria, as we have previously demonstrated that Cx32 localizes to the IMM of hepatic mitochondria (Chapter 2). First, isolated mitochondria were purified in OP density gradients, and immobilized onto PLL-coated coverslips. Evaluation of confocal Tom20 fluorescence (Figure 3.3A) was overlaid with the corresponding DIC image. Figure 3.3B indicated that Tom20 protein is present around the circumference of each spherical organelle (~700-1200 nm in diameter). Subfractionation of isolated mitochondria into OM and IM fractions confirmed an IMM-localization for Cx32 (Figure 3.3C). Here, sensitized and digitonin-solubilized OMM fractions were enriched in Tom20, and devoid of CoxIV, while the remaining WT IMM fractions were positive for CoxIV and Cx32, as also described in Chapter 2 (Fowler et al. 2013) (Figure 3.3C). With this proof of concept experiment, we further identified that Cx32 protein was present in isolated mitochondrial organelles, as shown by microscopy of purified mitochondrial preparations. Here, confocal imaging indicated that Cx32 protein was present at the circumference of WT (Figure 3.3D), but not Cx32 KO (Figure 3.3E) mitochondria. Super-resolution STED-CW imaging of the mitochondrion in Figure 3.3D revealed a circumferential punctate staining pattern for Cx32, with each spot being approximately <80 nm in diameter (Figure 3.3F).

Figure 3.2: Biological pathways associated with loss of Cx32.

REACTOME pathway analysis of all significantly increased and decreased proteins following proteomic perturbation due to loss of Cx32. Pathways are displayed in order of IV ($\% \text{ identities} * \log_{10} (1/(p - \text{value}))$). Red = increased in Cx32 KO, cyan = decreased in Cx32 KO.

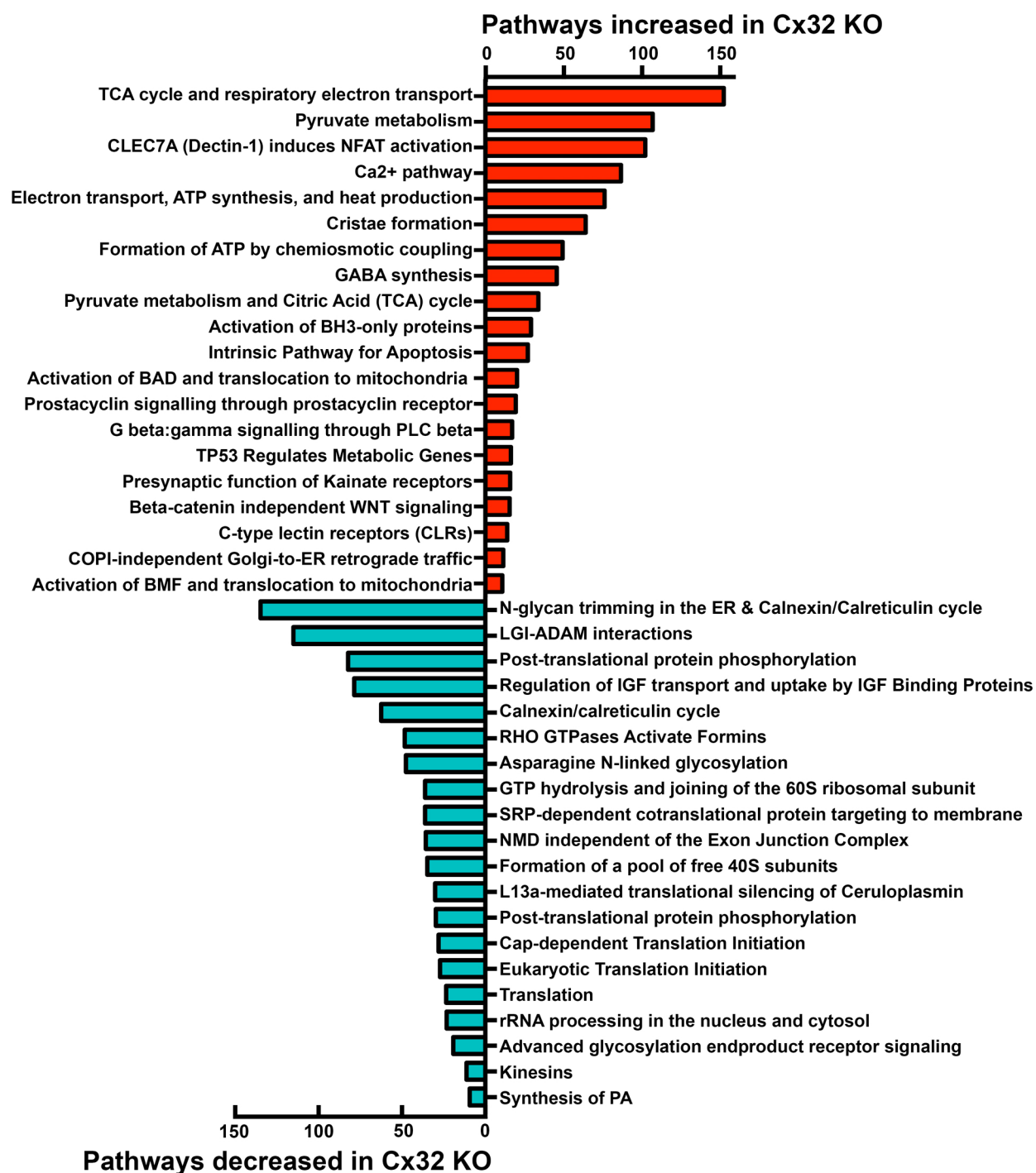


Figure 3.2

Table 3.1: Significantly regulated proteins in WT and Cx32 KO Membranes.

Protein Name	Gene Names	Uniprot Identity	Coverage [%]	Log2 Ratio (KO/WT)	P-value
Cytochrome b-c1 complex subunit 1, mitochondrial	Uqcrc1	Q9CZ13	46	0.60	0.0100
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	Pdhb	Q9D051	34	0.62	0.0200
ATP synthase subunit beta, mitochondrial	Atp5b	P56480	76	0.65	0.0200
Probable G-protein coupled receptor 158	Gpr158	Q8C419	8.3	0.69	0.0300
10 kDa heat shock protein, mitochondrial	Hspe1	Q64433	87.3	0.62	0.0200
Elongation factor 1-alpha 1	Eef1a1	P10126	41.1	0.78	0.0300
AP-2 complex subunit mu	Ap2m1	P84091	41.6	0.81	0.0200
Peroxiredoxin-1	Prdx1	P35700	55.3	0.85	0.0200
NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial	Ndufs2	Q91WD5	28.1	0.88	0.0100
L-lactate dehydrogenase B chain	Ldhb	P16125	50	0.88	0.0100
Peroxiredoxin-5, mitochondrial	Prdx5	P99029	45.7	0.94	0.0010
G protein-regulated inducer of neurite outgrowth 1	Gprin1	Q3UNH4	17.9	0.98	0.0400
Cytochrome c oxidase subunit 5A, mitochondrial	Cox5a	P12787	39	1.00	0.0300
14-3-3 protein eta	Ywhah	P68510	63	1.10	0.0200
Receptor expression-enhancing protein 5	Reep5	Q60870	24.3	1.20	0.0400
Acetyl-CoA acetyltransferase, mitochondrial	Acat1	Q8QZT1	47.6	1.24	0.0200
Glutamate decarboxylase 1	Gad1	P48318	4.6	1.30	0.0090
Peptidyl-prolyl cis-trans isomerase A	Ppia	P17742	80.2	1.35	0.0020
Oligodendrocyte-myelin glycoprotein	Omg	Q63912	16.5	1.35	0.0300
Cytochrome b-c1 complex subunit 7	Uqcrb	Q9CQB4	23.4	2.05	0.0200
Dynein light chain 2, cytoplasmic	Dynll2	Q9D0M5	12.4	2.15	0.0100
Serine/threonine-protein phosphatase 2B catalytic subunit beta isoform	Ppp3cb	P48453	16	2.41	0.0400
Myogenin	Myog	P12979	4	2.89	0.0400
Cytochrome c oxidase subunit 6B1	Cox6b1	P56391	20.9	3.17	0.0400
Platelet-activating factor acetylhydrolase IB subunit alpha	Pafah1b1	P63005	26.6	3.23	0.0100
Protein DJ-1	Park7	Q99LX0	27.5	4.36	0.0030
Signal transducing adapter molecule 1	Stam	P70297	4.5	7.26	0.0020
Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12	Gng12	Q9DAS9	25	7.62	0.0100
ATP synthase protein 8	Mtstp8	P03930	14.9	8.25	0.0090
Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-2	Gng2	P63213	19.7	10.31	0.0300
Formin-like protein 2	Fmn12	A2APV2	2.1	-8.03	0.0100
60S ribosomal protein L9	Rpl9	P51410	10.4	-7.67	0.0040
Uncharacterized protein KIAA0513	Kiaa0513	Q8R0A7	8.4	-7.15	0.0010
Microtubule-associated protein RP/EB family member 3	Mapre3	Q6PER3	10	-6.72	0.0001
Glucosidase 2 subunit beta	Prkcsh	O08795	12.7	-2.15	0.0300
Disintegrin and metalloproteinase domain-containing protein 23	Adam23	Q9R1V7	5.5	-0.95	0.0400
Kinesin-like protein KIF21A	Kif21a	Q9QXL2	3.2	-8.57	0.0100

Figure 3.3: Purified mitochondria contain Cx32 protein.

(A,B) Confocal imaging of WT purified mitochondrial preparations. Tom20 protein was detected using an Alexa488 secondary antibody **(A)**, and the fluorescent signal was overlaid onto the corresponding DIC image **(B)**. Scale bars, 1000 nm. **(C)** Immunoblotting of mitochondrial subfractions verified by enrichment of VDAC-1 in OMM fractions, and enrichment of CoxIV in IMM fractions. WT mitochondrial IMM subfractions contain Cx32 protein, while no signal is present in the WT OMM fraction, or in Cx32 KO mitochondria (20 µg of protein/lane). **(D)** Confocal image of Cx32 protein in a WT purified mitochondrial preparation, detected using an anti-mouse Atto488 antibody. **(E)** An identically prepared sample of Cx32 KO mitochondria indicates the specificity of the mouse Cx32 antibody **(F)** Super-resolution STED-CW imaging of the Cx32 Atto488 fluorescent signal from the mitochondrion in **(D)**. Scale bars, 700 nm and 80 nm as indicated.

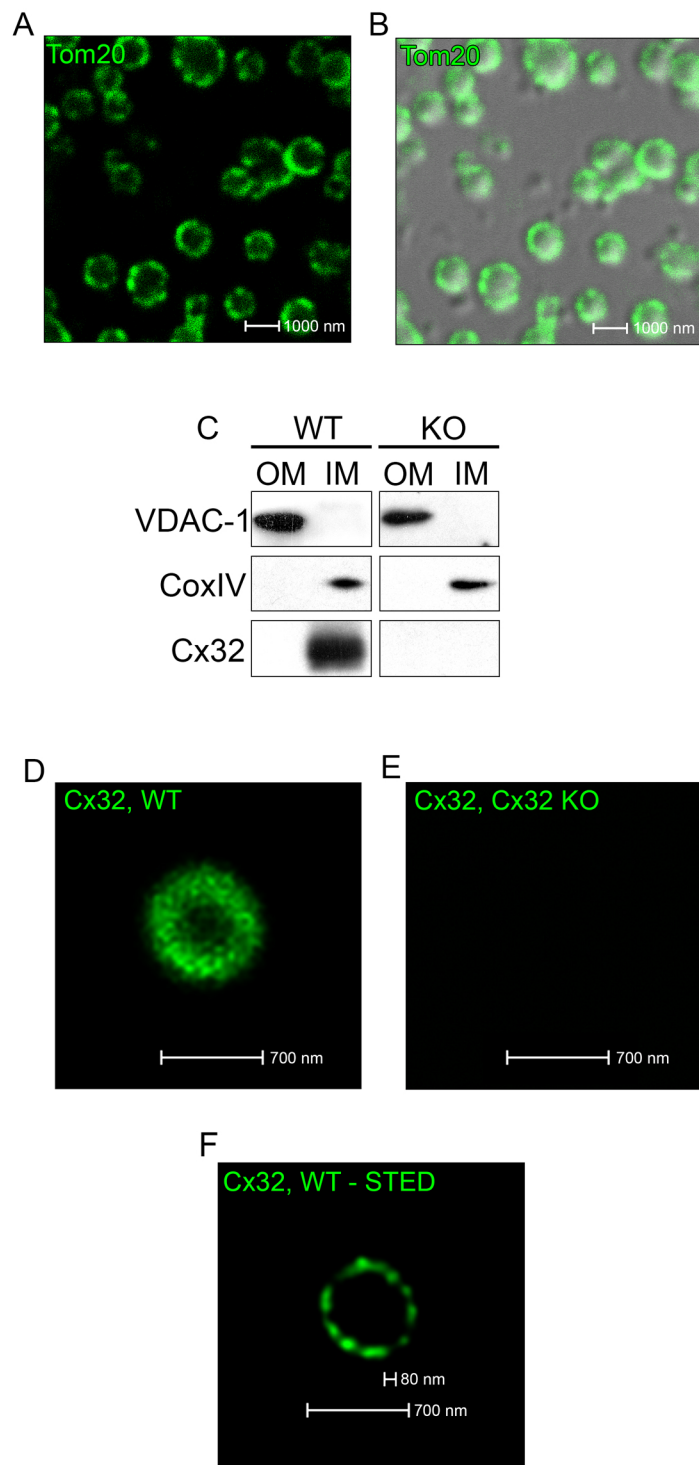


Figure 3.3

3.6.4. Mitochondrial bioenergetics are altered in Cx32 KO isolated mitochondria

Given that loss of Cx32 induces upregulation of mitochondrial pathways, and that Cx32 protein is present in WT mitochondria, we hypothesized that the loss of Cx32 from hepatic mitochondria would interfere with OCRs. We used the relatively high throughput microplate assay of the Seahorse XF Analyzer to measure OCRs of freshly-isolated WT and Cx32 KO hepatic mitochondria. In these experiments, the complex I substrates pyruvate and malate polarize mitochondria in state 2 respiration, as H^+ ions are shuttled from the matrix into the intermembrane space. ADP addition activates the ATP synthase, resulting in the flow of H^+ ions back into the mitochondrial matrix, and a marked increase in the OCR (state 3 respiration). Subsequent addition of oligomycin inhibits the ATP synthase, and any residual oxygen consumption is a result of proton leaks across the IMM (state 4o). Maximal OCRs can then be produced from the addition of the proton ionophore FCCP, which disrupts the proton gradient across the IMM, effectively uncoupling oxidation from phosphorylation, as protons flow freely back into the matrix (state 3 μ respiration). Finally, addition of AntA inhibits complex III, which shuttles protons across the IMM. Loss of this function prevents maintenance of the electrochemical gradient, and inhibits proton flow through the ATP synthase. Any oxygen consumed under AntA treatment represents non-mitochondrial respiration, and these values were subtracted from all experiments where AntA was used.

We found that state 2, state 4o, and state 3 μ OCRs were elevated in freshly-isolated Cx32 KO liver mitochondria (Figure 3.4A), indicating that basal, leak, and maximal respiratory states are increased with loss of Cx32. The gold standard evaluation of general mitochondrial function is the respiratory control ratio (RCR)

Figure 3.4: Cx32 KO mitochondria consume more oxygen than WT mitochondria

(A) OCRs for WT and Cx32 KO isolated mitochondria following the addition of ADP (0.1 mM), oligomycin (3.2 μ M), FCCP (8 μ M), and AntA (4 μ M). Vertical dotted lines indicate the time of injection of each chemical modifier. Asterisks indicate significant differences between WT and Cx32 KO OCRs ($p=0.05$, 2-tailed Student's t-test, $n=4$, $*p<0.05$). Data are presented as pmol/min/ μ g mitochondrial protein, and are corrected for AntA background fluorescence. **(B,C)** state 3/state 4o **(B)** and state 3 μ /state4o **(C)** RCRs ($p=0.05$, 2-tailed Student's t-test, $n=4$, $*p<0.05$).

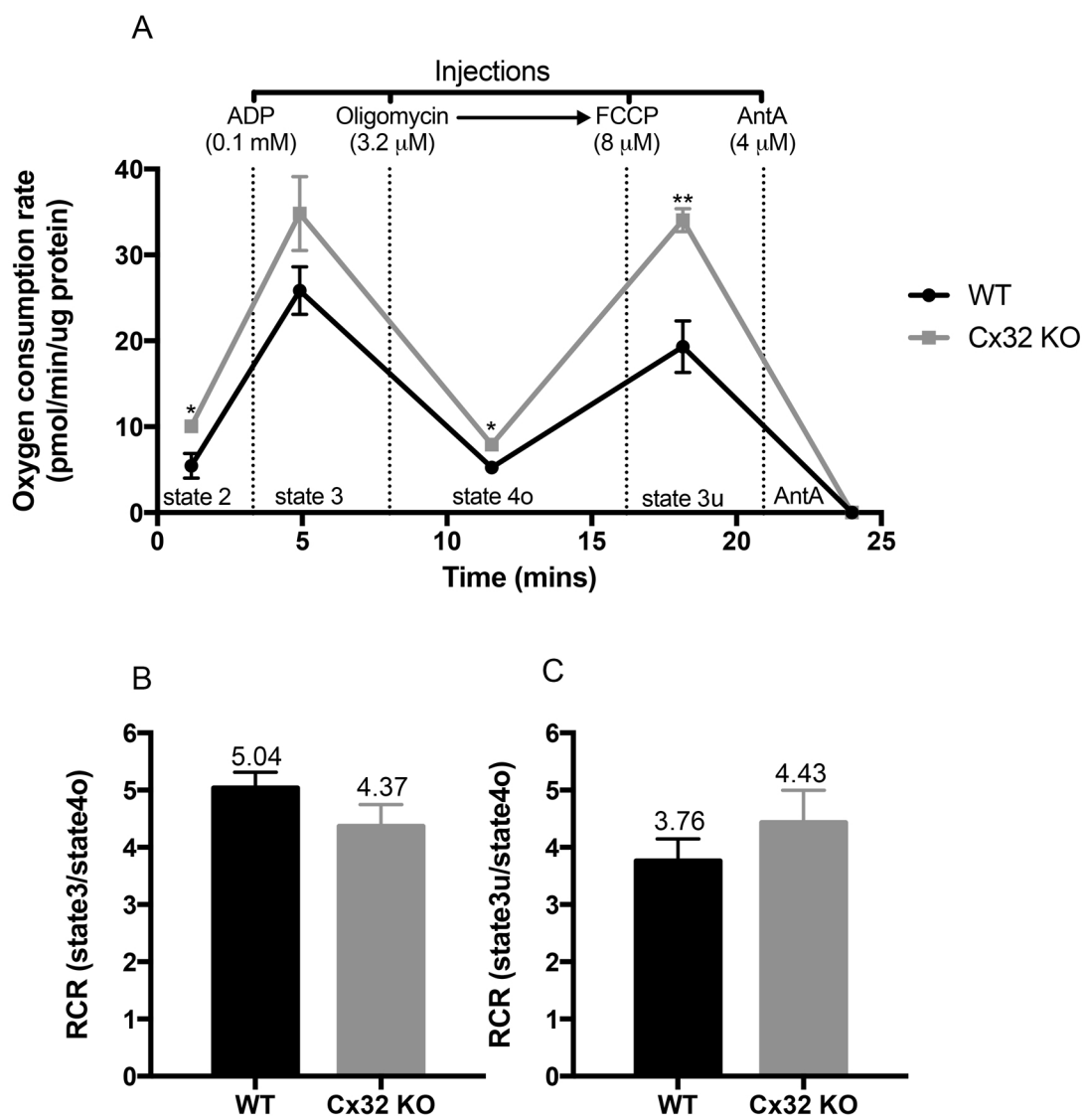


Figure 3.4

(Brand and Nicholls 2011). These ratios can be calculated from both state 3/state 4o and/or state 3_μ/state 4o OCRs, and generally, higher values indicate expected function, while lower values indicate dysfunction. Here, despite significant elevations in the absolute OCRs between WT and Cx32 KO mitochondria, we present no significant differences between RCRs (Figure 3.4B,C). The slightly lower $RCR_{(state\ 3/state\ 4o)}$ is representative of increased proton leak in Cx32 KO mitochondria (Figure 3.4B), while the trend for an increased $RCR_{(state\ 3_{\mu}/state\ 4o)}$ is representative of a higher FCCP-induced OCR in Cx32 KO mitochondria (Figure 3.4C). We confirmed that absolute bioenergetic differences between WT and Cx32 KO mitochondria were not artifacts of mitochondrial isolation. Isolated mitochondrial preparations from WT and Cx32 KO mitochondria (2 separate isolations each) were responsive to previously validated ADP concentrations (0.05 - 0.5 mM, Figure 3.5A), and showed the expected decrease in OCR upon CytC titration (Figure 3.5B). A decrease in the OCR following addition of exogenous CytC indicates that the OMM was not damaged during mitochondrial isolation, or during attachment to the Seahorse microplate. Despite healthy ADP/CytC responses in these optimization experiments, Cx32 KO mitochondria exhibited increased state 2 OCRs compared to WT mitochondria, as was also demonstrated in the bioenergetics determinations (Figure 3.5A). Finally, while WT mitochondria exhibited maximal ADP-stimulated state 3 respiration rates at 0.1 mM ADP, Cx32 KO mitochondria respired at higher rates when exposed to 0.5 mM ADP, suggesting an inherent capacity for increased respiration.

Figure 3.5: Isolated mitochondria are healthy and functional.

(A) WT and Cx32 KO mitochondria respond equally to 0.1 mM ADP, while Cx32 KO mitochondria are capable of elevated OCRs with 0.5 mM ADP. Vertical dotted lines indicate the ADP injections. Asterisks indicate significant differences between WT and Cx32 KO OCRs ($p=0.05$, Student's 2-tailed t-test, $n=4$, $*p<0.05$). Data are presented as pmol/min/ μ g mitochondrial protein. **(B)** CytC titration (5-10 μ M) of ADP-stimulated (0.1 mM ADP) mitochondria resulted in decreased in OCRs, indicating intact OMMs. Vertical dotted lines indicate ADP, CytC, and AntA injections. Asterisks indicate significant differences between WT and Cx32 KO OCRs ($p=0.05$, Student's 2-tailed t-test, $n=4$, $*p<0.05$). Data are presented as pmol/min/ μ g mitochondrial protein, and are corrected for AntA background fluorescence.

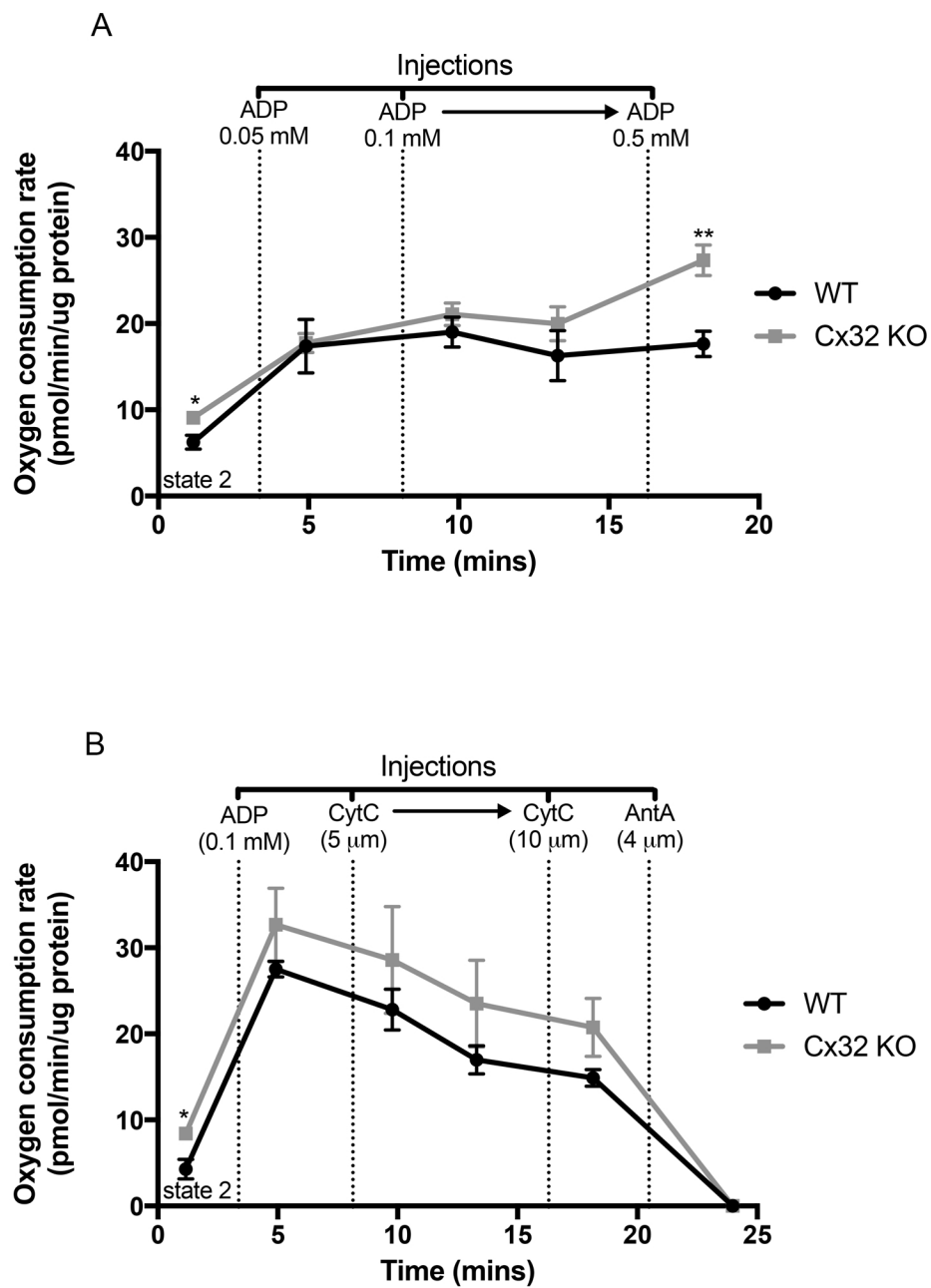


Figure 3.5

3.6.5. ROS levels and rate of ROS production are increased in Cx32 KO mitochondria

Given the significant increase in basal, maximal, and proton leak respiration in Cx32 KO mitochondria, we investigated the levels and rate of ROS generation using H₂DCFDA, a non-fluorescent dye that becomes fluorescent upon oxidation. We show that baseline fluorescence of H₂DCFDA was stable throughout the 2 min measurement period, and that it was equal between WT and Cx32 KO mitochondria (Figure 3.6A). Upon injection of complex I substrates (10 mM pyruvate/2 mM malate), ROS generation spiked, and steadily increased throughout the 2 min measurement period in both genotypes (Figure 3.6A). Quantitation of the average fluorescence intensity following addition of substrate indicated that Cx32 KO mitochondria generated larger absolute levels of ROS (H₂DCFDA fluorescence intensity), compared to WT mitochondria (Figure 3.6B). Further, calculation of the rate of H₂DCFDA increase over the measurement period (2 mins) indicated that ROS was also produced at an increased rate in Cx32 KO mitochondria (Figure 3.6C).

3.6.6. Cx32 KO mitochondria are more polarized than WT mitochondria

Increased respiration and ROS generation is usually accompanied by alterations in $\Delta\Psi$ M, therefore, we examined differences between WT and Cx32 KO mitochondria using the positively charged fluorescent dye, TMRE. TMRE readily accumulates into the negatively charged mitochondrial matrix, thus, changes in the fluorescent signal reflect $\Delta\Psi$ M. In isolated mitochondria, low concentrations of TMRE result in the quenching phenomenon, wherein accumulation and intercalation of TMRE molecules induces quenching of the fluorescent signal. Quenching continues until FCCP-induced

Figure 3.6: Cx32 KO mitochondria produce more ROS than WT mitochondria

(A) Kinetic assay of H₂DCFDA fluorescence in WT and Cx32 KO mitochondria. H₂DCFDA was added directly to mitochondrial suspensions at time zero, and the vertical dotted line indicates injection of 10 mM pyruvate and 2 mM malate. Data are presented as arbitrary fluorescence units (AU) per mg of mitochondrial protein. **(B)** Average fluorescence intensity (AU/mg) for substrate-stimulated mitochondria (Student's 2-tailed t-test, n=3, *p<0.05). **(C)** Rate of H₂DCFDA fluorescence elevation following substrate addition (AU/sec/mg) (Student's 2-tailed t-test, n=3, *p<0.05).

