EXAMINATION OF WNT/β-CATENIN SIGNALING IN THE BLOOD-BRAIN BARRIER: EXPLORATION IN A HUMAN BBB CULTURE MODEL UNDER NORMAL AND PATHOPHYSIOLOGICAL CONDITIONS

By

MARLYN D. LAKSITORINI

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Department of Pharmacology and Therapeutics

Max Rady Faculty of Health Sciences

University of Manitoba

Winnipeg, MB, Canada

ABSTRACT

Introduction. Wnt/β-catenin plays an essential role in the development and maintenance of the blood-brain barrier (BBB). Dysregulation of Wnt/β-catenin signaling in brain microvessel endothelial cells has been linked to BBB dysfunction observed in various pathological conditions such as multiple sclerosis, Huntington's disease and cerebral ischemia. However, the potential alterations in Wnt/β-catenin activity in brain endothelial cells upon chronic ethanol exposure have not been established. We hypothesized that chronic ethanol impaires Wnt/β-catenin signaling in the brain microvessel endothelial cells that form the BBB.

Objectives. The studies examined Wnt/β-catenin activity using a culture model of the BBB to determine the impact on barrier properties under normal conditions and following exposure to ethanol. In addition, various pharmacological agents were screened for their ability to activate Wnt/β-catenin in the brain endothelial cells and improve barrier function.

Methods. Wnt/β-catenin signaling under normal monoculture conditions and following ethanol exposure was examined using the human cerebral microvessel endothelial cell line (hCMEC/D3). Studies correlated changes in downstream genes important for establishing the BBB phenotype as well as functional assessment of transporter activity and paracellular diffusion. Additionally, Wnt/β-catenin signaling was examined in both brain homogenate and isolated brain microvessels from C57BL/6 mice receiving daily i.p injections of ethanol (2.0 g/kg for 7 days).

Result. The hCMEC/D3 expressed a nearly complete array of Wnt ligands, Wnt receptors, Wnt co-receptors and Wnt modulators. Despite expressing several Wnt ligands, pharmacological inhibition of endogenous Wnt ligand had minimal impact on the maintanence of blood-brain barrier phenotype in the hCMEC/D3 monolayers. However, external activation of Wnt/β-catenin signaling using lithium chloride 10 mM or Wnt3a 200 ng/ml significantly improved paracellular barrier function, Pgp and BCRP function as well as reduced vesicular transport activity at hCMEC/D3. Mice treated with ethanol for one week showed downregulation of Wnt/β-catenin signaling in the brain cortex as well as in isolated microvessels from brain cortex. Similar Wnt/β-catenin downregulation was also observed at hCMEC/D3 following ethanol exposure and external Wnt activation by LiCl (10 mM) improved the barrier restrictiveness of the monolayer. As LiCl 10 mM is clinically toxic, additional studies were conducted to explore clinically approved CNS drugs at therapeutically relevant concentrations to improve barrier function. Among the drugs examined, fluoxetine activated Wnt/β-catenin signaling and prevented barrier dysfunction following ethanol insult.

Conclusion. The studies suggest that Wnt/β-catenin signaling is downregulated at the blood-brain barrier following repeated exposure to ethanol. As brain endothelial cells appear to be more responsive to paracrine activation of Wnt/β-catenin, pharmacological interventions directed at activation of Wnt/β-catenin may counteract the negative effects of ethanol on the permeability of brain endothelial cells.

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Did We not expand for you your breast and We removed from you your burden which had weighed down upon your back.

And We exalted for you your reputation?

Then surely, with hardship there is an ease.

Surely, with hardship comes the ease.

So when you have finished with your immediate task, still strive hard (then toil).

And to your Lord direct your longing.

(Al Inshiroh: 1-8)

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5HT	: 5-hydroxytryptamine
ABC	: ATP Binding Cassette
AD	: Alzheimer's disease
AHP	: adult hippocampus progenitor cell
AMT	: adsorptive mediated transport
APC	: Adenomatous polyposis coli
APCDD1	: denomatosis polyposis coli down-regulated 1
AQP4	: Aquaporin-4
BAC	: blood alcohol concentration
BAEC	: bovine aortic endothelial cell
BBB	: blood-brain barrier
BCRP	: breast cancer related protein
BIO	: 6-bromoindirubin-3-oxime
BMP	: bone morphogenic factor
β-TrCP	: Beta-transducin repeats-containing proteins
CamK-II	: calcium calmodulin dependent kinase-II
CK-1	: casein kinase-1
CNS	: central neurosystem
CRD	: cystein rich domain
CRT	: creatine transporter

CVO : circumventricular organ

D2R : dopamine-2 receptor

DKK : Dikkopf

Dvl : dishevelled

EAE : experimental autoimmune encephalomyelitis

ER : Endoplasmic reticulum

ESCRT : endosomal sorting complex required for transport protein

FCCP : Carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone

FcRn : neonatal Fc receptor

Fzd : frizzled

FMT : fluid phase mediated transport

GFAP : Glial fibrillary acidic protein

Glut-DS : glut-1 DS

GPCR : G protein-coupled receptors

GPR124 : Probable G-protein coupled receptor 124

GSH : glutathione

GSK3 : Glycogen synthase kinase 3

hCMEC/D3 : Human cerebral microvascular endothelial cell

HD : Huntington's disease

HEK cell : human embryonic kidney cell

HSEC : hepatic sinusoidal endothelial cells

HUVEC : Human umbilical vascular endothelial cell

INVP : intra neural vascular plexus

IR : insulin receptor

JAM : junction adhesion molecules

JNK : c-Jun N-terminal kinases

LAT : large neutral amino acid transporter

LEF : lymphoid enhancer factor

LGR-4 : GPCR-4 with leucine-rich repeat

LiCl : Lithium Chloride

LRP : Low density lipoprotein receptor-related protein

MAO : Monoamine oxidase

MCT : monocarboxylate transporter

MLCK : Myosin light chain kinase

MMP : Matrix metallopeptidases

MRP : multidrug resistance-associated protein

MVB : multivesicular bodies

NDP : Norrie disease protein

NFAT : Nuclear factor of activated T-cells

NFκB : nuclear factor kappa-light-chain-enhancer of activated B cells

NLK : Serine/threonine protein kinase

NPC : neural progenitor cell

NSC : neural stem cell

OAP : orthogonal array of particles

OAT : organic anion transporter

OATP : organic anion polypeptide transporter

OCT : organic cation transporter

PAEC : porcine aortic endothelial cell

PCP : planar cell polarity

PDGF-β : platelet derived growth factor-β

PDGFRβ : platelet derived growth factor receptor-β

PFC : prefrontal cortex

Pgp : P-glycoprotein

PKC : protein kinase-c

PLC : phospholipase-c

PLVAP : plasmalemma vesicle associated protein

PNVP : perineural vascular plexus

PORCN : Porcupine

PP2A : Protein phosphatase 2A

RAGE : receptor for advance glycation end product

RMT : receptor mediated transport

ROCK : Rho-associated protein kinase

ROR2 : receptor tyrosine kinase-like orphan receptor -2

RRID : research resource identifier

RSPO : R-spondin

rtPA : tissue plasminogen activator

RYK : related to receptor tyrosine kinase

SERT : serotonin transporter

sFRP : soluble frizzled related proteins

Shh	: sonic hedgehog
SLC	: solute carrier mediated transport
SSRI	: selective serotonine reuptake inhibitor
SVZ	: subventricular zone
TCF	: T-cell factor
TfR	: transferrin receptor
TGF-β	: transforming growth factor beta
TOP Flash	: TCF reporter plasmid
TSPAN-12	: Tetraspan-12
VEGF	: Vascular endothelial growth factor
VEGFR	: Vascular endothelial growth factor receptor
Vps	: vacuolar protein sorting
WIF	: Wnt inhibitory factor
ZNRF3	: E3 ubiquitin ligase zinc and ring finger
ZO-1	: Zonule occludens

CHAPTER I:Wnt/β-Catenin signaling in the Blood-Brain Barrier

1.1 General features of the blood-brain barrier

The blood-brain barrier (BBB) refers to the specialized cerebral microvasculature that separates the blood and its constituents from the brain extracellular fluid and regulates the passage of substances into and out of the brain. The German scientist, Paul Ehrlich, is credited with the discovery of the BBB in 1885, based on his observation that mice receiving intra peritoneal injections of tryptan blue showed dye distribution to all organs and tissues except the brain and spinal cord[1]. This observation, along with the follow-up studies of his student, Edwin Goldmann, showing injection of trypan blue into the brain resulted in intense staining of the brain but not the peripheral tissue, provided the evidence for the existence of a BBB. While Erhlich originally attributed these barrier properties to the astrocytes, it is now understood that the specialized endothelial cells found within the brain microvasculature are responsible for the restricted passage of solutes into and out of the brain. The anatomical and biochemical features of the brain endothelial cells important for normal BBB function are described in more detail below.

1.2 Characteristics of the brain endothelial cells forming the BBB

1.2.1 Sealed intercellular junctions

Brain endothelial cells have different morphological features compared to endothelial cells from peripheral capillary beds. While the peripheral endothelial cells have an intercellular cleft that allows for paracellular diffusion of solutes in the water filled channels between the cells, the gap between brain endothelial cells is tightly sealed by various tight junction (TJ) and adherence junction (AJ) proteins. The proteins found in the TJ of brain endothelial cells include occludin, various claudins, ZO-1 and several junctional adhesion molecules (JAMs) (see Fig. 1.1). The AJ is located in close proximity to the TJ and includes VE-cadherin, and nectin (see Fig. 1.1). Together

the TJ and AJ proteins act to restrict paracellular diffusion of solutes between the brain endothelial cells forming the BBB.

At the mRNA level, brain endothelial cells have been reported to express claudin-1,-5, and -12[2]. However of the various claudins, claudin-5 is the most abundant isoform expressed with up to 600-times enrichment compared to other claudin isoforms[3,4]. Deletion of this particular isoform also impacts BBB integrity. This was demonstrated by the fact that *claudin-5*$^{-/-}$ knockout mice in which claudin-5 has been deleted showed normal angiogenesis and no brain edema but died within 10 hours after birth[5]. Examination of the BBB function via Caesarian section at embryonic day 18.5 showed partial weakening of the BBB[5]. Of note, the increased BBB permeability in the claudin-5 knockdown mouse was limited to smaller molecules (i.e. molecular weight less than 800 Dalton) and not the macromolecules, suggesting other tight junction proteins likely contribute the overall barrier restrictiveness[5].

The extent to which claudin-1, claudin-3, and claudin-12 are important for reduced paracellular diffusion observed in the BBB is less clear with some studies reporting low levels of expression[6] while others report no expression of claudin-1 and claudin-3 under normal conditions in the BBB[7-9]. Furthermore, while brain endothelial cells express low levels of claudin-12, the absence of this particular isoform had no impact on BBB dysfunction in an experimental autoimmune encephalomyelitis (EAE) mouse model. This demonstrated that claudin-12 was not essential for BBB maintenance[6]. Together, these studies suggest that claudin-5 is the main claudin isoform in the brain endothelial cells.

Similar to claudin-5, occludin deficient mice also show normal formation of tight junctions as assessed with electron microscopy and immunoflourescence microscopy, however, functionally

the occludin deficient mice have increased accumulation of calcium and phosphorus within the brain parenchyma[10]. Furthermore, under pathological conditions such as brain ischemia and ethanol-induced BBB dysfunction, occludin degradation from the isolated cerebral microvessels has been reported. The loss of occludin was associated with a compromised BBB and neuroinflammation[11-13].

Cadherin and nectin are additional structural proteins that form the adherens junction (AJ). Vascular endothelial cadherin (VE-cadherin) is the isoform expressed in brain endothelial cells[14]. It has five repeat extracellular domains forming both homo- and hetero-dimers in a calcium dependent process. While most of the attention has been on the interactions of the extracellular domains, it should be noted that the intracellular domain of VE-cadherin binds to β-catenin and P120[15]. The consequence of VE-cadherin and β-catenin interaction is the ability of VE-cadherin to sequester β-catenin within the plasma membrane and influence important signaling pathways that depend on cytosolic levels of β-catenin[16-18]. Thus alterations in VE-cadherin have potential impact on both the junctional complex as well as the cell signaling capacity of the brain endothelial cells.

Nectin is an immunogloblulin-like adhesion molecule with three extracellular loops that bind in homotypic and heterotypic fashion with adjacent nectin molecules[19]. The intracellular domain of nectin adhesion molecules also form connections to the actin cytoskeleton. Studies demonstrate that nectin plays an important role in determining cell polarity and establishment of the adherence junctions. Through a complex signaling process, the association of nectin with adjacent nectin molecules prevents the endocytosis of cadherin and leads to an accumulation of free cadherins within distinct sites on the plasma cell membrane[20,21]. Thus, it is the nectin interactions that trigger further cadherin-cadherin interactions resulting in the formation of the AJ complex.

Developmentally, homodimerization of the cadherin and nectin proteins between adjacent brain endothelial cells occurs first and allows the other tight junction protein interactions to take place[21,22]. While the AJ is essential for the formation of the TJ complex during development of the BBB, its role in the maintenance of BBB paracellular barrier properties has traditionally been considered to be minor compared to the TJ proteins. Studies in epithelial cell models support this view as deletion of cadherin in Madin-Darby canine kidney (MDCK) epithelial cells prevented the formation of intercellular junctions but had no significant impact on the maintenance of the junctional complex[23]. However, studies using cadherin binding peptides that prevent homo and hetero-dimer interactions between the extracellular regions of cadherin molecules, provide convincing evidence that the cadherin proteins can modulate BBB permeability to hydrophilic solutes[24-26].

While the tight junction (TJ) and adherence junction (AJ) molecules represent distinct processes within the junction of the brain endothelial cells they are physically linked to each other via ZO-1 and cytoskeleton proteins[27]. Recent studies highlight the interdependency between the TJ and AJ complexes[28,29]. From a cell-signaling standpoint, VE-cadherin (an AJ protein) is known to influence the expression of claudin-5 (a TJ protein) by removing the repressor activity of FoxO1 transcription factor[28]. Such interactions between these anatomically distinct sites demonstrates the inter-relatedness of the various proteins that form the complex junction between brain endothelial cells.

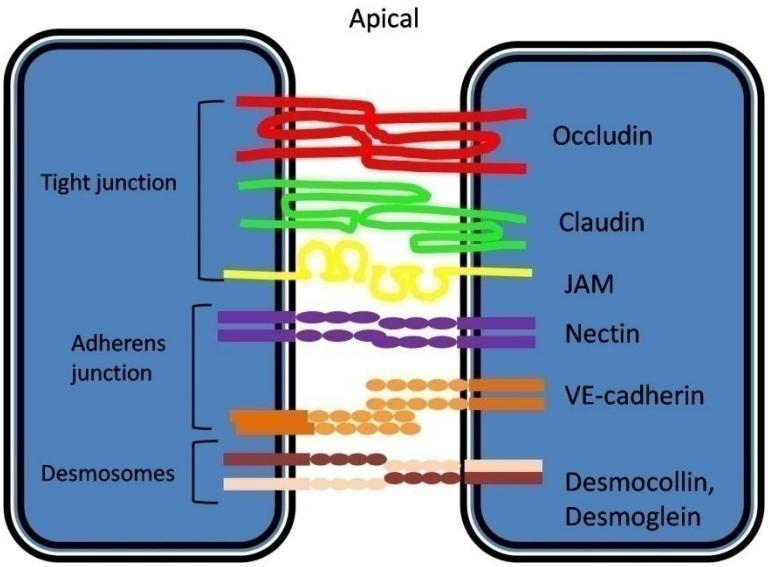

Figure 1.1: The intercellular junction in brain endothelial cells is sealed with three classes of protein; tight junction, adherens junction and desmosomal. The most apical layer is made up of tight junction proteins including occludin, claudin, and junctional adhesion molecules (JAMs). Both occludin and claudin have four transmembrane domains that form homotypic interactions via their two extracellular loops. The JAMs belong to the immunoglobulin superfamily and facilitate leukocyte extravasation across the endothelial cells. The adherens junction, directly adjacent to the tight junction, consists of VE-cadherin and nectin that span across the junction. VE-cadherin has five repeat extracellular domains that form both homo- and hetero-dimers in a calcium dependent process. Nectin is an immunogloblulin-like adhesion molecule with three loops that bind in homotypic and heterotypic fashion with adjacent nectin molecules. Desmosomes form the most abluminal intercellular junction molecules, with both desmoglein and desmocollin adhering the endothelial cells together.

1.2.2. The absence of fenestrae

Another morphological distinction between CNS endothelial and peripheral endothelial cells lies in the absence of endothelial fenestrations. Fenestrae is the term for a water-filled channel or pore (60-70 nm) that forms within the endothelial plasma membrane that enables blood-born protein to escape from vascular lumen[30]. The absence of fenestrations in the BBB has been directly correlated with reduced expression of plasmalemma vesicle associated protein (PLVAP)[31,32]. Homodimers of PLVAP form a radial wheel-like structure that span across the opening of the caveolae, fenestrae and transedothelial channels[33]. The presence of PLVAP appears to stabilize these formations within the cells as reductions in PLVAP expression in brain endothelial cells is associated with

decreased fenestrae and trans-endothelial channel (TEC) formation in brain endothelial cells[30,33]. Furthermore, upregulation of BBB PLVAP has also been reported following cerebral ischemia and brain tumors, and is associated with brain microvessel leakiness[32-35].

1.2.3. Reduced vesicular transport activity

Vesicular transport activity in brain endothelial cells can be generally categorized as either fluid-phase mediated transport (FMT), adsorptive mediated transport (AMT), or receptor mediated transport (RMT)[36]. Of the three vesicular transport routes, FMT is the least specific with endocytosis being driven by changes in the extracellular fluid environment. In contrast, AMT requires electrochemical interactions between cationic macromolecules and the negatively charged cell membrane[37]. For RMT, endocytosis is triggered by the binding of a macromolecule to a receptor present on the plasma membrane.

Within the brain microvasculature, endocytosis occurs at two distinct regions of the plasma membrane, either caveolae, or clathrin coated pit regions[38]. As both caveolae and clathrin coated pit regions are less abundant in healthy brain endothelial cells compared to peripheral endothelial cells[39], solute and macromolecule entry into the brain through FMT and AMT routes are limited in the BBB. Likewise, changes in FMT and AMT may underlie the leakiness of the BBB observed in various brain pathologies. The upregulation of caveolin-1 and -2 has been observed in cerebral ischemia and experimental autoimmune encephalomyelitis (EAE)[40,41].

Brain endothelial cells have reduced vesicular transport activity compared to endothelial cells from peripheral tissues. The low vesicular transport activity in the brain endothelial cells appears to be correlated with increased expression of Mfsd2a (a sodium dependent lysophosphatidylcholine symporter-1)[42]. Mfsd2a is a brain endothelial cell specific transporter as expression in peripheral

endothelial cells is not detected[42]. Mfsd2a mediates the transport of lysophosphatidylcholine (LPC)-esterified fatty acids. An example of a fatty acid transported by Mfsd2a is lysophosphatidylcholine docosahexaenoic acid (LPC-DHA)[43,44]. The Mfsd2a transporter activity results in a unique lipid environment in the plasma membrane of brain endothelial cells that is enriched with DHA[45]. It has been suggested that the presence of DHA within the brain endothelial plasma membrane interacts with caveolae and results in reduced caveolae-mediated transcytosis. The importance of Mfsd2a for BBB function is demonstrated in the Mfsd2a$^{-/-}$ mouse. While the mice lacking Mfsd2a showed unaltered CNS angiogenesis and normal tight junction formation, they exhibited a marked reduction in BBB integrity[46]. Upon further examination it was shown that the BBB dysfunction observed in the Mfsd2a$^{-/-}$ mice was not attributable to the opening of paracellular pathways but rather to an increase in transcytosis activity[42,45,46].

Although vesicular transport is reduced in the brain endothelial cells compared to the peripheral vasculature, both AMT and RMT pathways have important roles especially for large peptide and protein hormone transport in the BBB. Many important macromolecules including insulin, glucagon, vasopressin, natriuretic peptide, transferrin and epidermal growth factor rely on receptor mediated transport (RMT) routes to cross the BBB[47-49]. RMT allows specific entry to the BBB and minimizes a non-specific transport. Receptors participating in RMT in the BBB include the transferrin receptors (TfR), insulin receptors (IR), low density lipoprotein receptors (LRP) and neonatal Fc receptors (FcRn)[47]. Both AMT and RMT are saturable transport processes. However, RMT is considered to be higher-affinity and lower-capacity compared to AMT. The transport rate for both AMT and RMT is slower than solute carrier mediated transport that will be discussed in the next section[50].

There is currently much interest in utilizing AMT and RMT routes to deliver macromolecule drugs across the BBB[36,47-49,51]. In the case of AMT, the delivery system takes advantage of interactions between cationic charges on the macromolecule or its delivery system and the negatively charged regions of the plasma membrane[52]. Cationic cell penetrating peptide (CPP), and other positively charged proteins and polymers have been utilized to deliver nanoparticles, liposomes and therapeutic macromolecules[50,52]. Likewise, hexamethylenediamine or tetramethylene diamine has been used to increase the cationic charge of proteins such as insulin, albumin, sodium dismutase, and IgG. The resulting cationized proteins have significantly improved BBB passage compared to the parent proteins[53]. While further research is needed, the advantages of this approach for delivery of therapeutic agents and biologicals across the BBB are readily recognized.

RMT is the most selective and specific vesicular delivery route dependent on binding to various receptors expressed on the brain microvessel endothelial cells. This particular route has been targeted for delivery of therapeutic macromolecule to the brain[48,49,54]. A specific example is the use of transferrin functionalized liposomes to deliver ApoE2 plasmids for gene therapy[55]. Transferrin binds to specific receptors on the brain endothelial cells and triggers endocytosis. These BBB targeted liposome platforms were shown to successfully penetrate mouse brain endothelial cells and deliver ApoE2 plasmid and increase neuronal ApoE2 in a BBB-neuron co-culture model[55]. Furthermore, this liposomal formulation was successfully translated to an animal model where tail injections of the liposomes increased ApoE2 expression in the rat brain by 7-fold[55]. These studies demonstrate the ability RMT to aid in the delivery of DNA plasmids to the brain.

1.2.4 Transcellular uptake and efflux transporters

In addition to the morphological differences stated above, brain endothelial cells also express a wide range of plasma membrane transporters that regulate the entry of nutrients and solutes into the brain and the removal of unwanted brain metabolites to the blood. Generally referred to as solute carriers (SLCs), these membrane transporters facilitate movement of select solutes into and out of the brain. Important SLCs expressed in the BBB include Glut-1 (SLC2A1), organic cation transporter (OCT), organic anion transporter (OAT), organic anion polypeptide transporter (OATP), nucleotide transporter, monocarboxylate transporter (MCT), large neutral amino acid transporter (LAT), and creatine transporter (CRT)[37,51]. These transporters carefully regulate the level of neurotransmitter, ion, nutrient and other neuro-active agents in the brain.

In addition to the various SLCs that facilitate solute entry into the brain, there are multiple ATP Binding Cassette (ABC) proteins such as P-glycoprotein (Pgp/ABCB1), breast cancer related protein (BCRP/ABCG2) and multidrug resistance-associated protein-1, -3, -4, -5, and -6 (MRP) that function as efflux transporters to remove waste material or xenobiotics (foreign substance) from brain parenchyma[56,57]. These transporters are ATP-driven and recognize more diverse chemical structures compare to the SLCs mentioned above. Pgp and BCRP are the two main efflux transporters in the human BBB[58,59]. Due to the wide range of chemical structures recognized by Pgp and BCRP, the passage of many therapeutic agents to the brain is limited by the efflux activity of these two transporters[56]. The BBB also expresses several isoforms of MRP. While the expression of the various MRP isoforms are not as great as Pgp or BCRP[60,61] collectively the MRPs are important for transporting a variety of hydrophilic solutes. In addition, MRP-1 has an important role in the removal of phase II drug conjugates such as morphine-3-glucoronide from brain endothelial cells[37,62,63].

SLC and ABC transporter expression is an important feature of the brain endothelial cells that form the BBB. Studies demonstrated an enrichment of P-glycoprotein (ABCB1), BCRP (ABCG2), SLC7A5 (LAT-1), SLCO2B1 (Solute carrier organic anion transporter family member 2B1), SLC26 (multifunctional anion exchanger), SLC19A3 (thiamine transporter), SLC6A13 (GABA transporter), and SLC47A2 (multidrug and toxin extrusion protein-2) in isolated brain microvessels compared to whole cortical tissue. Similarly, the expression of P-glycoprotein transporter (ABCB1), MRP-5 (ABCC5), BCRP (ABCG2), SLC22A5 (carnitine symporter) and SLCO1A2 (solute carrier organic anion transporter family member 1A2) are 2-fold higher in the CNS microvessels compared to kidney microvessels[58]. Separate studies also identified 700-fold enrichment of BCRP (ABCG2) in isolated brain microvessels compared to cortical homogenates from mice[64]. The impact of Pgp efflux activity on drug and toxin penetration to the brain is significant. As an example, both ivermectin and cyclosporin are Pgp substrates. The brain permeation of ivermectin and cyclosporin was increased 70-and 10-fold respectively in P-glycoprotein deficient mice compared to wild-type mice with functional Pgp[65]. These studies illustrate the significant impact that efflux transporters in the BBB have in limiting the brain permeation of active pharmaceutical agents.

Similar to AMT and RMT, SLC and ABC transporters have also been targeted to aid drug delivery to the brain. The use of BBB influx transporters to aid in brain delivery was seen with L-Dopa, a pharmaceutical agent used in Parkinson's disease, whose brain entry is mediated by LAT-1 (SLC7A5)[66]. Modulation of ABC transporters has also been used to improve drug passage to the brain. For example, concomitant administration of the Pgp and BCRP inhibitor, elacridar and pantoprazole with imatinib improved imatinib brain permeation by 5.2 fold[67]. Imatinib is a tyrosine kinase inhibitor used as therapeutic agent to treat various cancers. However, the

effectiveness of imatinib in treating brain tumors is hampered by Pgp and BCRP efflux at the BBB[67]. Elacridar and pantoprazole inhibited Pgp and BCRP in the brain endothelial cells leading to improved passage of imatinib across the BBB[68]. These examples illustrate how SLC and ABC transporters have been targeted to aid in the brain delivery of therapeutic agents.

Besides being a prime target for improving the brain delivery of drugs, alterations in BBB receptors and transporters may also play a role in neurological disease progression. As an example, cerebral microvasculature from Alzheimer's (AD) patients have reduced LRP-1 (low density lipoprotein receptor related protein-1) and increased RAGE (receptor for advance glycation end-product) expression. Changes of the expression of these receptors in the BBB may contribute to disease progression by restricting amyloid-β clearance from the brain[69]. In addition, Alzheimer's disease patients exhibit reduced Glut-1 (SLC2A1) expression in the hippocampus and cortex[70,71]. Replication of this in an AD animal model, the $SLC2A1^{+/-}$-$APP^{Sw/0}$ mouse, resulted in reduced brain glucose metabolism, cerebral microvascular degeneration and BBB breakdown[72].

Another example of aberrant BBB transporter expression causing neuropathology is found in X-linked creatine deficiency syndrome. Clinically, X-linked creatine deficiency syndrome is characterized by developmental retardation, speech impairment, autism and epilepsy[73]. A subset of the X-linked creatine deficiency syndrome is attributable to a loss of function mutation in the creatine transporter, resulting in inefficient creatine transport to the brain[74]. The creatine transporter is expressed in neurons, brain endothelial cells and oligodendrocytes where it functions to transport creatine into the cell to provide an alternate energy source in tissues with high energy demand[75]. Examination of creatine deficient mice shows accelerated brain aging with synaptic loss, reduced neurogenesis, progressive memory impairment, activated microglia and neuroinflammation[76].

A third example of dysfunction of BBB transporters is the mutation of the SLC2A1 (Glut-1) gene in individuals having the rare genetic disease, Glut-1 deficiency syndrome (Glut-1 DS). Reduced expression of functional Glut-1 in the BBB is associated with infantile low brain glucose levels, retarded development, microcephaly, seizures, and movement disorders. Less severe clinical phenotypes such as epilepsy, dyskinesia and ataxia are associated with 25-35% reductions of Glut-1 function. Greater reductions of Glut-1 function (by 40-70%) can cause more severe effects[77,78]. Collectively, these examples illustrate the essential role of the BBB transporters in maintaining a tightly controlled brain microenvironment to support neuron function.

1.2.5. Increased mitochondrial activity

Brain endothelial cells have a higher mitochondrial content compared to non-CNS endothelial cells. Electron micrograph studies of brain capillary endothelial cells showed mitochondrial organelles reached 8-12% of total cytoplasmic volume compared to 2-5% observed in non-CNS endothelial cells[79]. Increased mitochondrial activity in the BBB is anticipated given the wide variety of transporters expressed and the need to maintain cellular ion gradients to insure their proper function. A reduction in mitochondrial content also appears to correlate with reduced barrier function. This is evidenced by the reduction in mitochondrial content (2.8% of the cytoplasmic volume) observed in cerebral microvessels of Alzheimer's patients, where BBB dysfunction-occurs[80,81]. Further evidence is demonstrated by the effects of various mitochondrial inhibitors on BBB integrity. Mitochondrial inhibitors such as rotenone, FCCP, and oligomycin increased BBB permeability *in vitro* and *in vivo*. Moreover, mitochondria inhibition during stroke aggravates BBB dysfunction[82]. Together these studies suggest the importance of mitochondria in supporting BBB function.

1.3. Blood-brain barrier development

In the early 1980s, scientists began to understand that the brain microenvironment influenced the differentiation of brain endothelial cells and allowed them to establish the anatomical and biochemical characteristics representative of the BBB. This was elegantly demonstrated in the studies of Stewart et al [1981] that examined vascularization of brain tissue fragments taken from young quail embryo and implanted into a chicken embryo host. The brain tissue fragment from quail embryo was isolated at an embryonic period before CNS angiogenesis had started (Stage 13). The brain tissue fragment was transplanted into the coleom cavity of a chicken embryo and the resulting vascularization examined. Somatic vessels from the chicken that vascularized the transplanted brain tissue displayed BBB-like characteristics. In contrast, the quail brain microvessels vascularizing the surrounding non-neural tissue lost their brain-barrier properties[83]. Separate studies by Ikeda (1996) showed the vasculature in the chicken coelom cavity differentiated to a BBB-like phenotype under the influence of a brain graft[84]. These studies were fundamental as they suggested that the neural microenvironment determined whether endothelial cells developed blood-brain barrier characteristics.