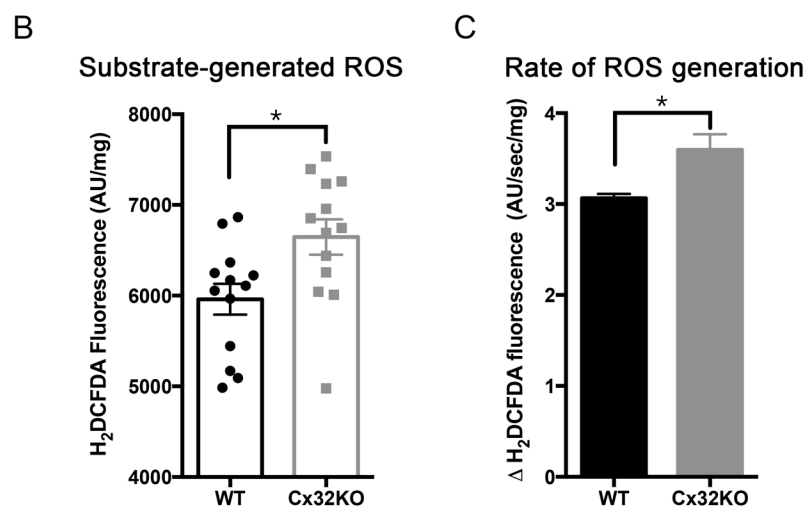
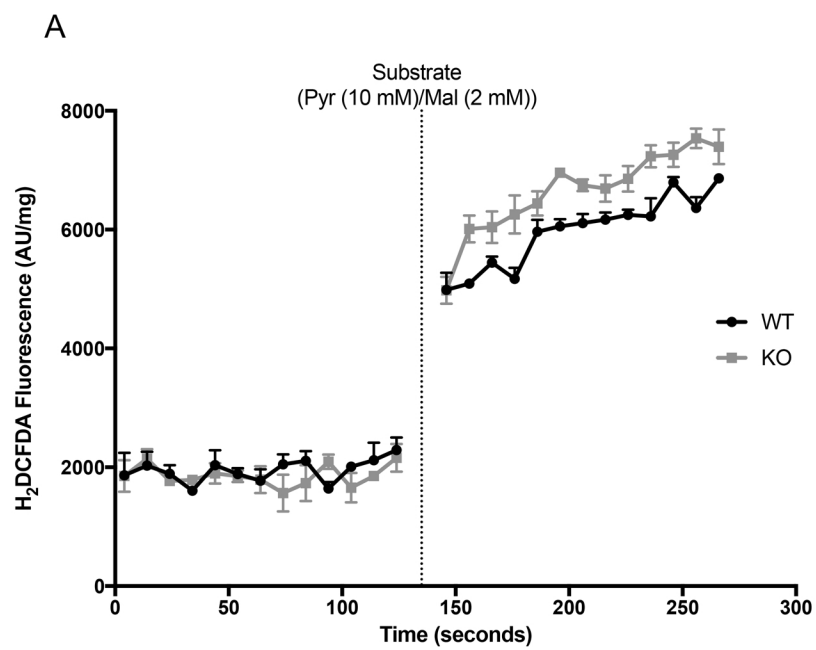


Figure 3.6

dissolution of $\Delta\Psi$ M results in its release from the mitochondrion. Here, addition of TMRE to the mitochondrial solution resulted in a decline in fluorescence as TMRE was taken up into the organelles over the 2 min measurement period (Figure 3.7A). The addition of complex I substrates pyruvate and malate contributed to a continued quenching of the fluorescent signal, as protons continued to be pumped across the IMM (Figure 3.7A). Finally, the addition of FCCP resulted in a rapid increase in TMRE fluorescence as $\Delta\Psi$ M was destroyed, followed by a steady dissipation to low levels as the dye equilibrated into the reaction medium (Figure 3.7A). The spike in fluorescence following addition of FCCP confirmed that the assay was operating in quench mode. Calculation of the mean fluorescent intensity between the two genotypes at each part of this kinetic assay indicated that more dye was accumulated into Cx32 KO mitochondria compared to WT mitochondria (lower fluorescence, more quenching), particularly upon addition of complex I substrates (Figure 3.7B, $p < 0.0001$).

Figure 3.7: Cx32 KO mitochondria are more polarized than WT mitochondria.

(A) TMRE fluorescence accumulation (AU/mg) in WT and Cx32 KO mitochondria during substrate-stimulated respiration, and following uncoupling with FCCP. Down-facing arrow indicates that TMRE was added just prior to data collection. Vertical dotted lines indicates the injection of 10 mM pyruvate and 2 mM malate, and 10 nM FCCP, respectively. A decrease in TMRE fluorescence intensity indicates increased $\Delta\Psi_M$ (more polarized mitochondria), as TMRE fluorescence is quenched upon accumulation into the mitochondrial matrix. Data are presented as arbitrary fluorescence units (AU/mg protein). Maximal TMRE accumulation points are indicated with * for WT and ♦ for Cx32 KO. **(B)** Average TMRE fluorescence values for TMRE alone, TMRE+substrates, and TMRE+FCCP (Student's 2-tailed t-test, n=3, **p<0.01, ***p<0.001, ***p<0.0001). Data are presented as arbitrary fluorescence units (AU/mg protein).

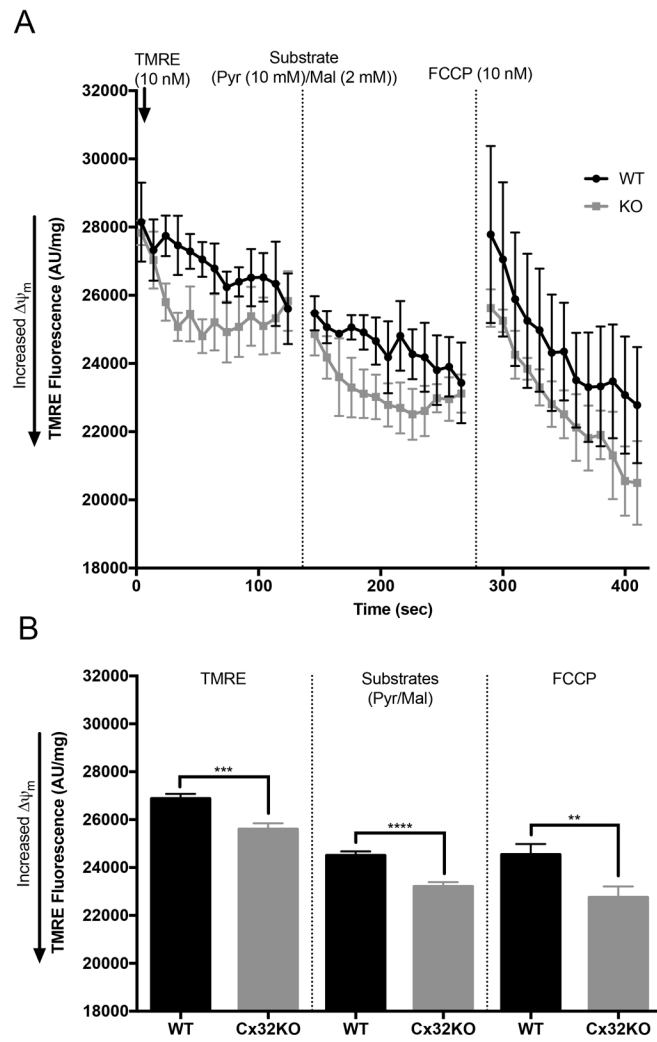


Figure 3.7

3.7. Discussion

Our previous work has shown that Cx32 interacts with mitochondrial proteins (Chapter 2). These observations indicated that mitochondrial function may be affected in the Cx32 KO mouse. To address this question, we performed unbiased proteomic profiling of WT and Cx32 KO brain membranes, employing LFQ and pathway analysis to determine the main effects of Cx32 loss. Our proteomic assessments indicate that loss of Cx32 alters mitochondrial TCA and respiratory electron transport pathways. We further provide the first direct super-resolution microscopy evidence of Cx32 protein in mitochondrial organelles, where Cx32 is present in ~80 nm wide puncta surrounding the periphery of isolated mitochondria. We also report that OCRs of freshly-isolated, healthy Cx32 KO mitochondrial preparations are elevated during state 2 (basal), state 4o (leak), and state 3 μ (FCCP-induced maximal) respiratory conditions. Finally, when stimulated with the complex I substrates pyruvate and malate, Cx32 KO mitochondria produced more ROS than WT mitochondria. Under conditions of both substrate starvation and substrate excess, Cx32 KO mitochondria were more polarized (possessed higher $\Delta\Psi$ M) than WT mitochondria. These findings provide converging evidence for an intrinsic role for Cx32 in restricting rates of oxidative phosphorylation. Indeed, loss of Cx32 results in higher mitochondrial respiration rates and ROS production, which may contribute to the phenotypic alterations seen in Cx32-related neuropathies and tumours.

To our knowledge, this report is the first quantitative LFQ proteomic profiling of Cx32 KO tissue. The pathways identified from the significantly regulated proteins are almost exclusively related to TCA cycle, pyruvate metabolism, electron transport, ATP

synthesis, and mitochondrial biogenesis (Atp5b, Cox6b1, Uqcrb, Ldhb, Ndufs2, Mtatp8, Uqcrc1, Pdhb, Cox5a, and Ppp3cb). These data suggest that Cx32 may be participating in these processes directly in mitochondrial membranes, as has been shown for Cx43. Indeed, biochemical confirmation of Cx32 at sub-fractionated mitochondrial IMMs, coupled with the novel STED-CW-microscopic identification of Cx32 protein in isolated mitochondria, provided further support for this hypothesis.

Bioenergetic analysis of isolated hepatic mitochondria indicated that basal, leak, and FCCP-induced OCRs were increased in Cx32 KO mitochondria. As basal respiration is predominantly controlled by ATP turnover, and partly by substrate oxidation and proton leak (Brand and Nicholls 2011), increased basal respiration in the presence of pyruvate and malate suggests that substrate oxidation is enhanced by complex I, resulting in the increased shuttling of H^+ ions across the IMM. Importantly, lactate dehydrogenase b and pyruvate dehydrogenase are significantly increased in Cx32 KO mitochondria, suggesting an enhanced capacity for interconversion of pyruvate and lactate, and enhanced production of Acetyl-CoA from pyruvate. These reactions are both coupled with reduction of NAD^+ to NADH, contributing electrons and protons to Complex I, and may contribute to higher mitochondrial oxygen consumption if elevated (Liang et al. 2016). Further, Ndufs2, a core subunit of NADH dehydrogenase (Complex I), is also significantly elevated in Cx32 KO membranes. Ndufs2, along with other Ndufs family members, transfers electrons from NADH to the respiratory chain through ubiquinone (Zickermann et al. 2009). Mutation of Ndufs2, and subsequent impairment of Complex I activity causes encephalomyopathy with hypertrophic cardiomyopathy, and Leigh Syndrome (Pagniez-Mammeri et al. 2012), thus, enhanced

expression of Ndufs1 may contribute to more efficient electron transfer, leading to enhanced proton pumping, and higher rates of basal proton leak respiration. Indeed, Cx32 KO mitochondria also exhibit significantly increased TMRE dye accumulation compared to WT mitochondria, suggesting that loss of Cx32 contributes to a more polarized mitochondrial phenotype characteristic of an enhanced electrochemical gradient across the IMM.

Interestingly, despite increased basal respiration, ADP-stimulated respiration was not significantly different in WT and Cx32 KO mitochondria under the current experimental conditions, and both values correspond well with similar studies of isolated mitochondrial respiration (Adjeitey et al. 2013; Mailloux et al. 2013; Mailloux et al. 2014). In contrast, isolated mitochondrial bioenergetics indicated that loss or inhibition of Cx43 causes reductions in ADP-stimulated respiration (2 μ M ADP/ μ g mitochondria) (Miro-Casas et al. 2009). We do not report any ADP-stimulated differences in respiration using similar amounts of ADP (5 μ M ADP/ μ g and 10 μ M/ADP/ μ g mitochondria, respectively), however, Cx32 KO mitochondria respired at significantly higher rates than WT mitochondria when 0.5 mM ADP was provided in the ADP titration experiment (50 μ M ADP/ μ g mitochondria). These data indicate that Cx32 KO mitochondria require more ADP to exhaust the activity of endogenous ATP synthase, and this may be due to increased expression of the ATP synthase subunits. Indeed, both Mtatp8 and Atp5b ATP Synthase subunits were significantly increased in Cx32 KO membranes via LFQ; however, these changes will require independent validation by Western analysis.

Application of oligomycin inhibits the ATP synthase, and allows the characterization of proton leak across the IMM, separate from leak across the ATP

synthase. As for basal respiration, we show similar significant differences in state4o OCRs between WT and Cx32 KO mitochondria following oligomycin injection. Interestingly, basal OCRs are significantly higher than state4o OCRs in Cx32 KO, but not in WT mitochondria (Supplemental Figure 1), indicating that leak through the ATPase partially contributes to elevated basal respiration in the Cx32 KO. This observation could be partially explained by increased ATP Synthase subunit expression in the Cx32 KO, yet again, these changes must be empirically determined, as leak respiration in state4o is predominantly controlled by the adenine nucleotide translocase (ANT) and the uncoupling proteins (UCPs) (Brand et al. 2005; Divakaruni and Brand 2011). There is no evidence that the ANT or UCPs are upregulated in the Cx32 KO, however, we have reported that Cx32 may interact with the ANT-1 (Fowler et al. 2013), which could stabilize the amount of leak occurring from the membrane/ANT protein interface (Brand et al. 2005). Alternatively, loss of Cx32 at IMMs may enable enhanced IMM localization of ANT proteins, thereby enhancing the amount of proton leak.

Cx32 KO mitochondria also exhibit enhanced maximal, FCCP-induced OCRs compared to WT mitochondria. Here, H^+ ions flow freely back into the matrix, and respiration is limited only by the rate of substrate oxidation and the activity of the respiratory chain (Brand and Nicholls 2011). These data are consistent with our findings that substrate oxidation is likely enhanced in Cx32 KO membranes, as *Ldhd* and *Pdhd* are both upregulated, yet, in addition to *Ndufs2* enhancement, Complex III subunit 1 (*Uqcrc1*) and Cytochrome c oxidase, subunit 5a (*Cox5a*) and Cytochrome c oxidase subunit 6B1 (*Cox6b1*) are also significantly upregulated in Cx32 KO membranes (KO/WT ratios: 1.51, 1.46, respectively). These findings provide evidence

that both substrate oxidation and activity of the respiratory chain proteins may contribute to higher OCRs in Cx32 KO mitochondria. It is important to note, however, that both calculated RCRs (state 3/state 4o, state 3 μ /state 4o) were not significantly different between WT and Cx32 KO, indicating that no gross dysfunctions in respiratory control were evident with loss of Cx32.

The enhancement of ROS production, and the elevation of $\Delta\Psi$ are phenotypes that align well with enhanced substrate oxidation and respiratory complex activity. Complex I and Complex III are the major sites of oxidant production, with other contributors including Pdh, and the electron transfer flavoprotein-ubiquinone oxidoreductase family (Holmstrom and Finkel 2014), of which multiple family members were detected in our dataset. Generally, an increase in $\Delta\Psi$ is associated with increased ROS production, whereas a decrease in the $\Delta\Psi$ results in decreased ROS generation (Mailloux and Harper 2012). The exact relationship between these two entities is not precisely understood, however, $\Delta\Psi$ can also be affected by the active transport of cations (Ca^{2+}), anions (ADP/ATP), and metabolites (aspartate and glutamate) across the IMM, and it is very possible that Cx32 plays a role in these processes (Hinkle 2005; Kadenbach 2003).

Higher $\Delta\Psi$ s and ROS production may also provide insight into the mechanism of enhanced carcinogenesis in the liver of the Cx32 KO mouse. Indeed, Cx32 KO mice are particularly sensitive to the development of spontaneous liver tumors, and are more susceptible to chemical and X-ray radiation-induced liver tumors. Cx32 KO mice also exhibit increased lung tumorigenesis, and on rare occasions develop invasive medullary adrenal tumors (pheochromocytoma) (King and Lampe 2004a; King and Lampe 2004b).

Increased ROS production activates the MAPK oncogenic pathway (Meves et al. 2001), which is directly responsible for promoting aberrant cell proliferation (Irani et al. 1997; Ogrunc et al. 2014). Accordingly, increased levels of activated MAP kinases ERK1 and ERK2 are present in tumors from Cx32 KO mice, and are hypothesized to underlie the propensity for oncogenesis in this mouse model (King and Lampe 2004a; King and Lampe 2004b).

Finally, we report here that loss of Cx32 causes significant increases in the mitochondrial antioxidants Peroxiredoxin 5 (Prdx5) and Peroxiredoxin 1 (Prdx1). These data are consistent with transcriptional analyses of hepatocytes that reported 10-fold higher levels of the antioxidant superoxide dismutase in Cx32 KO cells compared to WT cells (Stahl et al. 2005). Combined, these data suggest that baseline oxidant damage is elevated with loss of Cx32, and that differences in respiration, membrane potential, and ROS production from isolated liver mitochondria may contribute to the increased incidence of hepatocellular tumors and increased hepatocyte proliferation rates in the Cx32 KO mouse (Evert et al. 2002; King and Lampe 2004b; Temme et al. 1997).

Taken together, increases in basal, leak, and maximal respiration, combined with elevations in ROS and $\Delta\Psi_M$, are evidence of enhanced respiratory function with loss of Cx32. This finding directly opposes the proposed role for Cx43 in cardiac mitochondrial membranes. Here, loss or inhibition of cardiomyocyte Cx43 resulted in lowered ADP-stimulated Complex I OCRs, and significantly decreased citrate synthase activity (Boengler et al. 2012). It is proposed that Cx43 forms hemichannel-like hexamers at the IMM that contribute to mitochondrial K^+ uptake, thereby positively affecting mitochondrial oxygen consumption capacities (Boengler et al. 2013). Conversely, Cx32

appears to limit mitochondrial OCRs, and differences between the two connexins are evident in studies of genetic replacement of Cx32 into Cx43KO cardiac mitochondria, as Cx32 expression is not sufficient to functionally replace the CBX-sensitive K⁺ influx of WT, Cx43-containing mitochondria (Miro-Casas et al. 2009). Despite this clear tissue-specific requirement for mitochondrial connexin phosphorylation, interaction, or channel-dependent events, it is possible that enhanced respiration in Cx32 KO mitochondria is a direct result of Cx32 interaction with electron transfer chain proteins at the IMM, as has also been described for Cx43 (Denuc et al. 2016). Alternatively, Cx32 may form hemichannels in the IMM that passively allow the efflux of mitochondrial substrates from the matrix, preventing the higher levels of respiration and ROS production seen in the Cx32 KO.

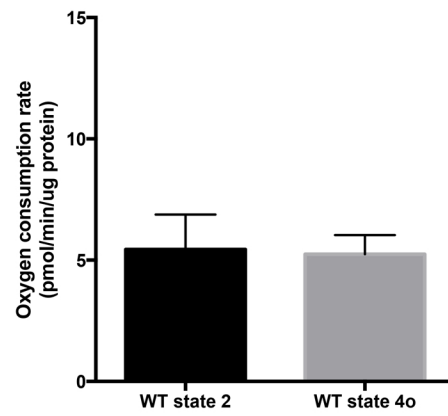
In summary, we have undertaken the first quantitative proteomic profiling of WT and Cx32 KO membranes. We show that the majority of differentially regulated proteins are increased with loss of Cx32, and correspond mainly to TCA cycle, respiratory electron transport, and ATP synthesis, while downregulated proteins are involved in a variety of synaptic functions. This study also provides the first evidence of Cx32 protein expression in mitochondria via super-resolution STED-CW microscopy imaging of intact mitochondrial organelles. The loss of Cx32 was sufficient to significantly alter several bioenergetic properties of isolated hepatic mitochondria, culminating in a subclinical phenotype characterized by enhanced basal and maximal OCRs, more polarized membrane potentials, and significantly increased ROS production. As such, the presence of Cx32 appears to limit the respiration rate of hepatic mitochondria, preventing the deleterious signalling consequences of ROS. Taken together, these

findings extend our understanding of intracellular connexins, and may help uncover novel pharmacological targets to reduce the severity of neuropathy and enhanced tumourigenesis due to Cx32 mutation and/or downregulation.

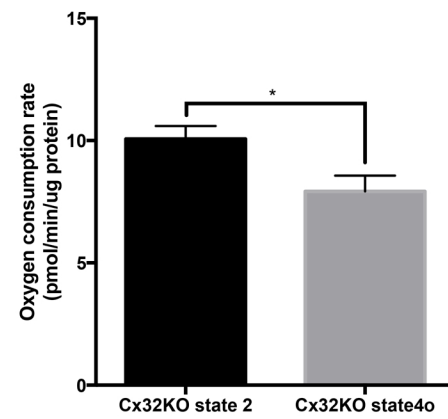
Supplemental Figure 1: State 4o respiration is significantly decreased compared to state 2 respiration in the Cx32 KO.

(A) There is no change in OCR (pmol/min/ μ g protein) between state 2 and state4o in WT isolated mitochondria (two-tailed t-test, $p=0.05$, $n=4$). **(B)** The State4o OCR is significantly decreased compared to the state 2 OCR, suggesting that ATP Synthase-related oxygen consumption contributes significantly to the basal respiration rate in Cx32 KO mitochondria, but not in WT mitochondria (two-tailed t-test, $p=0.05$, $n=4$) (Data from Figure 3.4A).

A



B



Chapter 4: A Connexin-Dependent Juxtacrine Signalling Network Regulates Adult Hippocampal Progenitor Neurogenesis

Stephanie Fowler^{1†}, Lysanne Melanson-Drapeau^{1†}, Mark Akins¹, Mario Y. Morin¹, Sophie Imbeault¹, Hadi Toeg¹, Claude Messier², Diane Lagace³, Akiko Nishiyama⁴, David L. Paul⁵, Steffany A.L. Bennett^{1*}

¹Neural Regeneration Laboratory, Ottawa Institute of Systems Biology, Centre for Catalysis Research and Innovation, Ottawa Brain and Mind Research Institute, Department of Biochemistry, Microbiology and Immunology, ²Department of Psychology, ³Department of Cell and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada, ⁴Department of Physiology and Neurobiology, University of Connecticut, Storrs, CT, USA, ⁵Department of Neurobiology, Harvard Medical School, Boston, MA, USA. †Both authors contributed equally.

Keywords: gap junction, connexin, Cx32, glial cell network, adult neurogenesis, kainic acid, hippocampus, BrdU, lineage analysis, neurogenic niche, regeneration

Chapter 4: A connexin-dependent juxtacrine signalling network regulates adult hippocampal progenitor neurogenesis

4.1. Objective of study

The objective of this chapter was to investigate the role of the glial network in neuronal regeneration following kainic acid-induced injury in WT and Cx32 KO mice. We identified permissive glial cell:cell interactions in intact brain PMs, and show that loss of Cx32 creates a microenvironment more permissive to neural regeneration following injury.

4.2. Author contributions

SF wrote the manuscript and performed the cell fractionation and IP analyses, analysis and graphics for Figure 4, immunohistochemistry for Fig 10, analysis and graphics for Figure 11, graphics for Supplemental Figure 1, and experiments for Supplemental Figure 5. SALB and LMD conceived and designed the experiments for Figures 5-10A. MA performed mouse husbandry and kainic acid experiments for the Nestin⁺/NG2⁺ marker mice, MM performed the Morris Water Maze behavioural tests, SI performed Cx47 confocal immunofluorescence, HT performed the co-culture experiments, CM kindly assisted in the Morris Water Maze interpretations, DL and AN provided the NesCre and NG2Cre ERT2 mouse models, DP provided crucial reagents and necessary interpretations.

4.3. Abstract

The adult human hippocampus is capable of significant neurogenesis; however, seizure-induced cell replacement produces ectopic granule neurons that impair functional and behavioural recovery. Here, we show that adult type 1 and type 2a NPCs are part of a pan-glial juxtacrine interactome coupled by heterotypic gap junction subunits Cx32:Cx30, Cx47:Cx43, Cx30:Cx43 and homotypic Cx30:Cx30 and Cx43:Cx43 channels. In uninjured hippocampus, we find that oligodendrocyte (Cx32):NG2-Glia (Cx32) regulates NG2-Glia proliferation while NG2-Glia (Cx32):NPC (Cx30) communication regulates type 1 and type 2a NPC proliferation. Further, we show that Cx30-mediated gap junctional intercellular communication between astrocytes and type 1 and 2a NPCs is sufficient to induce neurogenesis *in vitro*. *In vivo*, following kainic acid-induced seizures, we find that Cx32:Cx30-mediated NG2-Glia:astrocyte and NG2-Glia:NPC communication opposes the pro-neurogenic effects of Cx30-mediated astrocyte:NPC gap junction coupling in injured hippocampus. Unexpectedly, removing regulatory Cx32 NG2-Glia: Cx30 astrocyte and NPC coupling from this juxtacrine network is sufficient to promote functional neuronal replacement, prevent ectopic granule cell integration into damaged cornu ammonis (CA) fields, and reverse seizure-induced learning and memory deficits. These results describe a novel juxtacrine signalling interactome that can be modulated to instruct functional NPC-mediated cell replacement in the adult hippocampus following acute seizure.

4.4. Introduction

Two regions of the uninjured adult brain are capable of significant neurogenesis: the SVZ of the lateral ventricle and the SGZ of the DG (Arvidsson et al. 2002; Lindvall and Bjorklund 2004). In the adult SGZ, neurogenesis is a step-wise process involving activation of quiescent type 1 stem-like NPCs generating highly proliferative type 2a NPCs. Type 2a NPCs specify to neuronally committed type 2b NPCs that, in turn, generate type 3 neuroblasts. Under physiological conditions, Type 3 neuroblasts migrate into the granule cell layer (GCL) and can terminally differentiate into granule neurons or are deleted prior to functional integration (Kempermann et al. 2004a). This perpetual process contributes approximately 700 new neurons per day in adult humans, representing 2% annual neuronal turnover (Spalding et al. 2013). Neurogenesis is also increased by physiological events such as aerobic exercise (Nokia et al. 2016; van Praag et al. 1999; van Praag et al. 2005) as well as pathophysiological brain injury (Chirumamilla et al. 2002; Jin et al. 2006; Kernie et al. 2001).

Augmenting endogenous cell replacement represents a novel, potentially transformative, approach to brain repair. Successful strategies, however, must be able to instruct activated NPCs to adopt required cell lineages. Migration of type 3 neuroblasts, for example, is effectively redirected towards lesioned CA cell fields following acute seizures but progeny terminally differentiate into granule not pyramidal cells (Dashtipour et al. 2001; Jessberger et al. 2005; Scharfman and Pierce 2012). Integration of these hyperexcitable ectopic neurons into damaged pyramidal cell fields accelerates epileptogenesis by promoting spontaneous recurrent seizure activity (Hung et al. 2012; Scharfman 2000). For this reason, inhibiting resident NPC proliferation in

experimental seizure models has proven more effective at improving behavioural measures of hippocampal recovery than has endogenous cell replacement (Jessberger et al. 2007a; Jessberger et al. 2007b).

Direct cell:cell contact instructs NPC fate (Alvarez-Buylla and Lim 2004; Morrens et al. 2012; Song et al. 2002). Astrocytes have repeatedly been shown to regulate NPC specification, in part, through ephrin-B and beta-catenin-mediated signalling (Ashton et al. 2012; Song et al. 2002). The complete repertoire of juxtacrine instructive pathways, however, has only begun to be elucidated. Gap junctional communication represents a robust but unexplored means by which terminally differentiated cells may instruct adult neurogenesis. In embryonic brain, over the course of corticogenesis, radial glial NPCs are transiently gap junction-coupled across the cell cycle with these adhesive channels regulating their proliferation and migration (Bittman et al. 1997; Elias et al. 2007).

Connexins are the structural units of gap junction channels. Twelve connexins (Cx26, Cx29, Cx30, Cx31.9, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, Cx47, Cx57) are expressed in adult brain (Rouach et al. 2002). Ten connexins (Cx26, Cx29, Cx30, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, and Cx47) have been detected in postnatal NPCs cultured *in vitro* over the course of their specification (Imbeault et al. 2009). Hexamerization of compatible connexin partners forms a hemichannel (or connexon). Insertion of these connexons into non-junctional PMs forms single membrane voltage-gated pores. At junctional membranes, docking of compatible connexons between adjacent cells forms intercellular channels enabling cell:cell adhesion and direct exchange of ions, metabolites, and small signaling molecules between coupled cells (Bruzzone and Ressot 1997; Dermietzel 1998; Magnotti et al. 2011b; Nagy et al. 2004).

While A/O gap junctional communication in adult brain has been inferred by freeze-fracture immuno-electron-microscopy, confocal co-immunolocalization of compatible connexin partners, and dye-transfer studies (Magnotti et al. 2011b; Nagy et al. 2003b), it is not known whether adult hippocampal NPCs are capable of GJIC with adjacent instructive cells.

Here, we asked whether a pan-glial/NPC connexin interactome regulates adult hippocampal NPC activation and specification. We show that oligodendrocytes and NG2-Glia interact with type 1 and type 2a NPCs via heterotypic Cx32:Cx30 and Cx47:Cx43 channels, whereas astrocytes interact with type 1 and 2a NPCs via homotypic Cx30:Cx30 and Cx43:Cx43 and heterotypic Cx30:Cx43 channels. Further, our data indicate that Cx32 presented by NG2-Glia modulates proliferation of Cx30-expressing NPCs. Following acute seizure, preventing NG2-Glial Cx32-mediated communication with Cx30-expressing astrocytes and type 1 and 2a NPCs is sufficient to promote functional neurogenesis and prevent birth of ectopic granule neurons. These findings demonstrate that modulating gap junction coupling between networks of glia and multipotential NPCs can be used to instruct functional neurogenesis in the adult hippocampus following acute seizure.

4.5. Materials and Methods

4.5.1. Mice

Cx32^{Y/-} and Cx30^{-/-} animals (Nelles et al., 1996; Teubner et al., 2003) were kindly provided by Dr. Klaus Willecke (University of Bonn). Breeding pairs of Cx43^{ff} and Cx47^{-/-} animals were generated by our laboratories as described (Altevogt et al., 2002; Menichella et al., 2003; Nishii et al., 2003). Connexin KO animal strains and backcross

information is as follows: Cx43^{ff}: C57BL/6 x 129/SvJ x FVB/N, Cx30^{-/-}: C57BL/6 x 129/SvJ N3, Cx47^{-/-}: C57BL/6 x 129/SvJ N9, and Cx32^{Y/-}: C57BL/6 x 129/SvJ N12. Tamoxifen (TAM)-inducible NG2-Cre reporter animals were obtained from Akiko Nishiyama (Zhu et al. 2008). NG2-Cre^{ERT2} animals were crossed with Cx32^{Tom} animals, and backcrossed to C57BL/6 to an N3 generation in our laboratory to generate NG2-Cre^{ERT2} x Cx32^{Tom} N3 C57BL/6 animals. Nestin-Cre^{ERT2-IRES-hPLAP} reporter animals were obtained from S. Baker (Cicero et al. 2009), crossed in our laboratory with Cx32^{YFP} animals, then backcrossed with C57BL/6 animals to an N6 generation. Nestin-Cre^{ERT2} animals were obtained from P. Frankland, and their generation is described by (Imayoshi et al. 2008). Genotyping of all animals was performed as in (Imbeault et al. 2009).

4.5.2. OP Fractionation of whole brain membranes

Two WT brains (cerebrum + cerebellum) were homogenized in a glass Potter-Elvehjem homogenizer with teflon pestle using homogenization buffer without detergent (HB: 250 mM sucrose, 1 mM CaCl₂, 20 mM HEPES, pH 7.4, EDTA-free protease inhibitor tablet, SIGMA #11873580001). Tissues were homogenized using ~50 gentle, even strokes, lysates were passed through a 23 g needle 5 times, and centrifuged at 1000 rcf for 10 mins to pellet nuclei. The cleared supernatants were pooled to generate the PNS, of which 2.4 mL was added to 3.6 mL of 50% working solution of OP density gradient medium (50% working solution: 5 volumes of OP with 1 volume of diluent [250 mM sucrose, 120 mM HEPES, pH 7.4]) to generate 6 mL of 30% OP/PNS solution. Subsequent dilutions of OP were prepared by diluting the 50% working solution with HB. Discontinuous gradients were prepared on ice by slowly overlaying the 6 mL of

30% OP/PNS preparations with 1 mL each of 25%, 20%, 17.5%, 15%, and 0.75 mL of 10%, 5%, and 2% OP solutions into 13.2 mL (14 x 89 mm) polyallomer centrifuge tube. HB was added to a point one mm below the surface of the tubes (~1 mL). Tubes were carefully balanced and adjusted with HB before assembly into an SW41 Ti rotor (Beckman Coulter). Gradients were centrifuged at $200,000 \times g_{ave}$ for 16 hours at 4°C (acceleration = 1, deceleration = 0). The resulting gradients were collected in 1 mL fractions (12 fractions/tube). Each fraction was buffer-replaced with 1% NP-40 buffer (20 mM Tris, 137 mM NaCl, 1% NP-40, 2 mM EDTA, pH 7.4), and solubilized for 2 h at 4°C with inversion rotation. Samples were centrifuged at 16,100 rcf for 20 mins to pellet insoluble material, and supernatants were analyzed by immunoblotting and IP.