From the initial studies of Stewart (1981) and Ikeda (1996) it was soon determined that secreted factors in the brain promoted the conversion to a BBB-like phenotype[83]. Further studies identified substances called brain morphogens released from various brain cells that triggered signaling pathways and influenced differentiation of the endothelial cells to become the distinctive and specialized brain endothelial cells found in the BBB. Signaling pathways induced by BBB morphogens have been shown to regulate tight junction protein expression, transporter and vesicular activity and fenestration development in brain endothelial cells[57,85]. The brain

morphogens responsible for the development of the BBB include Wnt, sonic hedgehog (Shh), transforming growth factor-β (TGF-β), bone morphogenic factor (BMP) and retinoic acid[86].

Blood-brain barrier development in mice has been reported to occur in three sequential phases (summarized in Table 1.1). The first stage is the angiogenesis phase that in mice starts from gestational day 9.5 (E9.5) to gestational day 15.5 (E15.5). The angiogenesis phase involves radial vascular sprouting starting at the perineural vascular plexus (PNVP) and moving outward toward the ventricular zone[87]. In addition to vascular sprouting, the angiogenesis phase is also characterized by the appearance of pericytes along the blood vessels that can be detected as early as E12[88]. The vascular sprouting was stimulated by several factors secreted by the neural progenitor cells including VEGF and Wnt7a/7b/3a[85]. The Wnt/β-catenin signaling pathway has a significant role in BBB formation. Interestingly, Wnt/β-catenin signaling appears to be selective as it triggers CNS angiogenesis but has no affect on angiogenesis in non-CNS tissue[89]. Furthermore, Wnt/β-catenin signaling not only drives the vascular sprouting but also induces the expression of specific BBB-related genes such as tight junction proteins and transporters during the angiogenesis phase[90]. Despite expressing tight junction and transporter proteins, the brain capillaries are still considered leaky during most of the angiogenesis phase, becoming a functional barrier at around embryonic day 15.5[42,91]. In this regard the mouse studies are in agreement with other mammalian systems including human that indicate the BBB is functional and mature before birth[88,92-94].

The fact that developing BBB remains leaky during the angiogenesis phase despite the expression of junctional proteins could be attributed to the abundant expression of PLVAP in brain microvessels during E12-E15. Coincidently, PLVAP expression in brain endothelial cells significantly declines by E16 and cannot be detected during the remaining gestational periods as

well as in the adult brain microvasculature[95]. The time at which PLVAP expression is lost in the brain endothelial cells coincides with the establishment of barrier function.

Table 1.1: Timeline of blood-brain barrier development in rodents

Phase	Angiogenesis phase	Differentiation phase	Maturation phase
Time	E9.5 to E15.5	E15.5 to E18.5	Beyond E19.5 (or postnatal)
Key events occurring during each phase	Radial vascular sprouting from perineural vascular plexus (PNVP) toward brain ventricles, Pericytes are generated	Interaction of brain endothelial cells with parenchymal cells to strengthen the barrier	Generation of astrocytes Cross-talk between endothelial cells and pericytes, astrocytes, and neurons maintain the BBB phenotype
Paracellular barrier	Leaky	Functional	Functional
PLVAP expression	Present	Significantly reduced/not detected	Significantly reduced/not detected
Transporter Expression	Pgp and Glut-1 are expressed	Increased expression of all transporters	Maximal expression of BBB transporters achieved
Wnt signaling	High level	High level	Reduced level
Source of morphogen	Neuronal progenitor cells (NPC), neuroepithelium	NPC, pericytes, neurons	NPC, astrocytes, pericytes, neurons

The second stage is the differentiation phase where the brain endothelial cells further develop the barrier phenotype that is characteristic of BBB. The differentiation stage spans from embryonic day 15.5 to embryonic day 18.5. The hallmark feature of this phase is interaction of brain endothelial cells with parenchymal cells that result in a strengthening of the barrier[90]. Examples of cell communication critical to establishing the BBB include brain endothelial cell-pericyte cross-talk via PDGF-β/PDGFR-β signaling, TGF-β/TGF-βR signaling, and angiopoeitin-1-Tie-2 signaling. TGF-β/TGF-βR signaling and angiopoeitin-1-Tie-2 signaling strengthens the tight junctions in brain endothelial cells[90]. PDGF-β/PDGFR-β signaling contributes to pericyte recruitment in the developing vascular tube[96]. The brain endothelial cells secrete platelet derived growth factor-β (PDGF-β) which, in turn, activates PDGFβ receptors on pericytes[97]. The importance of this signaling pathway to BBB development has been shown in genetically modified mice that lack *Pdgfrb*. While the expression of tight junction proteins such as claudin-5 and occludin were unaffected, the *Pdgfrb*$^{-/-}$ mutant mice showed a dramatic increase in brain endothelial vesicle trafficking as well as increased expression of PLVAP[88]. The *Pdgfrb*$^{-/-}$ mice die at birth due to BBB dysfunction.

The last BBB development phase is the maturation phase. This phase starts at post-embryonic day 19.5. Similar to the differentiation phase, this phase is dependent on cross-talk between endothelial cells, pericytes, astrocytes, and neurons to maintain the BBB phenotype[85]. During this phase, astrocytes are generated and make contact with the vascular tube and begin to form the characteristic astrocyte foot processes around the capillaries[88]. As the BBB is functional beginning around E15.5, this suggests the astrocytes play a more important role in maintaining barrier properties by releasing various factors such as sonic hedgehog (Shh), VEGF, angiopoietin-1, TGF-β and retinoic acid[88,98].

Although the BBB is functional by E15.5, studies suggest that the expression of BBB transporters represents a continual and evolving process throughout postnatal life and adulthood[99]. Pgp expression is detectable in the brain endothelial cells as early as E.10.5 (angiogenesis stage) and is recognized as one of the earliest markers of BBB development[99,100]. Both Pgp and BCRP expression in the brain microvessels are significantly increased at P14 compared to P2. Similarly, OATP1A4 (SLCO1A4) expression in CNS microvessels continues to increase from P2 to P84. Blood-to-brain transport of bumetanide, an OATP1A4 substrate, was significantly decreased in rat at P84 compared to P21, suggesting a higher OATP1A4 activity at P84[101]. Collectively, maturation of BBB transporter function and expression continues well past birth.

1.4. Wnt/β-catenin signaling in the blood-brain barrier

Wnt/β-catenin signaling is important for BBB development, maturation and maintenance[87,89,102-104]. While Wnt ligand release in the brain is highest during development, a dysregulation of Wnt/β-catenin signaling has been reported in various CNS disorders that involve BBB breakdown including multiple sclerosis[105,106], Alzheimer's disease (AD)[107-109], Huntington's disease (HD)[110] and brain tumors[111-113]. In multiple sclerosis, it has been postulated that Wnt/β-catenin activity is upregulated as a counteracting response to BBB dysfunction[105]. On the other hand, BBB dysfunction in Alzheimer's disease coincides with a downregulation of Wnt/β-catenin signaling[107]. Wnt/β-catenin signaling in Huntington's disease appears more complex as Wnt signaling is upregulated in the blood brain barrier but is characterized by reductions in transporter function and increased vesicular transcytosis[110]. These studies provide compelling evidence that Wnt/β-catenin signaling is important for maintenance of the BBB.

Wnt/β-catenin signaling alters the BBB phenotype at the transcription level through changing the expression of a number of genes ranging from select transporters (both SLC and ABC), tight junctions as well as genes responsible for fenestrations and endocytosis in brain endothelial cells (Table 1.2). In the mouse, both *in vivo* and *in vitro* experiments suggested that Glut-1 expression in the CNS vasculature was regulated by Wnt/β-catenin signaling via Wnt7a/b[87,89]. Other studies confirmed that Pgp was another Wnt/β-catenin target in the human brain endothelial cell[110,114-116]. In addition to Pgp, other efflux transporters such as BCRP and MRP4 were also found to be a target for Wnt/β-catenin activation in human and rodent brain endothelial cell models[101,115,117].

Table 1.2: BBB-related genes responsive to Wnt/β-catenin transcription factors

BBB phenotypes	LEF1	TCF1 (TCF7)	TCF 3 (TCF7L1)	TCF4 (TCF7L2)	Comments
Transporters					
P-glycoprotein		√		√	
BCRP				√	
Glut 1	√			√	
OCTN1	√				
MRP 4		√		√	
Tight junction proteins					
Claudin 1		√		√	
Claudin 3	√			√	
Claudin 5	Na	Na	Na	Na	In vivo, TCF4-dominant negative mice show reductions in claudin-5 expression in brain microvessels[104]
Occludin		√		√	
Endothelial fenenstrations					
PLVAP	√			√	

Wnt/β-catenin signaling also regulates the expression of tight junction proteins such as claudin-1, claudin-3 and claudin-5 in the brain endothelial cells[118,119]. Among the tight junction proteins influenced by Wnt/β-catenin signaling, claudin-5 was shown to be a particularly robust marker for Wnt activation in brain vasculature *in vitro* and *in vivo*[104,119-122]. On the other hand, occludin expression under Wnt activation appeared minimal[102,105,117]. Similarly, ZO-1 and VE-cadherin were less likely influenced by alterations in Wnt activity through both pharmacological and gene modulation[87,89,117,119].

Besides controlling the expression of tight junction molecules and BBB transporters, Wnt/β-catenin signaling also regulates endothelial fenestrations and vesicular transport activity through altering PLVAP/Mecca-32/PV-1 and Mfsd2a expression. Plasmalemma vesicle-associated protein (PLVAP) is a component of endothelial fenestrae and a marker of BBB dysfunction in rodents[32,33,123]. In humans, PLVAP is designated as PAL-E, and is absent in most brain regions except the cerebral microvasculature in the choroid plexus and circumventricular organs where the vasculature is leaky[31,33]. Evidence that PLVAP is influenced by Wnt/β-catenin activity is the observation that Wnt3a conditioned media reduced PLVAP gene expression in cultured mouse brain microvascular endothelial cells while conditional β-catenin gene knockout at P14 upregulated PLVAP expression in the brain microvasculature[102]. Separate studies have also shown that systematic deletion of Wnt/β-catenin components in both developing and adult mice resulted in upregulated PLVAP expression in the brain vasculature (see Table 1.5)[104,120].

Wnt/β-catenin signaling also plays a role in minimizing vesicular transcytosis activity in brain endothelial cells by regulating Mfsd2a expression. As previously noted, Mfsd2a is an omega-3 fatty acid docosahexaenoic acid (DHA) transporter important for maintaining PUFA levels in the CNS[124]. In brain endothelial cells, Mfsd2a expression alters caveolae formation and results in

reduced endocytotic activity[19,45]. Wnt/β-catenin activity in brain endothelial cells is associated with high expression of Mfsd2a[31,125,126]. In contrast, peripheral endothelial cells and CVO capillaries that have reduced barrier properties have low or negligible Wnt/β-catenin activity and low Mfsd2a expression[31,127]. Taken together, these studies suggest that Wnt/β-catenin signaling regulates many of the genes responsible for brain-barrier restrictiveness ranging from tight junction molecules and efflux transporters to regulation of endothelial fenestrations and vesicular transport.

1.5. Wnt/β-catenin signaling cascade

Activation of Wnt/β-catenin signaling is done through two phases, an initiation and an amplification phase. During initiation, Wnt ligands bind to the Frizzled (Fzd) receptor and co-receptors, LRP-5 or LRP-6. The signal is then transduced to Dishevelled (Dvl), a membrane protein that facilitates Fzd and LRP5/6 multimerization. Subsequently, Dvl recruits the GSK3α/β, CK-1 and Axin complex resulting in the phosphorylation of LRP5/6. In the amplification phase, the phosphorylated LRP5/6 recruits additional Axin-bound GSK3α/β and CK-1 to the plasma membrane to further phosphorylate adjacent LRP-5/6. These two events mediate the formation of the Wnt signalosome in the plasma membrane (Fig.1.2a). Subsequently, the signalosome is internalized and sequestered within multivesicular bodies (MVB). The sequestration separates GSK3α/β from its substrate and results in cytosolic β-catenin stabilization (Fig.1.3). As free β-catenin accumulates in the cytosol, some of the β-catenin translocates to the nucleus and binds to TCF (T-cell factor) or LEF (lymphoid enhancer factor) transcription factors regulating the expression of multiple downstream Wnt target genes in the cell[18,128-132].

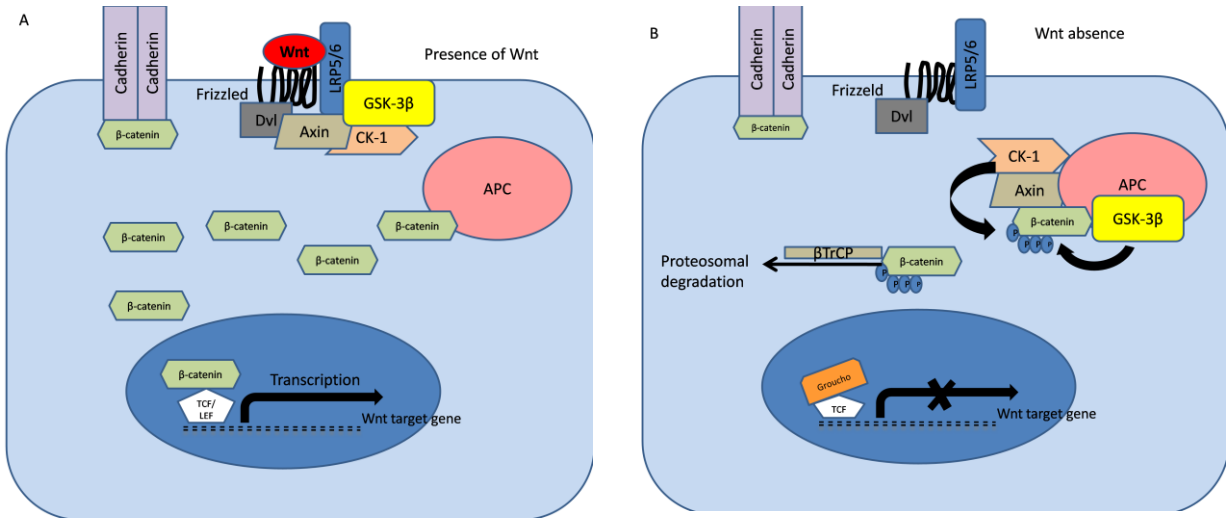

Figure 1.2: Schematic of Wnt/β-catenin signaling pathways. Panel A: Wnt ligand binds to Frizzled receptor and LRP5/6 co-receptor. The signal is transduced to Disheveled causing multimerization of frizzled and LRP5/6. Further, Dishevelled (Dvl) recruits Axin-bound GSK3α/β and CK-1 to phosphorylate LRP5/6 (initiation phase). Phospho LRP5/6 recruits another Axin-bound GSK3α/β and CK-1 to the plasma membrane to phosphorylate the adjacent LRP6/6 (amplification phase). This event mediates the formation of the Wnt signalosome in the plasma membrane. Further, the signalosome was internalized and sequestered in multivesicular bodies (more detail in figure 1.3). The translocation of Axin-bound GSK to the plasma membrane and entrapment in the MVB inhibits the activity of GSK-3α/β and CK-1 in phosporylating β-catenin. Inhibition of β-catenin phosphorylation leads to β-catenin stabilization in the cytosol and translocation to the nucleus. Transcriptional activity of β-catenin in canonical Wnt signaling was mediated by β-catenin binding to TCF/LEF transcription factor in the nucleus. Panel B: Under normal condition where there is no Wnt binding to the receptor, cytosolic β-catenin level was kept at a low level. Release of β-catenin to the cytosol leads to capture by the β-catenin destruction complex (Axin, GSK3α/β, CK-1 and APC) for tagging by phosphorylation. Phospho β-catenin is recognized by E3 ubiquitin ligase β-TrCP (β-transducin repeat-containing protein) for proteosomal degradation.

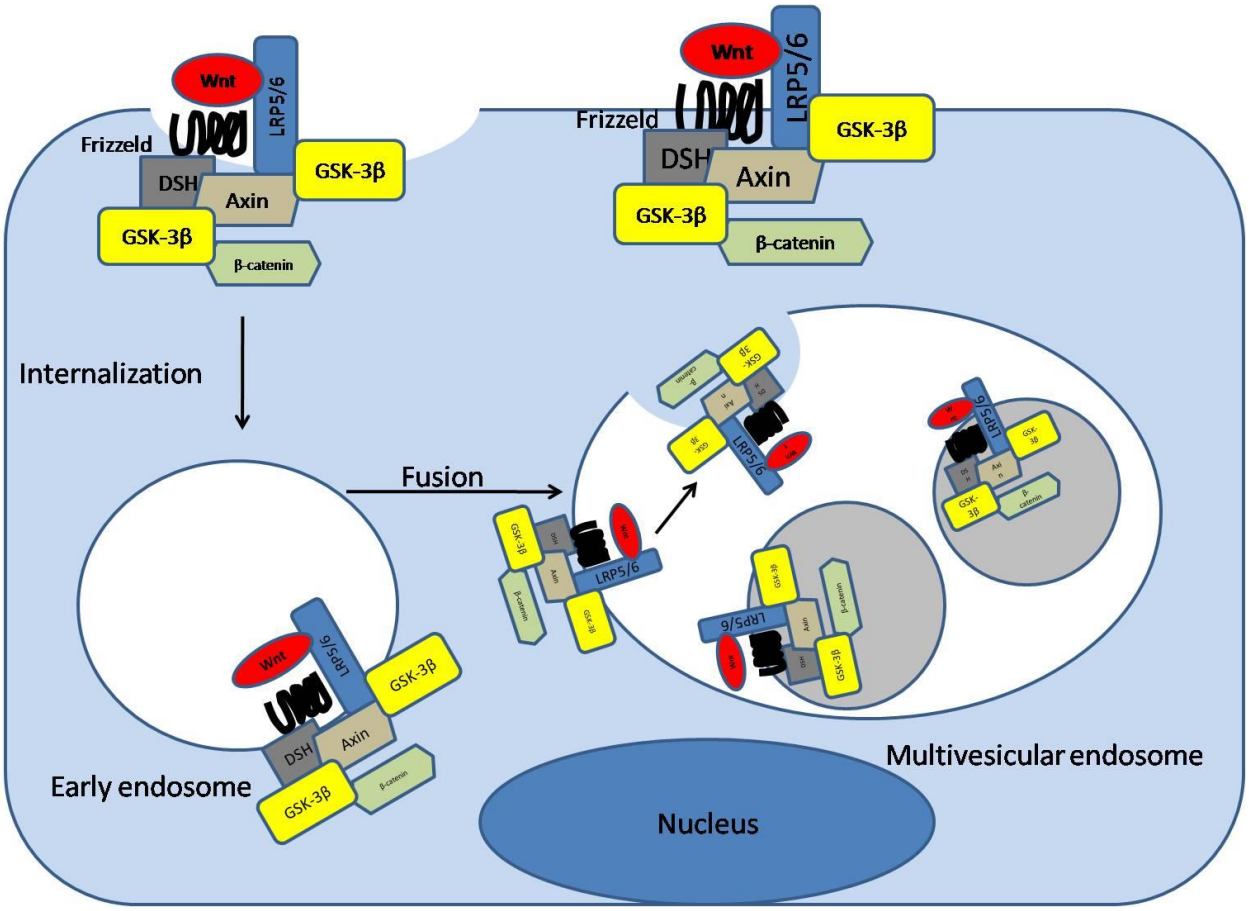

Figure 1.3: Internalization of the Wnt signalisome and its sequestration inside multivesicular bodies (MVB). Internalization of the Wnt signalosome is mediated by caveolin and dynamin. The early endosomes can enter multivesicular body (MBV) pathways. This process was mediated by vacuolar protein sorting-27 (Vps-27) and Vps-4. The sequestration of GSK3 in MVB sustains Wnt signaling activity by separating GSK3 from cytosolic β-catenin. Vps27 and Vps-4 are part of the endosomal sorting complex required for transport protein (ESCRT) that facilitates vesicle invagination.

In the absence of Wnt ligand, there is formation of the β-catenin destruction complex (consisting of Axin, APC, GSK3 and CK-1) that maintains a low level of β-catenin in the cytoplasm (see Fig. 1.2b)[16-18,133]. The destruction complex facilitates β-catenin phosphorylation by GSK3 and CK-1 proteosomal degradation of β-catenin[134]. In the absence of Wnt signaling, the half-life of β-catenin is less than an hour due to rapid β-catenin destruction by β-TrCP. In the presence of Wnt ligand and activation of Wnt/β-catenin signaling, β-catenin half-life increased to approximately 6 hours[135]. Another study reported stabilization of β-catenin using Wnt1 conditioned media with

increases in cytosolic β-catenin observed from 3 to 9 hours following treatment[136]. The reader is referred to several excellent reviews regarding the Wnt signaling cascade for further details[130,132,137,138].

Activation of Wnt follows multiple pathways, with various Wnt ligands associated with canonical Wnt signaling (known as the Wnt/β-catenin pathway) and others associated with the non-canonical Wnt signaling pathway. The non-canonical Wnt signaling pathway is independent of β-catenin and is referred to as either Wnt/planar cell polarity (Wnt/PCP signaling) or Wnt/Ca^{2+} utilization[132,139]. As the focus of this thesis is on Wnt/β-catenin signaling in the BBB discussion has been limited to the canonical Wnt pathway.

1.6. Cellular components of Wnt/β-catenin signaling

The complexity of Wnt signaling is attributable to multiple Wnt ligands, receptors, co-receptors, and transcription factors that have multiple isoforms and redundancy in terms of ligand and receptor interactions (summarized in Table 1.3). For example, there are ten Wnt receptors (Frizzled 1-10), four Wnt co-receptors (LRP5, LRP6, ROR2, and RYK), nineteen Wnt ligands (Wnt 1-16) and ten Wnt regulator peptides generated from three different proteins namely Dikkopf/Dkk, soluble frizzled related protein/sFRP, and Wnt inhibitory factor (WIF)[140]. Similarly, transcription factors in Wnt/β-catenin signaling include TCF-1 (Tcf7), TCF-3 (Tcf7l1), TCF-4 (Tcf7l2) or LEF-1[141].

Table 1.3: Cellular components of Wnt signaling

Wnt component				
Ligands	Receptors	Co-receptors	Wnt modulators	Other components
Wnt-1	Frizzled-1	LRP-5	Dkk-1	GPR124
Wnt 2	Frizzled-2	LRP-6	Dkk-3	Reck
Wnt-2b	Frizzled-3	ROR-1	Dkk-3	Norrin (NDP)
Wnt-3	Frizzled-4	ROR-2	Dkk-4	R-spondin (RSPO)
Wnt-3a	Frizzled-5	RYK	sFRP-1	Tspan12
Wnt-4	Frizzled-6		sFRP-2	LRG4
Wnt-5a	Frizzled-7		sFRP-3	ZNRF3
Wnt-5b	Frizzled-8		sFRP-4	
Wnt-6	Frizzled-9		sFRP-5	
Wnt-7a	Frizzled-10		WIF-1	
Wnt-7b				
Wnt-8a				
Wnt-8b				
Wnt-9a				
Wnt-9b				
Wnt-10a				
Wnt-10b				
Wnt-11				
Wnt-16				

1.6.1. Wnt Ligands

Wnt ligands are highly insoluble proteins between 350-400 amino acids in length that can act in either an autocrine or paracrine fashion. Ligands are divided into two general classes: non-canonical Wnt ligands (Wnt4, Wnt6, Wnt5a, Wnt5b, Wnt11 and Wnt16) and canonical Wnt ligands (Wnt3, Wnt3a, Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt9a, Wnt9b, Wnt10a and Wnt10b)[142,143]. Among canonical Wnt ligands, Wnt3 and Wnt3a are considered to be the most potent Wnt

activators[142,144,145]. However, Wnt3a expression is highly variable and cell-dependent. As an example, hippocampal astrocytes and brain cancer cells express high levels of Wnt3a[143,146-151], while in a variety of endothelial cells, such as lung endothelial cells, placental (HUVEC), adult dermal microvascular endothelial cells, neonatal dermal microvascular endothelial cells and hepatic sinusoid endothelial cell, Wnt3a is nearly absent[140,143,152,153].

After synthesis in the ribosome, Wnt ligands are stored and modified via glycosylation and palmitoylation in the endoplasmic reticulum (ER), an essential step for Wnt secretion and activity. N-glycosylation of Wnt ligand is important for proper folding and it is a pre-requisite step for palmitoylation[154]. Wnt palmitoylation occurs in the ER and is catalyzed by membrane bound O-acyltransferase (Porcupine/PORCN). All Wnt ligands require PORCN for proper processing and secretion from the cell. For intracellular trafficking, Wnt ligands use Evi/Wntless/WLS/WI/GPR177, transmembrane proteins that are found in the ER, Golgi apparatus, vesicles, plasma membrane and early endosomes. Evi binds palmitoylated Wnt ligand within its hydrophobic region[144,155,156]. Deletion of either Porcupine or Evi significantly reduced Wnt activity in the HEK cell[144]. Palmitoylated Wnt released from cells is sufficient for activating Wnt signaling within the cell (autocrine) and in adjacent cells (paracrine)[63,90,129]. Long range Wnt signaling required encapsulation of Wnt ligands in secreted vesicles or in association with a lipoprotein complex[129,157,158]. Examination of Wnt long range signaling in a live animal *C. Elegans* suggested that Wnt ligands can travel distances of 75 μm or greater activating Wnt pathway responses in cells that were not in direct cell contact with the native source[159]. Similarly, in Drosophila, Wnt/Wg can travel substantial distances (equivalent to 11 cell diameters) from the cell source, creating concentration gradients that act in a paracrine signaling fashion[160].

The Wnt secretion pathway has provided scientists with several ways to modulate the secretion of Wnt from cells and to study downstream Wnt processes. Compounds that have been identified that selectively inhibit porcupine activity include WntC59, IWP-2, Lgk974 and ETC-159[161,162]. These compounds prevent Wnt ligand release and inhibit autocrine Wnt signaling.

1.6.2. Wnt receptors

Frizzled receptors are classified into five subfamilies. Subfamily 1 includes frizzled-1, -2, and -7; subfamily 2 includes frizzled-5 and -8; subfamily 3 includes frizzled-9 and -10; subfamily 4 has only frizzled-4 as its member and subfamily 5 includes frizzled-3 and -6[163,164]. While frizzled-3, -6, and -9 mediate the binding of non-canonical Wnt ligands, the rest of the frizzled receptors facilitate binding to canonical Wnt ligands. Each of the frizzled isoforms has different specificities and affinities. For example Frizzled-1, -2 and -7 have high affinity for Wnt3 and Wnt3a, while frizzled-4 and -10 have high affinity for Wnt3, Wnt3a, Wnt9b and Wnt9b[142]. Separate studies demonstrated that frizzled-4 also interacts with Wnt1, Wnt3a, Wnt7a and Wnt7b suggesting the designation of frizzled-4 for canonical Wnt signaling[120]. Similar to frizzled-4, frizzled-5 can bind to all canonical Wnt ligands with high affinity. Frizzled-8 has high affinity for specific canonical Wnt ligands such as Wnt1, Wnt8a, Wnt8b and Wnt10 but not with Wnt3 or Wnt3a[142]. The fact that Wnt ligands have affinity to several frizzled receptors is an indication of the receptor redundancy that is present in the canonical Wnt pathway.

The membrane distribution of the frizzled receptor is determined by its cytoplasmic tail. Studies using fruit fly wings (Drosophila) revealed that frizzled receptors dedicated to Wnt/PCP; i.e. frizzled-1; were located at the apical site of the plasma membrane of cells, while those dedicated to canonical Wnt signaling; i.e. frizzled-2; were primarily basolaterally expressed[165]. Frizzled-2 but not frizzled-1 have 69 additional amino acids on their cytoplasmic tail that govern basolateral

location of frizzled-2[155,165]. Modification of the frizzled receptors cytoplasmic tail can be used to localize the receptor on the apical or basolateral surface.

1.6.3. Wnt Co-receptors

Low-density lipoprotein receptor-related protein-5 and-6 (LRP5/6) are specific co-receptors required for the canonical Wnt pathway. Both the extracellular and cytoplasmic domains of LRP5/6 are pivotal for signaling. The extracellular domain of LRP5/6 consists of four tandem β-propeller structures. The β-propeller repeat-1 and -2 interact with most of the canonical Wnt ligands (Wnt1, Wnt2, Wnt2b, Wnt6, Wnt8a, Wnt9a, Wnt9b, and Wnt10b). On the other hand, β-propeller repeat-2 and -4 interact with Wnt3 and Wnt3a. It should be noted that Dkk-1, which inhibits the action of all canonical Wnt ligands, interacts with all four β-propeller structures of the LRP5/6[166].

1.6.4. Wnt Modulators

Wnt/β-catenin signaling can be modulated by several secreted proteins including soluble frizzled related proteins (sFRP-1, -2, -3, -4, and -5), Dikkopf (Dkk-1, -2, -3, and -4) and Wnt inhibitory factor (WIF)[137]. The sFRPs act as classical antagonists by binding to Wnt ligands and preventing them from interacting with frizzled receptors on the cell. Because the cysteine rich domain (CRD) of sFRP has structural homology to frizzled CRD, sFRPs can bind to frizzled as well as to Wnt ligands. sFRP1 and sFRP2 both bind to Wnt3a and Wnt5a in the nanomolar range. As such, sFRP not only interferes with canonical Wnt signaling, but also with non canonical Wnt signaling[167]. Studies also suggest that Wnt3a has multiple binding sites on several sFRPs[168]. Normally acting as a tumor suppressor, downregulation of sFRP has been observed in several cancerous conditions where sFRP downregulation drives aberrant Wnt activation[167]. Understanding sFRP secretion

pathways and the cellular source will be valuable for developing pharmacological agents for Wnt modulation.

Unlike sFRP, Dkk-1 peptide specifically inhibits canonical Wnt signaling[115,137]. Dkk forms a complex with LRP5/6 and Kremen1/2 to trigger internalization of LRP5/6 from the plasma membrane[169,170]. LRP5/6 is an essential player for Wnt signaling and any reductions in LRP5/6 on the plasma membrane will significantly affect signaling activity[104]. Dysregulation of Wnt modulator secretion is associated with aberrant Wnt signaling in various diseases including cancer and Alzheimer's disease[171-173]. Upregulation of Dkk-1 expression in the brain of AD patients results in diminished Wnt activity, and increased GSK3-β phosphorylation of Tau protein[173]. Separate studies also observed elevations in Dkk-3 in the CSF of patients with Alzheimer's disease[172]. Together these studies suggest that changes in Wnt modulator levels can have a significant impact in contributing to pathological conditions.

1.7. Non-traditional β-catenin signaling

1.7.1. Wnt7a and Wnt 7b signaling

Different from other Wnt isoforms, β-catenin signaling triggered by Wnt7a and Wnt7a can be potentiated by GPR124 (Probable G-protein coupled receptor 124) and Reck (a multi-domain glycosyl phosphatidyl inositol (GPI)-anchored protein)[174]. The signaling pathway is initiated by the binding of Wnt7a or Wnt7b to GPR124 and Reck. The interactions of Wnt7a/b with GPR124 and Reck lead to an increased Wnt7a/b availability for binding to Frizzled and LRP receptors[175]. Mediated by Dvl polymerization, Wnt7a/7b-GPR124-Reck form clusters with Frizzled-LRP5/6[175]. Without binding to GPR124 and Reck, the Wnt7a/7b was rapidly degraded and inactivated[176]. Thus GPR124 and Reck can enhance Wnt signaling in response to Wn7a/7b and primarily with frizzled-5, -8 and -4[174,177]. Frizzled-4 and frizzled-8 are highly expressed in the developing brain[89].

More detail on 7a/7b-triggered β-catenin signaling will be discussed in sections 1.10 and 1.11 below.

1.7.2. R-spondin potentiates Wnt/β-catenin signaling

Normally, Wnt signaling is initiated by the binding of Wnt ligand to frizzled and its co-receptors. However, if R-spondin (RSPO) is present, Wnt activity can be potentiated[178]. R-spondin has 4 isoforms, RSPO-1, -2, -3 and -4. RSPO-3 appears to be an important mediator of angiogenesis as RSPO-3 deficient mice die at embryonic day 10.5 due to vascular defects[179]. R-spondin binding to LGR-4 (GPCR-4 with leucine-rich repeat) and ZNRF3 induces internalization of the RSPO-LGR-4-ZNRF3 complex[180]. In the absence of R-spondin, ZNRF3 (E3 ubiquitin ligase zinc and ring finger, a transmembrane ubiquitin machineries) ubiquinates frizzled receptor and LRP5/6 to promote their removal from the membrane[137,178]. Since R-spondin acts primarily by delaying Frizzled re-cycling, RSPO can potentiate not only Wnt/β-catenin but also Wnt/PCP signaling[181] and Wnt/Ca^{2+} signaling[182].

1.7.3. Activation of β-catenin signaling by Norrin (NDP)

Besides Wnt ligand, β-catenin signaling can also be activated by other morphogen such as Norrin (NDP)[183,184]. Activation of β-catenin signaling by Norrin requires LRP-5, Frizzled-4 and TSPAN-12. In Norrin/β-catenin signaling, TSPAN-12 serves as a co-receptor that facilitates ligand selectivity of Frizzled-4 to Norrin and stabilizes Norrin-Frizzled-4 interaction[185]. Following formation of Norrin-Frizzled-4-TSPAN-12 cluster, β-catenin destruction complex is inhibited resulting in the activation TCF/LEF transcriptional activity[120,137,186]. However, this process is specific to Frizzled-4 as among the ten Frizzled receptors, NDP shows affinity only for Frizzled-4[187].

1.8. Wnt receptor location in the BBB

While the expression of frizzled receptors in the brain microvasculature has been documented, the subcellular location of frizzled receptor and the source of Wnt ligand remain unaddressed from a BBB perspective. Although definitive studies determining the localization of frizzled receptors in the brain endothelial cells have not been done, a basolateral localization of the frizzled receptors would be consistent with the brain-centric source of Wnt ligands in the astrocytes[188], neurons[188], and pericytes[189]. For example studies on mature brain cortex revealed that Wnt7a and Wnt7b were expressed by astroglia, oligodendrocytes and neurons but was less abundant in brain endothelial cells themselves[190]. Furthermore, the presence of tight junction proteins within the brain endothelium would limit the passage of Wnt ligands across the BBB; minimizing the possibility of peripheral (non brain) sources of Wnt ligand.

1.9. Origin of Wnt ligand and evidence of paracrine and autocrine Wnt signaling in the BBB

Activation of Wnt/β-catenin pathways in brain endothelial cells is primarily through paracrine mechanisms[191]. During BBB development, neural progenitor cells produced Wnt ligands for CNS angiogenesis and the establishment of BBB characteristics[87,89]. Post-developmentally, sources of Wnt ligand for brain endothelial cells may include neurons, astrocytes and pericytes[86,110]. The evidence supporting astrocytes and pericytes as Wnt producing cells in the CNS is summarized in Table 1.4.

Table 1.4: Evidence for astrocytes and pericytes as important sources of Wnt ligand

Cells	Source	Wnt ligand produced	Experimental condition	Results	Reference
Astrocytes	Hippocampal astrocytes	Wnt3	*In vitro*	Co-culture with astrocytes increase adult hippocampal progenitor cell Wnt activity	[192]
Astrocytes	Ventral midbrain astrocytes	Wnt1	*In vivo*	Wnt1 from astrocytes support dopaminergic neuron survival and dopaminergic neurogenesis in the animal model of Parkinson's disease	[188]
Astrocytes	Hippocampal astrocytes	Wnt3 and Wnt3a	*In vivo* and *in vitro*	Wnt3 and Wnt3a expression in hippocampal astrocytes reduced with age	[149]
Astrocytes	Hippocampal astrocytes	Wnt3 and Wnt3a	*In vitro*	Hippocampal astrocytes isolated from old mice produced less Wnt ligand compared to young animal	[193]
Bergmann glia (radial astrocytes in the cerebellum)	Cerebellum	Wnt7a	*In vivo*	Bergman glia-specific Wnt7a elimination induced BBB dysfunction in the cerebellum	[120]
Pericytes	-	Wnt3a and Wnt7a	*In vitro*	Co-culture brain endothelial cells with pericytes improve barrier integrity	[189]

There is considerable evidence for astrocytes secreting Wnt ligands. Studies showed that hippocampal astrocytes expressed more Wnt3 mRNA compared to hippocampal homogenates. Co-culture of hippocampal astrocytes with adult hippocampus progenitor cells (AHP) exhibited elevated TCF transcriptional activity and neuron differentiation compared to AHP monocultures. Moreover, addition of Wnt inhibitor (i.e. sFRP-2 and sFRP-3) to the co-cultures reduced Wnt activity and neuron maturation in AHP[192]. Additional evidence of hippocampal astrocytes as sources of Wnt were seen in paracrine Wnt/β-catenin activation of NPC by astrocytes[193]. Conditioned media (CM) isolated from hippocampal astrocytes increases NPC Wnt activity. The

Wnt/β-catenin activity was correlated with upregulation of surivin expression and NPC proliferation. Addition of sFRP-2 and sFRP-3 to the astrocytes CM reversed NPC Wnt activity. Together these studies suggest that there is paracrine Wnt signaling between astrocytes and NPC.

More recent studies indicate that hippocampal astrocytes produced not only Wnt3 but also Wnt3a[149]. The studies identified that expression of these Wnt ligands declined with age. Additional support of astrocytes ability to produce Wnt ligand comes from studies reporting ventral midbrain astrocytes produced Wnt-1 to support neurogenesis and neuroprotective response following neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) insult in a mouse model of Parkinson disease[194]. These studies suggest that while Wnt ligand production and secretion may be highest during development, there astrocytes continue to secrete Wnt ligands after maturation of the BBB.

While hippocampal astrocytes secreted Wnt3 and Wnt3a, Bergmann glia, radial astrocytes located in the cerebellum, produced Wnt7. The importance of these cells as a source of Wnt ligand for BBB maintenance is demonstrated by the comparison of BBB changes in mice with astrocytes-specific deletion of NDP and Wnt7a using *Ndp^{KO}; $Wnt7a^{CKO/-;}$Gfap-Cre* mice versus global deletion (*Ndp^{KO}; $Wnt7a^{-/-}$* mice). In both the conditional and global deletion mutations, breakdown of the BBB was mainly observed in the cerebellum where microvessels were converted from Glut-1^{+}/PLVAP^{-} to Glut-1^{-}/PLVAP^{+}[120]. Collectively, these studies demonstrated that Wnt paracrine signaling between Bergmann glia and brain endothelial cells is important for regulating BBB maintenance in cerebellum.

The pericytes are another potential Wnt source for brain endothelial cells[86,189]. Cell lysates from pericyte monocultures show expression of both Wnt3a and Wnt7a. Co-culturing of brain

endothelial cells with pericytes improved the endothelial monolayer barrier function as demonstrated by reduced permeability to fluorescent marker Lucifer yellow. These improvements in endothelial barrier integrity in the co-culture model could be prevented by treatment with Wnt inhibitor XAV939[189]. These studies suggest that Wnt3a and Wnt7a secreted by pericytes elicited Wnt/β-catenin signaling in brain endothelial cells that resulted in an improved paracellular barrier.

Which brain cells provide the major source of Wnt ligand for Wnt/β-catenin signaling in the brain endothelial cell post BBB development is still a matter of debate. Pericytes are capable of producing Wnt ligand[189], but constitute of small portion of human brain. Glia and neuron cells are considered as the main cellular component of the human brain in term of cell number[195]. While neurons are also capable of producing Wnt ligand[105,151,196,197], a direct contribution of neurons to brain endothelial Wnt activity has not been demonstrated. However, a neuron-brain endothelial Wnt/β-catenin interaction is feasible as previous studies demonstrate the ability of Wnt ligand to travel as far as 75 μm distance in paracrine Wnt signaling[159]. Pericytes, astrocytes and neurons, present in close proximity to brain endothelial cells, are capable of producing Wnt ligands and could support a paracrine Wnt/β-catenin signaling pathway for brain microvessel endothelial cells.

In addition to paracrine activation of Wnt signaling in the BBB, there are some studies suggesting brain endothelial cells can themselves produce Wnt ligands. There are limited studies that document autocrine Wnt/β-catenin signaling in brain endothelial cells. On the other hand, information of autocrine Wnt/β-catenin signaling in peripheral endothelial cells has been reported. Autocrine Wnt activation has been observed in non-cerebral vascular preparations including bovine aortic endothelial cell (BAEC) and porcine aortic endothelial cell (PAEC) preparations where treatment with sFRP-1 reduced TCF transcriptional activity[140]. The possibility of autocrine Wnt signaling is further supported by the expression of Wnt2b in BAEC and Wnt-3, Wnt-4, Wnt-

5a, and Wnt7a mRNA expression in various peripheral endothelial cells[140]. Another example was the identification of Wnt2 at the cell surface of hepatic sinusoidal endothelial cells (HSEC) where the addition of sFRP-1 and WIF inhibited HSEC proliferation and *in vitro* tube formation[153]. Together these studies suggest that autocrine Wnt signaling in peripheral endothelial cells does exist. In terms of brain endothelial cells, addition of Dkk-1 in human cerebral microvessels endothelial cells (hCMEC/D3) reduced nuclear β-catenin[114,115,198]. Although the Wnt ligand concentration in the media was not measured, the reduction of Wnt activity with the addition of Dkk-1 strongly suggests the presence of autocrine Wnt signaling in brain endothelial cells[115].

1.10. Wnt/β-catenin signaling in BBB development

The uniqueness about Wnt as a BBB morphogen is its specificity for CNS vs. non-CNS vasculature. For example Wnt/β-catenin activity in the lung and heart microvessels were almost negligible compared to those in CNS microvessels examined at E12.5[89]. Additionally, endothelial-specific β-catenin deletion using *Ctnnb1$^{lox/lox;}$;TekCre* mice displayed significant vascular defects in CNS capillaries but had no effect in the peripheral vascular beds such as lung, skin, jaw and tail when examined at E11.5[89]. Separate studies reported that CNS microvasculature displayed more intense Wnt/β-catenin activities compared to non-CNS microvessels when examined at E13.5[127]. In agreement, elimination of LRP-5 and LRP-6 at E11.5 using *Lrp5$^{-/-}$ Lrp6$^{CKO/CKO}$; Tie2-Cre* mice negatively affected CNS vascular formation but did not alter non-CNS vascular architecture[104]. The specificity of Wnt/β-catenin action on the CNS vasculature was supported by the fact that LEF-1 and TCF-7 DNA binding motifs were significantly enriched in brain microvessels compared to lung, kidney, or liver vascular beds. Examination on LEF-1 reporter mice at E13.5 showed that LEF-1 expression is enriched in CNS endothelial cells but not in peripheral[126,127]. Interestingly, specificity of Wnt/β-catenin signaling in the CNS vasculature is not only seen in the

BBB development stage but also observed in adulthood and BBB maintenance. Conditional β-catenin knockout in adult mice alters CNS vascular permeability but did not change the permeability of lung capillaries[103].

β-catenin is a central player in canonical Wnt signaling. Among the various cellular components to canonical Wnt signaling, β-catenin is the only one that has no redundancy. Genetic mutations of β-catenin provide a powerful research tool for understanding canonical Wnt activity in the BBB as deletion of β-catenin would mean complete elimination of the signaling pathway (see table 1.5). Structural examination of the β-catenin mutant mice *(Flk1Cre$^{+/-}$; Ctnnb1$^{c/n}$)* at E11.5 and E12 indicated endothelial cells and pericytes were largely absent in CNS neural regions. Furthermore, in those areas where there was migration of brain endothelial cells, complete vascular structures were never observed. These studies suggested that Wnt/β-catenin signaling was important for migration of endothelial cells and pericytes from neuroepithelium to neural region during BBB development[87,89,104].

Table 1.5: Contribution of various Wnt/β-catenin components to blood-brain barrier function examined using genetically modified mice

Phenotype	Location of mutation	Time	Effect to BBB	Ref.
Flk1Cre$^{+/-}$; Ctnnb1$^{c/n}$	Deletion on vascular precursor	Embryonic	Not viable, lethal at E12, EC and pericytes absent from neuroepithelium, reduced Glut-1 expression on the perineural vascular plexus and neuroepithelium	87
Ctnnb1$^{lox/lox;}$ Pdgfb-iCreERT2	Conditional endothelial specific deletion	P4 and P7	↓Claudin-3 and ↑PLVAP in the brain vessel, ↑Evan blue extravasations	102
Ctnnb1-iCKO	Conditional endothelial specific knock out	Adult, 10-12 week age	Brain hemorrhage including hippocampus and basal ganglia, 10-fold increase in BBB albumin leakage, reduced expression of claudin-1 and claudin-3, lethality due to brain hemorrhage	103
Ctnnb1$^{lox/lox;}$;TekCre	Endothelial specific deletion	E11.5	Not viable, EC not able to invade the neuroepithelium, aggregation of EC in neuroepithelium	89
Ctnnb1$^{CKO/CKO;}$;Pdgfrb-CreER,	Postnatal conditional endothelial specific knock out	P22-23	BBB leakage to brain cortex, cerebellum, periventricular hypothalamus, ventral thalamus, pons and interpenduncular nuclei, ↓Claudin-5 and ↑PLVAP, induction of deletion earlier than P22 was fatal	104
Ctnnb1$^{CKO/CKO}$; Tie2-Cre	Endothelial specific knock out during embryonic stage	E11.5	Not viable, EC not able to invade the neuroepithelium, aggregation of EC in neuroepithelium	104
Ctnnb1$^{i\Delta EC}$	Postnatal conditional endothelial specific deletion	P6 (Induced 3 days before)	Reduced expression of Glut-1, Claudin-5 and Mfsd2a in the cortex tissue. Increased expression of PLVAP in cerebellar vasculature. Reduced expression of VEGFR-2 and -3 in cortex and cerebellum microvessels, reduce cerebellar angiogenesis (sprouting and tip cells), reduced DII4 expression at cortex	125
Lrp5$^{-/-}$	Global deletion	Postnatal	Viable and fertile but die after birth due to low bone mass Persistent eye vascularization	199
Lrp6$^{-/-}$	Global deletion	Postnatal	Die at birth	200

Phenotype	Location of mutation	Time	Effect to BBB	Ref.
Lrp5$^{-/-}$ Lrp6$^{CKO/CKO}$; Tie2-Cre	Endothelial specific deletion during embryonic	E 11.5	Not viable, EC not able to invade the neuroepithelium, aggregation of EC in neuroepithelium, bleeding in the hindbrain, midbrain, and forebrain	104
Lrp5$^{-/-}$ Lrp6$^{CKO/CKO}$;Pdgfrb-CreER,	Conditional endothelial specific knock out done at postnatal	P22-23	BBB leakage to brain cortex, cerebellum, periventricular hypothalamus, ventral thalamus, pons and interpenduncular nuclei ↓Claudin-5 and ↑PLVAP, earlier induction of deletion than P22 will die after day 3	104
Wnt7a$^{-/-}$; Wnt7b$^{c3/d3}$;Sox2Cre$^{+/-}$	Specific deletion on embryo	Embryonic stage (E 12.5)	Not viable, CNS hemorrhage and disorganization of neural tissue, the absence of EC and pericytes from ventral neural region	87
Wnt7a$^{-/-}$;Wnt7b$^{c3/d3}$;NestinCre$^{+/-}$	Specific deletion on neuroepithelium	Embryonic stage (E 12.5)	Not viable, CNS hemorrhage, malformation of blood vessels, reduced EC number on the intraneural vascular plexus (INVP)	87
Wnt7a knock out	Global deletion	E10.5 and E12.5	Viable and normal vascular pattern	89
Wnt7b knock out	Global deletion	E 10.5	Not viable, vascular malformation	89
Wnt7a/Wnt7b double knock out	Global deletion	E10.5	Not viable, vascular malformation	89
GPR124 $^{flox/flox}$; CMV- Cre	Global deletion	E12.5	Not viable, forebrain hemorrhage	201
GPR124$^{-/-}$	Global deletion	E13.5	Not viable, forebrain hemorrhage	202
GPR124 $^{flox/-}$;ROSACreER	Conditional endothelial deletion	P1 and adult (2-3 month of age)	Normal BBB phenotype and function	201
Reck$^{-/-}$	Global deletion	E10.5	Not viable, increased activity of matrix metalloproteinase and destruction of extracellular matrix in the basal lamina	203
Reck$^{flex2/\Delta ex2}$;Tie2-Cre	Endothelial specific deletion	E12.5	Forebrain hemorrhage, endothelial cell form a glomeruloid-like tuft instead of capillary network, Non CNS vascular was normal	174
Wnt7a$^{-/-}$	Global deletion	P30	No change on BBB dysfunction, Glut 1, PLVAP and Sulfo-NHS permeability	120
Ndp^{KO}	Global deletion	P30	Low level of barrier dysfunction on cerebellum and olfactory bulb, ↓Glut-1 and ↑PLVAP in cerebellum	120
Wnt7a$^{-/-}$; Fdz4$^{-/-}$	Global deletion	E17	Perinatally lethal, multiple cranial bleeding, ↑PLVAP with partial reduced	120

Phenotype	Location of mutation	Time	Effect to BBB	Ref.
			Glut 1 or claudin 5, the effect was milder compared to GPR124 deletion	
Ndp^{KO};$Wnt7a^{-/-}$	Global deletion	P30	Greater BBB dysfunction in the cerebellum, ↓Glut1 and ↑PLVAP in cerebellum and olfactory bulb	120
$Fz4^{-/-}$;	Global deletion	P1-P30	Develop normally up to P19 but show progressive cerebellum degeneration (granular cell death) at older age	204
$Fz4^{-/-}$;	Global deletion	P16 to 6 months	Viable but experience progressive abnormality of vascular cerebellum. P14: cerebellum vascular appears normal P30: cerebellum vascular is sparser 6 months: cerebellum vascular is sparse and irregular	184
$Fdz4^{CKOAP/-}$,Tie2Cre	Conditional endothelial specific deletion	P18	BBB leakage in cerebellum, ↓Claudin-5 and ↑PLVAP	183,205
$Fz4^{CKO/-}$ $Lrp5^{-/-}$;Tie2Cre	Conditional endothelial specific deletion	P10-11	Greater BBB breakdown including in the thalamus, brain stem, cortex, and pons/interpeduncular nuclei	104
$Tspan12^{-/-}$	Global deletion	P12	Normal BBB	120
$Tspan12^{-/-}$;$Wnt7a^{-/-}$	Global deletion	P12	Normal BBB	120
$Tspan12^{-/-}$;$Gpr124^{CKO/-}$;Pdgfb-CreER	Endothelial specific	Postnatal	Severe BBB leakage in hippocampus, superior colliculus, striatum, and brainstem, mild leakage at cerebral cortex, cerebellum, and hypothalamus. ↓Glut1 and ↑PLVAP	120
Ndp^{KO}; $Gpr124^{CKO/-}$;Pdgfb-CreER	Endothelial specific	Postnatal	Severe BBB leakage in cortex, hippocampus, superior colliculus, striatum, and brainstem, cerebellum, and hypothalamus. ↓Claudin-5 and ↑PLVAP	202
Ndp^{KO}; $Wnt7a^{CKO/-;}$Gfap-Cre	Astrocytes specific deletion	Postnatal	Severe BBB breakdown in the cerebellum	120
$Axin\text{-}1^{IEC\text{-}OE}$	Endothelial specific conditional deletion	P6 (Induced 6 days before)	Reduced expression of Glut-1, Claudin-5, Mfsd2a, VEGFR-2 and DII4 in brain cortex, reduced vascular sprouting and tip cells in cerebellum,	125
$Axin\text{-}1^{IEC\text{-}OE}$	Endothelial specific conditional deletion	E11.5 (Induced 2 days before)	Forebrain hemorrhage, vascular regression and dilation	206

The presence of LRP5 or LRP-6 is compulsory for Wnt/β-catenin signaling. Mice with deletion of both LRP5 and LRP6 using *Lrp5*$^{-/-}$ *Lrp6*$^{CKO/CKO}$*; Tie2-Cre* are embryonically non-viable. Examination performed on E11.5 embryos revealed an inability of endothelial cells to invade the neuroepithelium. Instead of forming a vascular network, the endothelial cells in the mutant mice formed endothelial aggregates. In addition, there is some bleeding in the hindbrain, midbrain, and forebrain. These phenotypes are similar to those in β-catenin mutant mice suggesting in dispensability of LRP5 and LRP-6 in BBB angiogenesis[104].

Despite the structural similarities in LRP-5 and LRP-6, LRP-6 appears to be more essential for fetal development. This is based on gene mutation studies in mice where LRP-5$^{-/-}$ was embryonic viable and fertile but showed impairment in retinal angiogenesis that was similar to familial exudative vitreo retinopathy (FEVR)[199]. In addition to that, many of the mice experienced death a month after birth due to low bone mass and bone fracture[199]. In contrast, LRP-6 global deletion resulted in death at birth with evidence of disorganized cerebellum and other organ malformations[200].

Although several canonical Wnt ligands are expressed in the developing CNS (i.e. Wnt1, Wnt3, Wnt3a, Wnt7a and Wnt7b), studies showed Wnt7a/b play an essential role in the CNS angiogenesis and development of BBB characteristics[87,89,174,177]. During the embryonic stage, the neuroepithelium secretes Wnt7a/b to direct the migration of endothelial cells from the perineural ventral plexus (PNVP) to neural regions; forming the intraneural vascular plexus (INVP). Similar to β-catenin or LRP-5/6 double-deletion, the conditional neuroepithelium-specific deletion of Wnt7a and Wnt7b in the Wnt7a$^{-/-}$;Wnt7b$^{c3/d3}$; NestinCre$^{+/-}$ mice is embryonically non-viable. Examination of brain tissue from E12.5 embryos showed malformation of blood vessels, reduced EC number on the intraneural vascular plexus (INVP) and CNS hemorrhage[87]. This suggested an

essential role of Wnt7a/b in CNS angiogenesis[87,89]. Interestingly, the role of Wnt7b in CNS angiogenesis more essential compared to Wnt7a. Wnt7b knockout mice display embryonic lethality at E10.5 and E12.5 due to CNS vascular malformations while Wnt7a knockout during angiogenesis phase were viable and showed normal CNS vascular development[89].

As expected, GPR124 and Reck also play essential role in CNS angiogenesis and BBB differentiation[174,177,207]. GPR124 or Reck global deletion is embryonic non-viable due to forebrain hemorrhage examined at E11.5-12.5[104,174,201,203]. GPR124 or Reck deletion is associated with a failure for vascular sprouting into neural tissue. This phenotype is similar to those in mice with β-catenin deletion, LRP-5 LRP-6 double deletion or Wnt7a/7b double deletion. Collectively, these studies suggest that Wnt7a/7b, LRP5/6, GPR124, Reck and β-catenin are all essential components for proper CNS angiogenesis.

Once BBB is functional (post E15.5), Wnt/β-catenin signaling remains important and indispensible for proper BBB function. *Wnt7a*$^{-/-}$; Fdz4$^{-/-}$ transgenic mice are embryonic lethal at E17 (before birth) due to multiple cranial bleeding with increased PLVAP and reduced Glut-1 and claudin-5 expression in CNS microvessels[120]. As Frizzled-4 is the isoform specific for Norrin signaling, the reduced BBB integrity observed in the *Wnt7a*$^{-/-}$; *Fdz4*$^{-/-}$ transgenic animals could reflect impairment in both Wnt7a/β-catenin signaling and Norrin/β-catenin signaling. These studies showed that Wnt7a and NDP are responsible for BBB maturation and maintenance.

Another intracellular component of Wnt/β-catenin signaling is Axin. Axin often referred to as a cytosolic scaffold protein required for the formation of β-catenin destruction complex[134,208]. Axin over-expression has been used as a tool to mimic *in vivo* Wnt/β-catenin inhibition[105]. Axin over-expression during the angiogenesis phase resulted in reduced Wnt/β-catenin activity in brain

microvessels by 40%. This conditional Axin over-expression model was associated with reduced radial vascular sprouting and forebrain hemorrhage. As expected, Axin over-expression during the angiogenesis phase resulted in a similar BBB phenotypes as observed with mice having embryonic β-catenin deletion. Conditional Axin over-expression during later angiogenesis phase (E11.5) or during the differentiation phase (E15.5) similarly reduced selected transporters (i.e. Glut-1, P-glycoprotein, Slco2b1, Slco1c1, Slc7a5) and tight junction molecules (i.e. occludin and claudin-5) within the cerebral vascular microvessels[206]. Together, these studies demonstrated the essential role of Axin as a cellular component of Wnt/β-catenin signaling in CNS angiogenesis and differentiation of brain endothelial cells.

1.11. Wnt/β-catenin signaling is important for BBB maturation and maintenance

Wnt/β-catenin signaling in brain microvessels is significantly reduced during BBB maturation phase and further reduced in adulthood[101,102,209]. Although compared to developing brain there is a reduction in activity, Wnt/β-catenin signaling remains operative and essential for normal BBB function[105,201]. It should be noted that although β-catenin deletion in mice is non-viable in both developing and mature animals, the underlying cause of postnatal mortality is not the dysregulation of angiogenesis but rather massive BBB dysfunction. Conditional β-catenin knockout in *Ctnnb1-iCKO* mice and *Cdh-Cre^{ERT2}; Ctnnb1$^{fl/fl}$* mice as well as double knockout of LRP-5 and LRP-6 using *Lrp5$^{-/-}$ Lrp6$^{CKO/CKO}$;Pdgfrb-CreER mice,* resulted in severe and widespread BBB dysfunction leading to lethality[103,104,113]. Studies by Zhou *et al* (2014) reported widespread compromised BBB in the brain cortex, cerebellum, periventricular hypothalamus, ventral thalamus, pons and interpenduncular nuclei when endothelial β-catenin was conditionally knocked out at P22 using *Ctnnb1$^{CKO/CKO;}$;Pdgfrb-CreER,* mice[104]. The BBB dysfunction following postnatal β-catenin deletion was mediated by upregulation of PLVAP and downregulation of

claudin-1, -3, and -5[102-104]. Similarly, a β-catenin conditional deletion in mature mice (2-3 months) resulted in BBB leakage leading to brain hemorrhage, brain inflammation, spontaneous seizure and death[103]. Altered BBB function was also observed in the hippocampus suggesting β-catenin signaling was crucial in this region to maintain BBB integrity[103]. This *in vivo* response of β-catenin deletion is in agreement with *in vitro* models of the BBB confirming the role of Wnt/β-catenin signaling on regulation of brain endothelial fenestration and tight junction expression[102]. Qualitatively similar responses were observed with conditional knockout of LRP6 and LRP5$^{-/-}$ mice (*Lrp5*$^{-/-}$ *Lrp6*$^{CKO/CKO}$*;Pdgfrb-CreER*). While conditional knockout of LRP6 and LRP5 was not viable at P3, conditional knockout at later periods (P22) were viable but experienced severe BBB breakdown in almost every brain region[104]. The fact that both LRP5/6 double deletion and β-catenin deletion show a similar widespread BBB dysfunction suggests that Wnt/β-catenin signaling is essential for BBB maintenance (see Table 1.5).

Postnatal removal of Wnt signaling components, other than β-catenin or LRP5 and LRP6, result in less severe and more localized impact on BBB function[120,183,201]. Conditional deletion of GPR124 (*GPR124* $^{flox/-}$*;ROSACreER*) or global deletion of Wnt7a postnatally were viable with the mice exhibiting a normal BBB phenotype[120,201]. Postnatal elimination of frizzled 4, a receptor for Norrin/β-catenin signaling, were also viable, although the mice showed a progressive loss of local BBB integrity, especially in the cerebellum[183,184,205]. This could be explained, by the fact that Frizzled-4 is a receptor specific for Norrin, which is highly expressed in the cerebellum[210]. In support of this, NDP knockout mice was viable but exhibited low level barrier dysfunction in the cerebellum and olfactory bulb[104]. However, the BBB dysfunction observed in the NDP (Norrin) knockout mice were further exasperated when Wnt7a was also deleted. The Ndp^{KO};Wnt7a$^{-/-}$ mice showed more dramatic BBB dysfunction in cerebellum and olfactory bulb compare to the

dysfunction in Ndp^{KO} *mice*[120]. BBB dysfunction *at* Ndp^{KO} mice is confined in the cerebellum[104]. Together, these studies suggested an interplay between Wnt7a and Norrin in maintaining cerebrovascular barrier integrity in the mature BBB.