4.5.2. Preparation of whole brain protein lysates for immunoblotting and IP

Whole brain (cerebrum + cerebellum) or dissected hippocampus was isolated from WT and Cx32^{Y/-} mice, and homogenized in 2 mL (brain), or 500 uL (hippocampus) fresh RIPA buffer (10 mM phosphate-buffered saline [PBS; 10 mM phosphate, 154 mM NaCl]), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitors (1 mM NaF, 50 g/mL aprotinin, 1 mM sodium orthovanadate, 1 mg/mL PMSF) using a tissue-tearor. Lysates were passed through a 26 g needle 6-8 times, and incubated on ice for 30 mins prior to centrifugation at 16,100 rcf for 30 mins. Supernatants were assayed for protein concentration using the Bio-Rad DC protein assay kit (Bio-Rad #5000116).

4.5.3. IP of glial connexin cell-cell interacting partners

Whole brain lysates were prepared as above, and diluted to 2.5 mg/mL in RIPA buffer with fresh protease inhibitors (Figure 4.1C). OP brain fractions 4 and pooled

fractions 5-7 were assayed for protein concentration, and for each IP, 250 µg of protein was pre-cleared with 50 µL PBS-washed protein A or protein G agarose beads for 1 h at 4°C. Bait antibody (1 µg) was added to the lysates and incubated with inversion overnight at 4°C. Complexes were captured onto 50 µL PBS-washed protein A or protein G agarose beads with inversion for 2 hr at 4°C, and collected by gravity at RT. Cx32 and Cx43 (rabbit polyclonal bait antibodies) were captured on protein G beads, while Cx47 and Cx30 were captured on protein A beads (mouse monoclonal bait antibodies). Bead complexes were washed 1X with 1 mL of cold RIPA buffer, and 3X with 1 mL of cold 10 mM PBS. Complexes were eluted from the beads in 100 µL prepared Bio-Rad sample buffer (as described in immunoblotting section), at 65°C for 10 mins. Beads were collected at 2000 rcf for 1 min, and bead supernatants were reserved. Immunoblots were prepared with 80% (Figure 4.1 B, D, E) or 10% (Figure 4.1C) of the total IP product.

4.5.4. Immunoblotting (Bio-Rad stain-free technology)

Protein samples (as prepared above) were diluted in 4X Laemmli sample buffer (Bio-Rad, #1610747) with 50 mM DTT and 10% BME, and solubilized at room temperature for 30 min. Ten µg of protein were resolved per lane on 4-15% Mini PROTEAN® TGX Stain-Free™ precast gels (Bio-Rad, #4568086 or #4568083), and transferred to Immun-blot® Low-Fluorescence PVDF membranes at 100V for 50 minutes as per manufacturer's instructions. Membranes were blocked in 5% (w/v) skim milk powder–PBS with 0.1% Tween-20 (PBST; blocking buffer) for 1 h, and incubated in primary antibody diluted in the same buffer for 2 hr at RT, or overnight at 4°C (see Table 4.1 for working concentrations of all antibodies). Membranes were rinsed twice in 0.1%

Table 4.1. Antibody Information

Antibody	Species	Company	Product #	*WB	**IHC	Used in Figure
Cx32	Mouse	Invitrogen	35-8900	1/500	N/A	4.1, 4.4
Cx32	Mouse	Invitrogen	13-8200	N/A	1 µg/mL	4.2
Cx30	Mouse	Santa Cruz	sc-514847	1/200	N/A	4.1, 4.2
Cx30	Rabbit	Invitrogen	71-2200	1/1000	N/A	4.1
Cx43	Rabbit	Invitrogen	71-0700	1/150	1:100	4.1, 4.2
Cx43	Mouse	Chemicon	05-763	1/1000	N/A	4.1
Cx47	Rabbit	D. Paul	N/A	1/1000	1:250	4.2
ATPase β2	Mouse	BD Bio	610915	1/5000	N/A	4.1
Flotillin	Mouse	BD Bio	610820	1/5000	N/A	4.1
Synaptophysin	Mouse	Abcam	ab8049	1/5000	N/A	4.1
Rab 5	Rabbit	Abcam	ab18211	1/1000	N/A	
CoxIV	Mouse	Molecular Probes	A21348	1/5000	N/A	4.1
MBP	Rabbit	Sigma	M3821	1/2000	N/A	4.1
ATP5A1	Rabbit	ProteinTech Group	14676-1-AP	1/500	N/A	4.1
Caspr	Rabbit	Abcam	ab34151	1/2000	N/A	4.1
AQP4	Rabbit	Millipore	AB2218	1:20000	N/A	4.1
Golgin97	Mouse	Molecular Probes	A21270	1/500	N/A	4.1
Actin	Mouse	Sigma	A4700	1/5000	N/A	4.4
CNPase	Mouse	Sigma	C5922	N/A	1:100	4.2
NG2	Rabbit	Chemicon	AB5320	N/A	1:200	4.2, 4.8
Nestin	Mouse	Chemicon	MAB353	N/A	1:50	4.2, 4.8
GFAP-Cy3	Mouse	Sigma	C9205	N/A	1:800	4.2, 4.8
RIP	Mouse	Chemicon	MAB1580	N/A	1:1000	4.2
PDGFαR	Rat	BD Bio	558774	N/A	1:333	4.2
NeuN	Mouse	Chemicon	MAB377	N/A	1:100	4.5, 4.6, 4.7, 4.8, 4.9
TOAD64	Mouse	Chemicon	C2993	N/A	1:1000	4.6
Tuj1	Mouse	RDI	MAB1195	N/A	1:250	4.6
NF160	Mouse	Sigma	N5264	N/A	1:200	4.6
Parvalbumin	Rabbit	SWANT	PV-28	N/A	1:1000	4.6
CaBP	Mouse	Chemicon	MABN849	N/A	1:200	4.6
BrdU	Mouse	Roche	11170376001	N/A	6 µg/mL	4.7, 4.8
DCX	GP	Chemicon	AB5910	N/A	1:4000	4.7
GFP	Rabbit	Abcam	ab290	N/A	1:2000	4.9
F4/80	Rat	Abcam	ab6640	N/A	1:100	4.8

*For all WB antibodies, the appropriate secondary antibody coupled to HRP was used at 1:2000 concentration (raised in mouse or rabbit). **For all immunochemistry, secondary antibodies were from Jackson ImmunoResearch Laboratories, and were tagged with Cy2 (rat IgG, 1:200), Cy3 (mouse IgG, 1:800; rabbit IgG, 1:600; guinea-pig IgG, 1:800; rat IgG, 1:400), FITC (mouse IgG, 1:100, rabbit IgG, 1:100), or AMCA (sheep IgG, 1:200). GP = guinea pig.

PBST, and twice in blocking buffer for 10 min prior to a 1 h incubation in HRP-conjugated anti-mouse or anti-rabbit secondary antibody diluted in blocking buffer. Chemiluminescent signal was detected using the ChemiDoc™ MP imaging system (Bio-Rad #1708280), and images were processed for densitometry analysis using ImageLab 5.2.1 software (Bio-Rad, build 11, Nov 14, 2015). Background-subtracted pixel densities for each band were corrected for loading variability by dividing by the pixel densities for the loading control actin (or by the stain-free gel signal). Corrected pixel densities were standardized to the WT value. Immunoblots in Figure 4.4 and Supplemental Figures 4.3 and 4.4 were performed as previously described (Fowler et al. 2009). Antibody details are found in Table 4.1.

4.5.6. Immunofluorescence

Mice were anesthetized with Euthansol (65 mg/mL) and intracardially perfused with 10 mM PBS (pH 7.2), followed by 3.7% molecular grade paraformaldehyde (Sigma) in 10 mM PBS (diluted immediately prior to use). Brains were removed and postfixed for 24 h at 4°C in the same fixative followed by 48 h of cryoprotection in 20% sucrose solution containing 0.001% sodium azide at 4°C. Serial cryostat sections (10 µm) were obtained (Leica), and sections were immunoreacted with primary antibodies (Table 4.1) diluted in antibody buffer (10 mM PBS, 0.3% Triton-X100, 3% bovine serum albumin). Details are as described in (Melanson-Drapeau et al. 2003). Sections were coverslipped in 0.05% p-phenylenediamine in PBS/glycerol, pH 8.0, or ProLong Gold (ThermoFisher Scientific, P36930), and imaged by epifluorescent microscopy using OpenLab 5.0.2 (Improvision) on a DMXRA2 epifluorescent microscope (Leica), or a

STED-CW Nanoscope (Leica) equipped with the Leica Application Suite Software v2.6.3.8173 (LAS).

4.5.7. Cx43 mRNA Detection

For *in situ* hybridization, the following DIG labeled oligoprobes for Cx43 were designed using Clone Manager (SciEd Software, Cary, NC, USA), and generated by Integrated DNA Technologies (Coralville, IA, USA): 3' labeled probe – CAGGCCGGAAGCACCAT-DIG and 5' labeled probe – DIG-GCTGTCGTCAGGGAAATCAA. 10 µm sections were hybridized with 100 pg/µL of a 1:1 mixture of the two probes in a humid chamber containing 50% formamide (EMD chemicals, Darmstadt, Germany) in a 1X salt solution (20 mM NaCl, 9 mM Tris-HCL, 1 mM Tris base, 5 mM dihydrous sodium phosphate, 5 mM disodium phosphate and 5 mM EDTA) at 65°C overnight. The hybridization solution contained 50% deionized formamide, 20% of a 50% dextran sulfate solution, 0.1 mg/mL yeast rRNA, and 1X Denhardt's solution (2% bovine serum albumin, 2% Ficoll 400, 2% polyvinyl pyrrolidone) in a 1X salt solution. Hybridization was detected using a sheep anti-DIG – alkaline phosphatase conjugated (Roche Diagnostics, Mannheim, Germany) antibody diluted 1:1500 in blocking solution (20% sheep serum, 2% blocking reagent (Roche Diagnostics) in 1X maleic acid buffer with 0.1% Tween-20). Alkaline phosphatase was developed using staining buffer (100mM NaCl, 100mM Tris-Cl pH9.5, 50mM MgCl_2 , 0.1% Tween-20) with 4.5 µL/mL 4-nitro-blue-tetrazolium chloride and 3.5 µL/mL 5-bromo-4-chloro-3-indoylphosphate (Roche Diagnostics). Unless otherwise noted all chemicals were obtained from Sigma (Sigma, Saint Louis, MO).

4.5.8. *BrdU birthdating*

BrdU (50 µg/g in sterile 10 mM phosphate buffered saline (PBS,) pH 7.0) was administered intraperitoneally to WT, Cx32 KO, Cx30 KO, and Cx43^{ff} animals. Unless otherwise indicated, animals received two daily injections (4-5 h apart) over two consecutive days, and a single injection on the third day. This delivery schedule enabled us to label not only more rapidly dividing Type 2a, Type 2b, and Type 3 neuroblasts known to be activated by acute seizure (Jessberger et al. 2005), but also the slowly dividing Type 1 population (Nowakowski et al. 1989; Pettit et al. 2012; Taupin 2007). At different survival times, BrdU immunostaining was performed as we have described (Melanson-Drapeau et al. 2003; Pettit et al. 2012). Quantification of the total number of BrdU-labelled cells in serial sections (n=8-10 sections per animal) along the entire anterior-posterior axis of the dorsal hippocampus, bregma -1.0 and -3.4 as defined by (Fanselow and Dong 2010) was performed as described (Pettit et al. 2012). Briefly, counts were restricted to the dorsal domain of the hippocampus as defined by (Fanselow and Dong 2010). The area of (a) the SGZ, defined as the region bordering the GCL and the hilus (3 cell widths into each), (b) the hilus, and (c) the entire GCL was established for each section using the Advanced Measurement Module of OpenLab 5.0.2 (Improvision) software. The number of labeled cells was assessed in both the left and right hippocampi by two independent observers blinded to the identity of the sections. These values were averaged to yield a single number per region, section, and animal. All labelled cells were counted within these defined areas in accordance with previous studies (Guo et al. 2010; Thallmair et al. 2006). Lineage of BrdU⁺ cells was determined by co-staining sections with BrdU and one or two lineage markers, to

determine the proportion of cycling cells of each lineage. Data were presented as % of proliferating cells that were lineage marker positive.

4.5.9. Kainic acid-induced seizures

To establish seizure sensitivity, cohorts of WT and Cx32 KO male mice (3 months of age) were injected intraperitoneally with 12.5-35 mg/kg kainic acid (Ocean Produce International). The optimal 25 mg/kg concentration producing stage 4 seizure with minimal mortality was used in all subsequent experiments. Seizure intensity was established according to behavioral criteria we have described previously (Cheung et al. 2005): stage 0, no response; stage 1, reduced locomotion and sedation; stage 2, automatisms including head bobbing, scratching, repetitive face washing, increased frequency of "freezing" and "wet-dog shakes"; stage 3, tremors and weak clonic convulsions; stage 4, intermittent followed by continuous rearing with forelimb clonus and loss of postural control; and stage 5, severe limbic seizures with respiratory difficulty. After 5 min of stage 4 seizure, mice were injected with diazepam (5 mg/kg) to limit convulsions, reduce mortality, and ensure equivalent CA3a/b loss in all cohorts. A total of n=51 WT and n=61 Cx32 KO mice survived stage 4 seizure at the optimal 25 mg/kg dose following this protocol. Mice were then anesthetized with euthanol (Bimeda-MTC Animal Health Ins., Cambridge, ON, Canada) prepared in sterile water to a final concentration of 65 mg/ml at 0.5 (3.5 days), 1, 2, 4.5, or 6 wks after kainic acid-induced seizure and euthanized by transcardial perfusion. Serial 10 μ m coronal cryosections were collected using a Leica CM1900 cryostat (Leica Microsystems, Wetzlar, Germany), throughout the dorsal domain of hippocampus from bregma -1.0 to -3.4 using the coordinates of Hof et al. (Hof et al. 2001) as we have described previously

(Pettit et al. 2012). Alternatively, mice lethally anesthetized with euthanol, brains removed, and hippocampus dissected for protein extraction. All procedures were in agreement with the guidelines of the Canadian Council for Animal Care and approved by the University of Ottawa Animal Care Committee.

4.5.10. Neurodegeneration measures

Serial sections were stained with Methyl Green Pyronine Y (MGPY), Hematoxylin and Eosin (H&E) and Cresyl Violet. Viable cells in the CA3a/b were defined and quantified described in (Bennett et al. 1995; Cheung et al. 2005; Cortez et al. 2010). Briefly, in MGPY-stained sections, DNA is stained blue and RNA is stained pink. Cells defined as viable exhibited a light blue nucleus, confirming nuclear integrity, and a pink cytoplasm, indicative of RNA transcription. Cells with hyperchromatic nucleus and/or lack of cytoplasmic (RNA) staining were identified as damaged cells (Al-Hazzaa and Bowen 1998; Cheung et al. 2005; Cortez et al. 2010). In H&E-stained sections, pyknotic, eosinophilic or hyperchromatic cells with amorphous or fragmented nuclei were defined as damaged cells. Cells with oval nuclei, prominent nucleoli lacking eosinophilic cytoplasm and homogenously stained by Hematoxylin or Cresyl Violet were defined as healthy cells (Bennett et al. 1998; Bennett et al. 1995; Cortez et al. 2010). Cells dying through an apoptotic-like mechanism were identified by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL). (Roche, catalog #11684795910, Montreal, Canada) as described in (Melanson-Drapeau et al. 2003). Briefly, coronal sections were permeabilized by a 5 min incubation in 0.1% Triton-X/0.1% sodium citrate on ice and a 1.5 min incubation in ethanol: acetic acid (2:1) on ice. Sections were rinsed for 2 min in 10 mM PBS (10 mM sodium phosphate, 150

mM NaCl) and reacted for 1 h at 37 °C with FITC–labeled dUTP in TdT buffer. Negative controls included sections incubated with FITC–labeled dUTP in the absence of TdT. Degenerating neurons were identified by fluorojade B labeling as described in (Cortez et al. 2010). Briefly, slides were incubated in a solution of 1% NaOH in 80% alcohol for 5 min, followed by 2 min washes in 70% alcohol and distilled water and a 10 min incubation in a 0.06% potassium permanganate solution. Slides were rinsed in distilled water and stained in a 0.0004% Fluorojade B staining solution for 20 min. All cells counts were performed using computer-assessed image analysis using a Leica DMXRA2 microscope with a XYZ computer-controlled motorized stage and the Advanced Measurement Module of Openlab 3.5 software (Improvision) as described in (Pettit et al. 2012). The number of cell profiles was counted at 400X magnification using a 150 X 150 µm counting frame. TUNEL⁺ and fluorojade⁺ counts were determined in bilateral hippocampi of two adjacent sections in 10 µm sections between bregma -1.7 and -2.1. NeuN⁺ neurons and MGPY-stained cells that met our morphological criteria of viable cells were counted stereologically within the defined CA3a/b counting frame over a minimum of 8 and maximum 14 serial sections. Quantification was performed by two independent evaluators blind as to the identity of the section and means averaged to yield a single parameter per animal. A minimum of 5 animals per time point was analyzed. Data were expressed either as the mean number of cells per 0.1 mm² in WT and Cx32 KO mice considered an estimate of the total cell population or as the percentage of positive cells per total number of cells considered an estimate of the percentage of dying (TUNEL) or degenerating (fluorojade) cells at a particular point in time as indicated.

4.5.11. Quantitative Cx32 immunofluorescence in hippocampus

Quantitative immunofluorescence confirmed qualitative assessment of changes within subregions of the hippocampus. Using the Advanced Measurement Module of Openlab v3.5, binary layers of the indicated regions of interest were created, and a density slice applied based on the average background fluorescence intensity. Protein expression in these density slices was established as the pixel intensity/pixel number*total pixel area. Data was expressed as fold-change relative to uninjured control tissue. All sections were processed at the same time to ensure consistent background. Data represent the mean of 3 animals per time point. Fluorescence was analyzed on a Leica DMXRA2 microscope, where exposure time and digital gain were kept constant, as well as on Olympus FV1000 confocal microscope with laser intensity for each fluorophore (measured as photomultiplier tube levels) kept within linear range. The fold-change established on each fluorescent system was averaged to yield a single data point per animal. Cell counts were performed as described above. To quantify double-labeled cells in Figure 7D, the number of double-labeled cell profiles per field was established, and expressed as a percentage of total cell number per field (cells were double-labeled with Cx32 and NG2, CNPase, GFAP, F4/80, DCX, Nestin, or NeuN). Antibody details found in Table 4.1.

4.5.12. TAM Injections in Nestin/NG2 Cre Mouse Models

Mice were injected with TAM (Sigma, T5648) dissolved in 10% EtOH and 90% autoclaved sunflower seed oil according to the following schedule. At time of kainate injection, mice received 120 mg/kg. Mice received two daily injections of 90 mg/kg separated by a 6 h time period on Day 1 and Day 2 after seizure induction. On Day 3,

mice received 120 mg/kg IP injection and were sacrificed 6 h or 31 days after the last injection. Control mice underwent the same injection protocol without seizure induction.

4.5.13. Astrocyte culture from SGZ

WT mice at the age of P7-P9 were sacrificed by lethal injection with sodium pentobarbital. Whole brains were dissected out and mounted for coronal sectioning. A Leica VT1000S vibratome was used to cut 500 μ m coronal sections, which were collected at the appropriate Bregma regions. With the use of a dissecting microscope and fine surgical instruments, the SVZ and DG were isolated. Tissue was minced with a scalpel and placed in dissociation media (26 mM NaHCO₃, 124 mM NaCl, 5 mM KCl, 0.1 mM CaCl₂·2H₂O, 3.2 mM MgCl₂·6H₂O, 10 mM D-glucose, 1% penicillin/streptomycin, 0.1% neural protease, 0.01% papain, 0.01% DNase I) for 45 min at 37 °C on a clinical rotator. 10 mM PBS was added to mixture followed by mild trituration and gentle centrifugation for 5 min at 1500 rpm. Supernatant was removed, and washed again with 10 mM PBS. After the last PBS wash, cells were dispersed in astrocyte maintenance media (DMEM/F12, 10% FBS, 1% penicillin/streptomycin), and viable cell counts were determined. Cells were plated at 5.0×10^5 cells per plate in a 10 cm Petri dish on PLL (0.1 mg/ml PLL in ddH₂O)-coated glass coverslips. Half of the media was replaced every three days until > 85% confluency was achieved 21 days after initial plating.

4.5.14. Neurosphere and astrocyte co-culture

All chemical reagents were obtained from Sigma-Aldrich (St.-Louis, MO, USA) and all cell culture reagents were obtained from Invitrogen (Burlington, ON, Canada) unless otherwise stated. Briefly, cells were isolated from the hippocampi of postnatal day 0-2 (P0-P2) mouse pups. Animals were sacrificed by lethal injection with Euthansol

(Schering-Plough Canada Inc., Pointe-Claire, QC, Canada) and 500 μ m sections between Bregma -1.7 mm and -2.2 mm were prepared on a VT1000S vibratome (Leica Microsystems Inc.) in ice-cold artificial cerebral spinal fluid (aCSF: 26 mM NaHCO₃, 124 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1.3 mM MgCl, 10 mM D-glucose, 100 U/ml penicillin and 100 ng/ml streptomycin). A total of n=6 hippocampi were pooled from n=3 animals/genotype in each experiment. Under a Leica MZ6 dissecting microscope, hippocampi were removed and dissected free of blood vessels and choroid plexus, minced with a scalpel, and enzymatically dissociated in: 26 mM NaHCO₃, 124 mM NaCl, 5 mM KCl, 0.1 mM CaCl₂, 3.2 mM MgCl₂, 10 mM D-glucose, 1% penicillin/streptomycin, 0.1 % neural protease, 0.01 % papain, and 0.01% DNase I for 45 minutes at 37°C. Single cells were resuspended in expansion media (DMEM/F12, 2 mM L-glutamine, 100 U/ml penicillin, 100 ng/ml streptomycin, 1X B27 supplement, 20 ng/mL human recombinant epidermal growth factor (EGF) and 10 ng/ml basic fibroblast growth factor (FGF-2)) in 60 mm Petri dishes (Fisher Scientific, Nepean, ON, Canada). Cell viability was established by Trypan Blue hemocytometer counts, and cells were plated at a density of 2.5×10^5 viable cells/dish. Cultures were maintained at 37°C in a 5% CO₂ atmosphere with fresh EGF and FGF-2 added every two days for 8 days in vitro (DIV). On DIV 8, neurospheres were plated in 10 cm tissue culture dishes on laminin-coated (15 μ g/mL) glass coverslips, or onto coverslips with a confluent WT or Cx30 KO astrocyte layer (see above for astrocyte culture). Cultures were pulsed with 20 μ g/mL BrdU 24 h prior to plating, to label actively dividing cells. The fate of neurospheres cultured on laminin were compared to that of cells co-cultured with astrocytes in the same co-culture media. To induce neurogenesis, neurospheres

cultured on laminin were cultured under neuronal driving conditions (NeuralBasal media (Gibco, Invitrogen), containing 0.5 μ M retinoic acid and 20 ng/mL brain derived neurotrophic factor (BDNF)) for 6 DIV. In one set of experiments, neurospheres co-cultured with WT astrocytes were also treated with 100 μ M of the gap junction blocker 18-alpha-glycyrrhetic acid (GRA), as described in (Imbeault et al. 2009). Neurospheres were not dissociated to maintain the 3D topography of the cultures, and within 24 h of transfer, the spheres had adhered to each substrate, and had begun to spread out as a monolayer.

4.5.15. Immunocytochemistry (Neurospheres)

After 14 DIV, neurospheres cultured onto laminin, or in astrocyte co-culture, were fixed in 3.7% formaldehyde solution in PBS for 20 min. Immunocytochemistry was carried out as previously described (Imbeault et al. 2009). Antibodies were diluted in 3% bovine serum albumin + 0.3% Triton X-100 in PBS, and Hoechst 33258 (1 μ g/mL) was used as a nuclear counterstain (Antibody details in Table 4.1). BrdU⁺ cells were established in five fields per neurosphere calculated for 5–10 neurospheres per connexin (n = 25–50 fields per condition). Cell counts were performed over five defined peripheral cell fields shot at 40X magnification as in (Imbeault et al. 2009). All cell images were taken with a Leica DMXRA2 epifluorescence microscope and analyzed using Openlab software v5.05 (Improvision, Lexington, MA, USA).

4.5.16. Behavioural assessment of hippocampal function

Uninjured mice (WT n=11, Cx32 KO n=8) and mice subjected to kainic acid seizure (WT n=7, Cx32 KO n=8) were tested in the Morris Water Maze. Apparatus and test details are as described (Mielke et al. 2006). Testing began 4 wk after seizure

induction when animals were 4 months of age. Each test day consisted of four trials of 60 s per day, with a 60 s rest period between each trial. On the 9th day, the platform was removed from the pool, and mice were placed in the water maze for a free swim period of 60 s. One hour after this probe trial, the platform was moved 180° from its original position and mice were submitted to the 4-trial per day paradigm for two consecutive days to assess reversal learning performance. All trials were recorded with a digital camera located above the water tank, and analyzed with Ethovision software (Noldus). Animals were sacrificed 24 h after the last test session 6 wk after induction of kainic acid seizures, and neuronal number was determined as described above

4.5.17. Statistics

Data are presented as the mean $\pm$ standard error of the mean (SEM) and were analyzed by Student's *t* test, ANOVA, or two-way ANOVA with or without repeated measures followed by Tukey, Dunnett, or Holm-Sidak *post hoc* analysis as appropriate. N refers to the total number of animals analyzed per genotype in each assay; n refers to the number of subjects per condition.

4.6. Results

4.6.1. Type 1 and 2 NPCs, NG2-glia, astrocytes, and oligodendrocytes form a connexin-mediated pan-glia network in the adult hippocampus

As has been previously suggested, oligodendrocytes (Cx32, Cx47), NG2-Glia (Cx32), and astrocytes (Cx30, Cx43) express connexins capable of interacting with adjacent Cx30⁺ and Cx43⁺ type 1 and 2 NPCs. To assess biochemically whether O:A connexin pairs are present at adjacent PM microdomains, subcellular localization was assessed by fractionation. Brain membranes were depleted of nuclei, and the post-nuclear supernatant (PNS) was fractionated into twelve fractions in an OP density gradient (Figure 4.1A). We found that Cx32 localized to myelin sheath (F1-3) and PM (F4) (Figure 4.1A). Cx30 co-fractionated with both Cx32 and Cx43 within the PM microdomains only (F4), and Cx47 co-fractionated with Cx32 in the myelin (F1-3) and PM domains (F4), and with Cx30 and Cx43 in the PM domains. A small amount of Cx47 was found in the most dense membrane domains containing Golgi and mitochondrial membranes (F8).

We used reciprocal co-IPs to provide direct biochemical evidence for glial connexin:connexin interactions in adult brain. Control lysates included a pooled sample of fractions 5-7 (depleted of connexin proteins, Figure 1B, D, E), and Cx32 KO whole brain lysates (Figure 1C). A separate control using the bait antibody and RIPA buffer instead of lysate was also used to confirm that blotting antibodies did not detect components of the bead/bait antibody complex. Cx32 interacted with Cx30 (Figure 4.1B,C) but not with Cx43 (Figure 4.1B) or Cx47 (Figure 4.1C). Cx30 interacted with Cx32 and Cx43 (Figure 4.1D). Cx43 interacted with Cx30, but not Cx32. These co-IPs

define the following direct connexin:connexin interacting pairs: Cx32:Cx30 (O:A), Cx30:Cx43 (A:A, NPC:NPC, A:NPC), but provide evidence precluding the existence of Cx32:Cx47 (O:O) and Cx32:Cx43 (O:A) interaction pairs.

These interacting connexins were localized in adult hippocampus by confocal immunofluorescence using established protein lineage markers distinguishing NPC populations, and terminally differentiated neurons and glia (Figure 4.2A). Cx32 co-localized with CNPase in subsets of mature oligodendrocytes (Figure 4.2B-i,i', arrows and insets). Large Cx32⁺ puncta, characteristic of elongated gap junction assemblies, were also observed at the PM of NG2-Glia (Figure 4.2B-ii,ii', arrows and insets). Cx32 was not found in nestin⁺ NPCs, GFAP⁺ astrocytes, or NeuN⁺ neurons (data not shown). Its interacting partner, Cx30, was not present in oligodendroglia but was expressed by nestin⁺/GFAP⁺ type 1 NPCs (Figure 4.2C-i, large arrow), nestin⁺/GFAP⁻ type 2 NPCs (Figure 4.2C-i, small arrowhead), and GFAP⁺/nestin⁻ astrocytes (Figure 4.2C-i, small arrows). Frequently, Cx30⁺/nestin⁺ NPCs were found in direct apposition with Cx30⁺/GFAP⁺ astrocytic processes (Figure 4.2C-i). Hippocampal astrocytic Cx30 protein expression was further confirmed in primary postnatal astrocyte cultures, where robust gap junction plaques were clearly evident between adjacent cells (Figure 4.2C-ii; large arrowheads) as well as at non-junctional membranes (Figure 4.2C-ii; asterisk). Likewise, Cx43 mRNA and protein was expressed by both GFAP⁺ astrocytes in the hilus (Figure 4.2D-i,ii arrows) and Type 1 NPCs in the SGZ (Figure 4.2D-i,i',ii,ii' arrowhead and insets). GFAP⁺ Type 1 cells were distinguished morphologically from astrocytes by their triangular soma in the SGZ, and single radial

Figure 4.1. Connexins localize to overlapping and distinct subcellular membrane microdomains, where Cx32:Cx30 and Cx30:Cx43 protein-protein interactions occur in adult mouse brain.