Although GPR124 is dispensable in the mature animal, the absence of GPR124 in various pathophysiological conditions such as stroke ischemia or glioblastoma results in BBB dysfunction[201]. $Gpr124^{flox/-}$;Cdh5-CreER mice subjected to ischemia displayed more severe cerebral hemorrhage, a worse neurological score and increased BBB permeability compared to control animals. Similarly, elimination of GPR124 reduced mice survival and increased brain tumor edema in a rodent model of glioblastoma. Collectively, these findings suggested that even though GPR124 deletion has minimal impact in normal adult animals, it remains important in maintaining BBB integrity during pathological conditions.

Under pathological conditions, Wnt/β-catenin can act as an adaptive defense mechanism during BBB breakdown. An increase in Wnt activity has been reported in some conditions that involve BBB breakdown such as experimental autoimmune encephalomyelitis (EAE) and cerebral ischemia[105,121]. Using, the EAE mouse as a multiple sclerosis disease model, increased activation of Wnt/β-catenin signaling was observed in the CNS vasculature. Furthermore, the upregulation of Wnt reporter genes such as, APCDD1 and Sox-17 observed in the EAE spinal cord vasculature increased as the disease progressed. The elevation of Wnt activity correlated with an upregulation of Wnt3 and Wnt5a expression in neurons, but not in the astrocytes. The re-emergence of Wnt/β-catenin activity during EAE disease progression was postulated as a response mechanism to restore BBB function. Endothelial Wnt/β-catenin inhibition via Axin overexpression exacerbated the disease score and increased immune cell entry to the spinal cord. In addition, brain microvessels displayed upregulation of caveolin-1 protein suggesting increased vesicular transport activity. In

agreement with EAE mouse model, increased activity of Wnt/β-catenin signaling was also observed in the white matter of MS patients[105]. The studies demonstrated that Wnt/β-catenin can act as an intrinsic compensatory mechanism in response to BBB insult.

The Wnt/β-catenin pathway is also impacted by other pathological conditions. Activation of Wnt/β-catenin was reported as a compensatory mechanism to help preserve BBB function following cerebral ischemia in mouse models. Three hours after the stroke, nuclear β-catenin levels increased in brain endothelial cells and remained elevated up to 24 hours after the insult[121]. Treatment with XAV939, a Wnt inhibitor, reduced nuclear β-catenin in the isolated brain microvessels of cerebral ischemic mice. These studies suggest that Wnt/β-catenin activation may be beneficial in restoring BBB function following cerebral ischemia.

1.12. Regional differences of Wnt/β-catenin signaling in the brain

Studies suggest that the profile of Wnt ligands, receptor and modulators vary as a function of specific brain regions[85,104]. However, it is not known if this diversity also governs differential BBB tightness across the brain. Differential Wnt/β-catenin activity across the postnatal mouse brain was elegantly studied by Zhou et al[104]. In these studies, systematic deletion or mutation of Norrin (NDP), Frizzled-4, LRP-5, LRP-6 and β-catenin displayed different degrees of BBB dysfunction across different BBB regions. Pons, interpeduncular nuclei and cerebellum were the most responsive to Wnt/β-catenin perturbation and showed the greatest BBB disruption. On the other hand, the cortex was the least sensitive to reductions in Wnt/β-catenin activity. The brainstem and thalamus displayed intermediate sensitivity. Even within the cerebellum, there were differences with the molecular layer displaying more sensitivity towards Wnt/β-catenin reductions compared to the granule layer[104,183]. These findings suggested differential Wnt/β-catenin activity at the BBB across different brain region.

Regional differences of Wnt/β-catenin signaling were also seen with regard to Norrin expression. The highest expression of NDP was seen in cerebellum followed by olfactory bulb and brainstem[210]. Norrin expression in those regions was higher than in hippocampus and cerebral cortex. In agreement with that, Frizzled-4, a receptor for Norrin, is most highly expressed in the cerebellum[210]. Mutation on Norrin gene (NDP) impairs the barrier integrity mainly in the cerebellum and olfactory bulb with minimal BBB loss at cerebral cortex, striatum or thalamus[104,183]. Norrin/β-catenin signaling pathway contributes to BBB maintenance in cerebellum and olfactory bulb but less/minimal role in the maintenance of cerebral cortex, striatum and thalamus BBB[183].

The BBB heterogeneity is influenced in part by astrocytes as this cell can secrete different morphogens such as Wnt, Norrin and Shh[85,86,149,211]. As discussed previously, astrocytes have different subtypes from region to region. Protoplasmic astrocytes, interlaminar astrocytes and varicose projection astrocytes reside in grey matter while fibrous astrocytes reside in white matter[212]. What morphogens or Wnt isoforms are secreted by each astrocyte subtype is not well understood.

Apart from differential expression of Wnt components across brain regions, astrocyte content across the brain regions is also varied. Studies showed astrocyte content between white matter and grey matter correlated with their BBB permeability. *In vivo*, examination on both porcine and mouse brain indicated higher astrocyte content in white matter compared to grey matters. *Ex vivo*, primary brain endothelial cells isolated from white matter exhibited higher trans-endothelial electrical resistance (TEER) compared to those isolated from grey matter. The primary brain endothelial cell isolated from white matter exhibited higher expression of β-catenin, occludin, and claudin-5[213]. Although the microvessels from white matter and grey matter have different astrocyte

content, studies suggest that the pericytes content from those two brain regions are similar[213]. This suggests that regional differences in BBB permeability correlate with astrocyte content and may help explain differences in cerebral vascular permeability in white and grey matter.

1.12.1. Wnt β-catenin signaling in the hippocampus

Wnt signaling in the hippocampus attracts much interest due to its role in adult neurogenesis[192]. Yet, little information is available towards the correlation between Wnt/β-catenin signaling in this region and hippocampus BBB permeability. Studies done with conditional endothelial β-catenin specific deletion in adult mice have shown severe hemorrhage in the hippocampus suggesting the contribution of β-catenin signaling in maintaining adult BBB in the hippocampus[103].

The hippocampus expresses some essential Wnt ligands for adult neurogenesis[149,193,214]. For example, NSC and astrocytes in denta gyrus secreted Wnt3 and Wnt3a. The capacity of hippocampal astrocytes to produce these Wnt ligands is reduced by 20-30 times with age. This reduction coincides with a decline in adult neurogenesis[149,193]. Interestingly, the hippocampus is the brain region that often displays the first indications of BBB breakdown during aging[69]. Wnt3 and Wnt3a synthesis in denta gyrus astrocytes can be re-stimulated by exercise[149]. It is currently unknown if the exercise-induced Wnt3 and Wnt3a synthesis could alleviate the barrier breakdown during aging.

Within CA1 and CA3 of the hippocampus, there is BBB variability. Following ischemic insult, the capillaries in the CA3 showed lower BBB impairment compared to those in the CA1[215]. A similar effect was also reported in aging where CA3 showing lower BBB dysfunction compared to the CA1[216]. These studies suggest local factors in CA3 region may be involved in regulating the tightness of BBB. Post ischemia, CA1 region exhibited up-regulation of Dkk-1, an endogenous

Wnt/β-catenin inhibitor. Reduction of Wnt/β-catenin activity CA1 post global ischemia, reduced surviving expression and increased neuronal death[217-220]. In contrary, Dkk-1 expression post global ischemia was not detectable in CA3. Further evidence linking this Wnt modulator to the differential response following ischemia was the ability of Dkk-1 antisense oligonucleotide to reduce neuronal death in the CA1. These studies highlight the differential Wnt/β-catenin activity in CA1 and CA3 of hippocampus that determine the impact of ischemic insult to neuron survival[217].

1.12.2. Wnt/β-catenin signaling in the choroid plexus and circumventricular organs.

The circumventricular organs (CVO) are specialized structures located around the third and fourth ventricles. The CVO plays a substantial role in regulating water and sodium balance, baroreflex responses in the cardiovascular system, gastrointestinal hormone release, and immune response. The CVO consists of sensory and secretory structures. The sensory CVO includes area postrema (AP), subfornical organ (SFO), and organum vasculosum of the lamina terminalis (OVLT). The secretory CVO includes the pineal gland, median eminence and neurohypophysis[221]. Although CVO microvessels are permeable, the transport of low molecular weight (LMW) and high molecular weight (HMW) compounds have different mechanism. The LMW compound can pass through the capillaries, the inner and outer basement membranes. On the other hand, HMW compound could cross the brain capillaries and inner membrane basement but restricted by the outer membrane basement[222]. Within sensory CVO, tight junction protein is heterogeneously expressed. For example claudin-5, occludin and ZO-1 are almost absent in all sensory CVO but present at lateral zone of OVLT and area postrema[222]. The implication of these features is a selective permeability of sensory CVO.

The choroid plexus (CP) is a cluster of ependymal cells present in lateral, third and fourth ventricles that are tasked with the production and secretion of cerebrospinal fluid (CSF)[223]. The epithelial cells of the CP also form the blood-CFS barrier (BCSFB). The CP controls CSF composition by regulating the movement of ions and solutes into and out of the CSF. The restrictive nature of the BCSFB is mainly attributed to the specialized tight junction proteins, such as occludin, claudin-2, -9, -19 and -22 that form the intercellular junctions between the epithelial cells in the CP[224]. Epithelial cells of the choroid plexus express Pgp, MRP-1, MRP-4, MRP-5, Slco1a4, Slco1a5, Slco2b1, insulin receptor, and transthyretin receptor that regulate the passage of solutes and macromolecules between the blood and CSF[225]. In terms of water, aquaporin-1 (AQP-1) is involved in the exchange of water from the blood to CSF[226].

In contrast to other brain regions, the capillaries in the CP and CVOs are leaky and do not form a BBB[222,227]. The endothelial cells in the CP and CVOs are fenestrated, readily permeable to solutes and macromolecules, and are enriched with plasmalemma vesicle-associated protein (PLVAP)[31,228]. Based on these morphological features, one might predict low Wnt/β-catenin activity in the microvasculature of the CP and CVOs[31,104,229]. Indeed under normal conditions, choroid plexus and CVO brain endothelial cells show undetectable expression of LEF-1 suggesting negligible Wnt/β-catenin activity in these regions[31,229]. Gene reporter assays show no β-catenin activation at the CVO of mice examined at E13.5 and P21[31]. Studies also identified high expression of WIF-1 (Wnt inhibitory factor) in the area postrema within the CVO that would likely work to reduce any basal Wnt/β-catenin activity in the region[31].

Leaky endothelial cells in CVO and choroid plexus maintain their plasticity. Endothelial specific β-catenin stabilization at postnatal life converts leaky endothelial cell in those two regions to be restrictive capillaries. The more restrictive CVO microvessels is due to an increase in endothelial

claudin-5 and reduction in PLVAP expression[29,99,224]. In these areas, endothelial specific β-catenin stabilization plays an important role in helping to convert the leaky endothelial cells in this region to a more restrictive capillary phenotype[29,99,224]. These studies illustrate the responsiveness of the cerebral microvasculature to β-catenin and the ability to regionally modulate BBB phenotypes through this important cell-signaling pathway.

1.13. Impairment of Wnt/β-catenin signaling in the brain following chronic ethanol exposure

1.13.1. Effects of chronic ethanol exposure on blood-brain barrier function

A considerable number of studies have reported the dysregulation of the BBB following ethanol exposure both *in vivo* and *in vitro*[230-232]. As the severity of BBB dysfunction caused by ethanol varies depending on the frequency and magnitude of ethanol exposure, some discussion of the classification of alcohol consumption and resulting blood alcohol concentrations (BACs) is in order. In general, alcohol consumption can be described as moderate, binge or heavy drinking. According to the National Institute of Alcohol Abuse and Addiction (NIAAA), moderate drinking is defined by daily alcohol consumption that is the equivalent of one drink, for women, or two drinks, for men[233]. Typically blood alcohol concentrations in moderate drinkers are less than 0.05 g/dL. Heavy drinking is defined as weekly consumption of 15 drinks or more for men, and 8 drinks or more for women with BAC is above 0.15 g/dL[234,235]. Binge alcohol drinking is considered when an individual consumes five or more (men) or four or more (women) drinks within a period of two hours[236,237]. This type of ethanol consumption can result in BAC levels above the 0.08 g/dL legal limit for operating a motor vehicle in the USA and Canada[238].

With regard to binge drinking status, there is some ambiguity between the NIAAA definition and societal definitions. Binge alcohol drinking in society is defined by several consecutive days of drinking resulting in BACs far above 0.08g/dL[239]. Within the general population there is a

surprisingly high incidence of people with BACs far above the 0.08g/dL legal limit of intoxication. Studies examining a total of 381 patients presenting to the emergency department of Henepin County Minnesota, reported a wide range of BACs from below 0.08 g/dL to 0.42 g/dL. Of the 374 patients, 36 (nearly 10%) presented with BAC<0.099 g/dL, 129 patients (approximately 34%) had BAC of 0.100-0.199 g/dL, and 161 patients (approximately 43%) had BAC of 0.200-0.299 g/dL. Furthermore, 55 patients (approximately 15%) had BAC 0.30-0.39 g/dL and 3 patients had BAC > 0,4 g/dL[240]. In Sweden, examination of special drunk driving cases from what period 1989-1997 identified over 81 cases where the reported BAC was 0.4 g/dL or higher[241]. As BAC above 0.299 g/dL has a high likelihood of unconsciousness and acute alcohol poisoning, and BACs of 0.15 g/dL can result in impairment of coordination, response time and cognitive function that increase risk of physical harm[234], the findings of these studies are concerning.

The BACs achieved with either binge alcohol drinking or heavy alcohol drinking are sufficient to cause BBB dysfunction. In adult mice, a forced 5% alcohol diet for two weeks (resulting in BAC 0.15-0.2 g/dL) caused a dramatic disruption of BBB. With this animal model, the ten-fold increase in albumin entry into the brain was attributed to an increase in BBB vesicular transport in mice receiving ethanol[231]. In newborn pups from alcoholic mothers receiving an ethanol diet in their last trimester (resulting in BAC of 0.3 g/dL) displayed postnatal (P4-P9) vascular damage in the cortex was also observed[242].

Ethanol exposure also leads to BBB dysfunction in humans. Exposure of primary cultured human brain microvascular endothelial cells to ethanol (0.23 g/dL) for 48 hours impaired BBB integrity and was linked with increased MMP activity and reduced claudin-5 expression[243]. Post-mortem examination of brain tissue from patients with chronic alcohol abuse showed an enhanced brain neuro-inflammatory state. Similar to the rodent studies, molecular examination of the human post-

mortem brain tissue suggested an increased MMP-9 activity, degradation of claudin-5 and collagen type IV as well as increased leukocyte infiltration in the prefrontal cortex of alcoholic subjects[230]. Together, these studies suggest that exposure to BAC in the 0.16 - 0.24 g/dL range, are associated with impaired BBB integrity.

While the impact of binge and heavy drinking on BBB integrity predominantly point to reduced barrier function, exposure to reduced amounts of ethanol associated with moderate levels of consumption appear to have a lesser impact on BBB permeability. Ethanol exposure in cultured brain endothelial cells, at concentrations ranging from 10-25 mM (0.05-0.115 g/dL), did not alter paracellular barrier[244]. In agreement with these cell culture studies, prenatal ethanol exposure in pregnant rats resulting in BACs of around 0.14 g/dL in the pups, had no effect on BBB permeability to horseradish peroxidase in any of the brain regions examined[245]. Similarly, rats with free choice of drinking ethanol (15%) or water for 70 days showed no alteration in BBB permeability to sucrose (340Da) and dextrans (50-100kDa)[246]. These studies suggest that ethanol-induced BBB dysfunction is less likely to be present with moderate alcohol consumption.

1.13.2. Chronic ethanol exposure interferes with Wnt/β-catenin signaling in the brain

Besides impaired BBB function, chronic consumption of alcohol can be deleterious to adult neurogenesis, a process that is partly regulated by Wnt/β-catenin signaling[192,193,247]. Alcohol exposure interfered with the division and migration of pre-progenitor neurons and altered cognitive ability in non-human primates[248,249]. Studies in neonatal mice treated with a single 1 g/kg i.p dose of ethanol that produced BAC of 0.21 g/dL 3-hrs after injection, exhibited reduced β-catenin and increased phopho β-catenin Ser33/37/Thr41 in the hippocampus and neurocortex. As expected, β-catenin translocation to the nucleus was significantly reduced in the ethanol-exposed mice suggesting downregulation of β-catenin signaling in the hippocampus[250]. Separate *in vitro* studies

in fetal human brain neuronal stem cells (NSC) exposed to 100 mM (0.46 g/dL) for 96 hours reported reductions in several important proteins involved in Wnt/β-catenin signaling including Wnt3a, LRP-6, phospho LRP-6, β-catenin and phospho GSK3-Ser9. Downregulation of these important canonical Wnt components is consistent with an ethanol-induced reduction in Wnt/β-catenin activity in NSC[251]. Additional evidence that ethanol exposure reduces neurogenesis are the studies using hippocampal NPC isolated from rats pups whose mother was chronically exposed to ethanol. The NPC from the ethanol treatment group exhibited a reduction in Wnt3a expression as well as β-catenin binding to TCF compared to the control group[252]. Ethanol also increased phospho GSK3-β Tyr216 levels resulting in more active GSK3 and more substantial β-catenin degradation in NPC[251]. Together, these studies suggest that ethanol impaired Wnt/β-catenin activity in NPC or NSC, and may help explain the deleterious effect of chronic alcohol exposure on adult neurogenesis.

Despite several studies demonstrating alterations of brain Wnt activity following ethanol exposure, there is no specific information regarding Wnt activity in the blood-brain barrier following ethanol exposure. Given the importance of the Wnt signaling pathway to brain endothelial cell function and the observations that prolonged ethanol exposure impacts blood-brain barrier integrity[230-232], these two may be causatively linked. Furthermore, if this is indeed the case, pharmacological interventions directed at the Wnt/β-catenin signaling pathway may provide a therapeutic approach for treating alcohol related responses in the brain.

1.14. Pharmacological interventions that impact on β-catenin signaling.

Given the influence of aberrant Wnt/β-catenin signaling in various CNS pathologies, there is interest in identification of pharmacological agents that target the Wnt/β-catenin signaling process. Exploration of clinically approved drugs to activate Wnt/β-catenin signaling may also prove

beneficial for enhancing BBB function in pathological conditions where Wnt signaling is downregulated. Repurposing of therapeutic agents has gained attention as an easier and more straightforward path towards drug discovery. It is based on the premise that a new indication for an already approved drug circumvents two of the more common issues in drug development, namely toxicity and inadequate pharmacokinetics. The section below discusses approved CNS drugs with potential Wnt/β-catenin activation properties.

1.14.1. Glycogen synthase kinase-3 as downstream effect among psychiatric medication.

Psychiatric disorders such as mood disorders, bipolar disorders, depression, and schizophrenia have been associated with dysregulation of GSK3[253,254]. Not surprisingly, alterations in GSK-3 activity have been reported for many pharmacological agents that are used to treat these conditions. Several psychiatric drugs display GSK3α/β inhibitory properties by increasing GSK3α/β phosphorylation at Ser21 or Ser9. Examples include lithium chloride, various antipsychotics (clozapine, quetiapine, risperidone), tricyclic antidepressants (imipramine) and selective serotonin reuptake inhibitors (such as fluoxetine)[255-260].

While the above agents inhibit GSK3 by increasing phopho-GSK3α/β at Ser21/9, this is mechanistically different from the GSK3 inhibition triggered by Wnt/β-catenin signaling. GSK3 inhibition in Wnt-triggered β-catenin signaling involves sequestration of GSK3 inside MVB. This sequestration prevents GSK3 from phosphorylating β-catenin[128,261]. Evidence of this is the fact that Wnt3a remains capable of increasing β-catenin accumulation in the cells following site directed mutation in Ser21 of GSK3α and Ser9 of GSK3β that prevent phosphorylation of the enzyme[262]. Due to the different mechanism of GSK3 inhibition, it is imperative to examine whether β-catenin stabilization caused with pharmacological GSK3 inhibitors would mimic activation of Wnt/β-catenin signaling.

Numerous psychiatric drugs have been reported to inhibit GSK3 and promote β-catenin stabilization (summarized at Table 1.6). However, the β-catenin stabilization observed with these psychiatric drugs often required supra-therapeutic doses or chronic exposure[263,264]. For example clozapine treatment using a10μM (supra-therapeutic) concentration increased nuclear β-catenin in cultured SH-SY5Y cells[264]. Likewise, chronic exposure to haloperidol or clozapine increased GSK3 inhibition, leading to stabilized β-catenin and upregulation of Dvl-3 and Axin in mouse PFC[263]. Collectively, it appears that some psychiatric drugs are capable of inducing β-catenin stabilization, but may require higher doses or long-term exposure to produce the desired effects.

Table 1.6: Studies on the effect of therapeutic relevance concentration of CNS drugs to Wnt/β-catenin

Drug	System	Concentration and duration of exposure	Experimental Model	Effect on Wnt	Transcriptional activity studies	References
LiCl	In vitro	1 mM, 96 h, media changed every 24h	Cultured SY5Y cell line	(Immunoblotting) β-catenin ↑1.4-fold, pGSK3-β↑50%	TCF transcriptional activity ↑ 29%	[263]
	In vitro,	1 mM, 96h, media changed every 24h	Cultured PC12 cell line	(Immunoblotting) β-catenin ↑40%, pGSK3-β↑50%	No changes	[263]
	Postmortem human brain received chronic lithium,	No information	Postmortem brain tissue from chronic LiCl patients PFC; n of 5	No changed on β-catenin expression and pGSK3-β	N/A	[265]
	Mice	0.2% for 5 day followed by 0.4% for 10 day resulting 1 mEq/l plasma concentration	In vivo mouse hypothalamus	β-catenin ↑30%	Using BAT-Galactoside mice Amygdala ↑3.7 fold Hippocampus ↑1.6 fold Denta gyrus (not quantified)	[266]
	Rats	2.5 g/kg, 14 days, harvested 2 hour post final injection	*In vivo* rat prefrontal cortex	(Immunoblotting) β-catenin ↑1.28-fold pGSK3-α ↑56%, pGSK3-β↑51%	N/A	[263]
Haloperidol	Rats	0.5mg/kg; 14 days; harvested 2 hour post final injection	*In vivo* rat prefrontal cortex	(Immunoblotting) Dvl-3 ↑4.0-fold, pGSK3-α ↑1.75-fold pGSK3-β↑320% β-catenin ↑2.7-fold	N/A	[259]
	Rats	0.5 mg/kg; 14 days; harvested 2	*In vivo* rat striatum	(Immunoblotting) Dvl-3 ↑1.4-fold, pGSK3-α ↑250%	N/A	[259]

Drug	System	Concentration and duration of exposure	Experimental Model	Effect on Wnt	Transcriptional activity studies	References
		hour post final injection		pGSK3-β↑250% β-catenin ↑1.6-fold		
	Rats	0.5 mg/kg; 14 week, harvested 2 hour post final injection	*In vivo* rat prefrontal cortex	(Immunoblotting) Dvl-3 ↑4-fold, Axin-1 ↑1.8-fold, β-catenin ↑2-fold, pGSK3-α ↑2-fold, pGSK3-β↑350%	N/A	[263]
	Rats	1 mg/kg; 7 days; harvested 2 hour post final injection	*In vivo* rat striatum	(Immunoblotting) β-catenin ↑1.9-fold,	N/A	[267]
	Rats	1 mg/kg; 7 days; harvested 2 hour post final injection	*In vivo* rat prefrontal cortex	(Immunoblotting) β-catenin ↑2.2-fold,	N/A	[267]
	Rats	1 mg/kg; 28 days; harvested 2 hour post final injection	Prefrontal cortex	(Immunoblotting) β-catenin ↑1.8-fold, Acute administration have no effect on β-catenin	N/A	[267]
Clozapine	Rats	25 mg/kg, 14 days, harvested 2 hour post final injection	Prefrontal cortex	(Immunoblotting) Dvl-3 ↑4-fold, Axin-1 ↑1.6-fold, β-catenin ↑1.5-fold, pGSK3-α ↑200%, pGSK3-β↑300%	N/A	[263]
Clozapine	Rats	25 mg/kg; 14 days; harvested 2 hour post final injection	Prefrontal cortex	(Immunoblotting) Dvl-3 ↑3.5-fold, pGSK3-α no change pGSK3-β↑370% β-catenin ↑2.0-fold	N/A	[259]
Clozapine	Rats	25 mg/kg; 14 days; harvested 2 hour post final injection	Striatum	(Immunoblotting) Dvl-3 ↑no change pGSK3-α ↑70% pGSK3-β↑70% β-catenin no change	N/A	[259]
Clozapine	Rats	25 mg/kg; 7 days; harvested 2 hour post final injection	Prefrontal cortex	(Immunoblotting) β-catenin ↑1.5-fold,	N/A	[267]
Risperidone	Rats	0.9 mg/kg; 7 days; harvested 2 hour post final injection	Prefrontal cortex	(Immunoblotting) β-catenin ↑1.5-fold, pGSK3-β↑250%	N/A	[267]
Risperidone	Rats	0.9 mg/kg; 28 days; harvested 2 hour post final injection	Prefrontal cortex	(Immunoblotting) β-catenin ↑3.0-fold, Acute have no effect	N/A	[267]
Risperidone	Rats	2.0 mg/kg;7 days; harvested 2 hour post final injection	Prefrontal cortex	(Immunoblotting) β-catenin ↑2.3-fold,	N/A	[267]

Drug	System	Concentration and duration of exposure	Experimental Model	Effect on Wnt	Transcriptional activity studies	References
Fluoxetine	In vitro	1.0 μM, 48 h exposure	hippocampal NPC	(Immunoblotting) pGSK3-β↑80% Nuclear β-catenin ↑2.0-fold No change on cytosolic β-catenin	TOP/FLASH assay ↑ transcriptional activity by 4-fold	[255]
Fluoxetine	In vivo	10 mg/kg, 14 days	Prefrontal cortex	(Immunoblotting) pGSK3-β↑80% pGSK3-α↑80%	N/A	[259]
Fluoxetine	In vivo	10mg/kg, 14 days	Prefrontal cortex	(Immunoblotting) pGSK3-β↑75% pGSK3-α↑75%	N/A	[259]

N/A: not available

One of the issues with using pharmacological inhibitors of GSK3 may center on achieving the level of inhibition required for activation of Wnt/β-catenin signaling, as to mimic GSK3 inhibition following Wnt ligand activation, a nearly complete and sustained level of GSK3 inhibition is required. This was demonstrated by the studies of Doble *et al* where activation of Wnt/β-catenin signaling was only observed when GSK3 expression was depleted by 75% or greater using $GSK3\alpha^{(+/-)}/\beta^{(-/-)}$ or $GSK3\alpha^{(-/-)}/\beta^{(+/-)}$ mice[268]. The studies suggest that the level and duration of GSK3 inhibition dictate the activation of β-catenin signaling.

1.14.2. Lithium chloride.

Lithium (LiCl) is a classic mood stabilizer used in bipolar disease[269]. Lithium inhibits GSK3 through two possible mechanisms. First, it directly inhibits GSK3α/β by competing for the magnesium binding site on the kinase and increasing inhibitory phosphorylation of Ser21 or Ser9[270]. A second indirect effect has been proposed involving lithium disruption of the Akt-β-arrestin-PP2A complex, which in turn, allows Akt activation and GSK3 inhibition[271-273].

Under therapeutic conditions, the serum concentrations of lithium range from 0.5-1.0 mM. Such levels of lithium have been shown in multiple *in vitro* and *in vivo* models to result in reductions in

GSK3 activity of 25-50%[257,263,270,274,275]. In light of the anticipated brain concentrations of lithium in patients receiving LiCl, it is likely that the level of GSK3 inhibition obtained under normal therapeutic dosing parameters is insufficient to interfere with Wnt/β-catenin signaling. However, there is some evidence showing that prolonged lithium exposure, at therapeutically relevant doses, can indeed activate β-catenin signaling (Table 1.6). *In vivo* studies using a 15-day lithium treatment (0.2% LiCl for 5 days followed by 0.4% LiCl for 10 days) yielded plasma concentrations of LiCl of 0.97 ± 0.07 mEq/L[266]. This level of LiCl exposure increased TCF transcriptional activity in the denta gyrus, amygdala (3.7-fold) and hippocampus (1.6-fold)[266]. Furthermore, nine days repetitive treatment with LiCl (1.2 g/kg BW) resulting in serum lithium concentrations of 0.8 mM, were able to increase cytoplasmic β-catenin levels in the cortex and hippocampus by 1.6 to 2 fold, confirming the ability of therapeutically relevant levels of LiCl to stabilize β-catenin[276]. Together, these studies suggest that chronic exposure to therapeutically relevant concentrations of lithium may activate β-catenin signaling.

1.14.3. Atypical antipsychotics

The downstream effects of many of the neurological agents are not limited to the neuron but include brain endothelial cells, activated microglia and astrocytes[277]. An example of this are the antipsychotics drugs that produce their desired response by binding to dopamine-2 receptor (D2R) and 5HT receptors and some side effects through low affinity binding to α-adrenergic, muscarinic and histamine receptor[278]. The D2Rs are expressed in several neuronal cells[279,280], astrocytes, microglia[277] and various endothelial cell preparations including (HUVEC)[281], renal vasculature[282], coronary heart vessel[283] and human cerebral microvascular endothelial cells[284]. The presence of D2R in brain endothelial cells would allow the atypical antipsychotic agents to potentially trigger downstream effects in the brain endothelial cells.

The mechanism(s) by which antipsychotic drugs alter Wnt signaling, have focused predominantly on Akt/GSK3 pathway. Under normal conditions, D2R binding to dopamine results in the formation of a complex between PP2A (protein phosphatase-2), β-arrestin and Akt. This complex allows the dephosphorylation of Ser473 on Akt and results in inactivation of Akt and conversely, the activation of GSK3. Antipsychotics, as D2R antagonists, prevent the formation of AKT, PP2A, and β-arrestin complex, allowing Akt to remain in its active state to phosphorylate GSK3α/β at Ser 21 or Ser9[271,285]. In this regard, the Ser21 or Ser9 phosphorylation of GSK3α/β observed with many of the antipsychotic behave as pseudo-substrates that prevent the actual substrate from reaching the catalytic domain[208]. Consequently inhibition of GSK3 by antipsychotic agents would prevent β-catenin degradation and promote β-catenin stabilization. Risperidone, clozapine, and haloperidol are antipsychotic drugs that exhibit this GSK3 inhibitoryactivity[263,267].