(A) OP density gradient fractions from WT membranes isolated from whole brain tissue. F1-F8 were isolated from the top (F1) and bottom (F8) of the fractionation tubes, respectively, and represent membrane domains of increasing density. Fractions 1-3 were harvested from the 0-5% OP densities, Fraction 4 includes membranes pooled from the 5/10% OP interfaces through to the 15%/17.5% interfaces, and fractions 5-8 were harvested from the 17.5-25% OP densities. Equal amounts (5 µg) of protein from the PNS and from each fraction were blotted for glial gap junctions (Cx32, Cx30, Cx43, and Cx47), ATPase (PM), synaptophysin (synaptic vesicle), Caspr (axonal membrane), AQP4 (astrocytic end-feet), flotillin (lipid raft), MBP (myelin), golgin 97 (Golgi body), ATP5A1 (mitochondria), and CoxIV (mitochondria). Three separate membrane domains were defined by these fractions: (1) myelin sheath (F1-F3), (2) PMs (F4-F8) and (3) synaptic, axonal, and intracellular membranes (F5-8). Antibody concentrations are located in Table 4.1. **(B)** Cx32 IP from F4 tissue using a rabbit anti-Cx32 (71-0600) bait antibody. IP product was blotted with mouse anti-Cx32 antibody (35-8900), mouse anti-Cx30 antibody (33-2500), and mouse anti-Cx43 antibody (05-763) **(C)** Cx32 IP from whole brain tissue using mouse anti-Cx32 (35-8900) bait antibody. IP product was blotted with mouse rabbit-Cx30 antibody (71-2200), and rabbit anti-Cx47 antibody (D. Paul). **(D)** Cx30 IP from F4 using a mouse Cx30 antibody (sc-514847). IP product was blotted with rCx30 antibody (71-2200), rabbit Cx32 antibody (71-0600), and mouse Cx43 antibody (05-763). **(E)** Cx43 IP using a rabbit anti-Cx43 antibody (71-0700). IP

product was blotted using a mouse anti-Cx43 antibody (05-763), a mouse Cx30 antibody (sc-514847), and a rabbit Cx32 antibody (71-0600). Each IP (Figure 1, D, E) was performed using 250 µg RIPA-extracted F4 (connexin-enriched sample) and F5-7 (connexin depleted sample). Figure 1C was performed using whole brain lysate from WT and Cx32 KO mice. For each IP, 1 µg of bait antibody, and 50 µL of protein A (for anti-rabbit bait antibodies) or protein G (for anti-mouse bait antibodies) agarose beads were used. IP complexes were eluted in 100 µL of 1X Laemmli sample buffer, and 10 µL was loaded onto each gel (10% of total IP product). Solid bars indicate lysates, Cx IP samples, or control IP samples. Control reactions (IP bait antibody) were performed using agarose beads coupled to the bait antibody, with RIPA buffer instead of protein. Antibody concentrations located in Table 5.1

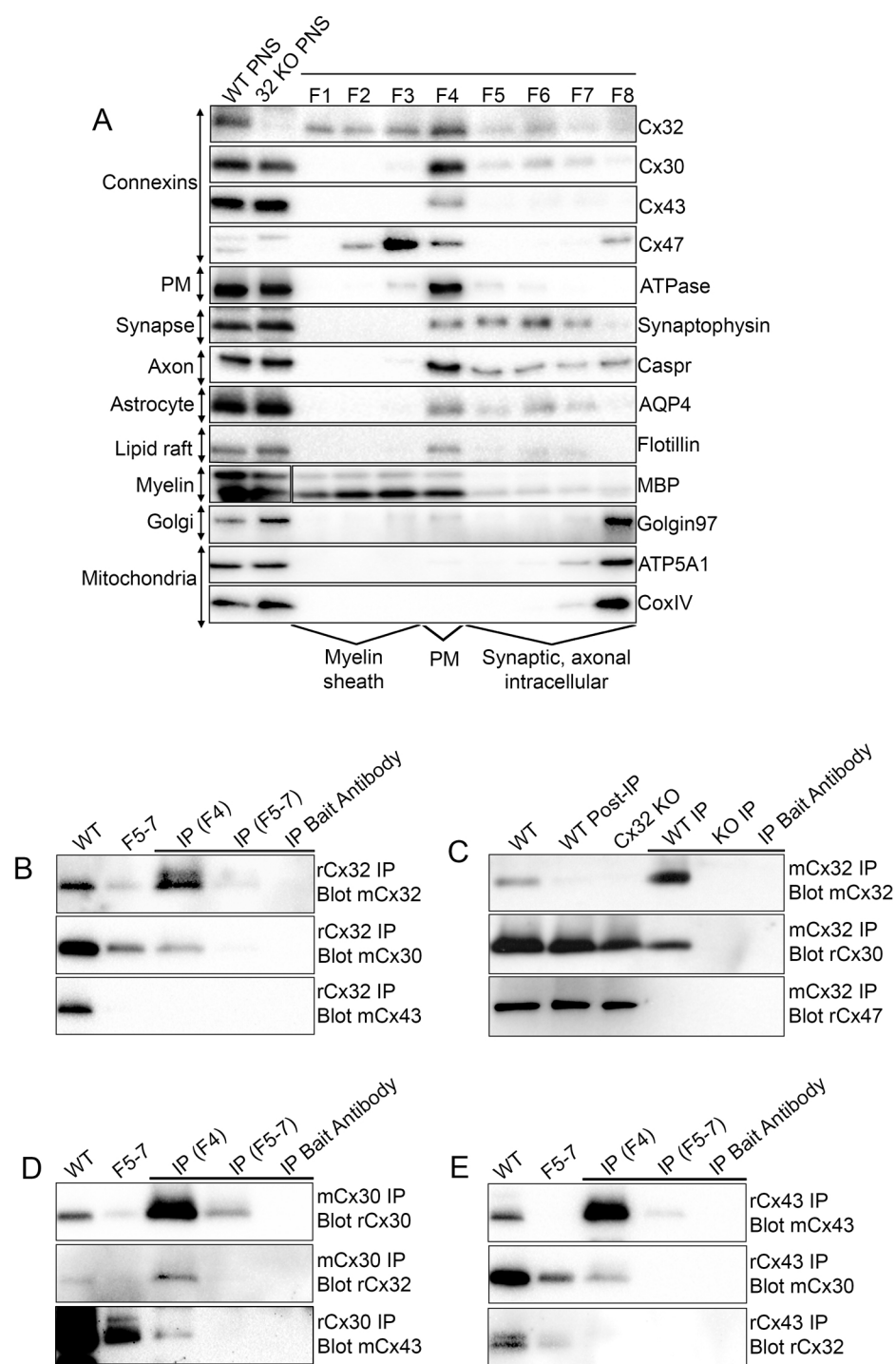


Figure 4.1

Figure 4.2. Localization of connexin partners at the PM of different cell types in adult hippocampus.

(A) Coronal schematic of the murine hippocampus indicating the location of photomicrographs: SGZ, subgranular zone; hilus; molecular layer; and GrDG, granular layer of the DG. Schematic of hippocampal neurogenesis and gliogenesis, indicating lineage markers representative of specifying NPCs and NG2-Glia: GFAP, glial fibrillary acidic protein; DCX, doublecortin; Tuj1, neuron-specific beta-III tubulin; NeuN, neuron-specific nuclear protein (Fox3); NG2, neural/glia antigen 2; PDGF α R, platelet-derived growth factor alpha receptor; CNPase, 2'3'-cyclic-nucleotide 3'-phosphodiesterase; RIP, RIP-antigen, CNPase (Watanabe et al. 2006); MBP, myelin basic protein) **(B)** Cx32 was expressed by mature, CNPase⁺ oligodendrocytes (4.2B-i,i'), and by NG2-Glia (4.2B-ii,ii'). Cx32 co-localized at PMs with both CNPase (i' panels, white arrows) and NG2 (ii' panels, white arrows). Scale bars, 50 μ m, insets 12.5 μ m. **(C)** Cx30 was expressed by nestin⁺/GFAP⁺ type 1 NPCs (Figure 4.2C-i, large arrow), nestin⁺ type 2 NPCs (Figure 4.2C-i, small arrowhead), and GFAP⁺/nestin⁻ astrocytes (Figure 4.2C-i, small arrow) in the SGZ and hilus. Dotted lines localize Z-axis sectioning. Scale bars, 25 μ m. Cx30 was also expressed by primary postnatal hippocampal astrocyte cultures at cell-cell junctions (Figure 4.2C-ii; large arrowheads, scale bars, 50 μ m). **(D)** Cx43 was expressed at the mRNA (Figure 4.2D-i,i') and protein level (Figure 4D-ii,ii') in the SGZ and hilus, in both Type 1 NPCs (arrowheads in Figure 4D-i' and ii' insets), and in mature astrocytes (small white arrows in Figure 4D-i and ii). Scale bars, 50 μ m. **(E)** Cx47 was expressed by mature, RIP⁺ oligodendrocytes only (Figure 4.2E-i), and signal was not detected in PDGF α R⁺ (Figure 4.2E-ii), NG2⁺ (Figure 4.2E-iii), or Nestin⁺ (Figure 4.2E-iv)

cells. White arrows indicate Cx47 puncta, and arrowheads indicate areas where Cx47 does not co-localize with immature lineage markers. Dotted lines localize Z-axis sectioning. Scale bars, 10 μm .

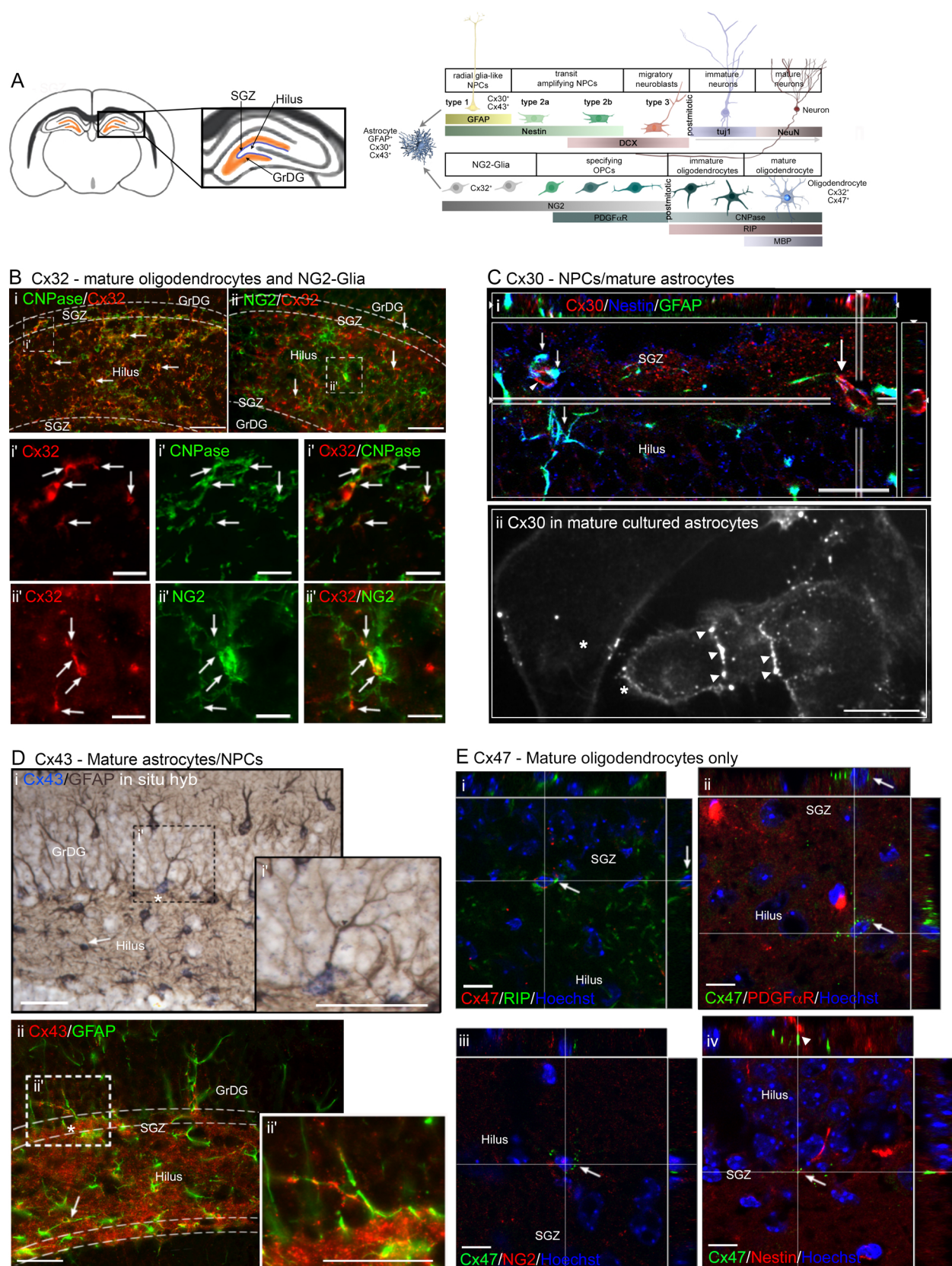


Figure 4.2

process spanning the entire granule layer of the DG (GrDG) (Figure 4.2D-i,ii). Finally, Cx47 was only detected in mature RIP⁺ oligodendrocytes and was not present in PDGF β R⁺ OPCs, NG2-Glia, GFAP⁺ astrocytes (data not shown) or nestin⁺ NPCs (Figure 4.2E-i-iv)

4.6.2. The connexin pan-glial/NPC network regulates NPC and NG2-glia proliferation in the uninjured hippocampus

These results provide converging evidence for a pan-glial juxtacrine network wherein oligodendrocytes, NG2-Glia, astrocytes communicate with type 1 and 2a NPCs in adult hippocampus through channels composed of Cx32:Cx32 (oligodendrocytes:NG2-Glia), Cx32:Cx30 (oligodendrocytes, NG2-Glia:NPCs), Cx47:Cx43 (oligodendrocytes:NPCs), Cx30:Cx30, Cx30:Cx43, and Cx43:Cx43 (astrocytes:NPCs) (Supplemental Figure 4.1). To address the functional consequences of these interactions, we used a loss of function approach to reduce and/or eliminate coupling between specific cell:cell pairings. Cx30, Cx32, Cx47 and brain-specific conditional Cx43 KO mice received three days of repeated equimolar BrdU intraperitoneal injections ensuring labelling of both rapidly dividing type 2 NPCs and slowly dividing NG2-glia and type 1 NPCs (Pettit et al. 2012; Psachoulia et al. 2009; Vega and Peterson 2005). Total numbers of BrdU-labelled nestin⁺ NPCs (type 1 and type 2) and NG2-Glia in the SGZ and hilus of the dorsal hippocampus were assessed stereologically 24 h after the last injection. Loss of Cx47, Cx43, or Cx30 had no effect on either proliferating nestin⁺ or NG2⁺ cell number (Figure 4.3A-C); however, a substantial increase in both BrdU-labelled nestin⁺ type 1 and 2 NPCs and NG2-Glia was evident in Cx32 KO mice (Figure 4.3D). Thus, in the absence of Cx47, Cx43, or Cx30,

Figure 4.3. Genetic dissection reveals that the connexin pan-glial/NPC network regulates NPC and NG2-Glial proliferation in uninjured hippocampus.

(A, B, C, D, left panels) Glial network connectivity with loss of glial connexins Cx47 (a) and Cx43 (b), Cx30 (c) and Cx32 (d). Glial connectivity is indicated by thickness and opacity of connecting lines, where thinner, less opaque lines indicate less connectivity.

(A, B, C, D, right panels) Stereological assessment of proliferation (BrdU⁺ cell number), and lineage of proliferating cells in the hippocampus following deletion of Cx47 (a), Cx43 (b), Cx30 (c), and Cx32 (d). Nestin/BrdU labels slower dividing Type 1 and actively proliferating type 2 NPCs, while NG2/BrdU labels slower dividing NG2-glia. Note the BrdU schematic in Figure 5A, entailing two twice-daily injections of BrdU, with a third injection on day 3, and sacrifice on day 4. Statistics are two-way ANOVA with Sidak post-hoc, **p<0.01, ****p<0.0001.

overall gap junctional communication is reduced, yet the complete pan-glial:NPC network remains intact without significant impact on NPC or NG2-Glia.

4.6.3. Seizure-induced neuronal replacement in adult hippocampus is more effective in the absence of Cx32

These results posit a model by which NG2-Glia and astrocytes reciprocally regulate NPC fate. To test the impact of this regulation on seizure-induced neurogenesis, WT and Cx32 KO mice were injected intraperitoneally with 25 mg/kg kainic acid to induce acute seizures. Both genotypes exhibited equivalent kainic acid sensitivity, seizure intensity, and mortality rates (Supplemental Table 4.1). To produce defined pyramidal cell lesions limited to the CA3a/b region, stage 4 seizure activity was restricted to a 5 min duration by injection with 5 mg/kg diazepam. This protocol elicits delayed excitotoxic loss of pyramidal and interneurons confined to the CA3a/b field as we have described (Cheung et al. 2005). Cell death is evident 0.5 wk (3.5 days) after kainic acid injection (Supplemental Figure 4.2). Dying neurons are cleared from the CA3a/b field within 2 wk of kainate injection. Endogenous cell replacement is complete 4.5 wk after seizure (Supplemental Figure 4.2).

We found that Cx32 expression transiently increased following acute seizure. Protein levels were maximal 0.5 wk (3.5 days) after kainic acid injection (Figure 4.4A). Significant increases in Cx32 fluorescence intensity were detected in the SGZ, hilus, and damaged CA3 cell field 0.5 and 1 wk after seizure but not in the GrDG (Figure 4.4B,C). Cx32 localized to CNPase⁺ oligodendrocytes and NG2-Glia (Fig 4.4D) with a statistically significant increase in the number of Cx32⁺ NG2-Glia in the CA3, but not GrDG, detected 0.5 wk after kainic acid injection (Figure 4.4D). There was no change

Figure 4.4. Seizure-activated NG2-glia express Cx32 at sites of injury.

(A) Cx32 protein levels increased in WT hippocampus following seizure. No signal was detected using Cx32 KO hippocampus (KO) confirming antibody specificity. Protein was extracted from hippocampus pooled from n=3 animals/time point. Immunoblot is representative of replicate experiments (n=6 animals/time point). U, uninjured. **(B)** Cx32 immunoreactivity in the CA3a/b and DG. Note the increase in fluorescence intensity and number of Cx32+ cell profiles 0.5 wk after seizure in the CA3a/b, SGZ, and hilus. All sections were immunoreacted at the same time. Scale bars, 50 μ m. Abbreviations: slu, stratum lucidum, remainder are as in Figure 1. **(C)** Quantification of Cx32 immunofluorescence in the GrDG, hilus and SGZ, and CA3a,b,c. Image locations in B are defined in schematic (green box, CA3a/b; red box, SGZ, GrDG, Hilus). A significant increase in Cx32 immunofluorescence was observed 0.5 and 2 wk after seizure in the CA3 and hilus/SGZ but not the GrDG. (N=15 WT, N=15 Cx32 KO, n=3 animals/time-point/genotype). Statistics were ANOVA followed by post-hoc Bonferroni test. *p<0.05, **p<0.01 compared to uninjured (U) controls. **(D)** Cx32 localized to CNPase+ oligodendrocytes and NG2+ OPCs. Images are from the SGZ and hilus of WT animals 0.5 wk after seizure. Scale bars, 50 μ m. **(E)** Quantification of Cx32/lineage marker double-positive cells. A transient increase in the number of Cx32+/NG2+ cells was detected in the CA3 0.5 wk after seizure. The number of Cx32+/CNPase+ oligodendrocytes did not change following seizure. At no point did we detect any other Cx32 immunopositive cell type.

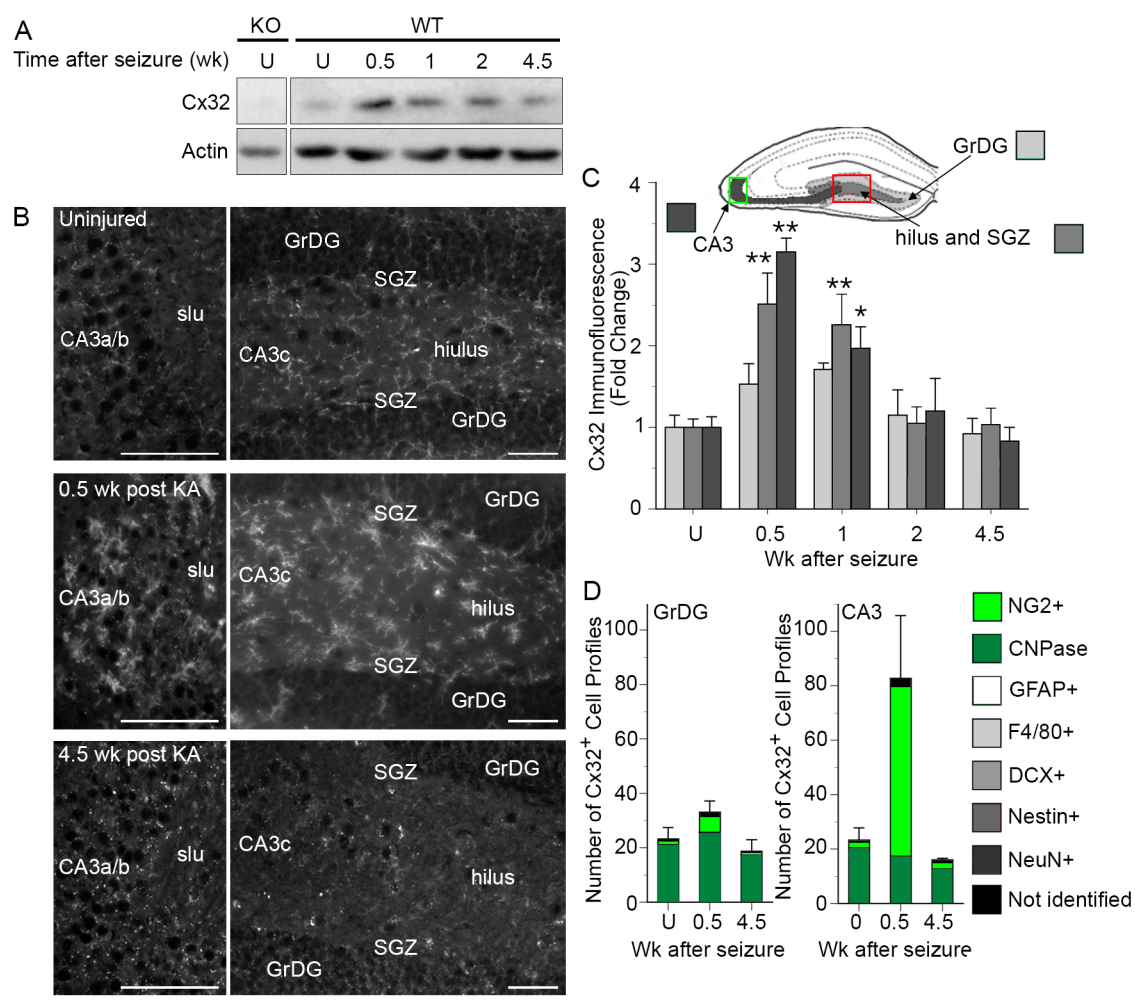


Figure 4.4

in the number of Cx32⁺/CNPase⁺ oligodendrocytes at any time point (Figure 4.4D). At no time was Cx32 detected in cells expressing antigenic markers of microglia (F4/80⁺), astrocytes (GFAP)⁺, type 1 or 2 NPCs (nestin⁺), type 3 neuroblasts (DCX⁺), or neurons (NeuN⁺) (Figure 4.4D).

Loss of Cx32 did not affect neuronal loss. NeuN⁺ neuronal number was significantly compromised in both WT and Cx32 KO mice (Figure 4.5A-E, NeuN⁺). Cell loss was further confirmed by quantitative assessment of histologically stained cells (MGPY, H&E, and cresyl violet, Figure 4.5A-D, H). Dying neurons were quantified using TUNEL and fluorojade. Fifty percent of cells in both WT and Cx32 KO CA3a/b were either TUNEL⁺ (~20%) or fluorojade⁺ (~30%) 0.5 wk after kainic acid seizures (Figure 4.5B,F,G).

Despite equivalent cell loss in the CA3a/b, neurons were regenerated more effectively in Cx32 KO mice (Figure 4.5D,E,H). MGPY-stained cells and NeuN⁺ neurons were restored to that of uninjured controls at 4.5 wk and 6 wk after seizure in Cx32 KO mice but not WT (Figure 4.5A,D,E,H). The appearance of new NeuN⁺ neurons was preceded by the appearance of cells expressing two antigenic markers of newly born neurons, the 64 kD Turned On After Division protein (TOAD-64) and the TuJ1 clone of Type III β -tubulin (Figure 4.6A, 2 wk). In WT mice, not only was neuronal number only partially restored (Figure 4.5D,E,H), but the kinetics of neuronal replacement were also delayed relative to Cx32 KO mice (Figure 4.5A, 4.5 wk).

Figure 4.5. Seizure-induced neuronal replacement in the CA3a/b is more efficient in Cx32 KO mice.

Neuronal loss is comparable but neuronal number is fully restored 6 wks after acute seizure in Cx32 KO but not WT mice. **(A)** Uninjured. **(B)** 0.5 wk post seizure. **(C)** 2 wk post seizure. **(D)** 4.5 week post seizure. Neuronal loss and cell replacement were assessed in serial sections throughout dorsal hippocampus of n=37 WT and n=40 KO by NeuN immunofluorescence, histological assessment of MGPY, H&E, and cresyl violet staining, TUNEL, and fluorojade (FJ) labeling. Scale bars, 150 μ m (**top panels**) or 50 μ m (**insets**). Stereological quantitation of **(E)** NeuN⁺ neurons, **(F)** TUNEL⁺ cells, **(G)** Fluorojade⁺ cells, and **(H)** MGPY-stained viable cells in Cx32 KO and WT mice prior to injury (uninjured, U) and up to 6 weeks following acute seizures was further performed. Total cell number in defined 150 X 150 μ m counting frame of CA3a/b in dorsal hippocampus between bregma -1.0 to -3.4 were quantified in serial sections and expressed as mean $\pm$ SEM cell number per 0.1 mm² or percentage of total number of cells. Statistics were one-way ANOVA (per genotype) followed by *post hoc* Dunnett's t test compared to uninjured WT or Cx32 KO controls. *p<0.05, **p<0.01. (n=37 WT, n=40 Cx32 KO, n=5-10 animals/time-point/genotype). Note the appearance of TUNEL⁺ and FJ⁺ cells and loss of NeuN⁺ neurons and histologically-stained cells 0.5-2 wk after acute excitotoxic seizures with cell partial cell replacement in WT mice and full restoration in Cx32 KO mice.

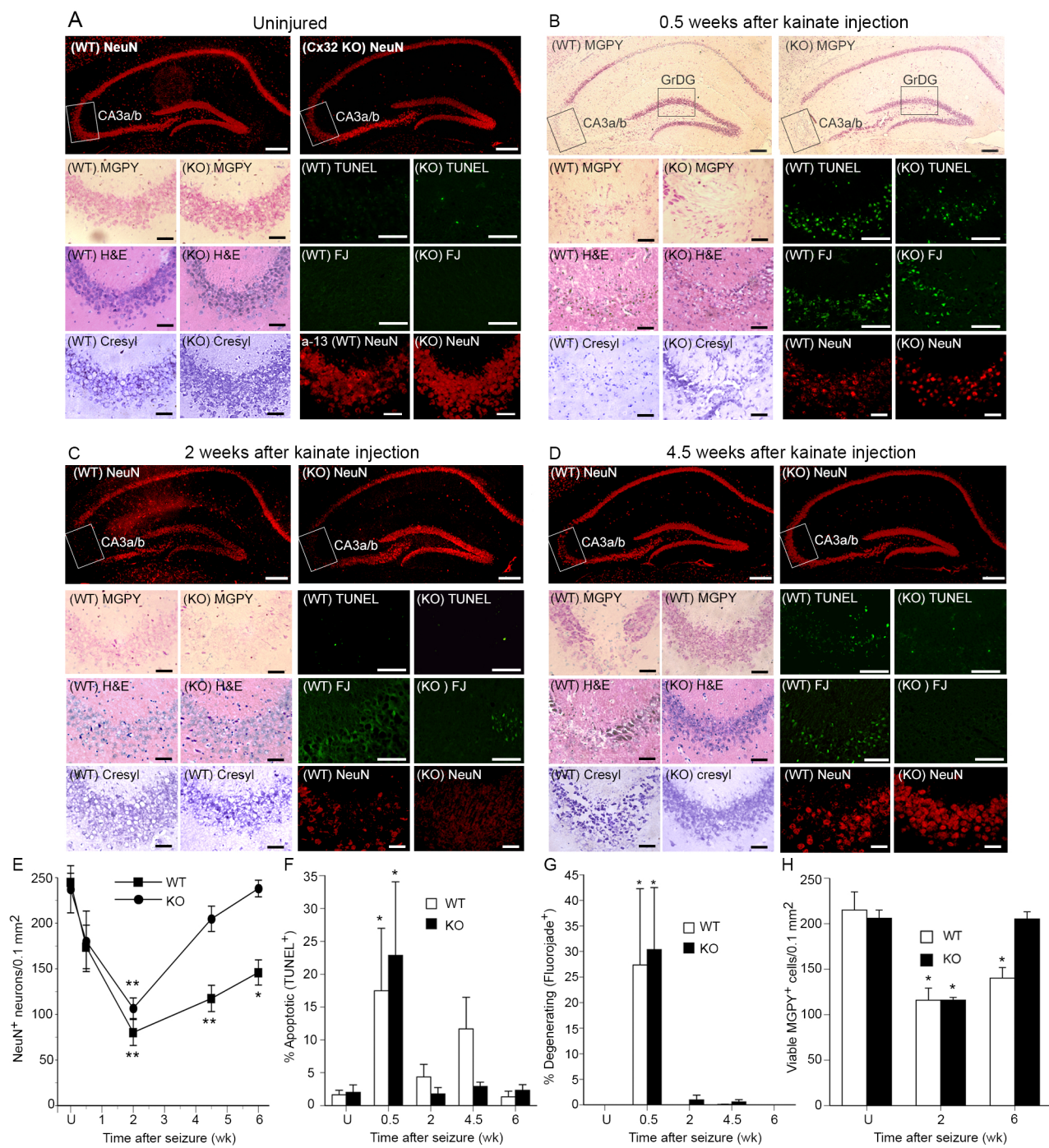
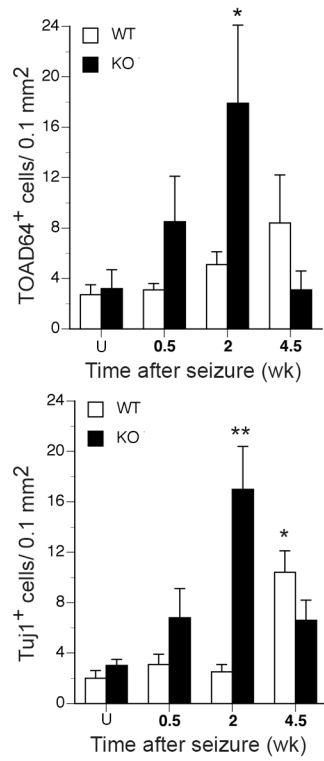


Figure 4.5

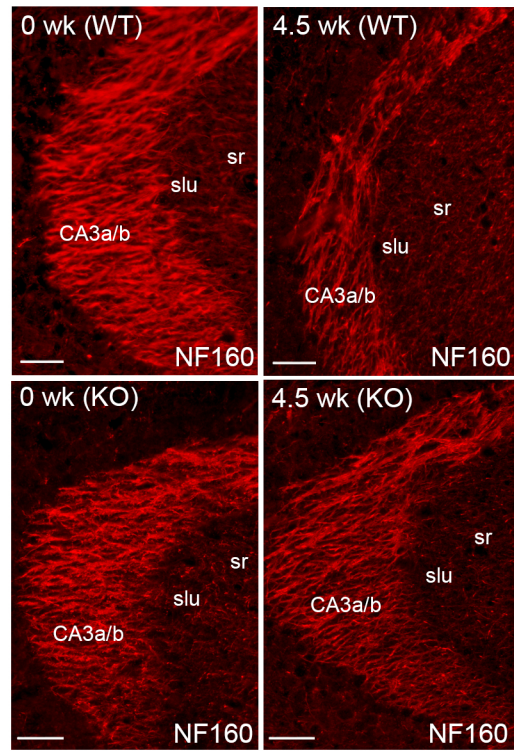
Figure 4.6. Pyramidal neurons are regenerated more effectively in the CA3a/b of Cx32 KO than WT mice following seizure.