Clozapine exhibited β-catenin stabilization activity *in vivo*[259,263,286]. GSK3 inhibition elicits by subcutaneous clozapine injection (dose 25 mg/kg, 7 days) is relatively high, reaching 3-3.7 fold [267]. *In vitro*, clozapine treatment at supra-therapeutic concentration (10 μM) increased nuclear β-catenin at SH-SY5Y cell[264]. GSK3 inhibition properties of risperidone are summarized at Table 1.6. Stabilization of β-catenin by risperidone has been reported following both chronic and sub-chronic treatment. For example, low dose (0.9 mg/kg) risperidone administration to rats increased prefrontal cortex (PFC) β-catenin level following 7 days and 28 days treatment but not in 24-hour treatment[267]. The GSK3 inhibition observed with risperidone was additive when imipramine or fluoxetine was concomitantly administered[256]. In the clinical setting, combination of two psychiatric drugs is a common practice when single therapy is insufficient in showing improvement[287]. However, the effect of psychiatric drug combination to the activity of Wnt/β-catenin signaling is currently unexplored.

1.14.4. Fluoxetine

Fluoxetine is SSRI drugs that have GSK3 inhibitory activity. The pharmacological target for fluoxetine is the serotonin transporter (SERT), where the agent acts by preventing serotonin reuptake by pre-synapstic neuron. The resulting increased availability of serotonin in the synapse leads to increased serotonin binding to 5HT receptor in post-synapse[288]. A downstream consequence of increased 5HT activation is GSK3 inhibition through the 5HT1A receptor/PI3K/Akt/GSK3β pathway[289].

The downstream effect of β-catenin stabilization by fluoxetine is better documented than risperidone and clozapine (summarized in Table 1.6). In embryonic NPC, fluoxetine (1 μM) increased GSK3 inhibition and nuclear β-catenin level. The activation of β-catenin signaling by fluoxetine was confirmed by TOP Flash assay where fluoxetine treatment increased TCF transcriptional activity by 4-fold. Furthermore, co-treatment of fluoxetine with XAV939, a Wnt inhibitor, reduced the TOP Flash/FOP Flash ratio[255]. *In vivo*, increased of GSK3 inhibition by fluoxetine was observed in the mice hippocampus, cortex, striatum and cerebellum of mice following i.p administration[256].

Previous studies suggest that fluoxetine can alleviate BBB dysfunction following transient global ischemia[288-290]. The mechanisms by which fluoxetine reduced BBB breakdown was attributed to a reduction in MMP activation and the prevention of laminin and occludin degradation[290]. Separate studies also showed that fluoxetine attenuated neuroinflammation following subarachnoid hemorrhage[291,292]. In this animal model, fluoxetine also inhibited MMP activation and reducing claudin-5 and occludin degradation[291]. These studies suggest, fluoxetine has a barrier protecting effect in various stroke models. However, it is currently unknown if the downstream β-catenin stabilization effect of fluoxetine would be observed in brain endothelial cells and could prove beneficial for restoring barrier properties

1.15. Statement of problem

Wnt/β-catenin signaling is essential in BBB development, maturation and maintenance. The signaling is complicated due to the redundancy of the ligands, receptors, co-receptors, modulators and transcription factors. Characterization of the essential Wnt signaling components in the BBB has been focused on the developmental phase. These studies have identified Wnt-7a and Wnt7b as important Wnt ligands in the developing brain and frizzled 4, -6, and -8 as important Wnt receptors in the developing BBB. However, the expression pattern of Wnt ligands, receptors and modulators in the postnatal brain and following the establishment of the BBB are currently unknown. As Wnt ligand production is diminished in the mature brain, the potential reliance on endothelial based Wnt ligands operating in an autocrine manner is a possibility. However critical studies determining whether brain endothelial cells are capable of sustaining Wnt/β-catenin activity and to what extent this endogenous Wnt activity can elicit a BBB phenotype remains to be established.

In vitro models of the BBB have been frequently used to answer various mechanistic questions related to BBB. The human cerebral microvessels endothelial cell line (hCMEC/D3) is one of the most common *in vitro* models used. While studies using the hCMEC/D3 culture model show Wnt/β-catenin activity[114,115,198], a complete profile of Wnt ligands, receptors and modulators and their correlation with barrier function remain to be determined. Thus this model provides a means to mechanistically address the various components involved in Wnt/β-catenin signaling and its role in maintaining barrier properties under normal and pathological conditions.

Binge and heavy alcohol drinking are associated with impairment of the blood-brain barrier. To date, scientists identified that prolonged ethanol exposure reduced Wnt/β-catenin in the brain in general. Diminished Wnt/β-catenin activity in the brain has also been observed in various

pathological conditions involving BBB breakdown. These include Alzheimer disease, Parkinson's disease and brain tumors. In addition to these conditions, ethanol abuse is associated with blood-brain barrier dysfunction and change in brain Wnt/β-catenin activity. However, the impact of ethanol to Wnt/β-catenin activity in the blood-brain barrier is currently unaddressed.

Wnt/β-catenin in the BBB is naturally re-activated following pathological conditions such as multiple sclerosis and cerebral ischemia as an adaptive response to BBB dysfunction. Furthermore, even in non-pathological settings, external activation of Wnt/β-catenin signaling has been shown to convert incompetent BBB in circumventricular organ and choroid plexus to be BBB competent. Together these findings suggest that activation of Wnt/β-catenin signaling can be used as a tool to improve BBB function during pathological conditions. However it is not known if Wnt/β-catenin activation can be used to reverse the negative effect of ethanol to BBB.

Activation of Wnt/β-catenin signaling can be achieved either using Wnt ligand or GSK3 inhibitor. To overcome the toxicity and pharmacokinetic issue, re-purposing of clinically approved drug can be used to identify an efficient and safe Wnt/β-catenin activator. Some antipsychotic drugs, SSRI and lithium chloride have been shown to inhibit GSK3 and increased nuclear β-catenin *in vivo*. However it is not known if therapeutic relevant concentrations of these drugs will be sufficient to activate Wnt/β-catenin signaling especially in the brain endothelial cells.

Based on current understanding of Wnt/b-catenin signaling in the brain the following hypotheses were postulated:

1. Brain endothelial cells have autocrine Wnt/β-catenin signaling that contributes to the maintenance of restrictive BBB permeability properties.
2. Chronic ethanol exposure leads to downregulation of Wnt/β-catenin activity in the BBB.

3. Activation of Wnt/β-catenin signaling can mitigate BBB breakdown following chronic ethanol exposure.

The main objectives of these studies were:

1. To establish an *in vitro* model for mechanistic examination of Wnt/β-catenin signaling and its impact on brain endothelial function and gene expression.
2. To examine Wnt/β-catenin activity at the blood-brain barrier following chronic ethanol exposure.
3. To examine the effects of Wnt/β-catenin modulation on barrier properties following ethanol exposure.
4. To examine the extent to which pharmacological agents used to treat neurological conditions maybe re-purposed for modulating Wnt/β-catenin signaling and BBB function.

1.16. References

1 Liddelow, S. A. Fluids and barriers of the CNS: a historical viewpoint. *Fluids and barriers of the CNS* **8**, 2, doi:10.1186/2045-8118-8-2 (2011).

2 Berndt, P. *et al.* Tight junction proteins at the blood-brain barrier: far more than claudin-5. *Cellular and molecular life sciences : CMLS* **76**, 1987-2002, doi:10.1007/s00018-019-03030-7 (2019).

3 Daneman, R. *et al.* The mouse blood-brain barrier transcriptome: a new resource for understanding the development and function of brain endothelial cells. *PloS one* **5**, e13741, doi:10.1371/journal.pone.0013741 (2010).

4 Ohtsuki, S., Yamaguchi, H., Katsukura, Y., Asashima, T. & Terasaki, T. mRNA expression levels of tight junction protein genes in mouse brain capillary endothelial cells highly purified by magnetic cell sorting. *Journal of neurochemistry* **104**, 147-154, doi:10.1111/j.1471-4159.2007.05008.x (2008).

5 Nitta, T. *et al.* Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *The Journal of cell biology* **161**, 653-660, doi:10.1083/jcb.200302070 (2003).

6 Castro Dias, M. *et al.* Claudin-12 is not required for blood-brain barrier tight junction function. *Fluids and barriers of the CNS* **16**, 30, doi:10.1186/s12987-019-0150-9 (2019).

7 Pfeiffer, F. *et al.* Claudin-1 induced sealing of blood-brain barrier tight junctions ameliorates chronic experimental autoimmune encephalomyelitis. *Acta neuropathologica* **122**, 601-614, doi:10.1007/s00401-011-0883-2 (2011).

8 Castro Dias, M. *et al.* Claudin-3-deficient C57BL/6J mice display intact brain barriers. *Scientific reports* **9**, 203, doi:10.1038/s41598-018-36731-3 (2019).

9 Steinemann, A., Galm, I., Chip, S., Nitsch, C. & Maly, I. P. Claudin-1, -2 and -3 Are Selectively Expressed in the Epithelia of the Choroid Plexus of the Mouse from Early Development and into Adulthood While Claudin-5 is Restricted to Endothelial Cells. *Frontiers in neuroanatomy* **10**, 16, doi:10.3389/fnana.2016.00016 (2016).

10 Saitou, M. *et al.* Complex phenotype of mice lacking occludin, a component of tight junction strands. *Molecular biology of the cell* **11**, 4131-4142, doi:10.1091/mbc.11.12.4131 (2000).

11 Abdul Muneer, P. M., Alikunju, S., Szlachetka, A. M. & Haorah, J. The mechanisms of cerebral vascular dysfunction and neuroinflammation by MMP-mediated degradation of VEGFR-2 in alcohol ingestion. *Arteriosclerosis, thrombosis, and vascular biology* **32**, 1167-1177, doi:10.1161/ATVBAHA.112.247668 (2012).

12 Liu, J., Jin, X., Liu, K. J. & Liu, W. Matrix metalloproteinase-2-mediated occludin degradation and caveolin-1-mediated claudin-5 redistribution contribute to blood-brain barrier damage in early ischemic stroke stage. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **32**, 3044-3057, doi:10.1523/JNEUROSCI.6409-11.2012 (2012).

13 Yang, Y. & Rosenberg, G. A. MMP-mediated disruption of claudin-5 in the blood-brain barrier of rat brain after cerebral ischemia. *Methods in molecular biology* **762**, 333-345, doi:10.1007/978-1-61779-185-7_24 (2011).

14 Vestweber, D. VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. *Arteriosclerosis, thrombosis, and vascular biology* **28**, 223-232, doi:10.1161/ATVBAHA.107.158014 (2008).

15 Pokutta, S. & Weis, W. I. Structure and mechanism of cadherins and catenins in cell-cell contacts. *Annual review of cell and developmental biology* **23**, 237-261, doi:10.1146/annurev.cellbio.22.010305.104241 (2007).

16 Heuberger, J. & Birchmeier, W. Interplay of cadherin-mediated cell adhesion and canonical Wnt signaling. *Cold Spring Harbor perspectives in biology* **2**, a002915, doi:10.1101/cshperspect.a002915 (2010).

17 Orsulic, S., Huber, O., Aberle, H., Arnold, S. & Kemler, R. E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation. *Journal of cell science* **112 (Pt 8)**, 1237-1245 (1999).

18 Nelson, W. J. & Nusse, R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* **303**, 1483-1487, doi:10.1126/science.1094291 (2004).

19 Rikitake, Y., Mandai, K. & Takai, Y. The role of nectins in different types of cell-cell adhesion. *Journal of cell science* **125**, 3713-3722, doi:10.1242/jcs.099572 (2012).

20 Hoshino, T. *et al.* Regulation of E-cadherin endocytosis by nectin through afadin, Rap1, and p120ctn. *The Journal of biological chemistry* **280**, 24095-24103, doi:10.1074/jbc.M414447200 (2005).

21 Sato, T. *et al.* Regulation of the assembly and adhesion activity of E-cadherin by nectin and afadin for the formation of adherens junctions in Madin-Darby canine kidney cells. *The Journal of biological chemistry* **281**, 5288-5299, doi:10.1074/jbc.M510070200 (2006).

22 Tunggal, J. A. *et al.* E-cadherin is essential for in vivo epidermal barrier function by regulating tight junctions. *The EMBO journal* **24**, 1146-1156, doi:10.1038/sj.emboj.7600605 (2005).

23 Capaldo, C. T. & Macara, I. G. Depletion of E-cadherin disrupts establishment but not maintenance of cell junctions in Madin-Darby canine kidney epithelial cells. *Molecular biology of the cell* **18**, 189-200, doi:10.1091/mbc.e06-05-0471 (2007).

24 Laksitorini, M. D. *et al.* Modulation of intercellular junctions by cyclic-ADT peptides as a method to reversibly increase blood-brain barrier permeability. *Journal of pharmaceutical sciences* **104**, 1065-1075, doi:10.1002/jps.24309 (2015).

25 On, N. H., Kiptoo, P., Siahaan, T. J. & Miller, D. W. Modulation of blood-brain barrier permeability in mice using synthetic E-cadherin peptide. *Molecular pharmaceutics* **11**, 974-981, doi:10.1021/mp400624v (2014).

26 Laksitorini, M., Prasasty, V. D., Kiptoo, P. K. & Siahaan, T. J. Pathways and progress in improving drug delivery through the intestinal mucosa and blood-brain barriers. *Therapeutic delivery* **5**, 1143-1163, doi:10.4155/tde.14.67 (2014).

27 Campbell, H. K., Maiers, J. L. & DeMali, K. A. Interplay between tight junctions & adherens junctions. *Experimental cell research* **358**, 39-44, doi:10.1016/j.yexcr.2017.03.061 (2017).

28 Taddei, A. *et al.* Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. *Nature cell biology* **10**, 923-934, doi:10.1038/ncb1752 (2008).

29 Tietz, S. & Engelhardt, B. Brain barriers: Crosstalk between complex tight junctions and adherens junctions. *The Journal of cell biology* **209**, 493-506, doi:10.1083/jcb.201412147 (2015).

30 Gordon, L. *et al.* The fenestrae-associated protein Plvap regulates the rate of blood-borne protein passage into the hypophysis. *Development* **146**, doi:10.1242/dev.177790 (2019).

31 Wang, Y. *et al.* Beta-catenin signaling regulates barrier-specific gene expression in circumventricular organ and ocular vasculatures. *eLife* **8**, doi:10.7554/eLife.43257 (2019).

32 Shue, E. H. *et al.* Plasmalemmal vesicle associated protein-1 (PV-1) is a marker of blood-brain barrier disruption in rodent models. *BMC neuroscience* **9**, 29, doi:10.1186/1471-2202-9-29 (2008).

33 Bosma, E. K., van Noorden, C. J. F., Schlingemann, R. O. & Klaassen, I. The role of plasmalemma vesicle-associated protein in pathological breakdown of blood-brain and blood-retinal barriers: potential novel therapeutic target for cerebral edema and diabetic macular edema. *Fluids and barriers of the CNS* **15**, 24, doi:10.1186/s12987-018-0109-2 (2018).

34 Leenstra, S. *et al.* Endothelial cell marker PAL-E reactivity in brain tumor, developing brain, and brain disease. *Cancer* **72**, 3061-3067, doi:10.1002/1097-0142(19931115)72:10<3061::aid-cncr2820721031>3.0.co;2-6 (1993).

35 Carson-Walter, E. B. *et al.* Plasmalemmal vesicle associated protein-1 is a novel marker implicated in brain tumor angiogenesis. *Clinical cancer research : an official journal of the American Association for Cancer Research* **11**, 7643-7650, doi:10.1158/1078-0432.CCR-05-1099 (2005).

36 Xiao, G. & Gan, L. S. Receptor-mediated endocytosis and brain delivery of therapeutic biologics. *International journal of cell biology* **2013**, 703545, doi:10.1155/2013/703545 (2013).

37 de Boer, A. G., van der Sandt, I. C. & Gaillard, P. J. The role of drug transporters at the blood-brain barrier. *Annual review of pharmacology and toxicology* **43**, 629-656, doi:10.1146/annurev.pharmtox.43.100901.140204 (2003).

38 Villasenor, R., Lampe, J., Schwaninger, M. & Collin, L. Intracellular transport and regulation of transcytosis across the blood-brain barrier. *Cellular and molecular life sciences : CMLS* **76**, 1081-1092, doi:10.1007/s00018-018-2982-x (2019).

39 Smith, M. W. & Gumbleton, M. Endocytosis at the blood-brain barrier: from basic understanding to drug delivery strategies. *Journal of drug targeting* **14**, 191-214, doi:10.1080/10611860600650086 (2006).

40 Shin, T. *et al.* Expression of caveolin-1, -2, and -3 in the spinal cords of Lewis rats with experimental autoimmune encephalomyelitis. *Journal of neuroimmunology* **165**, 11-20, doi:10.1016/j.jneuroim.2005.03.019 (2005).

41 Jasmin, J. F. *et al.* Caveolin-1 deficiency increases cerebral ischemic injury. *Circulation research* **100**, 721-729, doi:10.1161/01.RES.0000260180.42709.29 (2007).

42 Ben-Zvi, A. *et al.* Mfsd2a is critical for the formation and function of the blood-brain barrier. *Nature* **509**, 507-511, doi:10.1038/nature13324 (2014).

43 Nguyen, L. N. *et al.* Mfsd2a is a transporter for the essential omega-3 fatty acid docosahexaenoic acid. *Nature* **509**, 503-506, doi:10.1038/nature13241 (2014).

44 Wong, B. H. *et al.* Mfsd2a Is a Transporter for the Essential omega-3 Fatty Acid Docosahexaenoic Acid (DHA) in Eye and Is Important for Photoreceptor Cell Development. *The Journal of biological chemistry* **291**, 10501-10514, doi:10.1074/jbc.M116.721340 (2016).

45 Andreone, B. J. *et al.* Blood-Brain Barrier Permeability Is Regulated by Lipid Transport-Dependent Suppression of Caveolae-Mediated Transcytosis. *Neuron* **94**, 581-594 e585, doi:10.1016/j.neuron.2017.03.043 (2017).

46 Yang, Y. R. *et al.* Mfsd2a (Major Facilitator Superfamily Domain Containing 2a) Attenuates Intracerebral Hemorrhage-Induced Blood-Brain Barrier Disruption by Inhibiting Vesicular Transcytosis. *Journal of the American Heart Association* **6**, doi:10.1161/JAHA.117.005811 (2017).

47 Pardridge, W. M. Drug transport across the blood-brain barrier. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* **32**, 1959-1972, doi:10.1038/jcbfm.2012.126 (2012).

48 Pulgar, V. M. Transcytosis to Cross the Blood Brain Barrier, New Advancements and Challenges. *Frontiers in neuroscience* **12**, 1019, doi:10.3389/fnins.2018.01019 (2018).

49 Jones, A. R. & Shusta, E. V. Blood-brain barrier transport of therapeutics via receptor-mediation. *Pharmaceutical research* **24**, 1759-1771, doi:10.1007/s11095-007-9379-0 (2007).

50 Herve, F., Ghinea, N. & Scherrmann, J. M. CNS delivery via adsorptive transcytosis. *The AAPS journal* **10**, 455-472, doi:10.1208/s12248-008-9055-2 (2008).

51 Sanchez-Covarrubias, L., Slosky, L. M., Thompson, B. J., Davis, T. P. & Ronaldson, P. T. Transporters at CNS barrier sites: obstacles or opportunities for drug delivery? *Current pharmaceutical design* **20**, 1422-1449, doi:10.2174/13816128113199990463 (2014).

52 Lu, W. Adsorptive-mediated brain delivery systems. *Current pharmaceutical biotechnology* **13**, 2340-2348, doi:10.2174/138920112803341851 (2012).

53 Poduslo, J. F. & Curran, G. L. Increased permeability of superoxide dismutase at the blood-nerve and blood-brain barriers with retained enzymatic activity after covalent modification with the naturally occurring polyamine, putrescine. *Journal of neurochemistry* **67**, 734-741, doi:10.1046/j.1471-4159.1996.67020734.x (1996).

54 Pardridge, W. M. Molecular Trojan horses for blood-brain barrier drug delivery. *Discovery medicine* **6**, 139-143 (2006).

55 Dos Santos Rodrigues, B., Kanekiyo, T. & Singh, J. ApoE-2 Brain-Targeted Gene Therapy Through Transferrin and Penetratin Tagged Liposomal Nanoparticles. *Pharmaceutical research* **36**, 161, doi:10.1007/s11095-019-2691-7 (2019).

56 Mahringer, A. & Fricker, G. ABC transporters at the blood-brain barrier. *Expert opinion on drug metabolism & toxicology* **12**, 499-508, doi:10.1517/17425255.2016.1168804 (2016).

57 Miller, D. S. ABC transporter regulation by signaling at the blood-brain barrier: relevance to pharmacology. *Advances in pharmacology* **71**, 1-24, doi:10.1016/bs.apha.2014.06.008 (2014).

58 Geier, E. G. *et al.* Profiling solute carrier transporters in the human blood-brain barrier. *Clinical pharmacology and therapeutics* **94**, 636-639, doi:10.1038/clpt.2013.175 (2013).

59 Uchida, Y. *et al.* Quantitative targeted absolute proteomics of human blood-brain barrier transporters and receptors. *Journal of neurochemistry* **117**, 333-345, doi:10.1111/j.1471-4159.2011.07208.x (2011).

60 Ohtsuki, S. *et al.* Quantitative targeted absolute proteomic analysis of transporters, receptors and junction proteins for validation of human cerebral microvascular endothelial cell line hCMEC/D3 as a human blood-brain barrier model. *Molecular pharmaceutics* **10**, 289-296, doi:10.1021/mp3004308 (2013).

61 Qosa, H., Miller, D. S., Pasinelli, P. & Trotti, D. Regulation of ABC efflux transporters at blood-brain barrier in health and neurological disorders. *Brain research* **1628**, 298-316, doi:10.1016/j.brainres.2015.07.005 (2015).

62 Zelcer, N. *et al.* Mice lacking multidrug resistance protein 3 show altered morphine pharmacokinetics and morphine-6-glucuronide antinociception. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 7274-7279, doi:10.1073/pnas.0502530102 (2005).

63 Su, W. & Pasternak, G. W. The role of multidrug resistance-associated protein in the blood-brain barrier and opioid analgesia. *Synapse* **67**, 609-619, doi:10.1002/syn.21667 (2013).

64 Cisternino, S., Mercier, C., Bourasset, F., Roux, F. & Scherrmann, J. M. Expression, up-regulation, and transport activity of the multidrug-resistance protein Abcg2 at the mouse blood-brain barrier. *Cancer research* **64**, 3296-3301, doi:10.1158/0008-5472.can-03-2033 (2004).

65 Kwei, G. Y. *et al.* Disposition of ivermectin and cyclosporin A in CF-1 mice deficient in mdr1a P-glycoprotein. *Drug metabolism and disposition: the biological fate of chemicals* **27**, 581-587 (1999).

66 Kageyama, T. *et al.* The 4F2hc/LAT1 complex transports L-DOPA across the blood-brain barrier. *Brain research* **879**, 115-121, doi:10.1016/s0006-8993(00)02758-x (2000).

67 Breedveld, P. *et al.* The effect of Bcrp1 (Abcg2) on the in vivo pharmacokinetics and brain penetration of imatinib mesylate (Gleevec): implications for the use of breast cancer resistance protein and P-glycoprotein inhibitors to enable the brain penetration of imatinib in patients. *Cancer research* **65**, 2577-2582, doi:10.1158/0008-5472.CAN-04-2416 (2005).

68 Oostendorp, R. L., Buckle, T., Beijnen, J. H., van Tellingen, O. & Schellens, J. H. The effect of P-gp (Mdr1a/1b), BCRP (Bcrp1) and P-gp/BCRP inhibitors on the in vivo absorption, distribution, metabolism and excretion of imatinib. *Investigational new drugs* **27**, 31-40, doi:10.1007/s10637-008-9138-z (2009).

69 Montagne, A., Zhao, Z. & Zlokovic, B. V. Alzheimer's disease: A matter of blood-brain barrier dysfunction? *The Journal of experimental medicine* **214**, 3151-3169, doi:10.1084/jem.20171406 (2017).

70 Horwood, N. & Davies, D. C. Immunolabelling of hippocampal microvessel glucose transporter protein is reduced in Alzheimer's disease. *Virchows Archiv : an international journal of pathology* **425**, 69-72, doi:10.1007/bf00193951 (1994).

71 Kalaria, R. N. & Harik, S. I. Reduced glucose transporter at the blood-brain barrier and in cerebral cortex in Alzheimer disease. *Journal of neurochemistry* **53**, 1083-1088, doi:10.1111/j.1471-4159.1989.tb07399.x (1989).

72 Winkler, E. A. *et al.* GLUT1 reductions exacerbate Alzheimer's disease vasculo-neuronal dysfunction and degeneration. *Nature neuroscience* **18**, 521-530, doi:10.1038/nn.3966 (2015).

73 Poo-Arguelles, P. *et al.* X-Linked creatine transporter deficiency in two patients with severe mental retardation and autism. *Journal of inherited metabolic disease* **29**, 220-223, doi:10.1007/s10545-006-0212-4 (2006).

74 Salomons, G. S. *et al.* X-linked creatine transporter defect: an overview. *Journal of inherited metabolic disease* **26**, 309-318, doi:10.1023/a:1024405821638 (2003).

75 Beard, E. & Braissant, O. Synthesis and transport of creatine in the CNS: importance for cerebral functions. *Journal of neurochemistry* **115**, 297-313, doi:10.1111/j.1471-4159.2010.06935.x (2010).

76 Baroncelli, L. *et al.* A mouse model for creatine transporter deficiency reveals early onset cognitive impairment and neuropathology associated with brain aging. *Human molecular genetics* **25**, 4186-4200, doi:10.1093/hmg/ddw252 (2016).

77 De Giorgis, V. & Veggiotti, P. GLUT1 deficiency syndrome 2013: current state of the art. *Seizure* **22**, 803-811, doi:10.1016/j.seizure.2013.07.003 (2013).

78 Tang, M. *et al.* Brain microvasculature defects and Glut1 deficiency syndrome averted by early repletion of the glucose transporter-1 protein. *Nature communications* **8**, 14152, doi:10.1038/ncomms14152 (2017).

79 Oldendorf, W. H., Cornford, M. E. & Brown, W. J. The large apparent work capability of the blood-brain barrier: a study of the mitochondrial content of capillary endothelial cells in brain and other tissues of the rat. *Annals of neurology* **1**, 409-417, doi:10.1002/ana.410010502 (1977).

80 Claudio, L. Ultrastructural features of the blood-brain barrier in biopsy tissue from Alzheimer's disease patients. *Acta neuropathologica* **91**, 6-14, doi:10.1007/s004010050386 (1996).

81 Stewart, P. A., Hayakawa, K., Akers, M. A. & Vinters, H. V. A morphometric study of the blood-brain barrier in Alzheimer's disease. *Laboratory investigation; a journal of technical methods and pathology* **67**, 734-742 (1992).

82 Doll, D. N. *et al.* Mitochondrial crisis in cerebrovascular endothelial cells opens the blood-brain barrier. *Stroke* **46**, 1681-1689, doi:10.1161/STROKEAHA.115.009099 (2015).
83 Stewart, P. A. & Wiley, M. J. Developing nervous tissue induces formation of blood-brain barrier characteristics in invading endothelial cells: a study using quail--chick transplantation chimeras. *Developmental biology* **84**, 183-192 (1981).
84 Ikeda, E., Flamme, I. & Risau, W. Developing brain cells produce factors capable of inducing the HT7 antigen, a blood-brain barrier-specific molecule, in chick endothelial cells. *Neuroscience letters* **209**, 149-152 (1996).
85 Engelhardt, B. & Liebner, S. Novel insights into the development and maintenance of the blood-brain barrier. *Cell and tissue research* **355**, 687-699, doi:10.1007/s00441-014-1811-2 (2014).
86 Wevers, N. R. & de Vries, H. E. Morphogens and blood-brain barrier function in health and disease. *Tissue barriers* **4**, e1090524, doi:10.1080/21688370.2015.1090524 (2016).
87 Stenman, J. M. *et al.* Canonical Wnt signaling regulates organ-specific assembly and differentiation of CNS vasculature. *Science* **322**, 1247-1250, doi:10.1126/science.1164594 (2008).
88 Daneman, R., Zhou, L., Kebede, A. A. & Barres, B. A. Pericytes are required for blood-brain barrier integrity during embryogenesis. *Nature* **468**, 562-566, doi:10.1038/nature09513 (2010).
89 Daneman, R. *et al.* Wnt/beta-catenin signaling is required for CNS, but not non-CNS, angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 641-646, doi:10.1073/pnas.0805165106 (2009).
90 Obermeier, B., Daneman, R. & Ransohoff, R. M. Development, maintenance and disruption of the blood-brain barrier. *Nature medicine* **19**, 1584-1596, doi:10.1038/nm.3407 (2013).
91 Saunders, N. R., Liddelow, S. A. & Dziegielewska, K. M. Barrier mechanisms in the developing brain. *Frontiers in pharmacology* **3**, 46, doi:10.3389/fphar.2012.00046 (2012).
92 Ek, C. J., Dziegielewska, K. M., Habgood, M. D. & Saunders, N. R. Barriers in the developing brain and Neurotoxicology. *Neurotoxicology* **33**, 586-604, doi:10.1016/j.neuro.2011.12.009 (2012).
93 Ek, C. J., Dziegielewska, K. M., Stolp, H. & Saunders, N. R. Functional effectiveness of the blood-brain barrier to small water-soluble molecules in developing and adult opossum (Monodelphis domestica). *The Journal of comparative neurology* **496**, 13-26, doi:10.1002/cne.20885 (2006).
94 Johansson, P. A. *et al.* Blood-CSF barrier function in the rat embryo. *The European journal of neuroscience* **24**, 65-76, doi:10.1111/j.1460-9568.2006.04904.x (2006).
95 Hallmann, R., Mayer, D. N., Berg, E. L., Broermann, R. & Butcher, E. C. Novel mouse endothelial cell surface marker is suppressed during differentiation of the blood brain barrier. *Developmental dynamics : an official publication of the American Association of Anatomists* **202**, 325-332, doi:10.1002/aja.1002020402 (1995).
96 Hellstrom, M., Kalen, M., Lindahl, P., Abramsson, A. & Betsholtz, C. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development* **126**, 3047-3055 (1999).
97 Winkler, E. A., Bell, R. D. & Zlokovic, B. V. Pericyte-specific expression of PDGF beta receptor in mouse models with normal and deficient PDGF beta receptor signaling. *Molecular neurodegeneration* **5**, 32, doi:10.1186/1750-1326-5-32 (2010).
98 Daneman, R. & Prat, A. The blood-brain barrier. *Cold Spring Harbor perspectives in biology* **7**, a020412, doi:10.1101/cshperspect.a020412 (2015).
99 Strazielle, N. & Ghersi-Egea, J. F. Efflux transporters in blood-brain interfaces of the developing brain. *Frontiers in neuroscience* **9**, 21, doi:10.3389/fnins.2015.00021 (2015).
100 Qin, Y. & Sato, T. N. Mouse multidrug resistance 1a/3 gene is the earliest known endothelial cell differentiation marker during blood-brain barrier development. *Developmental dynamics : an official publication of the American Association of Anatomists* **202**, 172-180, doi:10.1002/aja.1002020209 (1995).
101 Harati, R., Benech, H., Villegier, A. S. & Mabondzo, A. P-glycoprotein, breast cancer resistance protein, Organic Anion Transporter 3, and Transporting Peptide 1a4 during blood-brain barrier

maturation: involvement of Wnt/beta-catenin and endothelin-1 signaling. *Molecular pharmaceutics* **10**, 1566-1580, doi:10.1021/mp300334r (2013).