(A) Quantification of TOAD64⁺ and Tuj1⁺ immature neurons in the CA3a/b pyramidal layer in uninjured (U) and up to 4.5 wk following seizure. (n=44 WT, n=54 Cx32 KO, n=4-16 animals/time-point/ genotype). Statistics were one-way ANOVA (per genotype) followed by *post hoc* Dunnett's t test compared to uninjured WT or Cx32 KO controls. *p<0.05, **p<0.01. **(B)** NF160 labeling of pyramidal neurons at 0 and 4.5 wk after seizure. NF160 immunoreactivity was comparable to controls 4.5 wk after seizure in Cx32 KO but reduced in WT mice. **(C)** Parvalbumin labeling of interneurons in the CA3a/b (arrows). Parvalbumin⁺ neurons were restored in higher frequency in Cx32 KO than WT mice 4.5 wk after seizure. **(D)** CaBP/NeuN double-labeling in the CA3a/b. Cells with small CaBP⁺ soma and large NeuN⁺ nuclei consistent with the morphology of ectopic granule neurons were detected in WT but not Cx32 KO mice 4.5 wk after seizure (arrows). Abbreviations: are as Fig 1 and slu, stratum lucidum; sr, stratum radiatum. All images were obtained at bregma -2.1 except for **d** (WT) where images are at bregma -2.5. Scale bars, 50 μ m.

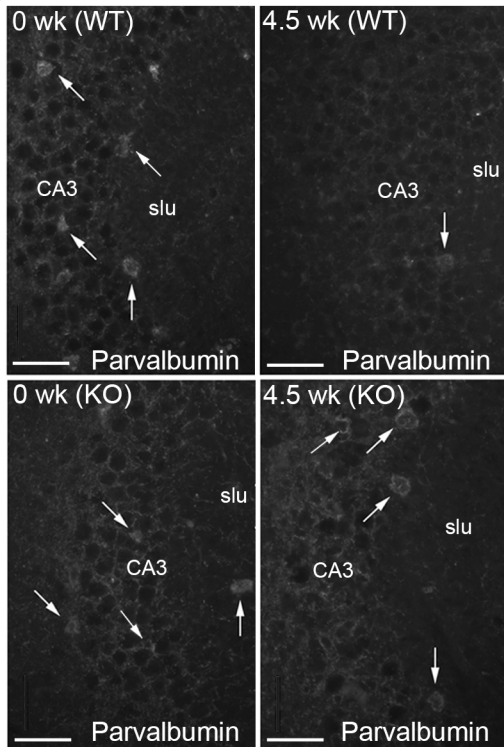
A



B



C



D

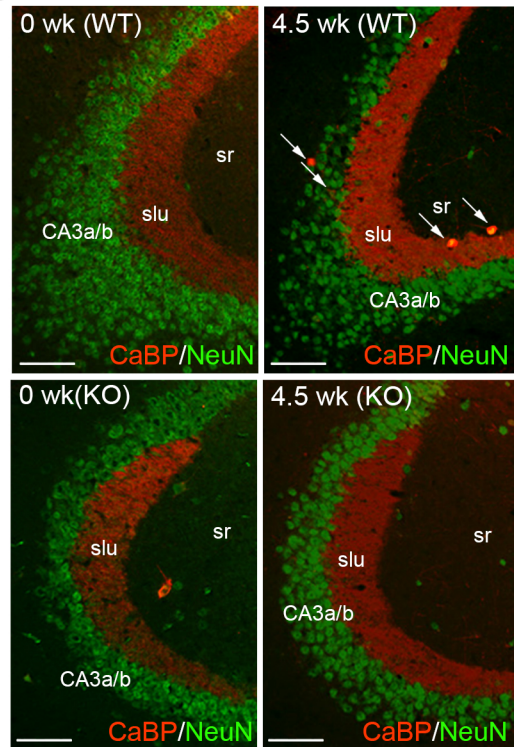


Figure 4.6

Because seizure-induced neurogenesis primarily produces ectopic granule neurons (Dashtipour et al. 2001; Jessberger et al. 2005; Scharfman and Pierce 2012), we assessed neurofilament 160 (NF160, Figure 4.6B), parvalbumin (Figure 4.6C), and calbindin (CaBP, Figure 4.6D) immunoreactivity in CA3a/b 4.5 wk after seizure in both WT and Cx32 KO mice. NF160 is a neuron-specific intermediate filament expressed at high levels by CA3 pyramidal neurons (Holopainen et al. 2001). Parvalbumin is a calcium-binding protein expressed by inhibitory basket and chandelier hippocampal interneurons in the pyramidal cell fields (Sloviter et al. 2003). NF160⁺ (Figure 4.6B) and parvalbumin⁺ (Figure 4.6C, arrows) immunoreactivity were comparable to uninjured controls in the CA3a/b of Cx32 KO animals, but only partially restored in WT mice. CaBP is a calcium-binding protein expressed by mature neurons in the GrDG (Nakamura and McEwen 2005). Both uninjured and injured WT and Cx32 KO mice exhibited the expected CaBP⁺ granule cell axons projecting along the mossy fiber pathway in the stratum lucidum (Figure 4.6D), but only injured WT mice exhibited ectopic CaBP⁺ cell soma in the CA3a/b and stratum lucidum, consistent with the aberrant integration of newly born neurons with granule cell phenotype (Figure 4.6D, arrows). No ectopic neurons were seen in Cx32 KO mice.

4.6.4. Nestin⁺ type 2a NPCs activated at the site of injury are the source of new neurons in Cx32 null-mutant mice; DCX⁺ type 3 neuroblasts activated in the SGZ are the source of new neurons in WT mice

Proliferating cells were labeled with BrdU at 0 (uninjured), 0.5, 1, 2, or 4 wk after kainic acid injection using the same injection schedule as Figure 4.3 to label slowly dividing type 1 NPCs as well as rapidly dividing Type 2 NPCs as well as Type 3 cells

and NG2-Glia activated following seizures (Figure 4.3A). Mice were sacrificed 24 h after the last BrdU injection to assess cell proliferation. BrdU-labeled cells in the SGZ and hilus (Figure 4.7B) and CA3a/b (Figure 4.8A) were identified by their expression of nestin, GFAP, NG2, DCX, PDGFR, or F4/80 (Figure 4.2A). In the SGZ and hilus, the number of proliferating cells peaked 1 wk after seizure in both genotypes, with fewer cells activated in Cx32 KO mice than WT (Figure 4.7C). The majority (> 60%) of BrdU⁺ cells labelled 1 wk after acute seizure were identified as nestin⁻/DCX⁺ Type 3 neuroblasts in the SGZ (Figure 4.7D,G,J arrows). Rare NeuN⁺/BrdU⁺ neurons, presumably undergoing DNA repair, were also detected (Figure 4.7D,F, arrowhead). In the CA3a/b field, significant cell proliferation was detected 0.5 wk post seizure (Figure 5.8B). In both Cx32 KO and WT mice, the majority of BrdU-labeled cells were NG2-Glia (~47%) or Type 2a nestin⁺/DCX⁻/GFAP⁻ (~33%) NPCs (Figure 4.8C,D,G,H,K, yellow arrowheads). Type 2b and 3 DCX⁺ NPCs were not present in significant numbers (Figure 4.8C). Although proliferating F4/80⁺ microglia and GFAP⁺ astrocytes were observed throughout the stratum radiatum, stratum lucidum, and stratum oriens, only a small proportion of glia in the CA3a/b were BrdU⁺ (Figure 4.8C,E,F,I,J). Some damaged NeuN⁺ neurons were BrdU⁺, consistent with DNA repair (Figure 4.8C,E,I). These cells were frequently surrounded by F4/80⁺ microglia phagocytosing dying neurons and neuronal debris (Figure 4.8F,J).

Figure 4.7. Type 3 DCX⁺ progenitor cells are activated in the SGZ of both WT and Cx32 KO mice following kainic acid-induced seizure.

(A) Schedule of BrdU injection and analysis following kainic acid injection. **(B)** Schematic of the dorsal hippocampus. BrdU⁺ cells in the DG were counted in the area indicated in grey. Location of images is indicated in red (**E, F, H, I**) and green (**G, J**). Abbreviations are as in Fig 6. **(C)** Quantification of BrdU-labeled cell number 0-4 wk after seizure. Cell proliferation peaked 1 wk after kainic acid-induced seizure in both genotypes. Statistics are two-way ANOVA followed by *post-hoc* Tukey test, * indicates a main effect of time and a statistically significant increase in progenitor cell proliferation at 1 wk after seizure induction in both genotypes compared to uninjured animals ($p < 0.05$). # indicates a statistically significant difference between Cx32 KO and WT mice ($p < 0.05$). **(D)** Histograms summarizing the antigenic identity of BrdU-labeled cells activated 1 wk after kainic acid-induced seizure. The majority of BrdU⁺ cells in the SGZ were double-labeled with DCX in both genotypes. **(E, F, H, I)** BrdU/NeuN double labeling at 0 and 1 wk after seizure induction. The majority of BrdU⁺ cells localized to the SGZ in both genotypes. Rare BrdU labeling of post-mitotic NeuN⁺ neurons observed was likely DNA repair (**F**, arrow). **(G, J)** Arrows point to representative BrdU⁺/DCX⁺ progenitor cells in the SGZ of WT and Cx32 KO mice. Scale bars, 50 μ m.

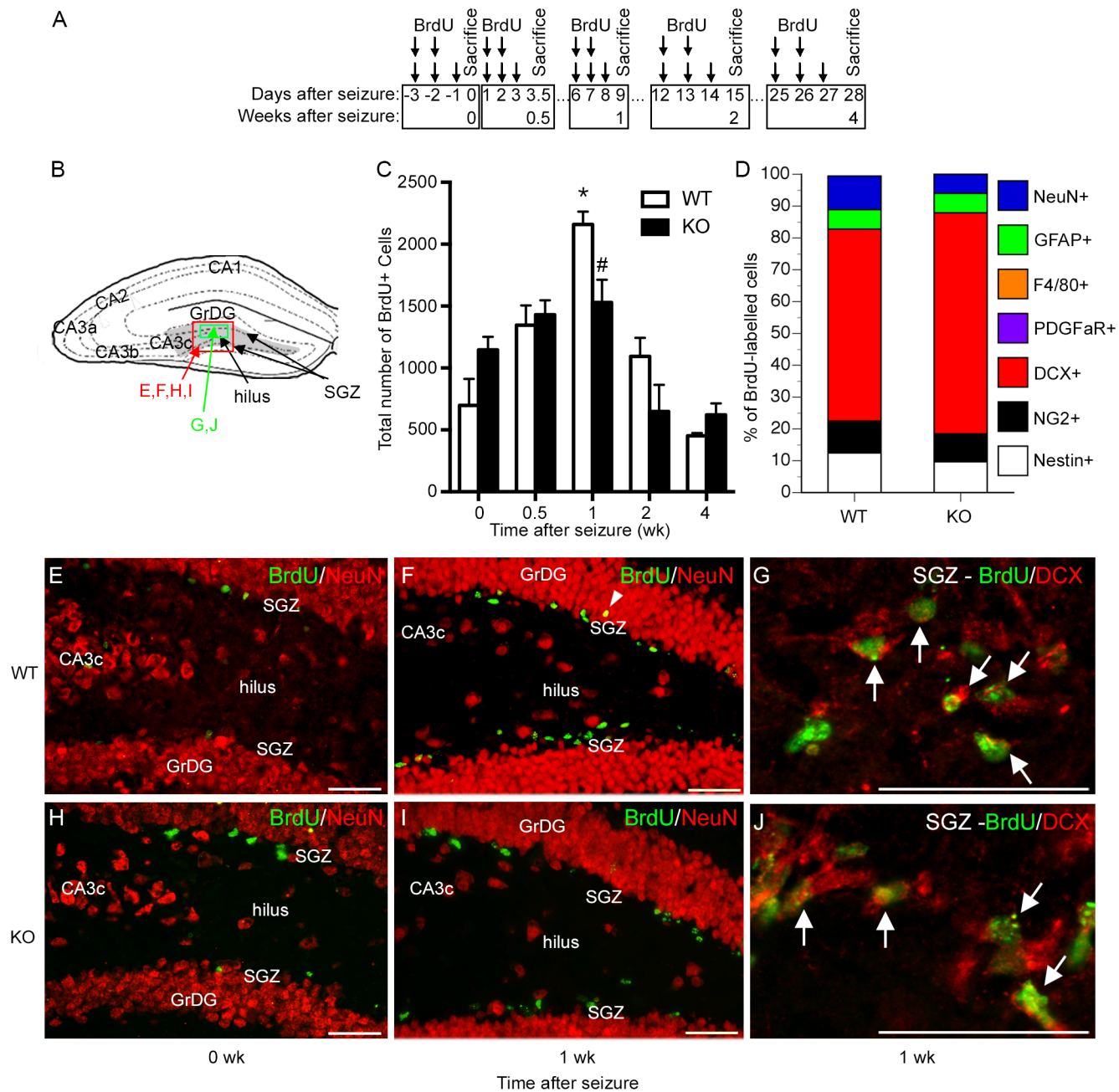
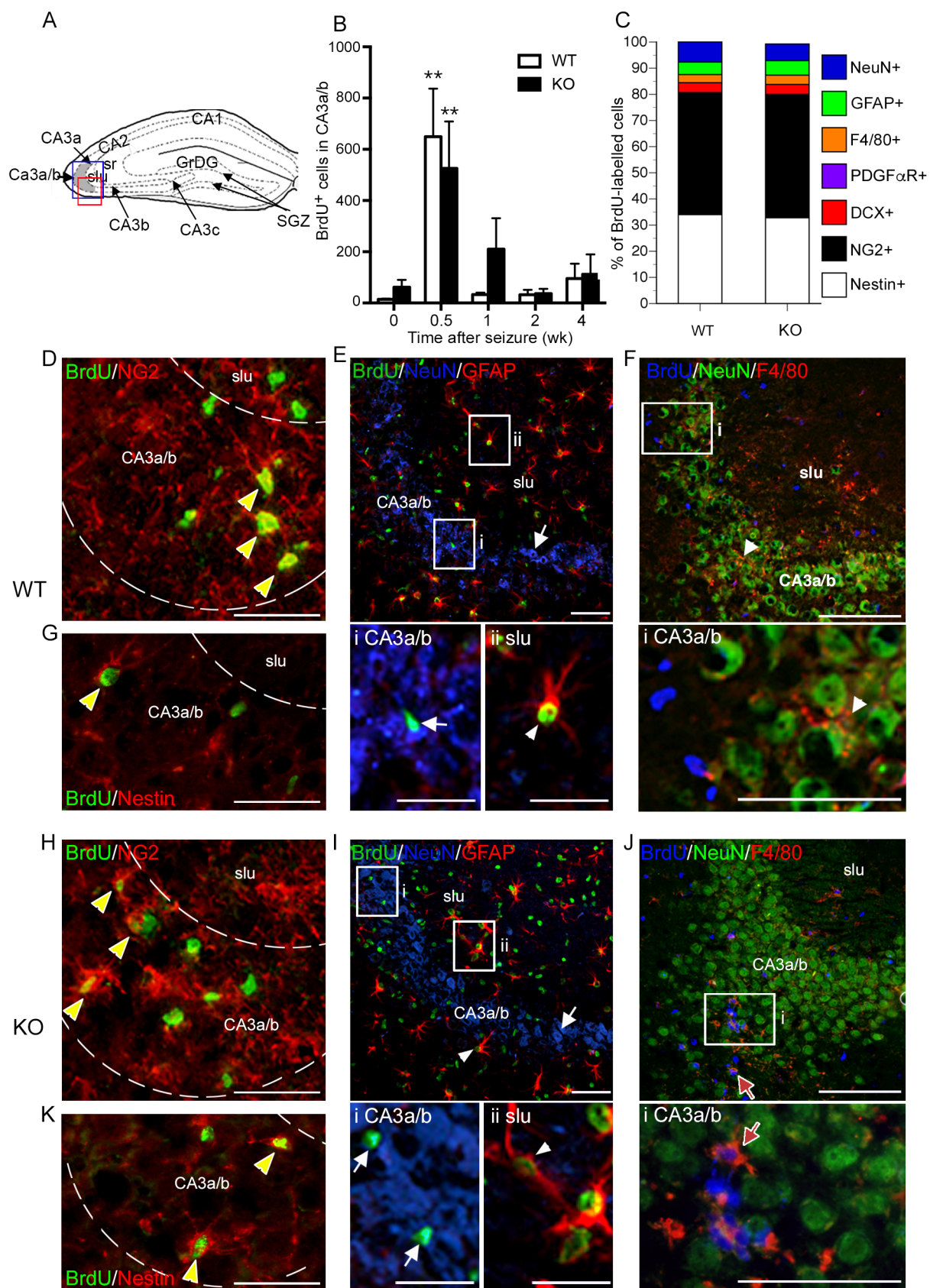


Figure 4.7

Figure 4.8. NG2-glia and Type 2a nestin⁺ progenitor cells are activated in the CA3a/b pyramidal layer of both WT and Cx32 KO mice 0.5 wk following seizure.

(A) Schematic of the dorsal hippocampus. BrdU⁺ cells were counted in the area indicated in grey. Location of images is indicated in red (**D, G, H, K**) and blue (**E, F, I, J**). Abbreviations are as in Fig 4.7. **(B)** Quantification of BrdU⁺ cell number labeled 0-4 wk after seizure. Injection schedule is shown in Fig 4.7A. Statistics are two-way ANOVA and *post-hoc* Tukey test, ***p*<0.01 compared to uninjured (0 wk) controls. **(C)** Histograms summarizing the antigenic identity of BrdU-labeled cells at 0.5 wk after kainic acid-induced seizure. The majority of BrdU⁺ cells were Type 2a DCX⁻/nestin⁺ or NG2⁺. In **D, G, H, K**, white dotted lines indicate the boundary of the CA3a/b pyramidal layer. **(D, H)** BrdU/NG2 double-labeling. **(G, K)** BrdU/nestin double-labeling. Yellow arrowheads indicate representative BrdU⁺/NG2⁺ or BrdU⁺/nestin⁺ progenitor cells. **(E, I)** BrdU/NeuN/GFAP triple-labeling. Boxes in **E** and **I** indicate location of insets in **Ei, Eii, li, lii**. White arrows point to damaged NeuN neurons labeled with BrdU. White arrowheads point to BrdU-labeled astrocytes. BrdU⁺ astrocytes were detected in the slu but not in significant numbers in the CA3a/b pyramidal cell layer. **(F, J)** BrdU/NeuN/F4/80 triple-labeling. Boxes in **F** and **J** indicate location of insets **Fi and Ji**. White arrowheads point to F4/80⁺ microglia (red) in close proximity to damaged neurons exhibiting nuclear vacuolization (green). Red arrows point to microglia apparently phagocytosing BrdU-labeled cells. All images were obtained at bregma -2.1 except for **F** and **J** where images were obtained at bregma -2.5. Scale bars, 50 μ m.



To establish whether the activated type 2a progenitor populations detected 0.5 wk after seizure in the CA3a/b field or the activated type 3 neuroblasts detected 1 wk after seizure in the SGZ were responsible for the increase in neurogenesis in Cx32 KO mice, we performed BrdU birthdating and cell survival analyses (Figure 4.9A). Animals were injected with BrdU at 0.5 wk or 1 wk after seizure. The number of proliferating cells throughout the DG and CA3 field was established 24 h after the last BrdU injection (Figure 4.9B). In separate cohorts of animals, the survival of these cells born 0.5 and 1 wk after seizure was assessed 28 days after the last BrdU injection in the injured CA3a/b (Figure 4.9C). No difference in percent survival of cells born 1 wk after seizure repopulating the CA3a/b 28 days later was detected, suggesting that Cx32 deletion does not enhance seizure-induced survival or migration of Type 3 DCX⁺ neuroblasts from the SGZ into the CA3a/b (Figure 4.9C, 1 wk). By contrast, cells born 0.5 wk after seizure at the site of injury survived in higher numbers in the CA3a/b of Cx32 KO mice (Figure 4.9C, 0.5 wk). In the absence of Cx32, the majority of cells labelled with BrdU 0.5 wk after kainate injection differentiated to NeuN⁺ neurons (Figure 4.9D). In WT mice, cells born 0.5 wk after seizure differentiated primarily to CNPase⁺ oligodendrocytes (Figure 4.9D).

While these data demonstrate that Cx32 null mutation enhances neurogenesis from cells activated 0.5 and not 1 week after seizure, it was unclear whether activated type 2a or NG2-Glia were the source of new neurons. To establish definitively that newly born neurons were the progeny of activated nestin⁺ NPCs and not trans-differentiation of activated NG2-Glia as has been previously suggested

Figure 4.9. Nestin⁺ NPCs, activated 0.5 weeks after seizure, are the source of new CA field neurons in Cx32 KO mice.

(A) Schedule of BrdU injections and analysis following kainic acid injection. **(B)** Quantification of BrdU-labeled cell number throughout the CA3, SGZ, PMNL, and GrDG (area indicated in grey in schematic) 0.5 and 1 wk after seizure. **(C)** Survival of cells born 0.5 wk after seizure is significantly enhanced in Cx32 KO mice. No difference in survival of cells born 1 wk after seizure was detected. Statistics are ANOVA and *post-hoc* Tukey test, # ($p < 0.05$) and ## ($p < 0.01$) indicates a statistically significant difference between Cx32 KO and WT mice. **(D)** Fate of cells born 0.5 wk after seizure. The antigenic identity of BrdU⁺ cells labeled 0.5 wk after seizure and assessed 28 days later was determined by double-labeling with NeuN (neurons), CNPase (oligodendrocytes), or GFAP (astrocytes). Note the increase in neurogenesis and decrease in oligodendrogenesis in Cx32 KO mice. **(E)** Schedule of TAM injections used to map the fate of activated NG2⁺ OPCs or nestin⁺ NPCs. **(F)** WT NG2⁺ OPCs activated immediately after seizure induction specify primarily to oligodendrocytes; KO OPCs remain OPCs. Large arrow points to a WT mG⁺ cell with characteristic oligodendrocyte morphology. Large arrowhead indicates a KO mG⁺ cell with representative OPC morphology (top panel) 31 days after KA injection. Scale bars, 100 μ m. **(G)** WT nestin⁺ NPCs activated immediately after KA insult specify to astrocytes in injured CA3a/b or granule neurons in DG; KO nestin⁺ NPCs specify to neurons in the injured CA3a/b. Small arrows point to mG⁺ cells with characteristic astrocytic morphology (top panel). In KO, mG⁺ expression is detected at the PM of NeuN⁺ CA3a/b neurons (top panel, scale bar 100 μ m, inset, scale bar 25 μ m). Identities were confirmed in nestin-

CreER^{T2}/R26R-YFP/Cx32^{-/y} mice wherein YFP is cytosolically expressed and not membrane bound. Arrows identify the progeny of nestin⁺ cells as NeuN⁺/YFP⁺ cells 31 days after KA injection (bottom panel, scale bar 100 μ m).

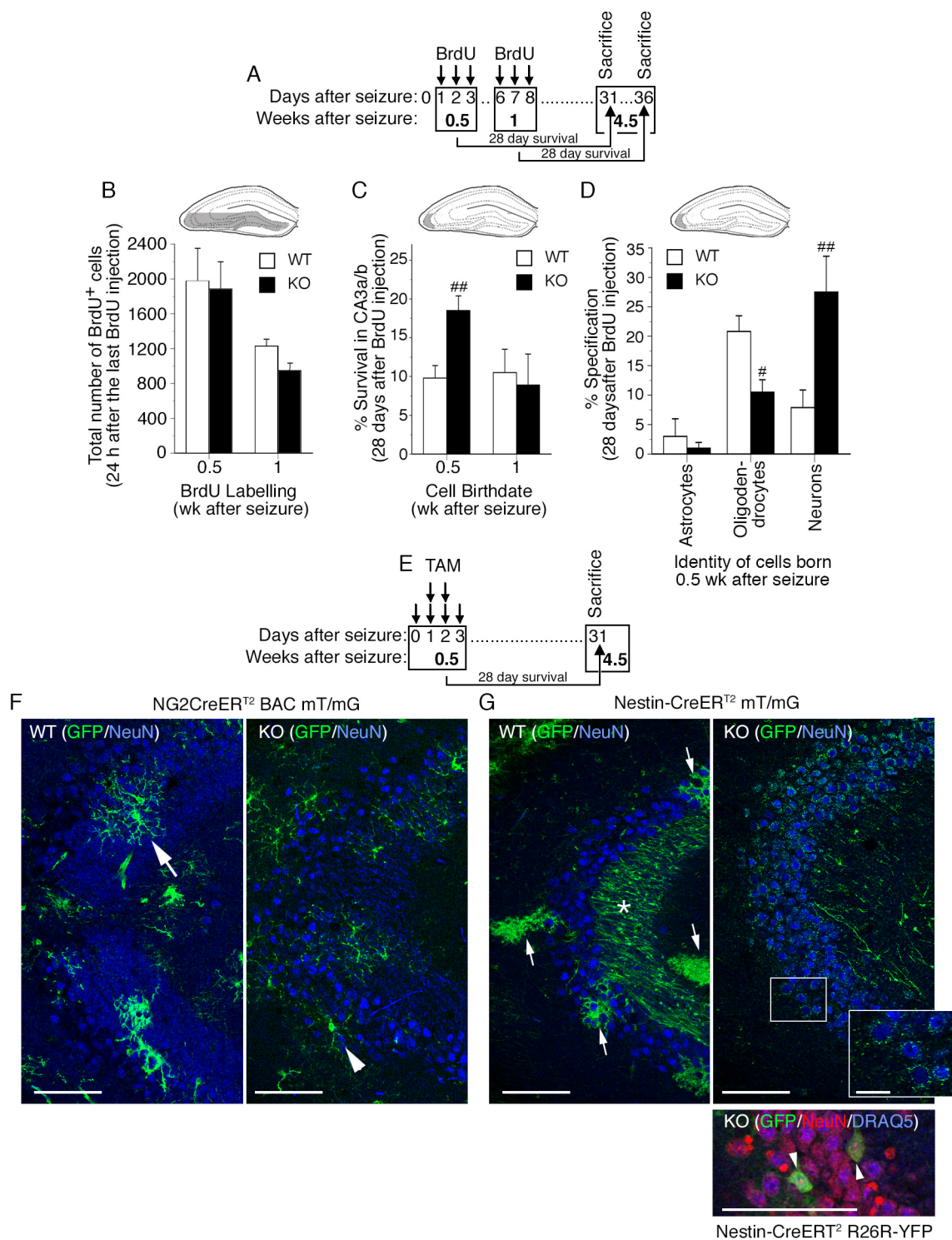


Figure 4.9

(Aguirre et al. 2004; Belachew et al. 2003), albeit with controversy (Komitova et al. 2009; Tognatta et al. 2016; Zhu et al. 2011), we crossed NG2CreERTM BAC transgenics (Zhu et al. 2011) and nestin-CreER^{T2} transgenics (Imayoshi et al. 2008) with double-fluorescent tandem dimer Tomato (mT)/membrane targeted green fluorescent reporter (mG) reporter mice (Muzumdar et al. 2007). These lines allow TAM-induced cre-recombinase to replace mT with mG, and the fate of mG⁺ cells to be tracked over time. To further confirm identity of the progeny of nestin⁺ NPCs, we used a second nestin-CreER^{T2}/R26R-yellow fluorescent protein (YFP) reporter line (Cicero et al. 2009). Here, YFP is not membrane-bound, but labels the entire cell body. All three reporter lines were placed on a Cx32 null-background. Seizures were elicited in adult male littermates. The fate of cells labeled acutely after seizure were tracked 4.5 wk later (Figure 4.9E). In WT mice, surviving NG2-Glia activated 0.5 weeks after seizure remained NG2-Glia or terminally differentiated to mature oligodendrocytes; nestin⁺ NPCs specified to astrocytes or granule neurons extending axons to CA3a/b through the mossy fibre pathway (Figure 4.9F). In the absence of Cx32, surviving NG2-Glia remained NG2-Glia, while nestin⁺ NPCs activated at time of injury effectively regenerated new neurons. (Figure 4.9G).

4.6.5. Instructive Cx30-mediated astrocyte:NPC communication directs neurogenesis

Based on these data, we hypothesized that removing the influence of Cx32-mediated NG2-Glia intercellular communication on Cx30-expressing type 1 and 2a NPCs would favour coupling of Cx30-expressing astrocytes with Cx30-expressing NPCs. To test whether Cx30-mediated astrocyte:NPC intercellular communication

enhanced neurogenesis, we tracked cell fate *in vitro* in post-natal neurospheres co-cultured with WT or Cx30 KO astrocytes. Single NPCs were dissociated from the dorsal hippocampus of WT mice, and clonally expanded for 8 DIV in mitogenic media (Figure 4.10A). Cultures were labeled with BrdU for 24 hours before plating onto laminin-coated glass coverslips (no astrocyte instruction), exposed to neurogenic paracrine factors (0.5 μ M retinoic acid (RA) and 20 ng/ml brain derived neurotrophic factor (BDNF), or placed in direct contact with a monolayer of primary WT astrocytes (astrocyte instruction). Neurogenesis was assessed on DIV 14 (Figure 4.10A). As previously reported (Song et al. 2002), co-culture with WT astrocytes markedly enhanced neurogenesis, with the number of newly born neurons comparable to that obtained by paracrine stimulation with neuron-inducing agents (Figure 4.10B). To test whether this induction required GJIC, cultures were treated with the gap junction blocker GRA (100 μ M) for six days. Blocking gap junctional channels inhibited instructive neurogenesis (Figure 4.10C). Because WT astrocytes express both Cx43 and Cx30, capable of coupling to Cx43 and Cx30 presented by NPCs cultured *in vitro* (Imbeault et al. 2009), we tested which cell:cell gap junction network was pro-neurogenic by co-culture of WT NPCs with Cx30-deficient astrocytes (Figure 4.10C). Cx30 deletion was sufficient to abrogate astrocyte:NPC neurogenic instruction. These data identified Cx30-mediated astrocyte:NPC gap junctional communication as the critical effector of instructive neurogenesis.

Figure 4.10: Instructive input from Cx30-expressing astrocytes promotes neurogenesis of NPCs.

(A) Methodological schematic of neurosphere generation and co-culture assay. Neurospheres were expanded for 8 DIV, and plated on laminin coated, or astrocyte-coated coverslips for an additional 6 DIV (14 DIV total). Both mature astrocytes and NPCs were isolated from the SGZ/dorsal hippocampus. **(B)** Lineage of BrdU⁺ cells following 7-day specification under neuronal driving conditions on WT astrocyte co-culture or laminin substrate alone. **(C)** Tuj1 lineage was also examined from neurospheres co-cultured with WT astrocytes treated with the gap junction blocker 18-alpha-glycyrrhetic acid (GRA), and from Cx30 KO astrocytes. Statistics were 1-way ANOVA with post-hoc Dunnett's, *p<0.05, ****P<0.0001.

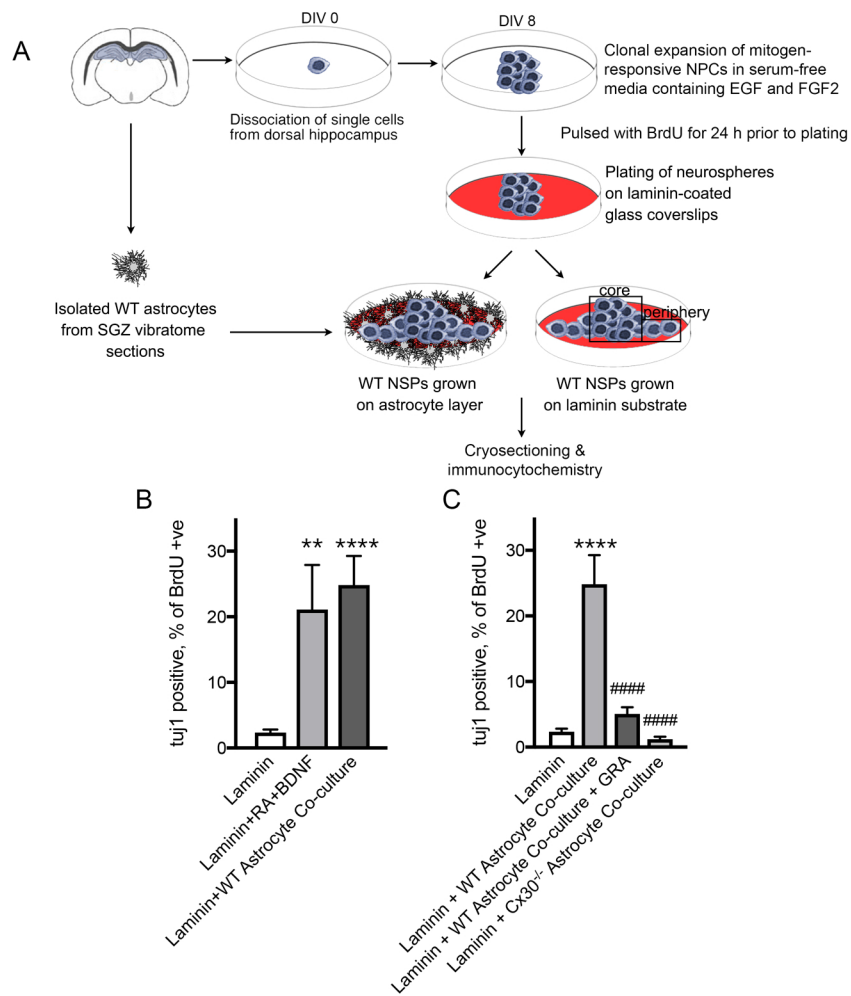


Figure 4.10

4.6.6. *Neurons generated in the absence of Cx32:Cx30 NG2-Glia:*

Astrocyte:NPC coupling restore hippocampal function

Finally, we asked whether the newly born progeny of type 2a NPCs were functional *in vivo*. Spatial memory and learning was tested in the Morris Water Maze 4.5 wk after kainic acid-induced seizure. Performance was compared to age-matched uninjured WT and Cx32 KO control mice. Testing occurred in two phases (Figure 4.11A). During the acquisition phase, mice learned to use minimal external visual cues to find a hidden escape platform submerged in a water pool. During the reversal phase, the platform location was moved 180° and mice were required to use their previous memory of existing spatial cues to locate the new position. Uninjured WT and Cx32 KO mice located the platform location with equal efficiency, navigating with increasingly shorter escape latencies over the acquisition test days (Figure 4.11B). By contrast, 4.5 wk after acute seizure, WT animals exhibited significant learning deficits (Figure 4.11B). Seizure-injured mice spent less time searching the maze quadrant containing the platform across the eight acquisition days than uninjured controls (Figure 4.11C). During the reversal phase, injured WT mice also failed to adapt to the new platform location (Figure 4.11B). In Cx32 KO mice, the only impairment following seizure was seen on the first test day (Figure 4.11B). Subsequent escape latencies and learning curves during both acquisition and reversal were comparable to uninjured WT and Cx32 KO mice and significantly faster than injured WT mice (Figure 4.11B). Moreover, injured Cx32 KO mice spent the same amount of time in the platform zone on average over the entire acquisition phase as uninjured mice, indicating equally effective search strategies (Figure 4.11C). No difference in motoric capacity was observed given that

Figure 4.11. Cx32 KO mice exhibited enhanced behavioural recovery compared to WT mice in the Morris Water Maze test of learning and memory.

(A) Schematic of kainic acid-induced seizure recovery and Morris Water Maze assessment of learning and memory. The behavioural paradigm included 8 test days initiated 4.5 wk post seizure, followed by a probe test wherein the platform position was reversed to the opposite side of the pool. **(B)** Average escape latency of WT and Cx32 KO uninjured and injured mice to reach the hidden pool platform for acquisition and probe test days. Both WT and Cx32 KO animals exhibited shorter escape latencies across the 8 test days, indicating that they were able to learn the test during the acquisition period (main effect of days, $F[7,210]=24.91$, $p<0.01$). WT injured animals exhibited a deficit in learning to finding the platform (days x genotype x injury interaction, $F[7,210]=2.53$, $p<0.05$, Tukey *post-hoc*), and failed to adapt to the new platform location during the reversal phase (main effect of genotype, $F[1,30]=7.6$, $p=0.01$). **(C)** Percent time spent searching for the platform in the correct platform zone (genotype x injury interaction $F[1,30]=4.1$, $p<0.05$, *post-hoc* Tukey, $p<0.05$), and **(D)** Swimming distance in cm (ANOVA $F[3,30]=0.8$, $p>0.05$).

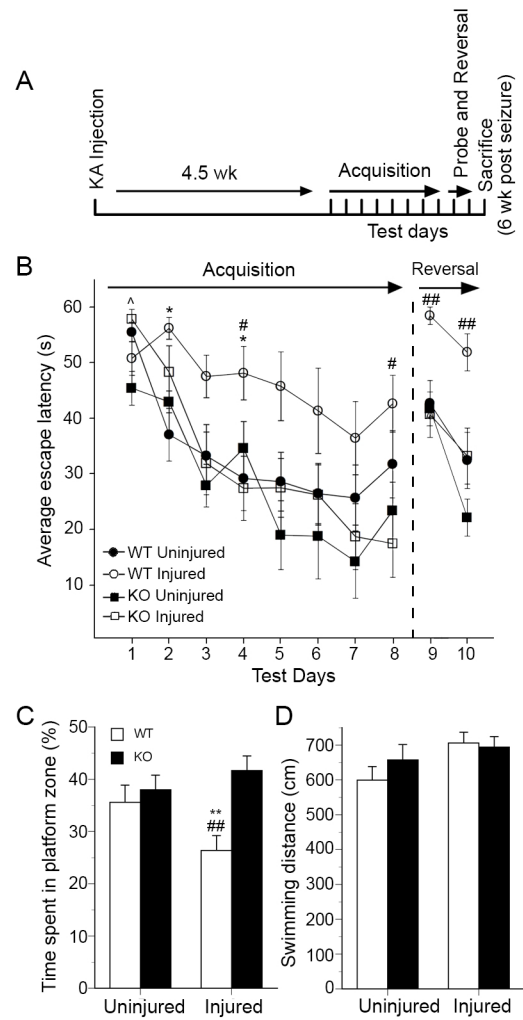


Figure 4.11

average swim distances were the same between all four groups (Figure 4.11D). Taken together, these results strongly indicate that pan-glial:NPC Cx32:Cx30 and Cx30:Cx30 gap junction coupling directs neurogenesis and that modulating this network can release the nascent neurogenic potential of type 2a NPCs activated at site of injury following acute seizure endogenously restricted by activated Cx32-expressing NG2-Glia.

4.7. Discussion

Our results provide direct evidence that a pan-glial:NPC GJIC directs NPC activation and specification in adult hippocampus. We show that Cx32 protein expressed by NG2-Glia at junctional membranes is capable of interacting with Cx30 protein present at the PM of both astrocytes and type 2a NPCs. In uninjured hippocampus, Cx32:Cx30 NG2-Glia/Oligodendrocyte:NPC coupling regulates the proliferation of nestin⁺ type 1 and 2a NPCs. Following kainic acid-induced seizure, removal of Cx32 from this pan-glial:NPC network enhances CA3a/b neuronal replacement and restores behavioural indices of learning and memory in the Morris Water Maze. Our *in vitro* results suggest that these pro-neurogenic effects result from the elimination of regulatory NG2-Glia/Oligodendroglial:NPC communication thereby favouring pro-neurogenic Cx30:Cx30 astrocyte:NPC coupling. We further show that loss of Cx32 is sufficient to increase the percentage of type 2a NPCs capable of producing new pyramidal cells and interneurons directly at sites of injury, as well as decrease the numbers of DCX⁺ neuroblasts that migrate from the SGZ and produce ectopic granule neurons.

This is the first report that endogenous pan-glial:NPC gap junctional communication directs NPC fate in injured hippocampus. These results are consistent with converging evidence that juxtacrine cell:cell contact is an important mediator of adult NPC fate in both embryonic (Huang et al. 1998a; Xu et al. 2001a; Lo Turco and Kriegstein 1991; Elias et al. 2007; Rash et al. 2016; Duval et al. 2002; Elias and Kriegstein 2008; Bittman et al. 1997; Bittman and LoTurco 1999; Xu et al. 2006) and postnatal NPCs (Cheng et al. 2004; Hartfield et al. 2011; Jaderstad et al. 2010; Kunze

et al. 2009; Ravella et al. 2015; Sommer and Rao 2002; Swayne and Bennett 2016; Lemcke et al. 2013; Freitas et al. 2012; Lacar et al. 2011; Talaveron et al. 2015; Dyce et al. 2014; Naus et al. 2016; Venance et al., 1995). Indeed, approximately 50-75% of Type 1 radial glial cells form functional gap junctions composed of Cx43 and Cx30 in the juvenile and adult SGZ (Imbeault et al. 2009; Kunze et al. 2009), and these communication networks are responsible for maintaining NPC proliferative capacity (Cheng et al. 2004; Dyce et al. 2014; Sommer and Rao 2002), survival (Ravella et al. 2015), and differentiation potential (Hartfield et al. 2011; Kunze et al. 2009; Lemcke et al. 2013).

The current study describes the *in vivo* effects of single connexin loss on both NPC (nestin⁺) and glial (NG2⁺) progenitor proliferation in the DG. No alteration of progenitor proliferation or lineage was evident in the Cx47 conditional KO, presumably because no cells of either lineage express Cx47 (Imbeault et al. 2009). As Cx47 contributes significantly to inter-oligodendrocyte and inter-astrocyte coupling (Maglione et al. 2010; Orthmann-Murphy et al. 2007), these data indicate that loss of Cx47 paracrine signaling and reduction of O:O, and O:A coupling does not impact NPC behaviour. Alternatively, compensatory increases in Cx32 along myelinated fibers are sufficient to restore coupling (Li et al. 2008).

Conditional deletion of Cx43 in the nestin lineage (NesCre⁺ Cx43 cKO) resulted in a trend for decreased NPC proliferation. Indeed, NPC coupling by Cx43 has been consistently implicated in the maintenance of NPC proliferation (Bittman and LoTurco 1999; Cheng et al. 2004; Duval et al. 2002; Lemcke et al. 2013; Weissman et al. 2004) (Figure 5B). Liebmann and colleagues report that loss of Cx43 in both GFAP⁺

astrocytes and in Type 1/2a NPCs (Zhuo et al. 2001) results in a significant decrease of both Ki67 and BrdU immunoreactivity in the SGZ and GCL (Liebmann et al. 2013). Deletion of Cx43 in the current study was limited to the nestin⁺ radial glial lineage (Type 1 and 2 NPCs), indicating that Cx43 may still be present in subsets of mature astrocytes, as has been reported in other nestin-Cre transgenic mouse models (Aoki et al. 2000; Walker et al. 2010). Thus, paracrine signaling in the niche through astrocytic Cx43 may have blunted the anti-proliferative effects of uncoupling amongst NPCs in our model (Ehret et al. 2015; Song et al. 2002). Loss of Cx43 does not appear to alter the lineage of the proliferating cells, as we report no changes in the numbers of BrdU⁺/nestin⁺ or NG2⁺ immunoreactivity. Loss of Cx30 induced a non-significant increase in NPC proliferation, with no alteration in the proportion of dividing nestin⁺/NG2⁺ progenitors. Liebmann and colleagues report similar proliferation trends for the astrocyte-specific Cx43 cKO and the Cx30KO, with further observations that proliferating DCX⁺ or NeuN⁺ cells are not altered, and there are no defects in the production of neurons in either mouse (Liebmann et al. 2013).

In striking contrast with the loss of Cx47, Cx43, or Cx30, the loss of Cx32 induces a drastic upregulation in the number of proliferating cells in the hippocampus, with significant increases in both the nestin⁺ and NG2⁺ cell populations (Figure 5D). We have previously shown that the majority of these hyperproliferative progenitor cells exhibit poor survival, fail to terminally differentiate, and are deleted by an apoptotic-like mechanism in uninjured hippocampus (Melanson-Drapeau et al. 2003). The mechanism by which loss of Cx32 results in hyperproliferation of both neuronal and glial progenitor cells is unknown, yet may result from subclinical CNS pathology in the Cx32

KO animal, consistent with the role of Cx32 in buffering neuronal extrusion of K^+ . Indeed, loss of Cx32 results in peripheral neuropathy in mice and humans (CMTX), yet sometimes presents with subclinical or mild CNS involvement (Gutmann and Shy 2015). On rare occasions overt CNS dysfunction occurs, and is characterized by transient stroke-like episodes (hemiparesis, sensory loss, and expressive aphasia) and white matter hyperintensity on magnetic resonance imaging (Basri et al. 2007; Kassubek et al. 2005; Sagnelli et al. 2014). I also present evidence in Chapter 4 of this thesis that loss of Cx32 results in upregulation of mitochondrial proteins involved in TCA cycle and oxidative phosphorylation, and culminates in enhanced mitochondrial ROS production, and more polarized mitochondrial phenotypes. It is possible that enhancement of these respiratory functions enables NPC/NG2-glial hyperproliferation. Further, Cx32 KO peripheral nerves constitutively express a similar transcriptome to WT nerves subjected to crush injury (Freidin et al. 2015), and such inflammatory environments are known to induce both NPC and NG2-glial proliferation (Arnett et al. 2001; Whitney et al. 2009).

In addition to the potential for subclinical CNS pathology in the Cx32 KO mouse, we hypothesize that WT expression of Cx32 exerts anti-proliferative and anti-neuronal (or pro-oligodendrogenic) effects on NPC and NG2-glial, given that Cx32 expression increases following the post-mitotic stages of oligodendrogenesis (Imbeault et al. 2009; Venance et al. 1995). Similarly, insight gained from models of demyelination indicate that the transition of adult NG2-glial to a proliferative state is accompanied by decreases in gap junction expression and coupling, which again increases upon specification to new oligodendrocytes (Li et al. 2014; Markoullis et al. 2012). These data further outline a novel hypothesis that Cx32 encourages the generation of oligodendrocytes by

enabling NG2⁺ cells to respond to oligodendrogenic stimuli. Indeed, the Cx32 nerve-specific promoter (P2) contains binding sites for the pro-myelination transcription factors Egr2/Knox20 and Sox10 (Bondurand et al. 2001; Houlden et al. 2004). These data suggest that NG2-glial activation of pro-myelination transcription factors recruits Cx32 expression at the same developmental time point as the myelin basic proteins, myelin protein zero, and the peripheral myelin protein 22, and that these proteins act in concert to encourage NG2-glial specification to a myelinating phenotype (Emery 2010). Taken together, I propose that WT gap junctional coupling limits the proliferation and specification of NPCs, and that loss of Cx32 removes the negative regulation on cell number and neurogenic fate, enabling NPCs to generate neurons more effectively following injury.

Neurogenesis is further impeded in the injured microenvironment, as hippocampal Cx32 protein is increased following kainic acid injury. Indeed, Cx32 mRNA and protein are also upregulated in other models of kainic acid induced seizures (Condorelli et al. 2003; Li et al. 2001), seizure induced by 4-aminopyridine (Laura et al. 2015), and seizures generated by chronic administration of bicuculline (Samoilova et al. 2003). Despite this consensus for an increase in Cx32 protein levels, there are conflicting reports about astrocytic Cx30 and Cx43 levels following seizure activity (Mylvaganam et al. 2014). We have preliminary data suggesting that hippocampal Cx30 protein is upregulated ~1.4X 3-42 days post-injury, while Cx43 protein levels do not change, or are decreased in WT mice (Supplemental Figure 5.5A). Similar results have been reported in rats for both glial connexins (Condorelli et al. 2003; Sohl et al. 2000). Combined, these increases in both Cx32 and Cx30 would enhance NG2-glial and

oligodendrocyte coupling with all cells in the network, allowing them to play larger instructive roles in the recovery process in WT hippocampus. Indeed, WT neurogenesis was not sufficient to restore TOAD64⁺/Tuj1⁺ neuronal number in the CA3a/b, as contributions from Cx32 signaling are likely anti-proliferative, and/or prevent NPCs from responding to the appropriate neurogenic stimuli.

Conversely, nestin⁺ NPCs in Cx32 KO mice are effectively recruited to an appropriate neuronal lineage in the damaged CA3a/b. These NPCs likely do not receive Cx32-mediated contact-dependent or paracrine cues from mature oligodendrocytes or from NG2-glia due to the loss of Cx32:Cx30 NPC:O and A:O junctions. Further, Nagy has described that a loss of Cx32 is accompanied by a loss of Cx30 puncta at A:O gap junctions (Nagy et al. 2003b), without significant downregulation of Cx30 or Cx43 protein levels, which we have also confirmed (Supplemental Figure 5.5B). Given this, we propose that hippocampal A:O and NPC:O Cx30 is not lost, but re-organized to strengthen A:A and A:NPC coupling, thereby increasing nestin⁺ NPC proliferation, and encouraging their specification to neurons. Indeed, it is well-known that neurogenic niche astrocytes exhibit near complete functional coupling with NPCs, provide potent pro-neurogenic fate instruction, and enable calcium wave propagation amongst and between discrete networks of NPCs and astrocytes (Lacar et al. 2011; Oh et al. 2009; Song et al. 2002; Talaveron et al. 2015). In this report, we provide further *in vitro* data supporting the pro-neurogenic effect of astrocytes on NPCs specification, and identify for the first time that signaling through astrocytic Cx30 is essential for this regulation.

Historically, the regulatory contributions of Cx30 have been undervalued, as hippocampal Cx43 is expressed at much higher levels (Cotrina et al. 2001; Nagy et al. 1999; Nakase et al. 2004; Sohl et al. 2000). These assertions are perpetuated by the finding that loss of Cx30 only results in a 22% decrease in inter-astrocytic coupling, with no compensatory increase in Cx43 (Gosejacob et al. 2011; Unger et al. 2012). Conversely, Cx30 is upregulated 22 fold in the GFAP-Cre astrocyte-specific Cx43 cKO hippocampus (Nakase et al. 2004; Unger et al. 2012), yet is only able to restore inter-astrocytic coupling to 50% of WT levels (Theis et al. 2003). Excitingly, recent work has discovered that Cx30 controls hippocampal excitatory synaptic transmission by mediating morphological changes that guide the insertion of astroglial processes into synaptic clefts. In this way, Cx30 modulates astrocytic glutamate transport, which plays an important role in synaptic plasticity and hippocampal contextual memory (Pannasch et al. 2014). Glutamate signaling is also associated with increased survival and proliferation of progenitor cells in the SVZ (Brazel et al. 2005), and SGZ (Di Giorgi-Gerevini et al. 2005), and is mediated through a variety of metabotropic glutamate receptors expressed by radial glial cells (Nochi et al. 2012). Taken together, these findings indicate that Cx30 participates less in physical cell:cell coupling than Cx43, but suggests that its roles in promoting neurogenesis from NPCs are related to glutamate signaling and/or astrocyte process proximity to NPCs in the hippocampal niche.

Our data also demonstrate that targeting the latent neurogenic potential of specific progenitor populations within the injured microenvironment can enhance brain repair. In C57BL/6 mice, loss of CA3 pyramidal neurons results in lasting impairment of hippocampal function (Brown-Croyts et al. 2000). In WT animals, neuronal replacement

following seizure is characterized by a higher frequency of ectopic granule neurons detrimental in and of themselves to long-term hippocampal function (Jessberger et al. 2005; Scharfman 2000). To our knowledge, no juxtacrine or paracrine signalling system is capable of inhibiting this aberrant specification of activated DCX⁺ type 3 neuroblasts. Here, we show that latent neurogenic potential of type 2a cells can be unlocked by modulating the balance of their connexin coupling with activated NG2-Glia and astrocytes infiltrating damaged CA cell fields following acute seizure. Cell:cell interaction with Cx32-expressing NG2-Glia inhibits type 2a neurogenesis. Removing this restriction favours pro-neurogenic Cx30-mediated astrocytic coupling. This study does not, however, address why type 2a but not type 3 neuroblasts can terminally differentiate into pyramidal cells and interneurons at sites of injury. Because activation of type 2a cells in injured pyramidal cell fields precedes peak amplification of Type 3 DCX⁺ cells in the SGZ, it may be that the neurogenic cues released within the damaged CA3 niche directs cells to adopt the appropriate neuronal subtype more effectively once neurogenesis is initiated. In support of this hypothesis, we found that new neurons expressing immature neuronal markers are detected earlier in the damaged CA3a/b field of Cx32 null-mutants than WT mice, whereas the expansion of Type 3 DCX⁺ cells observed 1 wk after seizure was reduced in Cx32 KO mice. The earlier type 2a neurogenic response in Cx32 KO mice may reduce the expansion and specification pressure placed on Type 3 DCX⁺ cells in the SGZ of the Cx32 KO that are normally fated to become the CaBP⁺ granule cell lineage. The signalling systems that ensure type 2a specification following pro-neurogenic Cx30-mediated astrocytic instruction remain to be elucidated.

In summary, we show that the hippocampal connexin-mediated glial network is restrictive to the proliferative and neurogenic capacity of NPCs contained within the neurogenic niche. Alteration of this network by genetic deletion of Cx32 removes instructive pressures from NG2-glia and mature oligodendrocytes, and strengthens coupling amongst astrocytes and NPCs. Using membrane fractionation and IP techniques, we show that PM-localized connexins physically interact in brain lysates, and are permissive to Cx32:Cx30, Cx30:Cx43, but not Cx32:Cx43, or Cx47:Cx32 cell:cell coupling. We show for the first time that astrocyte Cx30 is essential for the neurogenic instruction of Type 1 and 2 NPCs, and propose that glutamatergic signaling through astrocytic Cx30 encourages their specification to regionally-appropriate Tuj1⁺ neurons. We also provide evidence that Cx32-mediated networks amongst NG2-glia and mature oligodendrocytes are associated with reduced NG2⁺ and nestin⁺ progenitor proliferation. Further, we suggest that the presence of Cx32 provides pro-oligodendrogenic stimuli, and/or prevents NPCs from responding appropriately to neurogenic stimuli. As such, these data also hint at a potential role for Cx32 in promoting oligodendrogenesis from the injury-expanded pool of NG2⁺ progenitors. Taken together, this study provides convincing evidence that alteration of the glial connexin cell:cell interactome has important implications for brain repair; yet, targeted pharmacological interventions are lacking. Blood brain barrier-permeable Cx-specific modulators are still required to test these hypotheses, however, the current findings represent exciting mechanisms for the specific enhancement of neural and oligodendrogenic brain repair.

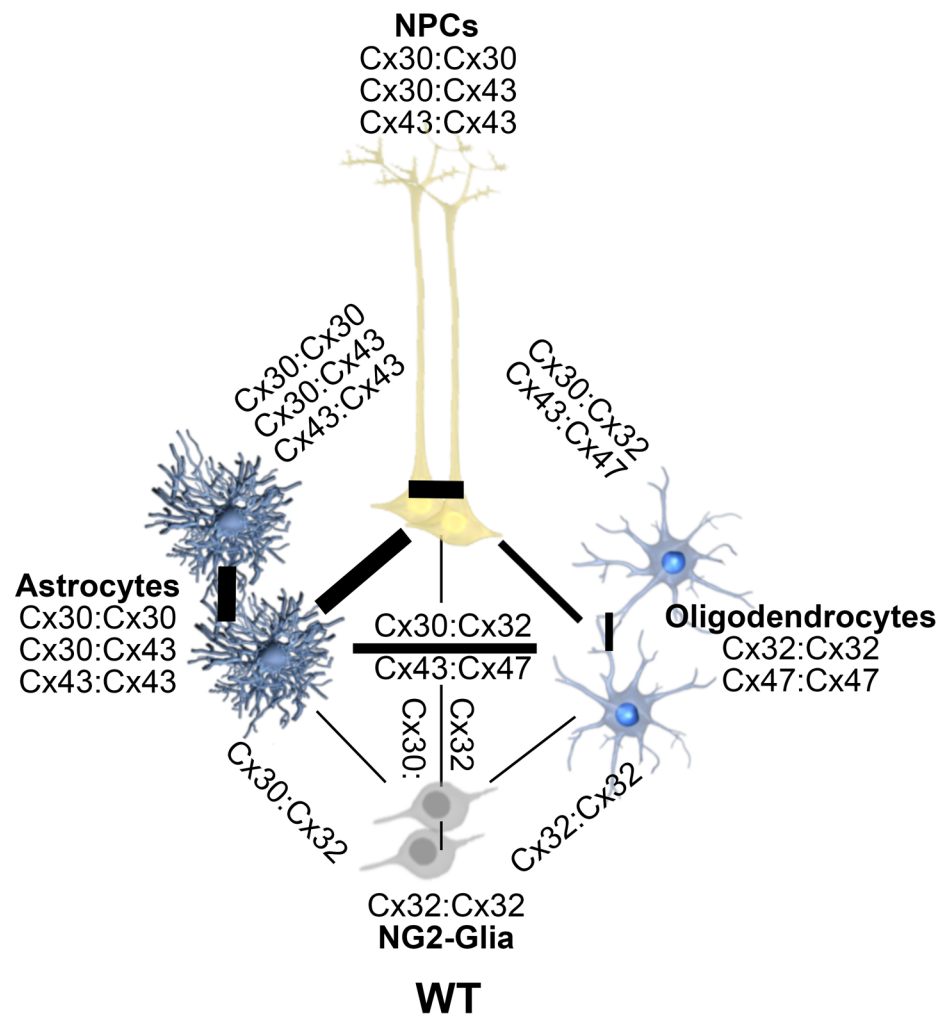
Supplemental Table 4.1. N13 WT and Cx32 KO mice exhibit comparable kainic acid sensitivity.

Genotype	Behavioral scale					Total number of mice
	1	2	3	4	5 (Mortality)	
WT	2 (3%)	3 (4%)	1 (2%)	51 (72%)	14 (19%)	72 (100%)
Cx32 KO	2 (2%)	1 (1%)	6 (7%)	61 (73%)	14 (17%)	84 (100%)

Animals were injected intraperitoneally with 25 mg/kg kainic acid to establish drug sensitivity. Seizure intensity was staged using a standard five point behavioral scale as described in Methods. Data represent the number of animals (and percentage of total number of subjects) that attained each stage without further progression.