102 Liebner, S. *et al.* Wnt/beta-catenin signaling controls development of the blood-brain barrier. *The Journal of cell biology* **183**, 409-417, doi:10.1083/jcb.200806024 (2008).

103 Tran, K. A. *et al.* Endothelial beta-Catenin Signaling Is Required for Maintaining Adult Blood-Brain Barrier Integrity and Central Nervous System Homeostasis. *Circulation* **133**, 177-186, doi:10.1161/CIRCULATIONAHA.115.015982 (2016).

104 Zhou, Y. *et al.* Canonical WNT signaling components in vascular development and barrier formation. *The Journal of clinical investigation* **124**, 3825-3846, doi:10.1172/JCI76431 (2014).

105 Lengfeld, J. E. *et al.* Endothelial Wnt/beta-catenin signaling reduces immune cell infiltration in multiple sclerosis. *Proceedings of the National Academy of Sciences of the United States of America* **114**, E1168-E1177, doi:10.1073/pnas.1609905114 (2017).

106 Vallee, A., Vallee, J. N., Guillevin, R. & Lecarpentier, Y. Interactions Between the Canonical WNT/Beta-Catenin Pathway and PPAR Gamma on Neuroinflammation, Demyelination, and Remyelination in Multiple Sclerosis. *Cellular and molecular neurobiology* **38**, 783-795, doi:10.1007/s10571-017-0550-9 (2018).

107 Liu, L., Wan, W., Xia, S., Kalionis, B. & Li, Y. Dysfunctional Wnt/beta-catenin signaling contributes to blood-brain barrier breakdown in Alzheimer's disease. *Neurochemistry international* **75**, 19-25, doi:10.1016/j.neuint.2014.05.004 (2014).

108 Purro, S. A., Galli, S. & Salinas, P. C. Dysfunction of Wnt signaling and synaptic disassembly in neurodegenerative diseases. *Journal of molecular cell biology* **6**, 75-80, doi:10.1093/jmcb/mjt049 (2014).

109 Tapia-Rojas, C. & Inestrosa, N. C. Loss of canonical Wnt signaling is involved in the pathogenesis of Alzheimer's disease. *Neural regeneration research* **13**, 1705-1710, doi:10.4103/1673-5374.238606 (2018).

110 Lim, R. G. *et al.* Huntington's Disease iPSC-Derived Brain Microvascular Endothelial Cells Reveal WNT-Mediated Angiogenic and Blood-Brain Barrier Deficits. *Cell reports* **19**, 1365-1377, doi:10.1016/j.celrep.2017.04.021 (2017).

111 Inestrosa, N. C. & Arenas, E. Emerging roles of Wnts in the adult nervous system. *Nature reviews. Neuroscience* **11**, 77-86, doi:10.1038/nrn2755 (2010).

112 Zuccarini, M. *et al.* The Role of Wnt Signal in Glioblastoma Development and Progression: A Possible New Pharmacological Target for the Therapy of This Tumor. *Genes* **9**, doi:10.3390/genes9020105 (2018).

113 Huang, M. *et al.* Wnt-mediated endothelial transformation into mesenchymal stem cell-like cells induces chemoresistance in glioblastoma. *Science translational medicine* **12**, doi:10.1126/scitranslmed.aay7522 (2020).

114 Pinzon-Daza, M. L. *et al.* The cross-talk between canonical and non-canonical Wnt-dependent pathways regulates P-glycoprotein expression in human blood-brain barrier cells. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* **34**, 1258-1269, doi:10.1038/jcbfm.2014.100 (2014).

115 Lim, J. C. *et al.* Activation of beta-catenin signalling by GSK-3 inhibition increases p-glycoprotein expression in brain endothelial cells. *Journal of neurochemistry* **106**, 1855-1865, doi:10.1111/j.1471-4159.2008.05537.x (2008).

116 Kania, K. D., Wijesuriya, H. C., Hladky, S. B. & Barrand, M. A. Beta amyloid effects on expression of multidrug efflux transporters in brain endothelial cells. *Brain research* **1418**, 1-11, doi:10.1016/j.brainres.2011.08.044 (2011).

117 Shawahna, R. *et al.* Effect of Long-term In Vitro Lithium Exposure on mRNA Levels of Claudin-3, CYP1A1, ABCG2 and GSTM3 Genes in the hCMEC/D3 Human Brain Endothelial Cell Line. *European journal of drug metabolism and pharmacokinetics* **42**, 1013-1017, doi:10.1007/s13318-017-0412-3 (2017).

118 Ramirez, S. H. *et al.* Inhibition of glycogen synthase kinase 3beta promotes tight junction stability in brain endothelial cells by half-life extension of occludin and claudin-5. *PloS one* **8**, e55972, doi:10.1371/journal.pone.0055972 (2013).

119 Paolinelli, R. *et al.* Wnt activation of immortalized brain endothelial cells as a tool for generating a standardized model of the blood brain barrier in vitro. *PloS one* **8**, e70233, doi:10.1371/journal.pone.0070233 (2013).

120 Wang, Y. *et al.* Interplay of the Norrin and Wnt7a/Wnt7b signaling systems in blood-brain barrier and blood-retina barrier development and maintenance. *Proceedings of the National Academy of Sciences of the United States of America* **115**, E11827-E11836, doi:10.1073/pnas.1813217115 (2018).

121 Jean LeBlanc, N. *et al.* Canonical Wnt Pathway Maintains Blood-Brain Barrier Integrity upon Ischemic Stroke and Its Activation Ameliorates Tissue Plasminogen Activator Therapy. *Molecular neurobiology* **56**, 6521-6538, doi:10.1007/s12035-019-1539-9 (2019).

122 Laksitorini, M. D., Yathindranath, V., Xiong, W., Hombach-Klonisch, S. & Miller, D. W. Modulation of Wnt/beta-catenin signaling promotes blood-brain barrier phenotype in cultured brain endothelial cells. *Scientific reports* **9**, 19718, doi:10.1038/s41598-019-56075-w (2019).

123 Herrnberger, L. *et al.* Lack of endothelial diaphragms in fenestrae and caveolae of mutant Plvap-deficient mice. *Histochemistry and cell biology* **138**, 709-724, doi:10.1007/s00418-012-0987-3 (2012).

124 Chan, J. P. *et al.* The lysolipid transporter Mfsd2a regulates lipogenesis in the developing brain. *PLoS biology* **16**, e2006443, doi:10.1371/journal.pbio.2006443 (2018).

125 Martowicz, A. *et al.* Endothelial beta-Catenin Signaling Supports Postnatal Brain and Retinal Angiogenesis by Promoting Sprouting, Tip Cell Formation, and VEGFR (Vascular Endothelial Growth Factor Receptor) 2 Expression. *Arteriosclerosis, thrombosis, and vascular biology* **39**, 2273-2288, doi:10.1161/ATVBAHA.119.312749 (2019).

126 Sabbagh, M. F. & Nathans, J. A genome-wide view of the de-differentiation of central nervous system endothelial cells in culture. *eLife* **9**, doi:10.7554/eLife.51276 (2020).

127 Sabbagh, M. F. *et al.* Transcriptional and epigenomic landscapes of CNS and non-CNS vascular endothelial cells. *eLife* **7**, doi:10.7554/eLife.36187 (2018).

128 Taelman, V. F. *et al.* Wnt signaling requires sequestration of glycogen synthase kinase 3 inside multivesicular endosomes. *Cell* **143**, 1136-1148, doi:10.1016/j.cell.2010.11.034 (2010).

129 Mikels, A. J. & Nusse, R. Wnts as ligands: processing, secretion and reception. *Oncogene* **25**, 7461-7468, doi:10.1038/sj.onc.1210053 (2006).

130 Gordon, M. D. & Nusse, R. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. *The Journal of biological chemistry* **281**, 22429-22433, doi:10.1074/jbc.R600015200 (2006).

131 Valkenburg, K. C., Graveel, C. R., Zylstra-Diegel, C. R., Zhong, Z. & Williams, B. O. Wnt/beta-catenin Signaling in Normal and Cancer Stem Cells. *Cancers* **3**, 2050-2079, doi:10.3390/cancers3022050 (2011).

132 Niehrs, C. The complex world of WNT receptor signalling. *Nature reviews. Molecular cell biology* **13**, 767-779, doi:10.1038/nrm3470 (2012).

133 Kam, Y. & Quaranta, V. Cadherin-bound beta-catenin feeds into the Wnt pathway upon adherens junctions dissociation: evidence for an intersection between beta-catenin pools. *PloS one* **4**, e4580, doi:10.1371/journal.pone.0004580 (2009).

134 Stamos, J. L. & Weis, W. I. The beta-catenin destruction complex. *Cold Spring Harbor perspectives in biology* **5**, a007898, doi:10.1101/cshperspect.a007898 (2013).

135 Hinck, L., Nelson, W. J. & Papkoff, J. Wnt-1 modulates cell-cell adhesion in mammalian cells by stabilizing beta-catenin binding to the cell adhesion protein cadherin. *The Journal of cell biology* **124**, 729-741, doi:10.1083/jcb.124.5.729 (1994).

136 Ding, V. W., Chen, R. H. & McCormick, F. Differential regulation of glycogen synthase kinase 3beta by insulin and Wnt signaling. *The Journal of biological chemistry* **275**, 32475-32481, doi:10.1074/jbc.M005342200 (2000).
137 Cruciat, C. M. & Niehrs, C. Secreted and transmembrane wnt inhibitors and activators. *Cold Spring Harbor perspectives in biology* **5**, a015081, doi:10.1101/cshperspect.a015081 (2013).
138 Logan, C. Y. & Nusse, R. The Wnt signaling pathway in development and disease. *Annual review of cell and developmental biology* **20**, 781-810, doi:10.1146/annurev.cellbio.20.010403.113126 (2004).
139 Oliva, C. A., Vargas, J. Y. & Inestrosa, N. C. Wnts in adult brain: from synaptic plasticity to cognitive deficiencies. *Frontiers in cellular neuroscience* **7**, 224, doi:10.3389/fncel.2013.00224 (2013).
140 Goodwin, A. M., Sullivan, K. M. & D'Amore, P. A. Cultured endothelial cells display endogenous activation of the canonical Wnt signaling pathway and express multiple ligands, receptors, and secreted modulators of Wnt signaling. *Developmental dynamics : an official publication of the American Association of Anatomists* **235**, 3110-3120, doi:10.1002/dvdy.20939 (2006).
141 Cadigan, K. M. & Waterman, M. L. TCF/LEFs and Wnt signaling in the nucleus. *Cold Spring Harbor perspectives in biology* **4**, doi:10.1101/cshperspect.a007906 (2012).
142 Voloshanenko, O., Gmach, P., Winter, J., Kranz, D. & Boutros, M. Mapping of Wnt-Frizzled interactions by multiplex CRISPR targeting of receptor gene families. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **31**, 4832-4844, doi:10.1096/fj.201700144R (2017).
143 Chavali, M. *et al.* Non-canonical Wnt signaling regulates neural stem cell quiescence during homeostasis and after demyelination. *Nature communications* **9**, 36, doi:10.1038/s41467-017-02440-0 (2018).
144 Najdi, R. *et al.* A uniform human Wnt expression library reveals a shared secretory pathway and unique signaling activities. *Differentiation; research in biological diversity* **84**, 203-213, doi:10.1016/j.diff.2012.06.004 (2012).
145 Kilander, M. B., Halleskog, C. & Schulte, G. Recombinant WNTs differentially activate beta-catenin-dependent and -independent signalling in mouse microglia-like cells. *Acta physiologica* **203**, 363-372, doi:10.1111/j.1748-1716.2011.02324.x (2011).
146 He, S. *et al.* Wnt3a: functions and implications in cancer. *Chinese journal of cancer* **34**, 554-562, doi:10.1186/s40880-015-0052-4 (2015).
147 Lee, S. M., Tole, S., Grove, E. & McMahon, A. P. A local Wnt-3a signal is required for development of the mammalian hippocampus. *Development* **127**, 457-467 (2000).
148 Qi, L. *et al.* Wnt3a expression is associated with epithelial-mesenchymal transition and promotes colon cancer progression. *Journal of experimental & clinical cancer research : CR* **33**, 107, doi:10.1186/s13046-014-0107-4 (2014).
149 Okamoto, M. *et al.* Reduction in paracrine Wnt3 factors during aging causes impaired adult neurogenesis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **25**, 3570-3582, doi:10.1096/fj.11-184697 (2011).
150 Zhang, H. *et al.* Expression profile and clinical significance of Wnt signaling in human gliomas. *Oncology letters* **15**, 610-617, doi:10.3892/ol.2017.7315 (2018).
151 Chen, J., Park, C. S. & Tang, S. J. Activity-dependent synaptic Wnt release regulates hippocampal long term potentiation. *The Journal of biological chemistry* **281**, 11910-11916, doi:10.1074/jbc.M511920200 (2006).
152 Korn, C. *et al.* Endothelial cell-derived non-canonical Wnt ligands control vascular pruning in angiogenesis. *Development* **141**, 1757-1766, doi:10.1242/dev.104422 (2014).
153 Klein, D. *et al.* Wnt2 acts as a cell type-specific, autocrine growth factor in rat hepatic sinusoidal endothelial cells cross-stimulating the VEGF pathway. *Hepatology* **47**, 1018-1031, doi:10.1002/hep.22084 (2008).

154 Komekado, H., Yamamoto, H., Chiba, T. & Kikuchi, A. Glycosylation and palmitoylation of Wnt-3a are coupled to produce an active form of Wnt-3a. *Genes to cells : devoted to molecular & cellular mechanisms* **12**, 521-534, doi:10.1111/j.1365-2443.2007.01068.x (2007).

155 Yamamoto, H. *et al.* Basolateral secretion of Wnt5a in polarized epithelial cells is required for apical lumen formation. *Journal of cell science* **128**, 1051-1063, doi:10.1242/jcs.163683 (2015).

156 Das, S., Yu, S., Sakamori, R., Stypulkowski, E. & Gao, N. Wntless in Wnt secretion: molecular, cellular and genetic aspects. *Frontiers in biology* **7**, 587-593, doi:10.1007/s11515-012-1200-8 (2012).

157 Coudreuse, D. & Korswagen, H. C. The making of Wnt: new insights into Wnt maturation, sorting and secretion. *Development* **134**, 3-12, doi:10.1242/dev.02699 (2007).

158 Gross, J. C., Chaudhary, V., Bartscherer, K. & Boutros, M. Active Wnt proteins are secreted on exosomes. *Nature cell biology* **14**, 1036-1045, doi:10.1038/ncb2574 (2012).

159 Pani, A. M. & Goldstein, B. Direct visualization of a native Wnt in vivo reveals that a long-range Wnt gradient forms by extracellular dispersal. *eLife* **7**, doi:10.7554/eLife.38325 (2018).

160 Chaudhary, V. *et al.* Robust Wnt signaling is maintained by a Wg protein gradient and Fz2 receptor activity in the developing Drosophila wing. *Development* **146**, doi:10.1242/dev.174789 (2019).

161 Zimmerli, D., Hausmann, G., Cantu, C. & Basler, K. Pharmacological interventions in the Wnt pathway: inhibition of Wnt secretion versus disrupting the protein-protein interfaces of nuclear factors. *British journal of pharmacology* **174**, 4600-4610, doi:10.1111/bph.13864 (2017).

162 Tran, F. H. & Zheng, J. J. Modulating the wnt signaling pathway with small molecules. *Protein science : a publication of the Protein Society* **26**, 650-661, doi:10.1002/pro.3122 (2017).

163 Huang, H. C. & Klein, P. S. The Frizzled family: receptors for multiple signal transduction pathways. *Genome biology* **5**, 234, doi:10.1186/gb-2004-5-7-234 (2004).

164 Wang, Y., Chang, H., Rattner, A. & Nathans, J. Frizzled Receptors in Development and Disease. *Current topics in developmental biology* **117**, 113-139, doi:10.1016/bs.ctdb.2015.11.028 (2016).

165 Wu, J., Klein, T. J. & Mlodzik, M. Subcellular localization of frizzled receptors, mediated by their cytoplasmic tails, regulates signaling pathway specificity. *PLoS biology* **2**, E158, doi:10.1371/journal.pbio.0020158 (2004).

166 MacDonald, B. T. & He, X. Frizzled and LRP5/6 receptors for Wnt/beta-catenin signaling. *Cold Spring Harbor perspectives in biology* **4**, doi:10.1101/cshperspect.a007880 (2012).

167 Bovolenta, P., Esteve, P., Ruiz, J. M., Cisneros, E. & Lopez-Rios, J. Beyond Wnt inhibition: new functions of secreted Frizzled-related proteins in development and disease. *Journal of cell science* **121**, 737-746, doi:10.1242/jcs.026096 (2008).

168 Wawrzak, D. *et al.* Wnt3a binds to several sFRPs in the nanomolar range. *Biochemical and biophysical research communications* **357**, 1119-1123, doi:10.1016/j.bbrc.2007.04.069 (2007).

169 Mao, B. *et al.* Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. *Nature* **417**, 664-667, doi:10.1038/nature756 (2002).

170 Sakane, H., Yamamoto, H. & Kikuchi, A. LRP6 is internalized by Dkk1 to suppress its phosphorylation in the lipid raft and is recycled for reuse. *Journal of cell science* **123**, 360-368, doi:10.1242/jcs.058008 (2010).

171 Niehrs, C. Function and biological roles of the Dickkopf family of Wnt modulators. *Oncogene* **25**, 7469-7481, doi:10.1038/sj.onc.1210054 (2006).

172 Zenzmaier, C., Marksteiner, J., Kiefer, A., Berger, P. & Humpel, C. Dkk-3 is elevated in CSF and plasma of Alzheimer's disease patients. *Journal of neurochemistry* **110**, 653-661, doi:10.1111/j.1471-4159.2009.06158.x (2009).

173 Caricasole, A. *et al.* Induction of Dickkopf-1, a negative modulator of the Wnt pathway, is associated with neuronal degeneration in Alzheimer's brain. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **24**, 6021-6027, doi:10.1523/JNEUROSCI.1381-04.2004 (2004).

174 Cho, C., Smallwood, P. M. & Nathans, J. Reck and Gpr124 Are Essential Receptor Cofactors for Wnt7a/Wnt7b-Specific Signaling in Mammalian CNS Angiogenesis and Blood-Brain Barrier Regulation. *Neuron* **95**, 1221-1225, doi:10.1016/j.neuron.2017.08.032 (2017).

175 Eubelen, M. *et al.* A molecular mechanism for Wnt ligand-specific signaling. *Science* **361**, doi:10.1126/science.aat1178 (2018).

176 Vallon, M. *et al.* A RECK-WNT7 Receptor-Ligand Interaction Enables Isoform-Specific Regulation of Wnt Bioavailability. *Cell reports* **25**, 339-349 e339, doi:10.1016/j.celrep.2018.09.045 (2018).

177 Cho, C., Wang, Y., Smallwood, P. M., Williams, J. & Nathans, J. Molecular determinants in Frizzled, Reck, and Wnt7a for ligand-specific signaling in neurovascular development. *eLife* **8**, doi:10.7554/eLife.47300 (2019).

178 Hao, H. X. *et al.* ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. *Nature* **485**, 195-200, doi:10.1038/nature11019 (2012).

179 Kazanskaya, O. *et al.* The Wnt signaling regulator R-spondin 3 promotes angioblast and vascular development. *Development* **135**, 3655-3664, doi:10.1242/dev.027284 (2008).

180 Li, Z., Zhang, W. & Mulholland, M. W. LGR4 and Its Role in Intestinal Protection and Energy Metabolism. *Frontiers in endocrinology* **6**, 131, doi:10.3389/fendo.2015.00131 (2015).

181 Glinka, A. *et al.* LGR4 and LGR5 are R-spondin receptors mediating Wnt/beta-catenin and Wnt/PCP signalling. *EMBO reports* **12**, 1055-1061, doi:10.1038/embor.2011.175 (2011).

182 Scholz, B. *et al.* Endothelial RSPO3 Controls Vascular Stability and Pruning through Non-canonical WNT/Ca(2+)/NFAT Signaling. *Developmental cell* **36**, 79-93, doi:10.1016/j.devcel.2015.12.015 (2016).

183 Wang, Y. *et al.* Norrin/Frizzled4 signaling in retinal vascular development and blood brain barrier plasticity. *Cell* **151**, 1332-1344, doi:10.1016/j.cell.2012.10.042 (2012).

184 Xu, Q. *et al.* Vascular development in the retina and inner ear: control by Norrin and Frizzled-4, a high-affinity ligand-receptor pair. *Cell* **116**, 883-895, doi:10.1016/s0092-8674(04)00216-8 (2004).

185 Lai, M. B. *et al.* TSPAN12 Is a Norrin Co-receptor that Amplifies Frizzled4 Ligand Selectivity and Signaling. *Cell reports* **19**, 2809-2822, doi:10.1016/j.celrep.2017.06.004 (2017).

186 Ye, X., Wang, Y. & Nathans, J. The Norrin/Frizzled4 signaling pathway in retinal vascular development and disease. *Trends in molecular medicine* **16**, 417-425, doi:10.1016/j.molmed.2010.07.003 (2010).

187 Smallwood, P. M., Williams, J., Xu, Q., Leahy, D. J. & Nathans, J. Mutational analysis of Norrin-Frizzled4 recognition. *The Journal of biological chemistry* **282**, 4057-4068, doi:10.1074/jbc.M609618200 (2007).

188 L'Episcopo, F. *et al.* A Wnt1 regulated Frizzled-1/beta-Catenin signaling pathway as a candidate regulatory circuit controlling mesencephalic dopaminergic neuron-astrocyte crosstalk: Therapeutical relevance for neuron survival and neuroprotection. *Molecular neurodegeneration* **6**, 49, doi:10.1186/1750-1326-6-49 (2011).

189 Cecchelli, R. *et al.* A stable and reproducible human blood-brain barrier model derived from hematopoietic stem cells. *PloS one* **9**, e99733, doi:10.1371/journal.pone.0099733 (2014).

190 Zhang, Y. *et al.* An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **34**, 11929-11947, doi:10.1523/JNEUROSCI.1860-14.2014 (2014).

191 Phoenix, T. N. *et al.* Medulloblastoma Genotype Dictates Blood Brain Barrier Phenotype. *Cancer cell* **29**, 508-522, doi:10.1016/j.ccell.2016.03.002 (2016).

192 Lie, D. C. *et al.* Wnt signalling regulates adult hippocampal neurogenesis. *Nature* **437**, 1370-1375, doi:10.1038/nature04108 (2005).

193 Miranda, C. J. *et al.* Aging brain microenvironment decreases hippocampal neurogenesis through Wnt-mediated survivin signaling. *Aging cell* **11**, 542-552, doi:10.1111/j.1474-9726.2012.00816.x (2012).

194 L'Episcopo, F. *et al.* Reactive astrocytes and Wnt/beta-catenin signaling link nigrostriatal injury to repair in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. *Neurobiology of disease* **41**, 508-527, doi:10.1016/j.nbd.2010.10.023 (2011).
195 von Bartheld, C. S., Bahney, J. & Herculano-Houzel, S. The search for true numbers of neurons and glial cells in the human brain: A review of 150 years of cell counting. *The Journal of comparative neurology* **524**, 3865-3895, doi:10.1002/cne.24040 (2016).
196 Zhang, W. *et al.* Neuron activity-induced Wnt signaling up-regulates expression of brain-derived neurotrophic factor in the pain neural circuit. *The Journal of biological chemistry* **293**, 15641-15651, doi:10.1074/jbc.RA118.002840 (2018).
197 Yu, X. & Malenka, R. C. Beta-catenin is critical for dendritic morphogenesis. *Nature neuroscience* **6**, 1169-1177, doi:10.1038/nn1132 (2003).
198 Riganti, C. *et al.* Temozolomide down-regulates P-glycoprotein in human blood-brain barrier cells by disrupting Wnt3 signaling. *Cellular and molecular life sciences : CMLS* **71**, 499-516, doi:10.1007/s00018-013-1397-y (2014).
199 Kato, M. *et al.* Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. *The Journal of cell biology* **157**, 303-314, doi:10.1083/jcb.200201089 (2002).
200 Pinson, K. I., Brennan, J., Monkley, S., Avery, B. J. & Skarnes, W. C. An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* **407**, 535-538, doi:10.1038/35035124 (2000).
201 Chang, J. *et al.* Gpr124 is essential for blood-brain barrier integrity in central nervous system disease. *Nature medicine* **23**, 450-460, doi:10.1038/nm.4309 (2017).
202 Zhou, Y. & Nathans, J. Gpr124 controls CNS angiogenesis and blood-brain barrier integrity by promoting ligand-specific canonical wnt signaling. *Developmental cell* **31**, 248-256, doi:10.1016/j.devcel.2014.08.018 (2014).
203 Oh, J. *et al.* The membrane-anchored MMP inhibitor RECK is a key regulator of extracellular matrix integrity and angiogenesis. *Cell* **107**, 789-800, doi:10.1016/s0092-8674(01)00597-9 (2001).
204 Wang, Y., Huso, D., Cahill, H., Ryugo, D. & Nathans, J. Progressive cerebellar, auditory, and esophageal dysfunction caused by targeted disruption of the frizzled-4 gene. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **21**, 4761-4771 (2001).
205 Ye, X. *et al.* Norrin, frizzled-4, and Lrp5 signaling in endothelial cells controls a genetic program for retinal vascularization. *Cell* **139**, 285-298, doi:10.1016/j.cell.2009.07.047 (2009).
206 Jensen, L. D. *et al.* Disruption of the Extracellular Matrix Progressively Impairs Central Nervous System Vascular Maturation Downstream of beta-Catenin Signaling. *Arteriosclerosis, thrombosis, and vascular biology* **39**, 1432-1447, doi:10.1161/ATVBAHA.119.312388 (2019).
207 Vanhollebeke, B. *et al.* Tip cell-specific requirement for an atypical Gpr124- and Reck-dependent Wnt/beta-catenin pathway during brain angiogenesis. *eLife* **4**, doi:10.7554/eLife.06489 (2015).
208 Patel, P. & Woodgett, J. R. Glycogen Synthase Kinase 3: A Kinase for All Pathways? *Current topics in developmental biology* **123**, 277-302, doi:10.1016/bs.ctdb.2016.11.011 (2017).
209 Corada, M. *et al.* Fine-Tuning of Sox17 and Canonical Wnt Coordinates the Permeability Properties of the Blood-Brain Barrier. *Circulation research* **124**, 511-525, doi:10.1161/CIRCRESAHA.118.313316 (2019).
210 Luhmann, U. F. *et al.* Vascular changes in the cerebellum of Norrin /Ndph knockout mice correlate with high expression of Norrin and Frizzled-4. *The European journal of neuroscience* **27**, 2619-2628, doi:10.1111/j.1460-9568.2008.06237.x (2008).
211 L'Episcopo, F. *et al.* Targeting Wnt signaling at the neuroimmune interface for dopaminergic neuroprotection/repair in Parkinson's disease. *Journal of molecular cell biology* **6**, 13-26, doi:10.1093/jmcb/mjt053 (2014).
212 Tabata, H. Diverse subtypes of astrocytes and their development during corticogenesis. *Frontiers in neuroscience* **9**, 114, doi:10.3389/fnins.2015.00114 (2015).