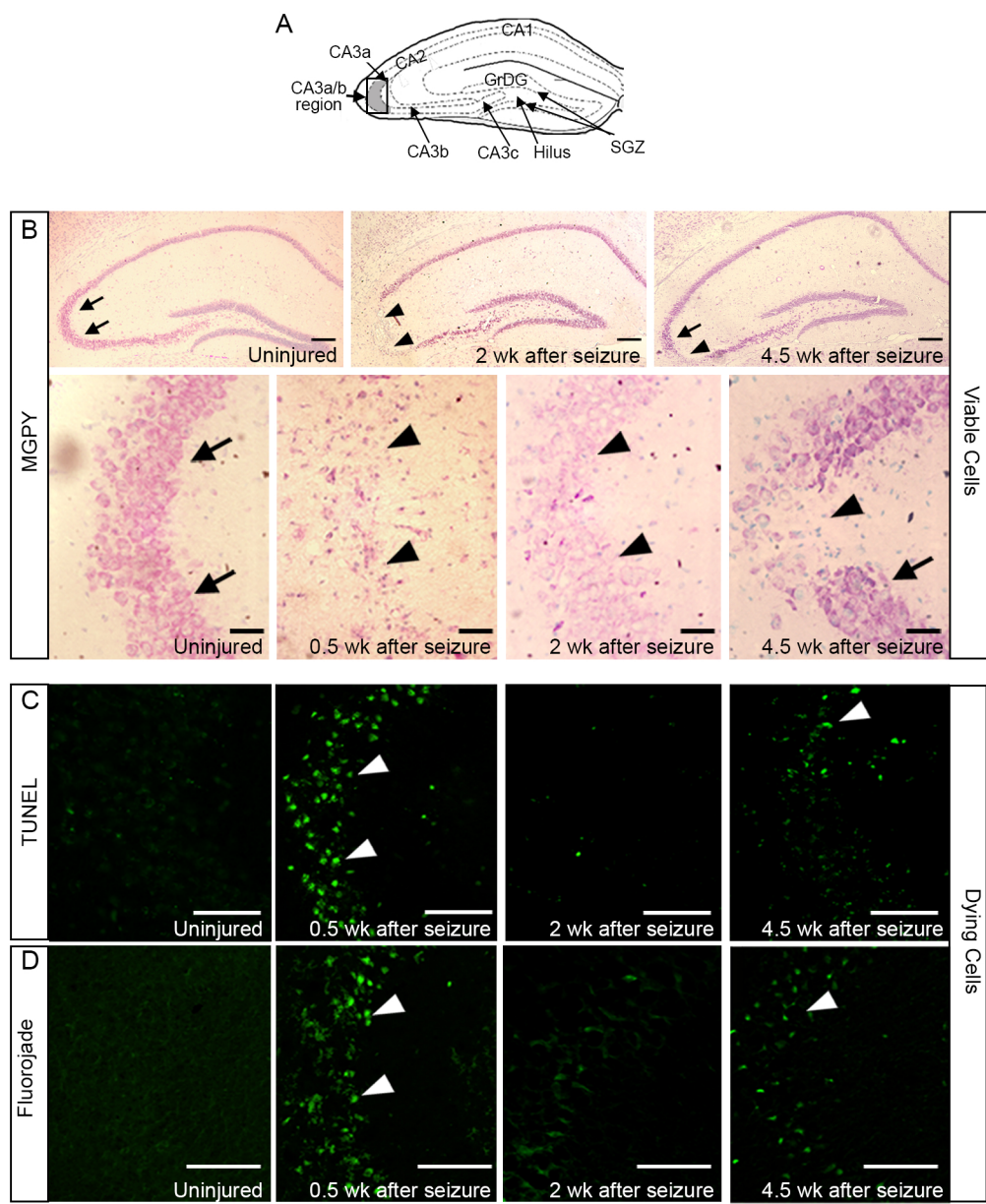
Supplemental Figure 4.1. WT Connectivity

Glial network connectivity in WT mice between oligodendrocyte connexins Cx47 and Cx32 and astrocyte connexins Cx30 and Cx43. Glial connectivity is indicated by thickness and opacity of connecting lines, where thinner, less opaque lines indicate less connectivity due to fewer gap junction partners.



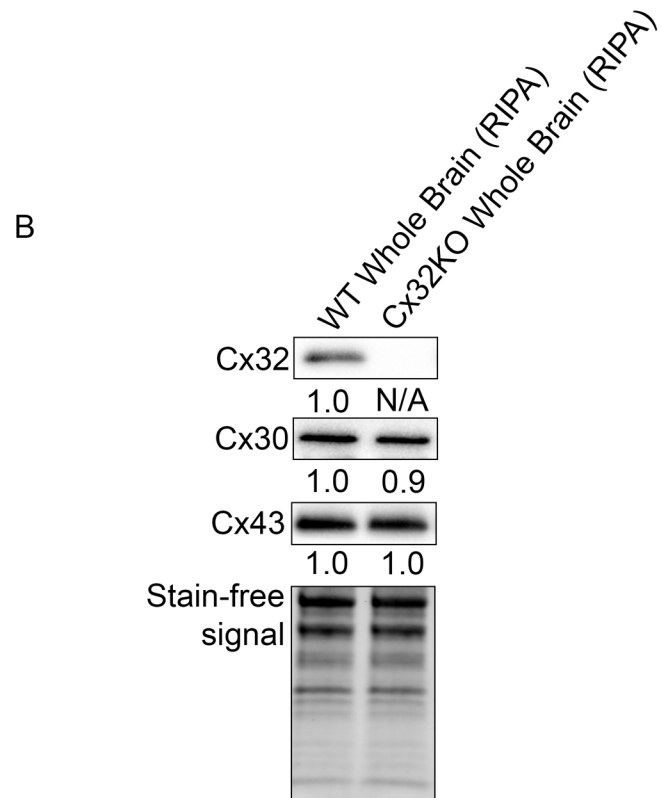
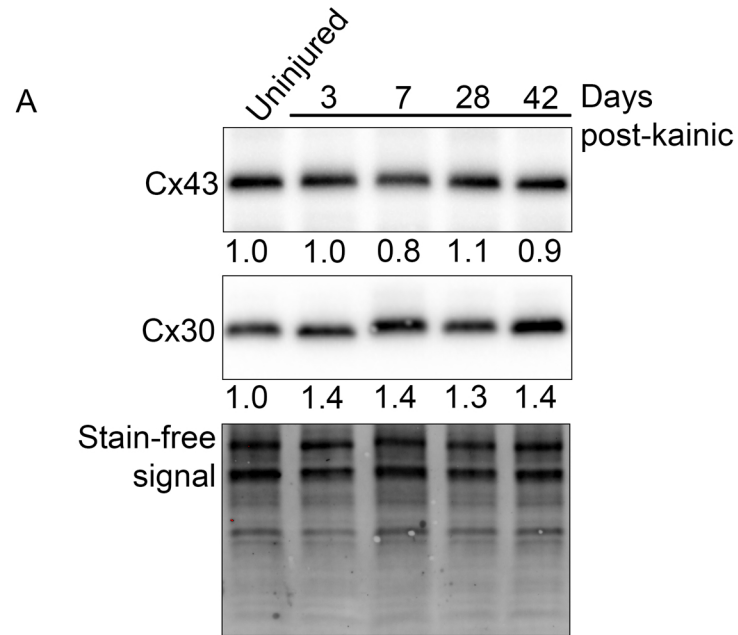
Supplemental Figure 4.2. Acute seizures trigger degeneration and partial cell replacement in the CA3a/b pyramidal cell field of 3 month adult WT mice.

(A) Hippocampal schematic indicating the primary lesion site (CA3a/b) (grey box). Abbreviations: CA1,2,3a,b,c, CA pyramidal cell fields; GrDG, granule cell layer of the DG; Hil, hilus; SGZ, subgranular zone. **(B-H)** MGPY staining in the hippocampus of uninjured animals **(B,E)** and mice 0.5 **(F)**, 2 **(C, G)**, and 4.5 **(D, H)** wk after intraperitoneal injection of 25 mg/kg kainic acid. Scale bars in **B-D**, 150 μm . Scale bars in **E-H**, 50 μm . Arrows indicate healthy CA3a/b cell field morphology; arrowheads indicate compromised cell morphology with loss of MGPY-stained neurons. A marked loss of MGPY-stained cells (arrowheads) was evident at 0.5 wk and 2 wk (arrowheads) with partial cell restoration 4.5 wk post seizure induction (arrows). **(I-P)** Terminal neuronal damage was assessed by TUNEL **(I-L)** and fluorojade staining **(M-P)**. Arrowheads point to representative regions of cell death and degeneration in adjacent sections stained with MGPY. Scale bars in **I-P**, 50 μm .



Supplemental Figure 4.3. Cx30, but not Cx43 expression is elevated post kainic acid-induced seizure.

(A) Cx30 and Cx43 protein expression from WT and Cx32 KO mice in uninjured and injured animals at 0.5, 1, 2, and 4 wk post-seizure. 10 µg of protein was loaded in each lane, and quantification was performed by normalization to the stain-free signal, and standardization to the WT, uninjured control. **(B)** Cx30 and Cx43 compensatory expression in uninjured WT and Cx32 KO whole brain lysates. 10 µg of protein was loaded in each lane, and quantification was performed by normalization to the stain-free signal, and standardization to WT.



Chapter 5 - General Discussion

In this thesis, I combine proteomic strategies and membrane fractionation-IP biochemical assays to identify novel interactions of gap junctions with intracellular proteins and/or organelles. Specifically, data presented in this thesis identifies a novel PM/mitochondrial signalling nexus regulated by Cx32. I show that Cx32 localizes to both PMs and IMMs (Chapter 2), and that loss of Cx32 results in the upregulation of mitochondrial TCA cycle and oxidative phosphorylation pathways. These mitochondrial phenotypes are also reflected by enhanced OCRs and ROS production from isolated Cx32 KO mitochondria. In addition to novel intracellular connexin protein interactions, we hypothesized that connexin-mediated glial cell:cell interactions were responsible for mediating intercellular interactions in the complex hippocampal neurogenic niche environment. Indeed, we identified that Cx32-mediated glial cell:cell interactions exert significant proliferative and fate specifying pressures on hippocampal progenitor cell types, wherein the loss of Cx32 enables improved histological and functional regeneration following excitotoxic injury. Resulting avenues of discussion include a developing literature surrounding the role of gap junctions in mitochondrial physiology and pathology, as well as a beneficial role for specific connexin modulators in mediating brain repair. The goal of this general discussion is to place the data obtained in this thesis in context with the existing literature, and to speculate how these new findings may contribute to novel and testable hypotheses of connexin biology.

5.1. Contributions to the gap junction-mitochondrial connection

The unbiased liver proteomics performed in Chapter 2 replicated findings of previously-validated PM-localized Cx32 interacting proteins (eg. Cx26), and also expanded our knowledge of possible interactions to include mitochondrial proteins (eg. SFXN-1). SFXN-1 is a likely participant in mitochondrial iron regulation, thus, we have also described a possible link between loss of Cx32 and serum hyperferritinemia that may be associated with impaired iron regulation. Further, as has been demonstrated for Cx43, we present biochemical evidence that Cx32 is present at mitochondrial inner membranes. Prior to this thesis, while exploring mitochondrial Cx43 phenotypes, Miro-Casas et al had identified Cx32 protein in liver mitochondrial preparations by immunoblotting (Miro-Casas et al. 2009); however, they were unsuccessful in identifying Cx32 by LC-MS/MS. They concluded that mitochondrial Cx32 was below the limit of detection for MS identification in their preparations. The work presented in this thesis is therefore the first exploration of possible mitochondrial phenotypes in the Cx32 KO mouse.

Chapter 3 builds on the hypothesis that Cx32 is involved in mitochondrial functions, and outlines a series of significantly upregulated proteins that may contribute to the altered mitochondrial oxygen consumption and ROS production we have described. It is possible that some of these phenotypes in Cx32 KO mitochondria are a direct result of Cx32 interaction with IMM proteins identified in Cx32 IPs from Chapter 2, including ACAA2, SLC25A10, ATP Synthase F1 complex, O subunit, and SLC25A22 (Fowler et al. 2013). In particular, SLC25A10 overexpression causes membrane hyperpolarization and increased ROS production (Lin et al. 2005). Additionally, the

ADP/ATP translocase (SLC25A5) is linked to increased glycolytic metabolism, tumorigenicity, and inefficient proton leak across the IMM (Chevrollier et al. 2011; Jastroch et al. 2010). Interestingly, examination of Cx43 mitochondrial interactions has identified a distinct set of IMM proteins with known roles in the electron transport chain (Denuc et al. 2016). These data further suggest that cell-specific connexin functions in mitochondria are dictated by unique protein interaction networks (Figure 5.1). Future proteomic investigations should concentrate on mitochondrial Cx32 interactions to validate a role for intrinsic connexin expression in mitochondrial inner membranes.

It is also interesting to note that 17% of the Cx32-interacting proteins identified by LC-MS-MS in Chapter 2 were ER-resident proteins. This finding suggests that gap junctions also interact with ER membrane proteins, and that the ER may play a direct role in mediating mitochondrial approach to the PM. Accordingly, Cx43 co-precipitates with the transient receptor potential cation channel subfamily C member 4 calcium channel (Song et al. 2005), which interacts with ER-localized STIM1 to form functional store-operated Ca^{2+} entry channels (Sundivakkam et al. 2012). The same may be true for Cx32, which would place gap junctions close to store-operated calcium influx. Alternatively, formation of ER-PM contacts close to gap junctional plaques could assist in the recruitment of subplasmalemmal mitochondria to adjacent sites at the PM. Okeke and colleagues posit a similar model wherein mitochondria are tethered at the PM by unknown proteins/lipids via dual interaction with the ER and the PM at separate membrane sites from STIM/Orai1 contacts (Okeke et al. 2016). These hypotheses may reveal novel ER-dependent roles in mitochondrial localization at gap junctional plaques.

Figure 5.1. Proposed interactions and roles of IMM Cx32

(A) Electron microscopy image of a mitochondrion adapted from (Alberts B 2002).

(A') Proposed arrangement of Cx32 hemichannels in complex with IMM proteins as identified by MS analyses in Chapter 2 (ACAA2, SLC25A10, ATP Synthase F1 complex, O subunit, and SLC25A22, SLC25A5). Cx32 may form adhesive contacts at the OMM that interact with PM-or lipid raft localized proteins/lipids, and/or may form hemichannels in the IMM that act as ion channels in their own right, or that modify the function of interacting proteins that are involved in ion and metabolite transport across the IMM.

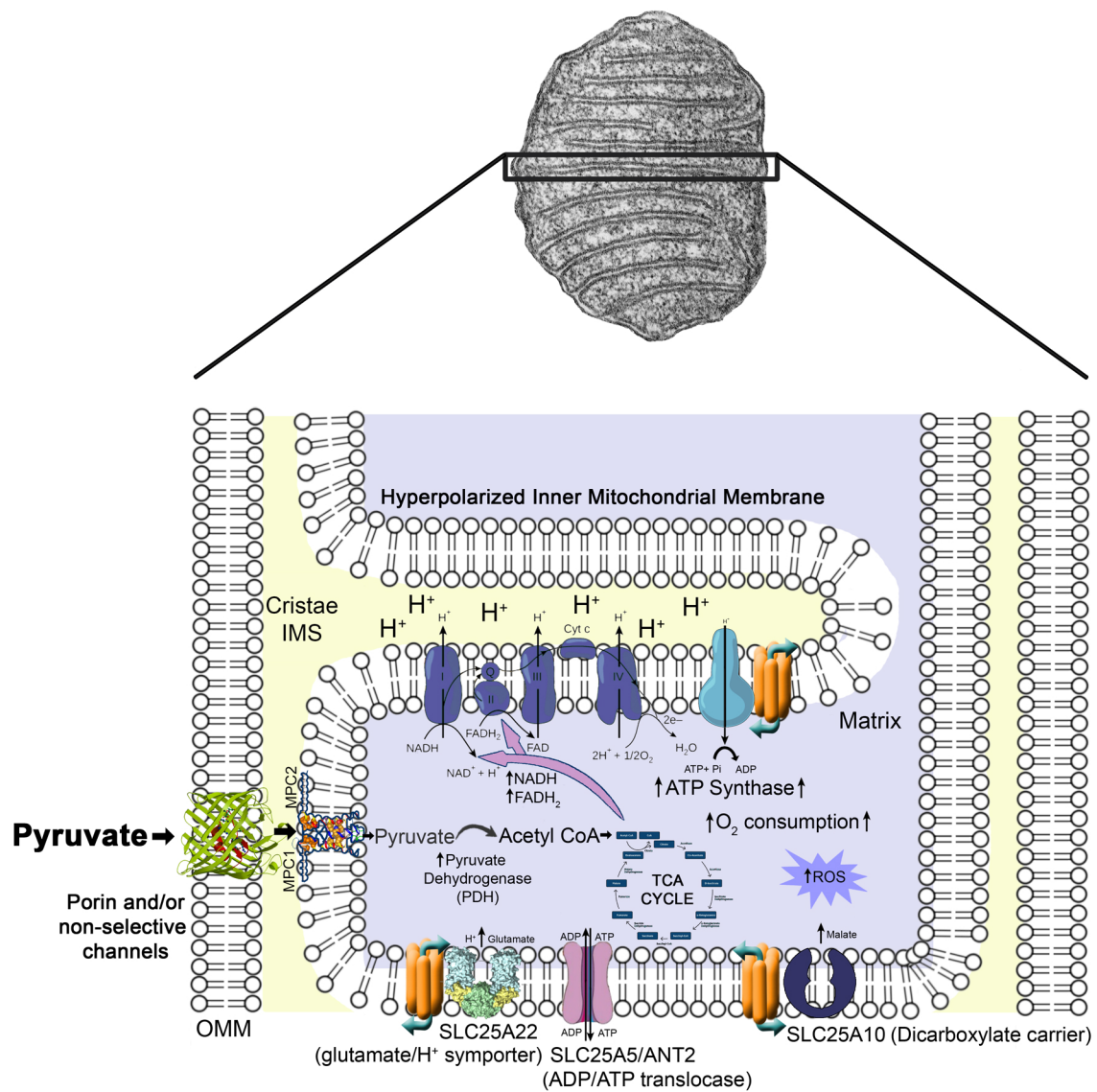


Figure 5.1

5.1.1. Evidence for the existence of lipid-protein organelle tethers at gap junctions

As alluded to above, mammalian tethers amongst intercellular organelles are not well described, and represent relatively undescribed phenomena. In this thesis, I have shown that mitochondrial SFXN-1 has the capacity to interact with PM-localized gap junction proteins, and suggest that liver Cx32 is part of a PM-mitochondrial interaction complex. While this is one of the few reports of a direct PM-mitochondrial interaction in mammals (Westermann 2015; Yu et al. 2004), mitochondria still associate close to the PM in cells lacking gap junctions (Varadi et al. 2004), indicating that gap junctions may not be required for PM-mitochondrial tethering, but rather, represent a tissue-specific modulator of the signaling environment at PM-mitochondrial interaction sites. In yeast, mitochondria are directly tethered to PM domains via bi-lateral lipid-protein interactions of the Num1/Mdm36 complex. Here, the N-terminal coiled-coiled domain of Num1 interacts with mitochondrial cardiolipin, and the C-terminal plextrin homology domain associates specifically with phosphatidylinositol 4,5-bisphosphate in the PM (Ping et al. 2016). Given the close proximity of gap junctions to specific lipid domains (Locke and Harris 2009) including lipid rafts (Locke et al. 2005), it may be possible that lipid-protein interactions with Cx32 also contribute to PM-mitochondrial tethering in mammalian cells. Indeed, similar lipid-binding complexes are also responsible for tethering at the yeast ER-mitochondrial encounter structure (ERMES), and are mediated by synaptotagmin-like mitochondrial lipid-binding protein domains on three of the five ERMES subunits (AhYoung et al. 2015). No ERMES-equivalent complex has yet to be identified in mammals, which likely reflects the inherent technical challenges to identifying lipid-protein interactions using conventional detergent-containing interaction assays (Smith

2012). Given the clear lack of data surrounding PM-mitochondrial contact sites, future work should prioritize the identification of the minimum complex requirements for PM-mitochondrial interaction, and determine how the Cx32-SFXN-1 interaction might contribute to unique liver-specific metabolic requirements at these contact sites.

5.1.2. Connexins and ferroptosis: an iron-dependent form of regulated cell death

A novel hypothesis concerning the mitochondrial-SFXN-1/Cx32 interaction involves the newly described form of regulated cell death (RCD) called ferroptosis. Ferroptosis is distinct from other forms of RCD including apoptosis, necroptosis, and autophagic cell death at the morphological, biochemical, and genetic levels, and is characterized by the presence of small mitochondria with condensed mitochondrial membrane densities, altered cristae, and OMM rupture. It is induced by experimental compounds in both normal and cancer cells, and is precipitated by activation of VDAC channels, MAP kinases, ER stress, and inhibition of the cysteine/glutamate antiporter. Ferroptosis is characterized by the accumulation of lipid peroxidation products, and ROS generated from iron metabolism. Given this, ferroptotic death can be pharmacologically inhibited by iron chelators, lipid peroxidation inhibitors, and a host of other factors that limit ROS production and/or reduce cellular iron uptake (Xie et al. 2016). It has recently been shown that an increase in mitochondrial ferritins is associated with ferroptosis, and is able to compensate for lethal doses of iron-generated ROS initiated by the small molecule erastin (Wang et al. 2016). The Cx32 interacting proteins, SFXN-1, is a carrier protein thought to be involved in transporting a component required for iron utilization (possibly pyridoxine) into or out of the mitochondria. As such, mice lacking the SFXN-1 gene (*flexed-tail* mutant mouse) present with

sideroblastic anemia characterized by pathological intramitochondrial iron deposits in fetal liver-derived erythrocytes, and increased serum ferritin levels (Cotter et al. 1999).

These data suggest that the presence of Cx32 might be protective against ferroptosis, potentially due to regulatory interactions with SFXN-1. Loss of interaction with PM and/or mitochondrially-localized Cx32 could impact SFXN-1 function, resulting in impaired mitochondrial iron utilization and hyperferritinemia, as seen in Cx32 KO mice. Evidence for connexin involvement in iron processing is apparent in diseases where connexin proteins are lost or mutated, including Oculodentodigital Dysplasia Syndrome (ODDD) and Multiple Sclerosis (MS). In ODDD, mutations in Cx43 result in demyelination and abnormal iron deposits in cerebral white matter (Gutmann et al. 1991), indicating a defect in iron utilization in oligodendrocytes (Todorich et al. 2009). Such white matter pathology resulting from loss of an astrocytic connexin also suggests that impaired cell-cell contact between astrocytes and oligodendrocytes (loss of Cx43:Cx47 signaling) may also contribute to iron-overload pathology. Similarly, white matter iron deposition is also a pathological hallmark of MS and Experimental Allergic Encephalomyelitis (EAE), wherein significant downregulation of Cx32 is also evident (LeVine and Chakrabarty 2004; Markoullis et al. 2012; Markoullis et al. 2014). NG2-glial oligodendrocyte precursors (prior to expression of Cx32) are particularly sensitive to oxidative lipid peroxidation induced by cysteine deprivation (Back et al. 1998), yet, given that ferroptosis has only been recognized as a distinct form of RCD since 2012, is it likely that many reports indicating oligodendrocyte sensitivity to iron or lipid-derived oxidants are actually describing classic forms of ferroptotic death. In light of converging molecular mechanisms linking gap junction and mitochondrial biology to ferroptosis,

future work from our laboratory will address these topics as we continue to investigate mitochondrial phenotypes in connexin-null animals.

5.1.3. Functional connexon hemichannels in mitochondrial membranes

While it is clear that PM membrane channels have the capacity to impact mitochondrial phenotypes, a more direct link between gap junctions and mitochondrial function was first described 12 years ago, when Boengler and colleagues demonstrated that Cx43 was capable of mitochondrial localization in cardiac myocytes (Boengler et al. 2005; Boengler et al. 2009). This finding suggested that connexins directly influence mitochondrial physiology by acting as K^+ and Ca^{2+} -permeable hemichannels in IMMs (Boengler et al. 2013; Srisakuldee et al. 2014). These concepts were highly debated until multiple laboratories replicated the original results, and mitochondrial connexins were found in many other cell types (Fowler et al. 2013; Kiec-Wilk et al. 2012; Kozoriz et al. 2010; Li et al. 2002a; Lu et al. 2012; Mohammad and Kowluru 2011; Sun et al. 2012; Vinken et al. 2013). Consistent with the role of cardiac Cx43 in mitochondrial respiratory functions, I have also demonstrated that mitochondrial oxygen consumption, membrane potential, and ROS production are altered in freshly-isolated hepatic mitochondria from Cx32 KO mice. It is still unknown how Cx32 might contribute to mitochondrial function, but it is very likely that Cx32 and Cx43 play very different roles in mitochondrial membranes, and these hypotheses must be rigorously tested.

5.1.4. Increased mitochondrial ROS and carcinogenesis in Cx32 KO mice

Cx32 KO mice are particularly sensitive to the development of various tumours (King and Lampe 2004a; King and Lampe 2004b). I hypothesize that increased Cx32 KO OCRs underlie increased rates of mitochondrial ROS production, which are known

to activate the MAPK oncogenic pathway (Meves et al. 2001). Activated MAPKs are directly responsible for promoting aberrant cell proliferation (Irani et al. 1997; Ogrunc et al. 2014), and are hypothesized to underlie the propensity for oncogenesis in the Cx32 KO mouse model (King and Lampe 2004a; King and Lampe 2004b)

Consistent with our data in isolated hepatic mitochondria, these data suggest that baseline oxidant damage is elevated in Cx32 KO cells, and is further corroborated by higher oxidant loads (as measured by increased hepatic glutathione disulfide/glutathione ratios) following acetaminophen-induced liver injury in Cx43KO^{+/-} animals (Maes et al. 2016). Combined, differences in respiration, membrane potential, and ROS production from isolated liver mitochondria may contribute to the increased incidence of hepatocellular tumors and hepatocyte proliferation rates described in the Cx32 KO mouse (Evert et al. 2002; King and Lampe 2004b; Temme et al. 1997).

5.1.5. Connexin loss or mutation precipitates mitochondrial dysfunction

Mitochondrial pathology has also been reported in humans with connexin mutations, or in models of disease involving connexins. For example, *GJB2* (Cx26) mutations enhance the severity of non-syndromic hearing loss associated with a mitochondrial mutation (1555A→G) in humans, suggesting a functional link between gap junction and mitochondrial physiology (Abe et al. 2001). Further, mutation of the *GJB1* (Cx32) gene underlies the development of CMTX. A similar presentation of CMT also results from mutations in the mitochondrial proteins Ganglioside Induced Differentiation Associated Protein 1 (GDAP1), and mitofusin 1/2 (MTF1/2), and presents with defects in mitochondrial organization and localization. As of yet, there have been no direct investigations of mitochondrial defects in CMT caused by *GJB1* mutation,

however, Abrams and Freidin hypothesize that mitochondrial dysfunction is likely to play a role in the pathogenesis of CMTX, as loss of Cx32 impedes the removal of K^+ from the subaxonal space, placing increased stress on sensitive K^+ -sequestering mitochondria (Freidin et al. 2015; Kozoriz et al. 2010). Similarly, in addition to increased c-Jun expression (Klein et al. 2014), transcriptional analysis of peripheral nerve revealed that the uninjured Cx32 KO nerve has alterations in genes related to mitochondrial function and apoptosis, and is transcriptionally similar to injured WT nerve, pointing to a basal injured state of gene expression in the Cx32 KO (Freidin et al. 2015). Likewise, transcriptional analyses of WT and Cx43-null astrocytes indicate that Cx43-null astrocytes experience significant alteration in genes related to apoptosis and energy metabolism (Iacobas et al. 2003). Similar analyses from Cx43 null and Cx32-null newborn brains reveal significant overlap in the type and number of similarly regulated genes in these two connexin-null genotypes, indicating conserved transcriptional activities amongst these two glial connexins (Iacobas et al. 2005). Here, altered genes corresponded more often with the cell cycle, apoptosis, and mitochondrial metabolism than to genes involved in transport into the cell and junction-adhesion-extracellular matrix (Iacobas et al. 2005). These data signify the importance of investigating mitochondrial function in diseases involving connexin protein loss and/or function, as many isoforms appear to be central transcriptional regulators, in addition to having specific effects on mitochondrial function (Iacobas et al. 2007).

5.2. Endogenous connexin-mediated cell:cell interaction networks influence neurogenic repair

As has been discussed above, the presence or absence of Cx32 impacts mitochondrial OCRs, and may play important roles in disease. These results have been discussed mainly in reference to intracellular pathology at the single cell level; yet, the intricate interaction networks enabled by gap junctional communication also affect groups of cells at the systems level, particularly in the heterogenous cellular environment of the brain. As such, investigation of connexin loss in an injury-repair model of the highly-coupled hippocampus provides an excellent model in which to study the effects of endogenous gap junctional communication networks. Here, cell:cell intercellular and protein:protein intracellular connexin interactions are affected, and the intracellular phenotypic alterations discussed in Chapters 2 and 3 can be extended to describe phenotypic alterations across heterogenous intercellular networks in a complex injury environment.

5.2.1. Mechanisms of enhanced neurogenic repair in the Cx32 KO mouse

Enhanced neuronal regeneration and behavioural recovery from kainic acid-induced seizures was evident in the Cx32 KO mouse. This outcome is likely made possible by synergistic processes regulating both the promotion of neurogenesis and the suppression of oligodendrogenesis. Indeed, reactive neurogenesis in the injured microenvironment produces mostly glial phenotypes; thus, successful brain repair strategies must employ multiple tactics to overcome this glial default (Bonfanti 2016; Peretto and Bonfanti 2015). Both promotion of neurogenic and suppression of oligodendrogenic cues involve extrinsic and intrinsic factors that may be equally

important in achieving enhanced neuronal repair following injury, and I will discuss some possible mechanisms contributing to the improved regeneration and behavioural outcomes of Cx32 KO mice.

5.2.2. *Extrinsic promotion of neurogenesis*

Cx30-mediated astrocyte:NPC synapse remodeling

As outlined in Chapter 4, I hypothesize that the glial network is altered in the Cx32 KO animal such that Cx30 is re-localized to NPC:astrocyte junctions, thereby enhancing Cx30-mediated signaling with newly generated nestin⁺ progenitors at the injury site. We provide *in vitro* evidence that astrocyte Cx30 is required for enhanced neurogenic signaling from isolated mouse hippocampal progenitor cells, and that this effect is prevented in the presence of CBX. This hypothesis represents an extrinsic promotion of neurogenesis, and may involve Cx30-mediated remodeling of astrocyte process architecture through alteration of cell adhesion and migration. More specifically, loss of Cx30 induced astrocyte process elongation, ramification, and invasion into excitatory neuronal synapses, where astrocyte glutamate receptors competed with post-synaptic AMPAR receptors for synaptic glutamate. Astrocyte synapse invasion was sufficient to decrease AMPAR-dependent glutamate signaling, yet this remodeling occurred independent of the C-terminal tail of Cx30, suggesting that these remodeling capabilities are channel-independent (Pannasch et al. 2014). Enhancing Cx30-mediated signaling would prevent astrocyte filopodia from encroaching on newly-born neurons, allowing them unfettered access to glutamatergic inputs, which are known to regulate proliferation, specification, and synaptic integration. In addition to reducing glutamate clearance by astrocytes, I suggest that channel function of Cx30 also

mediates transfer of neurogenic cues to the developing cells (Oh et al. 2010; Oh et al. 2009), as inactivation of Cx30 channel function was sufficient to inhibit the neurogenic effect of astrocyte:NPC co-culture in our *in vitro* experiments.