213 Nyul-Toth, A. *et al.* Differences in the molecular structure of the blood-brain barrier in the cerebral cortex and white matter: an in silico, in vitro, and ex vivo study. *American journal of physiology. Heart and circulatory physiology* **310**, H1702-1714, doi:10.1152/ajpheart.00774.2015 (2016).
214 Chen, M. & Do, H. Wnt Signaling in Neurogenesis during Aging and Physical Activity. *Brain sciences* **2**, 745-768, doi:10.3390/brainsci2040745 (2012).
215 Cavaglia, M. *et al.* Regional variation in brain capillary density and vascular response to ischemia. *Brain research* **910**, 81-93, doi:10.1016/s0006-8993(01)02637-3 (2001).
216 Montagne, A. *et al.* Blood-brain barrier breakdown in the aging human hippocampus. *Neuron* **85**, 296-302, doi:10.1016/j.neuron.2014.12.032 (2015).
217 Cappuccio, I. *et al.* Induction of Dickkopf-1, a negative modulator of the Wnt pathway, is required for the development of ischemic neuronal death. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **25**, 2647-2657, doi:10.1523/JNEUROSCI.5230-04.2005 (2005).
218 Zhang, Q. G., Wang, R., Khan, M., Mahesh, V. & Brann, D. W. Role of Dickkopf-1, an antagonist of the Wnt/beta-catenin signaling pathway, in estrogen-induced neuroprotection and attenuation of tau phosphorylation. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**, 8430-8441, doi:10.1523/JNEUROSCI.2752-08.2008 (2008).
219 Liu, B. *et al.* Involvement of the Wnt signaling pathway and cell apoptosis in the rat hippocampus following cerebral ischemia/reperfusion injury. *Neural regeneration research* **8**, 70-75, doi:10.3969/j.issn.1673-5374.2013.01.009 (2013).
220 Scott, E. L., Zhang, Q. G., Han, D., Desai, B. N. & Brann, D. W. Long-term estrogen deprivation leads to elevation of Dickkopf-1 and dysregulation of Wnt/beta-Catenin signaling in hippocampal CA1 neurons. *Steroids* **78**, 624-632, doi:10.1016/j.steroids.2012.11.004 (2013).
221 Benarroch, E. E. Choroid plexus--CSF system: Recent developments and clinical correlations. *Neurology* **86**, 286-296, doi:10.1212/WNL.0000000000002298 (2016).
222 Miyata, S. New aspects in fenestrated capillary and tissue dynamics in the sensory circumventricular organs of adult brains. *Frontiers in neuroscience* **9**, 390, doi:10.3389/fnins.2015.00390 (2015).
223 Liddelow, S. A. Development of the choroid plexus and blood-CSF barrier. *Frontiers in neuroscience* **9**, 32, doi:10.3389/fnins.2015.00032 (2015).
224 Liddelow, S. A. *et al.* Mechanisms that determine the internal environment of the developing brain: a transcriptomic, functional and ultrastructural approach. *PloS one* **8**, e65629, doi:10.1371/journal.pone.0065629 (2013).
225 Klas, J., Wolburg, H., Terasaki, T., Fricker, G. & Reichel, V. Characterization of immortalized choroid plexus epithelial cell lines for studies of transport processes across the blood-cerebrospinal fluid barrier. *Cerebrospinal fluid research* **7**, 11, doi:10.1186/1743-8454-7-11 (2010).
226 Johansson, P. A. *et al.* Aquaporin-1 in the choroid plexuses of developing mammalian brain. *Cell and tissue research* **322**, 353-364, doi:10.1007/s00441-005-1120-x (2005).
227 Bentivoglio, M., Kristensson, K. & Rottenberg, M. E. Circumventricular Organs and Parasite Neurotropism: Neglected Gates to the Brain? *Frontiers in immunology* **9**, 2877, doi:10.3389/fimmu.2018.02877 (2018).
228 Strazielle, N. & Ghersi-Egea, J. F. Physiology of blood-brain interfaces in relation to brain disposition of small compounds and macromolecules. *Molecular pharmaceutics* **10**, 1473-1491, doi:10.1021/mp300518e (2013).
229 Benz, F. *et al.* Low wnt/beta-catenin signaling determines leaky vessels in the subfornical organ and affects water homeostasis in mice. *eLife* **8**, doi:10.7554/eLife.43818 (2019).
230 Rubio-Araiz, A. *et al.* Disruption of blood-brain barrier integrity in postmortem alcoholic brain: preclinical evidence of TLR4 involvement from a binge-like drinking model. *Addiction biology* **22**, 1103-1116, doi:10.1111/adb.12376 (2017).
231 Pan, W., Barron, M., Hsuchou, H., Tu, H. & Kastin, A. J. Increased leptin permeation across the blood-brain barrier after chronic alcohol ingestion. *Neuropsychopharmacology : official*

publication of the American College of Neuropsychopharmacology **33**, 859-866, doi:10.1038/sj.npp.1301452 (2008).

232 Haorah, J., Knipe, B., Leibhart, J., Ghorpade, A. & Persidsky, Y. Alcohol-induced oxidative stress in brain endothelial cells causes blood-brain barrier dysfunction. *Journal of leukocyte biology* **78**, 1223-1232, doi:10.1189/jlb.0605340 (2005).

233 Health risks and benefits of alcohol consumption. *Alcohol research & health : the journal of the National Institute on Alcohol Abuse and Alcoholism* **24**, 5-11 (2000).

234 Brick, J. & Erickson, C. K. Intoxication is not always visible: an unrecognized prevention challenge. *Alcoholism, clinical and experimental research* **33**, 1489-1507, doi:10.1111/j.1530-0277.2009.00979.x (2009).

235 Baker, S. P., Braver, E. R., Chen, L. H., Li, G. & Williams, A. F. Drinking histories of fatally injured drivers. *Injury prevention : journal of the International Society for Child and Adolescent Injury Prevention* **8**, 221-226, doi:10.1136/ip.8.3.221 (2002).

236 Wechsler, H., Lee, J. E., Kuo, M. & Lee, H. College binge drinking in the 1990s: a continuing problem. Results of the Harvard School of Public Health 1999 College Alcohol Study. *Journal of American college health : J of ACH* **48**, 199-210, doi:10.1080/07448480009599305 (2000).

237 Wechsler, H. & Nelson, T. F. Binge drinking and the American college student: what's five drinks? *Psychology of addictive behaviors : journal of the Society of Psychologists in Addictive Behaviors* **15**, 287-291, doi:10.1037//0893-164x.15.4.287 (2001).

238 Hingson, R., Heeren, T. & Winter, M. Effects of recent 0.08% legal blood alcohol limits on fatal crash involvement. *Injury prevention : journal of the International Society for Child and Adolescent Injury Prevention* **6**, 109-114, doi:10.1136/ip.6.2.109 (2000).

239 Piano, M. R., Mazzuco, A., Kang, M. & Phillips, S. A. Binge Drinking Episodes in Young Adults: How Should We Measure Them in a Research Setting? *Journal of studies on alcohol and drugs* **78**, 502-511, doi:10.15288/jsad.2017.78.502 (2017).

240 Olson, K. N., Smith, S. W., Kloss, J. S., Ho, J. D. & Apple, F. S. Relationship between blood alcohol concentration and observable symptoms of intoxication in patients presenting to an emergency department. *Alcohol and alcoholism* **48**, 386-389, doi:10.1093/alcalc/agt042 (2013).

241 Jones, A. W. The drunkest drinking driver in Sweden: blood alcohol concentration 0.545% w/v. *Journal of studies on alcohol* **60**, 400-406, doi:10.15288/jsa.1999.60.400 (1999).

242 West, J. R. & Goodlett, C. R. Teratogenic effects of alcohol on brain development. *Annals of medicine* **22**, 319-325, doi:10.3109/07853899009147914 (1990).

243 Haorah, J., Schall, K., Ramirez, S. H. & Persidsky, Y. Activation of protein tyrosine kinases and matrix metalloproteinases causes blood-brain barrier injury: Novel mechanism for neurodegeneration associated with alcohol abuse. *Glia* **56**, 78-88, doi:10.1002/glia.20596 (2008).

244 Haorah, J. *et al.* Ethanol-induced activation of myosin light chain kinase leads to dysfunction of tight junctions and blood-brain barrier compromise. *Alcoholism, clinical and experimental research* **29**, 999-1009 (2005).

245 Phillips, D. E., Krueger, S. K., Wall, K. A., Smoyer-Dearing, L. H. & Sikora, A. K. The development of the blood-brain barrier in alcohol-exposed rats. *Alcohol* **14**, 333-343, doi:10.1016/s0741-8329(96)00180-2 (1997).

246 Singh, A. K., Jiang, Y., Gupta, S. & Benlhabib, E. Effects of chronic ethanol drinking on the blood brain barrier and ensuing neuronal toxicity in alcohol-preferring rats subjected to intraperitoneal LPS injection. *Alcohol and alcoholism* **42**, 385-399, doi:10.1093/alcalc/agl120 (2007).

247 Mardones, M. D. *et al.* Frizzled-1 receptor regulates adult hippocampal neurogenesis. *Molecular brain* **9**, 29, doi:10.1186/s13041-016-0209-3 (2016).

248 Taffe, M. A. *et al.* Long-lasting reduction in hippocampal neurogenesis by alcohol consumption in adolescent nonhuman primates. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 11104-11109, doi:10.1073/pnas.0912810107 (2010).

249 Ikonomidou, C. *et al.* Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science* **287**, 1056-1060, doi:10.1126/science.287.5455.1056 (2000).

250 Subbanna, S. & Basavarajappa, B. S. Postnatal Ethanol-Induced Neurodegeneration Involves CB1R-Mediated beta-Catenin Degradation in Neonatal Mice. *Brain sciences* **10**, doi:10.3390/brainsci10050271 (2020).
251 Vangipuram, S. D. & Lyman, W. D. Ethanol affects differentiation-related pathways and suppresses Wnt signaling protein expression in human neural stem cells. *Alcoholism, clinical and experimental research* **36**, 788-797, doi:10.1111/j.1530-0277.2011.01682.x (2012).
252 Singh, A. K., Gupta, S., Jiang, Y., Younus, M. & Ramzan, M. In vitro neurogenesis from neural progenitor cells isolated from the hippocampus region of the brain of adult rats exposed to ethanol during early development through their alcohol-drinking mothers. *Alcohol and alcoholism* **44**, 185-198, doi:10.1093/alcalc/agn109 (2009).
253 Jope, R. S. & Roh, M. S. Glycogen synthase kinase-3 (GSK3) in psychiatric diseases and therapeutic interventions. *Current drug targets* **7**, 1421-1434 (2006).
254 Li, X. & Jope, R. S. Is glycogen synthase kinase-3 a central modulator in mood regulation? *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **35**, 2143-2154, doi:10.1038/npp.2010.105 (2010).
255 Hui, J. *et al.* Fluoxetine regulates neurogenesis in vitro through modulation of GSK-3beta/beta-catenin signaling. *The international journal of neuropsychopharmacology* **18**, doi:10.1093/ijnp/pyu099 (2014).
256 Li, X., Rosborough, K. M., Friedman, A. B., Zhu, W. & Roth, K. A. Regulation of mouse brain glycogen synthase kinase-3 by atypical antipsychotics. *The international journal of neuropsychopharmacology* **10**, 7-19, doi:10.1017/S1461145706006547 (2007).
257 Ahn, M. *et al.* Potential involvement of glycogen synthase kinase (GSK)-3beta in a rat model of multiple sclerosis: evidenced by lithium treatment. *Anatomy & cell biology* **50**, 48-59, doi:10.5115/acb.2017.50.1.48 (2017).
258 Noble, W. *et al.* Inhibition of glycogen synthase kinase-3 by lithium correlates with reduced tauopathy and degeneration in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 6990-6995, doi:10.1073/pnas.0500466102 (2005).
259 Sutton, L. P. & Rushlow, W. J. The effects of neuropsychiatric drugs on glycogen synthase kinase-3 signaling. *Neuroscience* **199**, 116-124, doi:10.1016/j.neuroscience.2011.09.056 (2011).
260 Zhang, F., Phiel, C. J., Spece, L., Gurvich, N. & Klein, P. S. Inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3) in response to lithium. Evidence for autoregulation of GSK-3. *The Journal of biological chemistry* **278**, 33067-33077, doi:10.1074/jbc.M212635200 (2003).
261 Vinyoles, M. *et al.* Multivesicular GSK3 sequestration upon Wnt signaling is controlled by p120-catenin/cadherin interaction with LRP5/6. *Molecular cell* **53**, 444-457, doi:10.1016/j.molcel.2013.12.010 (2014).
262 McManus, E. J. *et al.* Role that phosphorylation of GSK3 plays in insulin and Wnt signalling defined by knockin analysis. *The EMBO journal* **24**, 1571-1583, doi:10.1038/sj.emboj.7600633 (2005).
263 Sutton, L. P., Honardoust, D., Mouyal, J., Rajakumar, N. & Rushlow, W. J. Activation of the canonical Wnt pathway by the antipsychotics haloperidol and clozapine involves dishevelled-3. *Journal of neurochemistry* **102**, 153-169, doi:10.1111/j.1471-4159.2007.04527.x (2007).
264 Kang, U. G. *et al.* The effects of clozapine on the GSK-3-mediated signaling pathway. *FEBS letters* **560**, 115-119, doi:10.1016/S0014-5793(04)00082-1 (2004).
265 Lesort, M., Greendorfer, A., Stockmeier, C., Johnson, G. V. & Jope, R. S. Glycogen synthase kinase-3beta, beta-catenin, and tau in postmortem bipolar brain. *Journal of neural transmission* **106**, 1217-1222, doi:10.1007/s007020050235 (1999).
266 O'Brien, W. T. *et al.* Glycogen synthase kinase-3beta haploinsufficiency mimics the behavioral and molecular effects of lithium. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **24**, 6791-6798, doi:10.1523/JNEUROSCI.4753-03.2004 (2004).

267 Alimohamad, H., Rajakumar, N., Seah, Y. H. & Rushlow, W. Antipsychotics alter the protein expression levels of beta-catenin and GSK-3 in the rat medial prefrontal cortex and striatum. *Biological psychiatry* **57**, 533-542, doi:10.1016/j.biopsych.2004.11.036 (2005).
268 Doble, B. W., Patel, S., Wood, G. A., Kockeritz, L. K. & Woodgett, J. R. Functional redundancy of GSK-3alpha and GSK-3beta in Wnt/beta-catenin signaling shown by using an allelic series of embryonic stem cell lines. *Developmental cell* **12**, 957-971, doi:10.1016/j.devcel.2007.04.001 (2007).
269 Young, W. Review of lithium effects on brain and blood. *Cell transplantation* **18**, 951-975, doi:10.3727/096368909X471251 (2009).
270 Ryves, W. J. & Harwood, A. J. Lithium inhibits glycogen synthase kinase-3 by competition for magnesium. *Biochemical and biophysical research communications* **280**, 720-725, doi:10.1006/bbrc.2000.4169 (2001).
271 Beaulieu, J. M., Del'guidice, T., Sotnikova, T. D., Lemasson, M. & Gainetdinov, R. R. Beyond cAMP: The Regulation of Akt and GSK3 by Dopamine Receptors. *Frontiers in molecular neuroscience* **4**, 38, doi:10.3389/fnmol.2011.00038 (2011).
272 Beaulieu, J. M. *et al.* A beta-arrestin 2 signaling complex mediates lithium action on behavior. *Cell* **132**, 125-136, doi:10.1016/j.cell.2007.11.041 (2008).
273 O'Brien, W. T. *et al.* Glycogen synthase kinase-3 is essential for beta-arrestin-2 complex formation and lithium-sensitive behaviors in mice. *The Journal of clinical investigation* **121**, 3756-3762, doi:10.1172/JCI45194 (2011).
274 Stambolic, V., Ruel, L. & Woodgett, J. R. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Current biology : CB* **6**, 1664-1668 (1996).
275 Kato, T., Takahashi, S. & Inubushi, T. Brain lithium concentration by 7Li- and 1H-magnetic resonance spectroscopy in bipolar disorder. *Psychiatry research* **45**, 53-63 (1992).
276 Gould, T. D., Chen, G. & Manji, H. K. In vivo evidence in the brain for lithium inhibition of glycogen synthase kinase-3. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **29**, 32-38, doi:10.1038/sj.npp.1300283 (2004).
277 Xia, Q. P., Cheng, Z. Y. & He, L. The modulatory role of dopamine receptors in brain neuroinflammation. *International immunopharmacology* **76**, 105908, doi:10.1016/j.intimp.2019.105908 (2019).
278 Gareri, P. *et al.* Use of atypical antipsychotics in the elderly: a clinical review. *Clinical interventions in aging* **9**, 1363-1373, doi:10.2147/CIA.S63942 (2014).
279 Missale, C., Nash, S. R., Robinson, S. W., Jaber, M. & Caron, M. G. Dopamine receptors: from structure to function. *Physiological reviews* **78**, 189-225, doi:10.1152/physrev.1998.78.1.189 (1998).
280 Jaber, M., Robinson, S. W., Missale, C. & Caron, M. G. Dopamine receptors and brain function. *Neuropharmacology* **35**, 1503-1519, doi:10.1016/s0028-3908(96)00100-1 (1996).
281 Chakroborty, D. *et al.* Dopamine stabilizes tumor blood vessels by up-regulating angiopoietin 1 expression in pericytes and Kruppel-like factor-2 expression in tumor endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 20730-20735, doi:10.1073/pnas.1108696108 (2011).
282 Lokhandwala, M. F. & Amenta, F. Anatomical distribution and function of dopamine receptors in the kidney. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **5**, 3023-3030, doi:10.1096/fasebj.5.15.1683844 (1991).
283 Tonnarini, G. *et al.* Dopamine receptor subtypes in the human coronary vessels of healthy subjects. *Journal of receptor and signal transduction research* **31**, 33-38, doi:10.3109/10799893.2010.506878 (2011).
284 Bacic, F., Uematsu, S., McCarron, R. M. & Spatz, M. Dopaminergic receptors linked to adenylate cyclase in human cerebromicrovascular endothelium. *Journal of neurochemistry* **57**, 1774-1780 (1991).

285 Freyberg, Z., Ferrando, S. J. & Javitch, J. A. Roles of the Akt/GSK-3 and Wnt signaling pathways in schizophrenia and antipsychotic drug action. *The American journal of psychiatry* **167**, 388-396, doi:10.1176/appi.ajp.2009.08121873 (2010).

286 Roh, M. S. *et al.* Haloperidol and clozapine differentially regulate signals upstream of glycogen synthase kinase 3 in the rat frontal cortex. *Experimental & molecular medicine* **39**, 353-360, doi:10.1038/emm.2007.39 (2007).

287 Tapp, A. *et al.* Combination antipsychotic therapy in clinical practice. *Psychiatric services* **54**, 55-59, doi:10.1176/appi.ps.54.1.55 (2003).

288 Beasley, C. M., Masica, D. N. & Potvin, J. H. Fluoxetine: a review of receptor and functional effects and their clinical implications. *Psychopharmacology* **107**, 1-10, doi:10.1007/bf02244958 (1992).

289 Polter, A. M. & Li, X. Glycogen Synthase Kinase-3 is an Intermediate Modulator of Serotonin Neurotransmission. *Frontiers in molecular neuroscience* **4**, 31, doi:10.3389/fnmol.2011.00031 (2011).

290 Lee, J. Y. *et al.* Fluoxetine inhibits transient global ischemia-induced hippocampal neuronal death and memory impairment by preventing blood-brain barrier disruption. *Neuropharmacology* **79**, 161-171, doi:10.1016/j.neuropharm.2013.11.011 (2014).

291 Liu, F. Y. *et al.* Fluoxetine attenuates neuroinflammation in early brain injury after subarachnoid hemorrhage: a possible role for the regulation of TLR4/MyD88/NF-kappaB signaling pathway. *Journal of neuroinflammation* **15**, 347, doi:10.1186/s12974-018-1388-x (2018).

292 Hu, H. M. *et al.* Fluoxetine is Neuroprotective in Early Brain Injury via its Anti-inflammatory and Anti-apoptotic Effects in a Rat Experimental Subarachnoid Hemorrhage Model. *Neuroscience bulletin* **34**, 951-962, doi:10.1007/s12264-018-0232-8 (2018).

CHAPTER II: Modulation of Wnt/β-catenin signaling promotes blood-brain barrier phenotype in cultured brain endothelial cells

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Modulation of Wnt/β-catenin signaling promotes blood-brain barrier phenotype in cultured brain endothelial cells

Marlyn D. Laksitorini[1,4], Vinith Yathindranath[1], Wei Xiong[1], Sabine Hombach-Klonisch[3], Donald W. Miller[1,2]*
[1]*Department of Pharmacology and Theurapetics, Max Rady College of Medicine, University of Manitoba, Winnipeg, R3E 0T6, Canada.*
[2.] *Kleysen Institute of Advanced Medicine, Health Sciences Center, Winnipeg, R3E 0T6, Canada.*
[3.]*Department of Human Anatomy and Cell Science, Max Rady College of Medicine, University of Manitoba, Winnipeg, R3E 0J9, Canada.*
[4]*Department of Pharmaceutics, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, 55281, Indonesia.*
**corresponding author: Donald.Miller@umanitoba.ca*

2.1 Abstract

Wnt/β-catenin signaling is important for blood-brain barrier (BBB) development and is implicated in BBB breakdown under various pathophysiological conditions. In the present study, a comprehensive characterization of the relevant genes, transport and permeability processes influenced by both the autocrine and external activation of Wnt signaling in human brain endothelial cells was examined using hCMEC/D3 culture model. The hCMEC/D3 expressed a full complement of Wnt ligands and receptors. Preventing Wnt ligand release from hCMEC/D3 produced minimal changes in brain endothelial function, while inhibition of intrinsic/autocrine Wnt/β-catenin activity through blocking β-catenin binding to Wnt transcription factor caused more modest changes. In contrast, activation of Wnt signaling using exogenous Wnt ligand (Wnt3a) or LiCl (GSK3 inhibitor) improved the BBB phenotypes of the hCMEC/D3 culture model, resulting in reduced paracellular permeability, and increased P-glycoprotein (P-gp) and breast cancer resistance associated protein (BCRP) efflux transporter activity. Further, Wnt3a reduced plasmalemma vesicle-associated protein (PLVAP) and vesicular transport activity in hCMEC/D3. Our data suggest that this *in vitro* model of the BBB has a more robust response to exogenous activation of Wnt/β-catenin signaling compared to autocrine activation, suggesting that BBB regulation may be more dependent on external activation of Wnt signaling within the brain microvasculature.

2.2 Introduction

Brain microvessel endothelial cells are the cellular interface separating the blood and its constituents from brain extracellular fluid. Together with astrocytic foot processes, pericytes, and basal lamina, the brain endothelial cells form the blood-brain barrier (BBB). Brain microvessel endothelial cells are characterized by the presence of tight junction proteins, an assortment of uptake and efflux transporters and reduced vesicular transport processes[1-5]. Together these

properties allow selective passage of molecules into and out of the brain and thus provides the proper microenvironment to support brain function[6].

Activation of the Wnt/β-catenin pathway promotes the formation of tight junction proteins and transporters in the developing brain capillaries[7-9]. Dysregulation of Wnt/β-catenin pathways has also been implicated in various CNS disorders that involve BBB breakdown including multiple sclerosis[10], stroke[11], Alzheimer's disease[12], Huntington's disease[13] and brain tumors[14]. Modulating Wnt signaling pathways is of interest therapeutically, however as a target, Wnt remains challenging due to the complexity of the pathways and its multiple players. There are two different signaling pathways that Wnt can activate. The first is the canonical Wnt pathway, also known as the Wnt/β-catenin signaling pathway. The second is the non-canonical Wnt pathway that is divided into a Wnt/planar cell polarity and Wnt/Ca^{2+} utilization pathway[15]. The complexity scales up as the proteins involved in Wnt/β-catenin signaling have multiple isoforms. For example, there are ten Wnt receptors (Frizzled 1-10), four Wnt co-receptors (LRP5, LRP6, ROR2 and RYK), nineteen Wnt ligands (Wnt 1-16) and ten Wnt modulator peptides (DKK, sFRP, and WIF)[16]. Little information is available regarding the distribution, function, and action of each isoform in the brain microvasculature.

Wnt canonical signaling involves binding of Wnt ligand to the Frizzled (Fzd) receptor and co-receptor LRP5/6. Upon binding, the cytoplasmic tail of LRP5/6 is phosphorylated. Binding of Wnt ligand initiates docking of Dishelleved (Dvl) to Fzd and further recruitment of the β-catenin destruction complex (Axin, CK-1, GSK3, APC) to the plasma membrane. In the inactive state, the β-catenin destruction complex is located in the cytosol where it can efficiently process β-catenin for proteosomal degradation. Recruitment of β-catenin destruction complex to the plasma membrane upon Wnt activation leads to a stabilization of β-catenin. The increased levels of β-

catenin in the cytosol results in its translocation to the nucleus where it acts as to modulate Wnt/β-catenin target gene transcription[17-20].

While previous studies have established the role of Wnt/β-catenin signaling in BBB development[7,9,11,21,22], less is known regarding the role of Wnt in the maintenance of BBB integrity in mature animals as well as Wnt/β-catenin activity in human BBB. The immortalized human brain microvessel cell line, hCMEC/D3, is a commonly used human in vitro BBB model[23-28]. Using hCMEC/D3, several laboratories have determined that Wnt/β-catenin signaling regulates P-glycoprotein (Pgp) expression[29,30]. However, comprehensive characterization of the extent that Wnt/β-catenin influences the barrier properties of the hCMEC/D3 model, beyond changing of Pgp drug efflux, has not been reported. In the present studies, the expression profile of Wnt components including Wnt ligands, receptors, co-receptors and modulators were characterized. The studies dissected the contribution of endogenous Wnt ligands released from hCMEC/D3 in establishment of BBB phenotype and compared the alteration in the BBB phenotype of hCMEC/D3 following activation through natural Wnt ligands and downstream kinase inhibition. While hCMEC/D3 produced Wnt ligand, the autocrine Wnt/β-catenin signaling contribution toward brain endothelial barrier function in the present study was minimal. In contrast, hCMEC/D3 were more responsive both in term of expression of genes known to contribute to BBB phenotypes, as well as functional barrier properties, following to exogenous activation of Wnt/β-catenin signaling through natural Wnt ligand or the inhibition of GSK activity. The studies suggest that autocrine activation of Wnt/β-catenin activation in the cerebral vasculature alone is insufficient to induce BBB phenotype. However, activation of Wnt/β-catenin through pharmacological means such as ligand stimulation or modulation of downstream elements in the signaling pathway can impact on the barrier properties of these cells.

2.3 Material and Methods

2.3.1 Material

hCMEC/D3 were obtained from Dr. Peter Couraud INSERM, France [59]. WntC59 was purchased from AdooQ Biosciences (Irvine, CA). Human Recombinant Wn3a, and GF120918 (GF) were purchased from R&D Systems (Minneapolis, MN). Rhodamine 800 and Rhodamine 123 were purchased from Sigma (St. Louis, MO). IRDye 800 CW PEG were purchased from LICOR (Lincoln, NB). EBM-2 media was purchased from Lonza (Walkersville, MD). The E-plates for RTCA were obtained from ACEA Biosciences (San Diego, CA). Transwell and tissue culture plates were purchased from Corning (Tewksbury, MA). Trizol and tetramethylrhodamine BSA was purchased from Life Technologies (CA, USA). P-glycoprotein and Claudin-1 antibodies were purchased from Abcam (Cambridge, MA). β-catenin, claudin-5 and β-actin antibodies were purchased from Sigma (St. Louis, MO). Human fetal brain total RNA was purchased from Takara Bio USA, Inc. (Madison, WI). Primers were obtained from Invitrogen (CA, USA) and primer sequence information is provided in table 2.1 supplementary information.

2.3.2 Cell Culture

For cell expansion, hCMEC/D3 were seeded at 10.000 cells/cm^2 onto T75 flasks (Corning Inc.) that had been coated with rat tail collagen 0.1mg/ml for an hour. Cells were cultured at 37^{o}C and 5% CO_2 in EBM-2 media supplemented with 5% FBS, 1% penicillin streptomycin, 1ng/ml bFGF, 10 mM HEPES, 5 µg/ml ascorbic acid, 1/100 CD lipid concentrate, and 1.4 µM hydrocortisone. Cells were passaged when reaching approximately 80% confluency using 1 ml Trypsin/EDTA. For expression and functional studies, cell were seeded onto culture plates coated with rat tail collagen at a density of 25,000 cells/cm^2 and used upon reaching confluency (usually within 4-5 days). To ensure maintenance of BBB properties the hCMEC/D3 were used at passage 29 to 34[23].

2.3.3 Immunoblotting

Cell were solubilized using RIPA buffer (Sigma) supplemented with protease and phosphatase inhibitors (Thermo Scientific). The lysates were subsequently centrifuged at 12.000 rpm for 8 minutes for isolating the whole cell lysate. The protein concentration was determined by Pierce BCA protein analysis (Thermo Scientific). Depending on the protein examined, 20-40 µg of protein was loaded into the SDS-polyacrylamide gel and run for separation at 110 Volt. The proteins were then transferred to PVDF membrane (BioRad) for 2 hour at 200 mA. The membrane was subsequently blocked in 5% non skim milk at TBST buffer for an hour at room temperature. Then, the membrane was incubated with the primary antibodies dissolved at 5% non skim milk TBST buffer at 4^{o}C on the rocking rack. Dilution 1:1000 were applied for Claudin-1, Claudin-5, P-glycoprotein, and β-catenin. In the next day, membranes were washed thrice with TBST buffer for ten minutes each before being incubated with secondary antibodies for 1 hour at room temperature. Bands were visualized using chemiluminescence at ChemiDoc Imager (Biorad) and analyzed using Image Lab software (Biorad).

2.3.4 RT-PCR

Expression of Wnt receptor, co-receptor, ligand, and modulator were examined in the human cerebral microvessels endothelial cell line (hCMEC/D3) using RT-PCR. Human fetal brain total RNA was used as a positive control for the primer as it expressed most of the Wnt component to identify the PCR product of the primer. Total RNA were isolated using Trizol®. RNA concentration was measured using Nanodrop UV Vis Spectrometer (Fisher Scientific). One microgram of total RNA was converted to cDNA using MLV Reverse Transcriptase enzyme (Invitrogen) with the final volume of 60 uL according to the manufacturer's protocol. One microliter of the cDNA were subjected to PCR using Platinum Taq polymerase (Invitrogen). The cycle was initial denaturation at 94^{o}C for 2 minutes followed by 30 PCR cycle that consist of 94^{o}C

for 30 seconds for denaturation, 60^{o}C for 45 second for annealing and 72 ^{o}C for 45 second for extension using programmable thermal controller (MJ Research Inc.). Ten microliter of PCR product were eluted in the 1% agarose gel at 104 Volt in 1x TAE buffer. Before the gel solidified, 10 uL of GelGreen (Biotium) were added to the agarose gel for PCR product visualization. The image was visualized using ChemiDoc (BioRad).

2.3.5 qPCR

RNA isolation was done similar to the previous section. Total RNA was subjected to qPCR using ITaq Universal Syber Green (BioRad) according to manufacturer protocol using QuantStudio 5 (Thermo Fisher).