Hyperexcitability of the Cx32 KO brain and microglial preconditioning

Glutamatergic signaling may also play an important role in the regenerative process of the Cx32 KO brain, as it is implicated both in the subclinical level of pathology present in the Cx32 KO brain, and in known protective preconditioning mechanisms. Preconditioning mechanisms function on the organelle/cell level (eg. generation of mitochondrial ROS), but also operate at the population level (recruitment/deployment of immune modulators), and are effective at both preventing lethal injury and enhancing repair processes (Chang et al. 2013; Jimenez-Mateos and Henshall 2009). Here we report that both WT and Cx32 KO mice are equally susceptible to the same dose of kainic acid, and experience similar cell loss in the CA3 region of the hippocampus. These data suggest that loss of Cx32 does not prevent neuronal damage, but rather encourages the regenerative process, and/or enhances the survival of newly-born cells. Electrophysiological examination of Cx32 KO neuronal function concluded that approximately 50% of cortical pyramidal neurons exhibited increased membrane input resistance, and altered synaptic responses characteristic of hyper-excitability. Further, afferent stimulation resulted in glutamatergic excitatory postsynaptic potentials that resembled the paroxysmal depolarizations seen in epilepsy. It was determined that this neuronal hyper-excitability was a result of impaired synchrony in GABA-induced inhibitory post-synaptic potentials. Finally, myelin sheaths were thinner in the Cx32 KO neocortex (Sutor et al. 2000). Together, these findings

suggest that the loss of Cx32 creates an environment in which cells are constitutively subjected to sub-lethal excitatory stressors that precondition the regenerative capacity of the Cx32 KO brain to respond more effectively following lethal stressors.

Indeed, preconditioning to kainic acid-induced hippocampal cell death can be accomplished by pretreatment with low, non-toxic doses of NMDA, which activate both synaptic and extrasynaptic NMDA receptors to induce enhanced neuronal firing. Sub-lethal NMDA treatment also results in significant neuroprotection by activation of phosphatidylinositol-4,5-bisphosphate 3-kinase - Akt signaling, forkhead box O export, glycogen synthase kinase 3 beta inhibition, and eventual cAMP response element binding protein-dependent activation of BDNF (Mohammadi et al. 2009; Ogita et al. 2003; Soriano et al. 2006). This finding strongly suggests that a similar constitutive phenotype in Cx32 KO mice could precondition the brain for enhanced neuronal survival and regeneration post-injury. Further investigation is required to determine if Cx32 KO mice exhibit increased levels of the preconditioning signaling mediators that result in increased BDNF signaling.

As determined in Chapter 3, Cx32 KO mitochondria generate more ROS than WT mitochondria, which is an important mediator of mitochondrial and cellular stress preconditioning. As complex I and complex III are the major sources of ROS generated from the electron transport chain, IMM Cx32 is perfectly positioned to interfere with the two main mitochondrial preconditioning pathways: ROS and mitochondrial ATP-sensitive potassium channels (mitoK_{ATP}) channels (Correia et al. 2010). Preconditioning is mediated by ROS production at the respiratory chain, which activates MitoK_{ATP} channels (Fornazari et al. 2008), allowing K⁺ to flow into mitochondria to

depolarize and alkalinize the mitochondrial matrix (Penna et al. 2009a). Finally, depolarization-induced ROS generation activates several kinases (eg. PKC ϵ) that prevent mitochondrial permeability transition pore (PTP) opening and death. These preconditioning events are reduced following ROS scavenging, and application of mitoK_{ATP} channel inhibitors (Penna et al. 2007). Cardiac Cx43 mediated-preconditioning is thought to be related to a drop in mitochondrial membrane potential, followed by K⁺ uptake through IMM Cx43 hemichannels, but not through mitoK_{ATP} channels (Ruiz-Meana et al. 2014). Similarly, Cx43 is found in rat brain mitochondria, and its phosphorylation status mediates Ca²⁺-induced PTP opening in both synaptic and non-synaptic mitochondria. When blocked with CBX, Ca²⁺-induced PTP opening occurs at a lower threshold, indicating that active mitochondrial Cx43 is protective (Azarashvili et al. 2011). We do not have evidence that Cx32 is present in oligodendrocyte mitochondria, nor can Cx32 provide the same preconditioning protection as Cx43 when “knocked-in” to cardiac Cx43-null mitochondria (Rodriguez-Sinovas et al. 2010). As Cx32 KO mitochondria generate more ROS than WT mitochondria, preconditioning may be possible by blockage of mitochondrial Cx32 channels in the WT brain. Despite this clear tissue-specific requirement for mitochondrial connexin phosphorylation or interaction events, Cx32 may modulate preconditioning through oligodendrocyte-specific protein interactions at the IMM. Alternatively, as protection is afforded in rat brain mitochondria expressing Cx43, mature astrocytes and NPCs may be preconditioned in the Cx32 KO brain, as mediators of oligodendrocyte stress (eg. K⁺, ROS) are easily transferred amongst cells in the glial network. Taken together, these data indicate that mitochondrial connexins are likely affected in the Cx32 KO

background, and that constitutive preconditioning stress in oligodendrocytes, astrocytes, and NPCs may contribute to improved cellular function post-injury.

Microglia activated in the Cx32 KO brain may also be preconditioned to provide better trophic support to newly-generated neurons, or preconditioned such that their response to kainic acid is reduced or altered (Lin et al. 2009). Indeed, microglia are polarized in their activation potential: M1 microglia secrete pro-inflammatory and anti-neurogenic mediators (tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), IL-12, IL-23, and inducible nitric oxide synthase) (Gordon and Martinez 2010), while M2 microglia secrete protective effectors (IL-10, Fizz1, Ym1, and arginase-1), and are associated with increased neurogenic and neuronal survival phenotypes (Ferrer-Martin et al. 2015; Hu et al. 2015). Compared to M1 microglia, M2 microglia provide superior trophic input for the resolution of inflammation, and exhibit enhanced phagocytotic and proteolytic recycling of dead cells/proteins (Balce et al. 2011; Sica and Mantovani 2012). In WT mice, microglial activation following kainic acid-induced seizures is of the M1 variety, and is neurotoxic (Andersson et al. 1991; Rogove and Tsirka 1998). Several lines of evidence support the hypothesis that microglia preconditioned to a variety of different stressors alter their response to subsequent insults, resulting in an M2 phenotype, suppression of pro-inflammatory responses (Qin et al. 2016; Schaafsma et al. 2015), and superior functional recovery from a variety of insults (Chen et al. 2012; Dmowska et al. 2010; Kanazawa et al. 2017; Lee et al. 2016; Marsh et al. 2009). In the uninjured Cx32 KO brain, hyper-activated Iba1⁺ microglia are evident across multiple specific Cx32 KO brain regions: spinal cord, brainstem, and cerebellum (Olympiou et al. 2016). Further, examination of inflammation in Cx32 KO spinal cord indicates that both CD8⁺

T-lymphocytes and F4/80⁺ macrophages are constitutively elevated, and that macrophages localize closely with degenerating myelin sheaths (Maurer et al. 2002).

These findings provide support for the hypothesis that the Cx32 KO brain continually experiences sub-clinical hyper-excitability-induced inflammatory stressors that activate microglia. Given this, I argue that this preconditioning environment of the uninjured Cx32 KO brain reduces the pro-inflammatory nature of microglia, shifting their activation to M2-like phenotypes, thereby contributing to improved cell replacement and behavioural outcomes. While microglial activation phenotypes have not been described in the uninjured or injured Cx32 KO brain, the constitutively enhanced hyperproliferation of NG2-glia in this model (Melanson-Drapeau et al. 2003) may actually assist in the promotion of an M2 microglial phenotype. Indeed, increased levels of the NG2-glia chondroitin sulfate proteoglycan (CSPG) at injury sites can activate microglia to non-toxic, beneficial cell types that display increased expression of neuroprotective IGF-1. Additionally, exposure of microglia to an endogenous degradation product of CSPG (CSPG-DS; a 6-sulfated disaccharide) dramatically induces the secretion of BDNF by microglia, which may further contribute to the enhanced repair capacity of Cx32 KO brains (Rolls et al. 2008).

Differentially activated microglia in the Cx32 KO brain may also provide insight into our finding that Cx32 KO brains do not support the survival or integration of seizure-derived ectopic neurons in the hippocampal hilus, or in the injured CA3a/b regions. Excitingly, Luo and colleagues have demonstrated that microglia engulf and phagocytose viable newborn cells born in the DG post kainic acid seizure activity. Inactivation of microglia with the drug minocycline resulted in a dramatic elevation of

hilar ectopic granule cells present in the DG, suggesting that microglial hyperactivation may completely prevent the integration of ectopic cells (Luo et al. 2016). Similarly, Matsuda and colleagues have identified that Toll-like receptor 9 (TLR-9) activation in microglia also prevents seizure-induced aberrant neurogenesis (Matsuda et al. 2015), however, their proposed mechanism is that TNF- α generated through TLR-9 activation inhibits neurogenesis, thereby reducing the number of new cells generated following seizure. Indeed, while both mechanisms may be at play in the injured hippocampus, precipitation of an M2-like, phagocytic microglial phenotype is also associated with secretion of protective cytokines and antioxidants that further encourage more complete brain regeneration (Schwartz 2010; Vilhardt et al. 2016). Thus, owing to the dominance of microglial activation in kainic acid-induced hippocampal injury, further investigation into microglial phenotypes should be undertaken to determine if the hyper-excitable Cx32 KO brain constitutively primes immune cells to enhance neuronal cell replacement/survival, and if microglia activated post-seizure in the Cx32 KO are indeed more effective at recognizing and preventing the integration of ectopic, seizure-inducing neurons.

5.2.3. Intrinsic promotion of neurogenesis

Hyperexcitability-driven neuronal integration in the Cx32 KO brain

Increased hyper-excitability in the Cx32 KO brain could also contribute to an intrinsic promotion of neurogenesis by enhancing survival and synaptic integration of newly-born neurons in the injured tissue. Glutamatergic signaling is known to enhance the survival and neuronal differentiation of NPCs (Brazel et al. 2005), thus, increased parenchymal glutamate concentrations may contribute to higher levels of baseline

progenitor proliferation in the Cx32 KO animal, and may continue to promote neurogenesis from newly-born cells. Indeed, new granule cells compete with, and invade synapses of mature neurons in an activity-dependent manner, and are more efficient at generating glutamatergic action potentials (Kee et al. 2007; Mongiat et al. 2009; Tashiro et al. 2007; Toni et al. 2007). Accordingly, NPCs receiving and responding to stimulatory inputs are less likely to be removed by microglial phagocytosis, which plays a large role in pruning of weaker, inactive synaptic inputs, thereby strengthening the remaining synapses (Schafer et al. 2012).

NG2-glial – neuronal synapses

Unlike the cells present within the canonical neurogenic niches of the SGZ and SVZ, NG2-glial are located uniformly throughout the adult brain parenchyma, and constitute 8-9% of the total cell population in white matter, and 2-3% of the total cells in grey matter (Dawson et al. 2003). While much controversy has surrounded the neurogenic potential of NG2-glial, fate mapping of NG2 and transgenic marker mice has definitively proven that NG2-glial do not contribute to new neurons in the intact brain; rather, NG2-glial self-renew, and constitutively contribute to the production of new oligodendrocytes (Huang et al. 2014; Nishiyama et al. 2009; Richardson et al. 2011). Further, while NG2⁺ cells contribute to the production of embryonic astrocytes, virtually no astrocytes are produced from post-natal NG2-glial *in vivo* (Zhu et al. 2011). Despite their role in generating new oligodendrocytes, no NG2⁺ cells are present in oligodendroglial and astroglial dye-coupled networks from hippocampal, thalamic or cortical brain regions (Griemsmann et al. 2015). Instead, every undifferentiated NG2 glial cell receives vesicular synaptic input from neighboring glutamatergic and

GABAergic neurons that results in nearly identical postsynaptic currents as those recorded in neurons (De Biase et al. 2010; Passlick et al. 2013). The functions of such synapses are proposed to regulate activity-dependent myelination (Gallo et al. 2008), but they may serve a more homeostatic role, as they are retained during NG2-glial cell division, and are passed on to daughter cells (Frohlich et al. 2011).

NG2-glia provide survival and extracellular matrix support to neurons and NPCs

While neuron-NG2-glial synapses may regulate NG2-glial proliferation and specification, the role of NG2-glial cell synapses on neurons is not well understood. Recent evidence supports the hypothesis that NG2-glia are required for neuronal survival; thus, as seen in this thesis, I hypothesize that enhanced NG2-glial cell activity in the Cx32 KO brain generates an environment more permissive to neuronal regeneration and NPC survival. Indeed, ablation of NG2-glia causes hippocampal atrophy, due to excess pro-inflammatory IL-1 β signaling (Nakano et al. 2017). Loss of NG2-glia also results in primary leptin resistance, as NG2-glia appear to support the survival of leptin receptor neurons in the median eminence (Djogo et al. 2016). Further, loss of NG2-glia impairs glutamatergic signaling in excitatory pyramidal neurons, and induces a depressive phenotype in mice by impairing postsynaptic, phosphorylation-dependent glutamate receptor trafficking (Birey et al. 2015). Here, homeostatic functions of NG2-glia support both astrocyte and neuronal functions, as Birey et al also report that glutamate scavenging by astrocytes is impaired. Homeostasis is facilitated through secreted factors from NG2-glia, particularly FGF-2, which mediates increased glutamate aspartate transporter levels, glutamate uptake, and phosphorylation of glutamate receptors. Additionally, NG2-glia actively secrete several extracellular matrix

molecules (hyaluronan, versican, phosphacan, neurocan, and tenascins) that stabilize synapses by providing anchors for ion channels and neurotransmitter receptors (Butt et al. 2005). Presumably, constitutive upregulation of NG2-glia factors in the Cx32 KO brain may also better support the survival of newly proliferating and specifying immature neurons at the site of injury in our model, yet this hypothesis remains to be tested.

These results demonstrate that early upregulation of CSPG following injury is beneficial for recovery of spinal cord regeneration, and recovery of motor function (Rolls et al. 2008). The CSPG NG2 is constitutively cleaved by α and γ secretases to produce a 290 kDa ectodomain, and a membrane-associated C-terminal fragment (CTF) of 12 kDa (Sakry et al. 2014). Inhibition of the α -secretase ADAM10 mimics the NG2-null phenotype, and recapitulates the loss of NMDAR-dependent long term potentiation (LTP) present in the NG2-null mouse model. Following chemical induction of LTP in Oli-neu cells and brain slices, ADAM10-dependent cleavage of NG2 is increased, indicating that NG2-cleavage is activity-dependent. NG2 contains two LNS (Laminin-alpha, Neurexin and Sex hormone-binding globulin) domains that are capable of restoring altered AMPAR characteristics in NG2-null mice. Further, LNS domains induce c-fos generation in glutamine-stimulated brain slices, suggesting a role for these domains in activity-dependent gene expression in target neurons (Sakry et al. 2014). A variety of other proteases cleave NG2 at various sites, leading to enhanced neuronal protection, and improved regenerative repair following injury (Rolls et al. 2004; Tauchi et al. 2012). Finally, NG2 cells secrete the neuromodulatory factors Prostaglandin D2 synthase (PTGDS) and neuronal Pentraxin 2 (Nptx2/Narp) (Sakry et al. 2015). PTGDS is neuroprotective at the D1 receptor following glutamatergic challenge (Liang et al.

2005), and following hypoxia (Taniguchi et al. 2007), while Nptx2 facilitates AMPA receptor aggregation at excitatory synapses (O'Brien et al. 1999), and promotes neurite outgrowth following neuronal activity (Tsui et al. 1996). Taken together, NG2-glia are important homeostatic and protective constituents of neuronal networks. Their capacity for proliferation and specification is modulated by neuronal activity, and in turn, NG2-glia secrete neuromodulatory factors and provide adhesive scaffolds to support neuronal outgrowth of existing and developing neurons. Hyperactivity in the Cx32 KO brain likely contributes to hyperproliferation of NG2-glia in an attempt to enhance the neuroprotective features of these cells. Thus, constitutive enhancement of NG2-glia prior to injury may underlie the improved regenerative capacity of the Cx32 KO brain, as well as the enhanced 28d survival of cells born 3.5 d post seizure (Figure 5.6).

5.2.4. Extrinsic suppression of oligodendrogenesis:

Distribution of fate-specifying molecules in the altered glial network

In addition to the extrinsic and intrinsic mechanisms regulating the promotion of neurogenesis, overcoming the glial default likely also involves suppression of glial-promoting signaling cues that reach injury-derived NG2⁺ and nestin⁺ cell types. Given that less oligodendrocytes are produced in the Cx32 KO

brain post-injury, we can assume that glial-promoting cues were suppressed. We know that inflammatory M2 activation of microglia (Miron et al. 2013), specific excitatory synaptic activity transduced from neurons (Wake et al. 2011), and astrocyte-derived BDNF (Miyamoto et al. 2015) encourage NG2-glia oligodendrogenesis, thus, I hypothesize that one or more of these pathways is affected by loss of Cx32. I have previously discussed how these three mechanisms may be altered in the Cx32 KO

brain, but it is also possible that loss of Cx32 from mature oligodendrocytes limits access of oligodendroglial cues to NG2-glia. In the Cx32 KO brain, O:A Cx32:Cx30 channel function is abrogated (Nagy et al. 2003b), and oligodendrocytes compensate by relying on Cx47 channels to share information amongst themselves and with astrocytes. Given differences between Cx47 and Cx32 channel permeability (Bedner et al. 2006), it is possible that certain metabolites produced in oligodendrocytes are not distributed properly throughout the glial network, and that the astrocyte gap junction-mediated secretome is altered (Choi et al. 2014). Conversely, mature oligodendrocytes account for a maximum of 14% of all dye-coupled cells in the hippocampus (Griemsmann et al. 2015), indicating that the glial network is dominated by astrocytes in this brain region. There is currently no evidence that secreted factors released from mature oligodendrocytes affect NG2-glia cell specification, thus, the loss of Cx32 in mature oligodendrocytes does not likely play an important role in restricting NG2-glia differentiation.

5.2.5. Intrinsic suppression of oligodendrogenesis

Delay in NG2-glia response to oligodendrogenic cues

As previously mentioned, Cx32 expression is turned on in pre-myelinating oligodendrocytes (Imbeault et al. 2009; Melanson-Drapeau et al. 2003). Given its expression prior to terminal differentiation, I hypothesize that the loss of Cx32 may result in a delay of injury-induced oligodendrogenesis in the Cx32 KO brain, as we provide data that Cx32 expression promotes specification of the NG2 lineage in proliferating mouse neurospheres. Indeed, Cx32 expression is transcribed through the actions of the Sox10 and Egr-2/Krox20 transcription factors, which also regulate the

expression of several key myelination gene products (Houlden et al. 2004; Mitew et al. 2014; Oyamada et al. 2013; Stolt et al. 2002). Appearance of these typical myelin proteins results in loss of the NG2-glial association with neurons, and the gradual recruitment of pre-myelinating and myelinating oligodendrocytes to the mature O:A network. There is no reduction in oligodendrocyte cell number in the Cx32 KO animal, and aside from thinner myelin sheaths, it is clear that mature oligodendrocytes are effectively produced, despite the loss of Cx32. Given this, I hypothesize that the inability of specifying NG2⁺ cells to express Cx32 may delay their ability to respond to oligodendrogenic stimuli, deferring their uncoupling from neuronal synapses and postponing the maturation of new premyelinating oligodendrocytes generated following injury. I provide evidence for this delay, as more BrdU⁺ cells are present in the Cx32 KO CA3a/b region 1 wk post injury compared to the WT, despite fairly equal proliferative post-injury responses in both genotypes. Indeed, more mature oligodendrocytes are produced in the WT CA3a/b following injury, while an increased number of neurons are produced in the Cx32 KO. A delay of oligodendrogenesis in this critical time window post injury (3.5 d-1 wk) may have significant effects in the injured microenvironment by enabling the sequestration of neuroprotective NG2 glia at neuronal synapses. Further, maintaining the undifferentiated state of NG2-glia may enhance their neuroprotective and matrix secretion abilities to better support the survival of newly-generated immature neurons, ensuring that nestin⁺ cells will commence successful neuronal differentiation and integration.

Finally, the presence of Cx32 in pre-myelinating NG2-glia may allow for more efficient differentiation to myelinating oligodendrocytes, given the permeability of Cx32

channels to adenosine released from neurons and paranodal astrocytes (Parkinson et al. 2002; Stevens et al. 2002; Wall and Dale 2013). Neuronal adenosine is released from equilibrative nucleoside transporters following induction of action potentials or Ca^{2+} stimulation (Wall and Dale 2013). Conversely, astrocyte-derived adenosine is produced from extracellular conversion of ATP released by exocytosis (Pangrsic et al. 2007), volume regulated anion channels (Anderson et al. 2004), or through connexin hemichannels (Pearson et al. 2005). Cx32 is highly permeable to adenosine (Goldberg et al. 2002; Goldberg et al. 2004), and may also signal to NG2-glia through a variety of endogenously-expressed adenosine receptors (Coppi et al. 2015). Treatment of NG2-glia with adenosine results in a concentration-dependent reduction in cell proliferation, and induction of MBP expression and myelination (Stevens et al. 2002). Expression of Cx32 may enable NG2-glia to uptake more extracellular adenosine, perpetuating receptor-mediated effects on differentiation. Excitingly, this finding provides support for the observation that myelination is an activity-dependent phenomenon, yet does not preclude the involvement of other Cx32-permeant signaling molecules involved in oligodendrogenesis.

5.3. Post-injury neuronal repair is mediated from parenchymal progenitor cells

5.3.1. CA3 hippocampal regeneration is not mediated by SGZ neurogenesis

This thesis provides evidence that CA3 regeneration following kainic acid-induced seizure is not mediated by SGZ neurogenesis, rather, is accomplished through activation of local neurogenesis directly at the site of injury. In our work, it is not plausible that regeneration of the injured CA3a/b region could have occurred from the SGZ, given that radial glial cells activated in the SGZ migrate only a short distance to

repopulate the GCL of the hippocampus. In the uninjured brain, $94.6 \pm 1.0\%$ of immature neurons migrate up to 50 μm (first 1/3 of the GCL) from their activation site, while no cells ever migrate further than 100 μm (outer 2/3 of the GCL) (Ibrahim et al. 2016). Migration of newly-activated immature neurons past 100 μm was detected at 1 wk post-injury, but not at 72 hrs post-injury (Ibrahim et al. 2016). Similarly, enhanced neuroblast migration was reported following seizure activity (Jessberger et al. 2005; Jessberger et al. 2007b; Scharfman 2000), however, it is very unlikely that these cells could migrate as far as the injured CA3 region ($\sim 1000 \mu\text{m}$ away). In our hands, dividing nestin⁺ and NG2⁺ cell numbers were maximal in the CA3a/b within 3.5 days post injury, confirming that SGZ-derived cells could not have contributed to this proliferating cell population, and that the cells must have come directly from the site of injury, from a region very close to the CA3a/b, or from a highly motile population of adjacent progenitor cells.

5.3.2. Endogenous parenchymal progenitor cells may contribute to regeneration in the injured microenvironment

Converging lines of evidence suggest that sustained neurogenesis occurs in “non-neurogenic” parenchymal areas of the brain, challenging the dogma that adult neurogenesis only occurs in the SGZ and SVZ. Indeed, mitotically active cells are often found outside of the classical neurogenic niches in rodents and primates, including the hippocampal CA1 and CA3 fields (Belmadani et al. 2015; Bonfanti and Nacher 2012; Bonfanti and Peretto 2011; Bonfanti et al. 2011; Hendrickson et al. 2011; Nakatomi et al. 2002; Ohira et al. 2010; Rietze et al. 2000). As discovered by Rietze and colleagues, proliferating cells in the uninjured hippocampus (including the CA fields and

Ammons's horn) do not express markers of mature neurons (NeuN, calbindin) or astrocytes (GFAP, S-100) immediately after BrdU-labeling, yet BrdU⁺ mature neurons and glia are present in the hippocampus 3 weeks later (Rietze et al. 2000). These findings suggest that new neurons and glia can be generated from populations of endogenous progenitor cells outside of the hippocampal SGZ in uninjured tissue; however, the injured microenvironment appears to better support regeneration. Unfortunately, the propensity for parenchymal progenitor cells to generate functional neurons in existing circuitry is debated, as their survival and terminal differentiation is limited (Bonfanti 2016; Peretto and Bonfanti 2014). For example, new neurons arising in degenerating mouse striatum initially show features of intermediate neuronal progenitors, yet exhibit poor survival, and fail to terminally differentiate to striatal projection neurons (Luzzati et al. 2011). Despite these setbacks, there is promising evidence from other parenchymal cell niches that certain endogenous progenitor cells do have the capacity to effectively regenerate damaged tissue.

5.3.3. Identity of parenchymal cells from “non-neurogenic” brain regions

The complete identity and source of proliferating parenchymal cells equilibrium and following different injury paradigms remains elusive. Nakatomi and colleagues describe significant cell loss in the CA1, coupled with local Pax6⁺, Emx2⁺, and Mash1⁺ cell proliferation 4 days post-ischemic injury (Nakatomi et al. 2002). Similarly, Ohira and colleagues report that ischemia-induced neurogenesis from Ki67⁺/GAD67⁺ progenitor cells resident in neocortical layer 1 significantly contribute to functional GABAergic interneuron cell replacement throughout cortical layers 1-6 (Ohira et al. 2010). Immature neurons from cortical layer II to type 3 immature neurons of the SGZ, and

demonstrate structural plasticity. These immature neurons are proliferative following various stimuli (Hayashi et al. 2001), and may provide a reservoir of new neurons should they be compelled to complete their differentiation programs (Bonfanti and Nacher 2012).

Most recently, Belmadani and colleagues have identified a third sustained neurogenic zone at the dorsal surface of the adult mouse hippocampus, and have named it the sub-hippocampal zone (SHZ) (Belmadani et al. 2015). Here, nestin⁺/GFAP⁺/brain lipid-binding protein⁺/sex-determining region Y-box 2⁺/T-box brain protein 2⁺ progenitor cells appear to originate from the medial walls of the LV, and exit through the fimbria-dentate junction. Cells then migrate along a “caudal migratory stream”, which includes the fimbria, and meninges, and terminates at the DG. Following insult to the DG, these highly-migratory cells proliferate, and are found in the DG within 1 wk post-injury, contributing granule cell neurons to both the GCL and the hilus. Given that the caudal migratory stream passes very close to the CA3a/b region of the hippocampus, SHZ cells may also contribute to the repair of this area in our model, yet, it is unknown whether SHZ progenitor cells can generate appropriate CA3 pyramidal cell neurons, or if they can only generate ectopic granule cells as seen in the hilus following injury to the DG (Belmadani et al. 2015).

In our work, injured WT animals do not effectively replace NeuN⁺ neurons to uninjured levels 4.5 wk post kainic acid-induced seizures, and significantly underperform in the Morris Water Maze test of learning in memory. Similar to some of the above reports that endogenous neurogenesis from non-neurogenic parenchymal brain regions does not effectively generate surviving, integrated, or regionally-

appropriate cell types, we also demonstrate that brain regeneration is incomplete and/or ectopic in WT animals. However, we also show that alteration of the injured microenvironment by loss of Cx32 better supports injury-induced survival and neuronal specification of newly-born cells, providing one of the first examples of functional regeneration from endogenously-generated neuroblasts. We further indicate that new neurons were born from cells that had once expressed nestin, while cells of the NG2 lineage produced only new oligodendrocytes, or remained NG2⁺ in the injured region. These endogenous nestin⁺ cells progenitors may be derived from one or more of the novel injury-induced cell populations described above. Additionally, regeneration of the CA3 may also rely on partial contributions from highly-migratory SVZ neuroblasts, as described by Luzzati and colleagues (Luzzati et al. 2011).

Based on the review of possible progenitor cell sources presented above, I propose that multiple populations of local progenitor cells may have contributed to the successful regeneration in our model, and that recruitment of a variety of cell types may better ensure infiltration of cells capable of specifying to the appropriate lineages. I further propose that one or more additional modifiers of the injured environment is required for optimal regeneration. This modifier may act directly on the production of new neurons, as was described for intraventricular growth factor infusions post-ischemic injury (Nakatomi et al. 2002). Alternatively, the modifiers may act indirectly to positively enhance the regenerative environment, as has been described for protective pre-conditioning paradigms, or in this thesis following genetic perturbation of the glial inter- and intracellular signalling network. These results provide evidence that specific pharmacological inhibition of Cx32 signalling could enhance endogenous NPC

recruitment, proliferation, and specification in injured tissue, providing a simple and reversible adjuvant therapy for effective tissue regeneration.

5.4. Summary

This thesis combines multiple techniques to describe the importance of both intracellular protein:protein and intercellular cell:cell interactions of hepatic and oligodendrocyte Cx32. I have identified that hepatic Cx32 interacts with a variety of mitochondrial, ER, cytoplasmic, and PM-localized proteins intracellularly, and confirm that Cx32 and Cx26 participate in cell-cell interactions, and/or interact heterotypically in gap junction channels. These data support evidence of a growing literature describing the role of connexin proteins in mitochondria, and provide evidence that Cx32 is present at the IMM in hepatic mitochondria, as has also been described for cardiac Cx43. Confirmation of a physical interaction between Cx32 and a relatively un-described mitochondrial carrier protein, SFXN-1, suggest that mitochondria may form contacts with PM-localized gap junction plaques, and that connexins may play a role in mitochondrial iron utilization and ferroptosis. We provide further evidence that loss of Cx32 alters mitochondrial phenotypes using LFQ of membrane proteomes in WT and Cx32 brain. Accordingly, isolated mitochondria from Cx32 KO animals consume significantly more oxygen, and produce more ROS than WT mitochondria. These data have pathological significance for carcinogenesis, and the health of myelinating cells in the CNS and PNS. Indeed, loss of Cx32 results in subclinical CNS pathology in mice and in humans affected by CMTX, yet, the Cx32 KO brain is more amenable to neuronal regeneration than the WT brain following significant cell loss in the hippocampal CA3a/b. I hypothesize that altered communication in the glial network

through increased Cx30-mediated signaling underlies the enhanced response of the Cx32 KO brain following injury, and that loss of Cx32 contributes to an environment of constant preconditioning stress that operates on a global level (altered recruitment of inflammatory cells, hyperexcitability), and on a cellular level (ROS-mediated mitochondrial preconditioning). This thesis lays the groundwork for more mitochondrial-centered exploration of connexin phenotypes, and suggests that targeting connexins in the glial network may be important for brain regeneration from endogenous progenitor cells. As transient pharmacological inhibition of Cx32 is not lethal, its selective inhibition may prove particularly effective in multiple types of parenchymal brain injury where recruitment of hippocampal and SVZ-derived neuroblasts is minimal. To this end, there is a still great need for the development of connexin-specific inhibitors of function and/or expression as pharmacological adjuvants in the treatment of brain injury.

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