2.3.6 P-glycoprotein and BCRP functional assay

Standard P-glycoprotein functional assay were done at 24 well plate using Rhodamine 123 (2.12 µM) as a Pgp substrate and GF120918 or GF (3.2 µM), as a Pgp inhibitor[60,61]. The cells were treated with Wnt3a, LiCl, WntC59 and ICRT-3 for 15-20 hour before the experiment under serum free media. On the day of experiment, the cells were washed with sterile PBS. Subsequently the cells were equilibrated in 0.5 ml assay buffer for 30 minutes. In this 30 minutes pre-incubation, only the GF group received 3.2 µM GF. The uptake were done for two hour at 37^{o}C and 5% CO_2 with 0.5ml sterile assay buffer (NaCl 150 mM, KCl 4 mM, $CaCl_2$ 3.2 mM, $MgCl_2$1.2 mM, HEPES 15 mM, Glucose 5 mM and 1% BSA) containing Rhodamine123 2.12 µM. At the end of the experiment, the plates were washed two times with 1 ml of ice-cold PBS. To solubilize intercellular Rhodamine123, 0.4 ml of 1% Triton X in PBS were added to each well and placed to the -20^{o}C freezer overnight. Following day, 100 µL of the lysate were transferred to black 96 well plate and were analyzed using Biotek Synergy HT Microplate Reader at excitation 485 nm and emission 528 nm. The amount of intercellular Rhodamine 123 was normalized to protein content using

Pierce BCA protein assay and expressed either as nanogram Rhodamine 123 per mg protein or as a percent of the control group.

Alternative P-glycoprotein functional assay were done using Rhodamine800 0.1 µM as Pgp substrate with GF120918 (GF) 3.2 µM as an inhibitor. The protocol were similar to Rhodamine123 studies. At the end of the experiment, the intercellular accumulation of Rhodamine800 was quantified using Odyssey Near Infrared Imager (Licor, Omaha, NE) using 700 nm channel.

BCRP functional studies used mitoxantrone 15 µM as a BCRP substrate and GF120918 (GF) 3.2 µM as a BCRP inhibitor[62] using similar protocol as Rhodamine123 and intracellular accumulation of mitoxantrone were quantified using Odyssey Near Infrared Imager (Licor) using 700 nm channel.

Rhodamine123, Rhodamine800, and mitoxantrone studies were done in separated sets of experiments. The assay was conducted at 15-20 hour post-treatment with Wnt ligands and modulators.

2.3.7 Endocytosis activity in hCMEC/D3 cell monolayers

Tetramethylrhodamine conjugated BSA 5 µg/ml was used to study vesicular transport mediated endocytosis[58]. Methyl β-cyclodextrin (MBCD; 0.5 mM) and genestein 200 µM were used as inhibitors of endocytic pathways[63]. Similar protocol as Pgp and BCRP functional studies were used. After uptake for two hours, intercellular accumulation of tetramethyl conjugated BSA was quantified using Biotek Synergy HT Microplate Reader at excitation 530 nm and emission 590 nm

2.3.8 Permeability studies

Permeability studies were done in the 6 well Transwell 0.4 µm pore polycarbonate membrane after pre-coated with rat tail collagen. hCMEC/D3 were seeded in the density of 25.000 cell/cm^2. Apical and basolateral chamber were filled with media 1.5 ml and 2.5 ml complete EBM-2 subsequently.

The monolayer was used upon reaching confluency, typically 4-5 days after seeding. For the permeability studies, the monolayers were treated with different Wnt activators or inhibitors for 15-20 hours in the absence of serum. On the day of experiment, the media was removed and replaced with assay buffer in both basolateral and apical compartments. The assay buffer in the apical compartment also contained 0.1 µM Rhodamine800, 0.1 µM IRdye 800CW PEG and 1 µM sodium fluorescein to assess monolayer permeability. A hundred microliters samples were removed from the basolateral compartment at various times (0, 15, 30, 60, 90 and 120 minutes) and replaced with equal volume of fresh assay buffer. Samples (10 µl) were also taken from apical compartment at the start and conclusion of permeability study. The samples from the apical and basolateral compartments were placed in black 96-well plates and diluted to 100 µl of assay buffer. Quantitative analysis of the various solutes were performed using an Odyssey Near Infrared Imager (Licor; 700 nm channel for Rhodamine800 and 800 nm channel for IRdye 800 CW PEG) and Biotek Synergy HT Microplate Reader at excitation 485 nm and emission 528 nm for sodium fluorescein. The amount of fluorescence activity was quantitated using standard curves for each fluorescent compound. Permeability data were presented as the percent flux.

2.3.9 Measurement of monolayer electrical impedance

Monolayer electrical impedance were measured using xCELLigence RTCA system. Briefly, the cell was seeded in 16 well E-plate (ACEA Biosciences) that have been coated with rat tail collagen with the density 14,000 cell/well. The E-plate surface was covered with microelectrode that measure the electrical impedance of the monolayer resulting a dimensionless value called Cell Index (CI). The cell was seeded in 200 µL EBM-2 media and been replaced with new media every other day. The impedance auto-mesurement interval was every 5 minutes. After the impedance reached the plateau for at least 24 hour, the media was removed and replaced with treatment drug with serum free EBM-2. Impedance monitoring was continued 24-48 hour post treatment.

2.4 Results:

2.4.1 Expression of Wnt receptors, ligands and modulators in hCMEC/D3.

Using PCR and qPCR, the various Wnt receptors, activators and modulators were profiled in hCMEC/D3 monolayers. As depicted in figure 2.1, hCMEC/D3 expressed not only Wnt receptors and co-receptors but also several Wnt ligands and Wnt modulators. For the Wnt receptors, Frizzled-3 and Frizzled-10 were undetectable while the other eight Frizzled isoforms were expressed (Fig. 2.1a). Analysis using qPCR showed a relatively similar expression level among the expressed frizzled receptors (see supplementary Fig. 2.S1a). However, Frizzled-2 and Frizzled-6 were slightly more abundant compared to the other Frizzled receptors. LRP-5 and LRP-6 were also expressed in the hCMEC/D3 functioning as co-receptors for Wnt/β-catenin signaling (Fig. 2.1a).

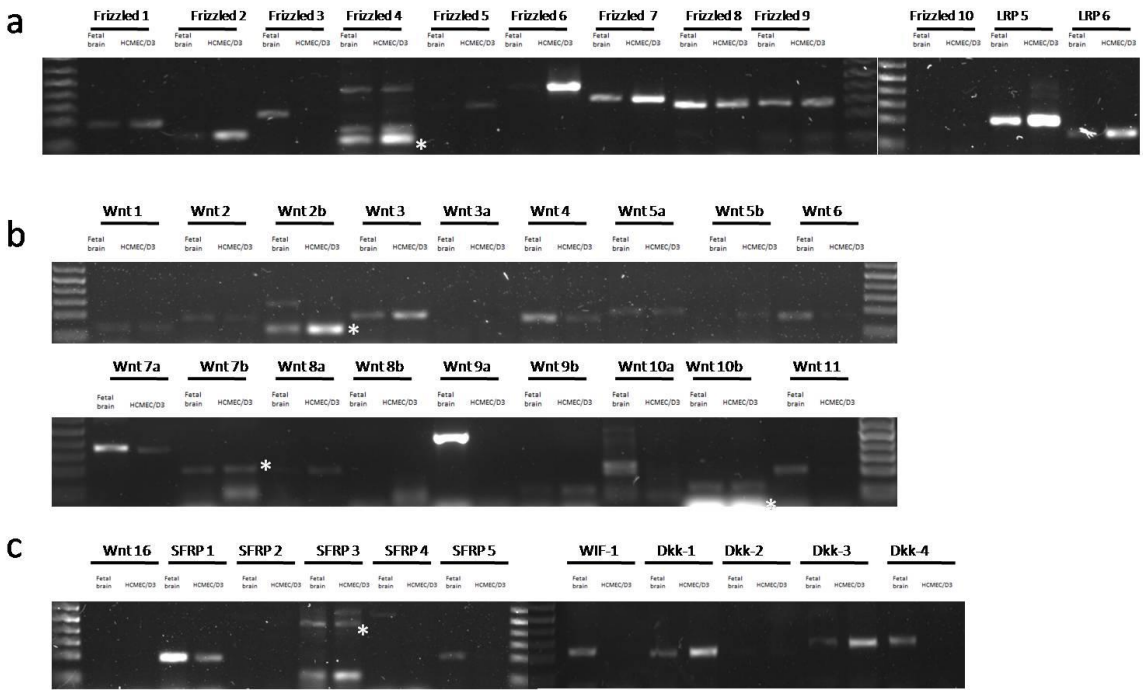

Figure 2.1: Expression of Wnt/β-catenin components in hCMEC/D3 cells. Expression of Wnt receptors and co-receptors (Panel A), Wnt ligands (Panel B) and Wnt modulators (Panel C) were examined by RT-PCR in confluent hCMEC/D3 monolayers. Total RNA were isolated for further PCR studies. Human Fetal Brain RNA were used as a control positive. Asterisk (*) is the correct PCR product in the primer that shown multiple bands.

Using the same method, the 19 Wnt ligands were also profiled. As depicted in Figure 2.1b, Wnt2b and Wnt3 were the most abundant endogenous canonical Wnt ligands expressed in hCMEC/D3. In addition to Wnt2b and Wnt3, hCMEC/D3 expressed lower levels of the canonical Wnt ligands, Wnt7a, Wnt7b, Wnt6 and Wnt10a. Non canonical Wnt ligands Wnt4, Wnt5a and Wnt5b were also expressed although in reduced amounts compared to Wnt2b and Wnt3 (Fig. 2.1b). Expression of Wnt3a was not detected in hCMEC/D3. Expression of several Wnt modulators were also identified such as Dkk-1, Dkk-3, sFRP-1 and sFRP-3 (Fig. 2.1c). Quantification using qPCR showed significantly lower CT number for Dkk-1 and Dkk-3 compared to Wnt2b and Wnt3 suggesting less expression of Wnt ligand compared to Wnt modulator (see supplementary Fig. 2.S1a). In addition, Dkk-2, Dkk-4, sFRP-2, sFRP-4 and sFRP-5 were not detected under the current experimental conditions.

Proteomics profiling of hCMEC/D3 lysates using SOMAscan assay confirmed the expression of Wnt7a, Dkk-1, Dkk-3, sFRP-1 and sFRP-3 proteins (see supplementary Fig. 2.S1b). Data analysis from proteomic profiling also indicated expression of RSPO-2, RSPO-3 and RSPO-4 in hCMEC/D3 monolayers (see supplementary Fig. 2.S1b). The R-spondin (RSPO) are Wnt agonists that have been shown to enhance and potentiate the strength of Wnt/β-catenin activity by preventing frizzled receptor internalization[31-33].

2.4.2 Intrinsic (autocrine) Wnt/β-catenin signaling in hCMEC/D3 cells

2.4.2.1 Effects of WntC59 on gene expression and barrier properties

With evidence for the expression of multiple Wnt proteins in the cell culture model of the BBB, the contribution of autocrine Wnt activation in the endothelial cells in establishing the BBB phenotype in hCMEC/D3 monolayers was examined. Treatment of cells with WntC59, an inhibitor of Wnt palmitoylation, an essential step for the secretion of Wnt proteins from cells[34,35], resulted

in a 40% reduction in β-catenin protein expression compared to control ($p<0.05$, Fig. 2.2a). Furthermore, WntC59 treatment resulted in reductions in Axin-2 mRNA expression by ~80% (Fig. 2.2b), indicative of reduced Wnt/β-catenin signaling in the hCMEC/D3 cells.

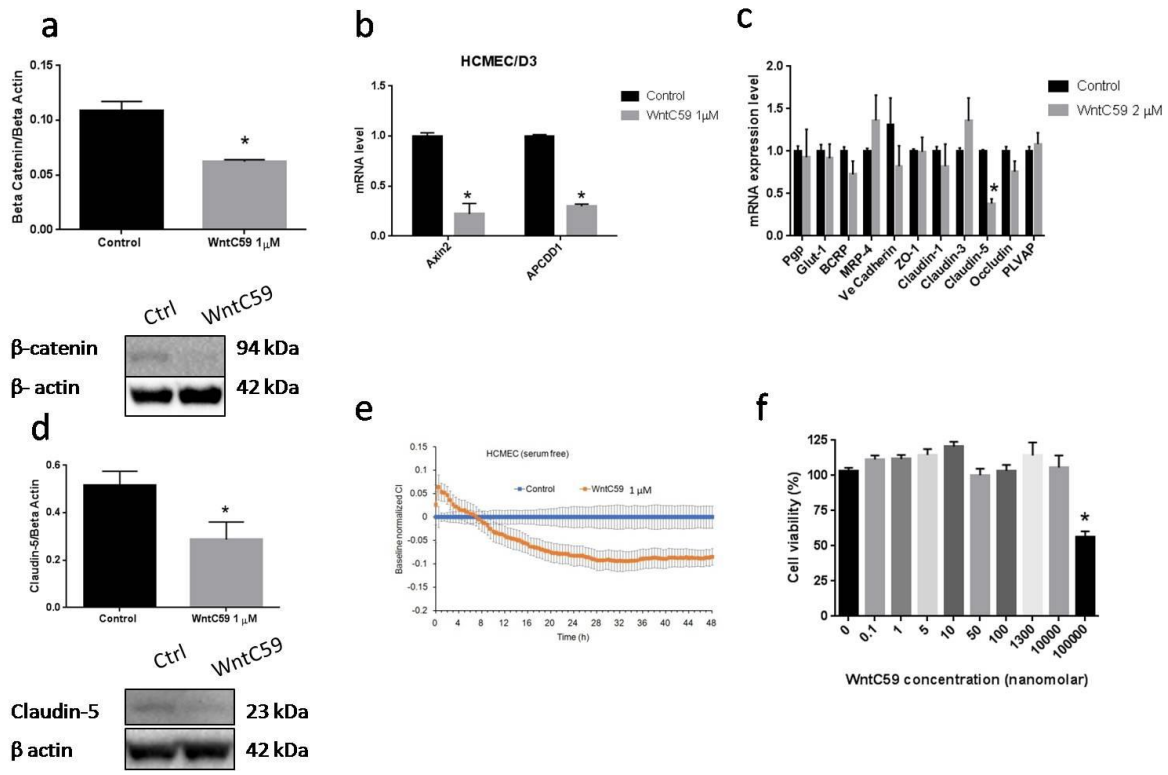

Figure 2.2: Evidence for autocrine activation of Wnt/β-catenin signaling in hCMEC/D3 monolayers. WntC59 1μM treatment for 15-20 hour reduced β-catenin abundance (Panel A), downregulate Axin-2 and APCDD1 mRNA expression (Panel B), diminished Claudin-5 mRNA expression (Panel C) and reduced Claudin-5 protein expression (Panel D). The decreased Claudin-5 expression associated with reductions in paracellular barrier were examined using real time cell analyzer (Panel E). hCMEC/D3 cell viability post 24 hours exposure to various concentrations of WntC59 (Panel F). Panel A and D show representative Western blotting of each protein. The cropped blot are used in the figure and full length blot are available at supplementary Figure 2.S5 and 2.S6. A and D: One tail t-test; n: 3-4. B and C: multitple t-test n: 4. E: n of 4. F: n of 8, One-way ANOVA followed by LSD Fisher's test. A B, C, D, E, F: *$p<0.05$. All value represent the mean±SEM except panel E: value is mean±SD.

However, examination of β-catenin responsive genes important in establishing the BBB phenotype showed only modest changes in tight junction and adherens junction molecules following WntC59

treatment (Fig. 2.2c). The most notable change in gene expression following WntC59 treatment was claudin-5 which was reduced approximately 50% at both the mRNA and protein levels compared to vehicle treated controls ($p<0.05$, Fig. 2.2c and 2.2d). The reductions in claudin-5 expression observed in the WntC59 treatment group were associated with a decrease in the electrical impedance of hCMEC3/D3 monolayers (Fig. 2.2e). The reduction in electrical impedance in the WntC59 treatment group was not due to cell toxicity as there was no difference on cell viability as examined by MTT assay (Fig. 2.2f). As electrical impedance is a surrogate marker for assessing tight junction integrity[36], the reductions observed in the WntC59 treated cells reflects reduced barrier properties of the cells. Despite the reduction in electrical impedance observed following treatment with WntC59, monolayer permeability assessed using both a small molecular weight marker, sodium fluorescein, and a large molecular weight marker, IRdye 800CW PEG, did not change significantly between control and WntC59 treatment groups (see supplementary Fig. 2.S2a and 2.S2b). Likewise, Pgp and BCRP transporter expression and function was also unaltered in the WntC59-treated hCMEC/D3 cells (Fig. 2.2c and Fig. 2.3e and 2.3f).

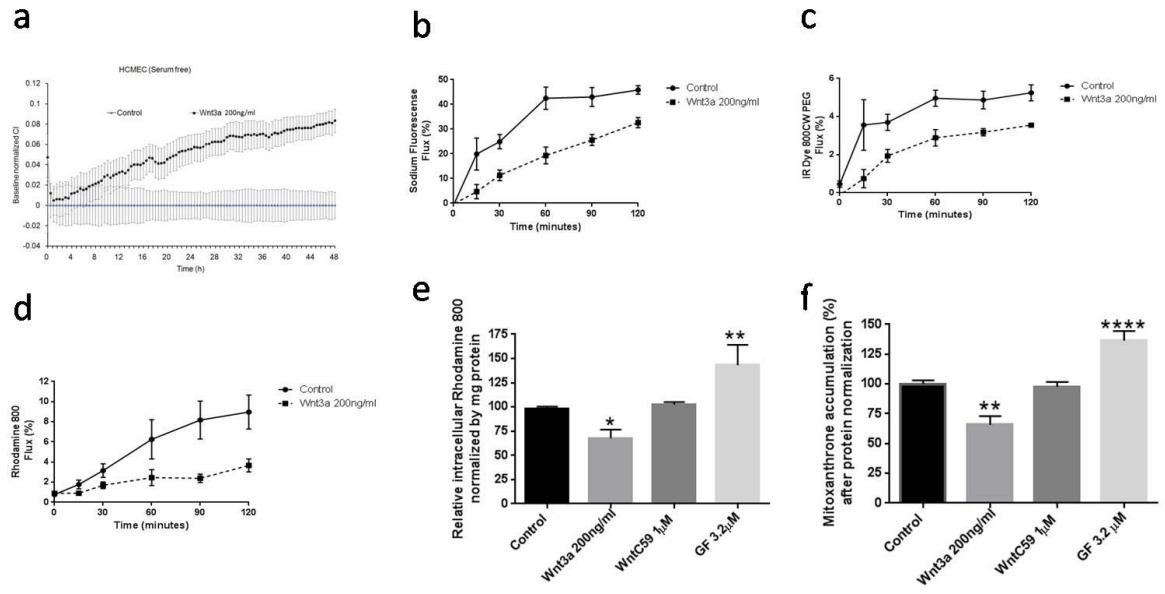

Figure 2.3: Functional impact of Wnt/β-catenin pathways modulation in hCMEC/D3 monolayers. Effects of Wnt3a exposure on electrical impedance (Panel A); transcellular permeability to small hydrophilic probe sodium fluorescein (Panel B), large hydrophilic probe IRdye 800 PEG (Panel C) and P-glycoprotein permeability probe, Rhodamine 800 (Panel D); and intracellular accumulation of P-glycoprotein and Breast Cancer Resistance Protein dependent fluorescent dyes (Panel E and F, respectively). Electrical impedance changes in response to Wnt3a were measured in real time and expressed as Cell index (CI). Influence of Wnt/β-catenin on transcellular permeability and transport were examined 15-hours following exposure to Wnt3a or WntC59. Values represent the mean ± SEM of 3-4 monolayers for the impendance and permeability studies and 8 monolayers for transporter studies. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ as determined using one-way ANOVA and LSD Fisher's test.

2.4.2.2 Effects of ICRT-3 on gene expression and barrier properties

In contrast, compared to WntC59, blocking the intrinsic activation of the Wnt/β-catenin in hCMEC/D3 cells at the transcription level using ICRT-3 resulted in more substantial changes in both gene expression and barrier properties. Using ICRT-3, a small molecule inhibitor of β-catenin binding to transcription factor TCF-4[37], significant reductions in claudin-3 as well as claudin-5, Pgp and BCRP at the mRNA expression level were observed (Fig. 2.4a). Confirmation of reductions in claudin-5, claudin-1 and Pgp expression were also observed at the protein level using immunoblotting (Fig. 2.4b-d). The altered expression of adhesion molecules and drug efflux transporters in hCMEC/D3 produced by ICRT-3 treatment was also correlated with changes in the functional properties of the cells. Monolayer electrical impedance measurements in ICRT-3 treated hCMEC/D3 were significantly lower than the control group (Fig. 2.4e). As the

concentrations of ICRT-3 did not change cell viability (Fig. 2.4f), these reductions in electrical impedance suggests reduced tight junction integrity. Similar to the responses to WntC59, no significant changes in NaF and IRdye 800CW PEG permeability were detected following ICRT-3 treatment (see supplementary Fig. 2.S2a and 2.S2b). P-glycoprotein efflux transporter functional studies using Rhodamine123 showed significant increases in cellular accumulation (~30%; $p<0.01$) following ICRT-3 treatment. Such increases were similar in magnitude to those observed following treatment with the Pgp transport inhibitor, GF120918 or GF (Fig. 2.4g). Together these studies suggest that inhibition of intrinsic Wnt/β-catenin activity with ICRT-3 has minimal impact on paracellular barrier properties, but significantly diminished Pgp transporter function in hCMEC/D3 monolayers.

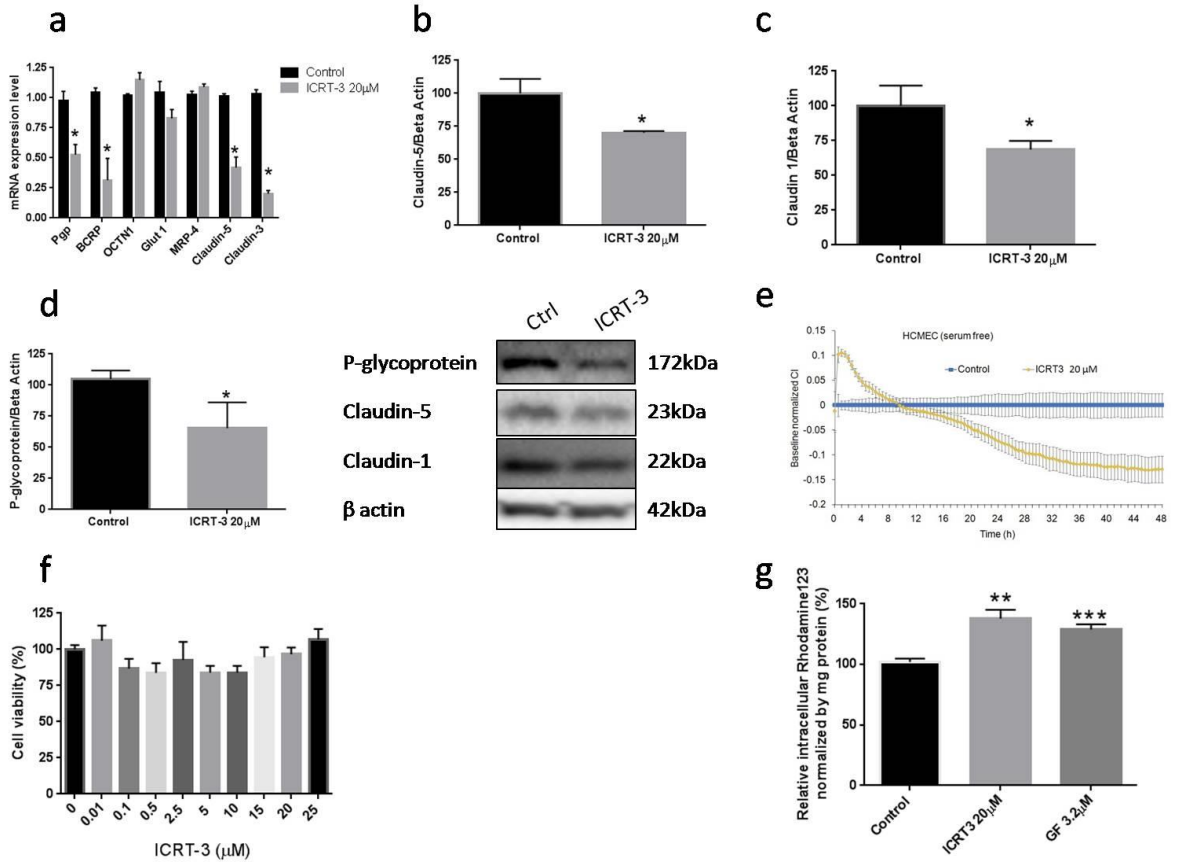

Figure 2.4: Modulation of Wnt/β-catenin response in hCMEC/D3 using TCF inhibitor ICRT-3. Effects of ICRT-3 treatment on select gene (Panel A) and protein expression (Panels B, C, and D) and endothelial cell function (Panels E and G). Viability of hCMEC/D3 after being exposed to various ICRT-3 concentrations for 24 hours (Panel F). The blots in the middle show representative Western blots for each protein. The cropped blots are used in the figure and full-length blots are available in supplementary Figure 2.S7. For gene and protein expression and functional studies, cells were exposed to ICRT-3 (20μM) for 15-hrs. For the electrical impedance studies readings were taken in real-time and expressed as the cell index (CI) normalized to control wells receiving no ICRT-3 (Panel E). Values in all panels represent the mean±SEM except panel E which is mean±SD. *p<0.05**p<0.01 ***p<0.001. Panel A was determined by multiple t-test; Panel B, C and D one tail t-test; Panel F and G: One-way ANOVA followed by LSD Fisher's test.

2.4.3 Extrinsic activation of Wnt canonical signaling in hCMEC/D3 cells

2.4.3.1 Effects of Wnt3a and LiCl on gene expression and barrier properties

Studies also examined the extent to which the resulting BBB phenotype of hCMEC/D3 could be altered by exogenous Wnt activators. This was done using Wnt3a, the most potent natural canonical Wnt activator, interacting with a wide range of frizzled receptors[38], and by treatment with LiCl, a GSK inhibitor that prevents activation of the β-catenin destruction complex. Both Wnt3a and LiCl treatments resulted in β-catenin stabilization and Axin-2 mRNA upregulation.

Axin-2, a commonly used marker to identify Wnt/β-catenin activation, was increased 9-fold following Wnt3a treatment while LiCl resulted in 5-fold increase in Axin-2 (Fig. 2.5a). Although the effects were less robust, both Wnt3a and LiCl increased APCDD1 and cyclin D1, additional target genes for Wnt activation (see supplementary Fig 2.S2c). The changes in Axin-2 expression observed with both Wnt3a and LiCl correlated with an increased β-catenin stabilization in the cells (Fig. 2.5b and 2.5c). The β-catenin protein levels were increased by ~2.0 and ~1.5 fold following Wnt3a and LiCl treatment, respectively (Fig. 2.5b and 2.5c).

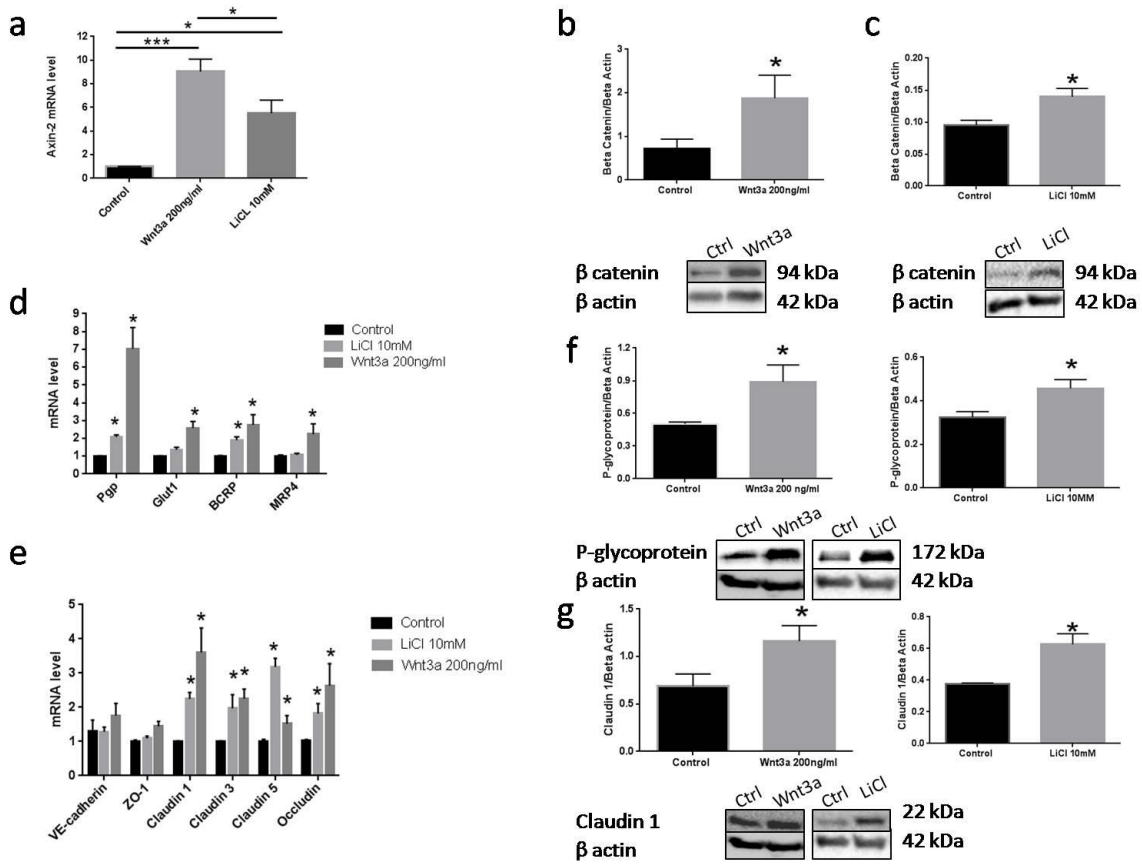

Figure 2.5: Modulation on Wnt/β-catenin signaling in hCMEC/D3. Natural ligand Wnt3a was a stronger Wnt/β-catenin activator compared to LiCl 10mM (Panel A). Wnt 3a and LiCl treatment increased β-catenin abundance in whole cell lysate (Panel B and C). The activation of Wnt/β-catenin signaling alters select transporters and intercellular junction proteins (Panel D and E). Wnt3a increased protein expression of Pgp and claudin-1 (Panel F and G). Panel B, C, F and G are representative Western blots of each protein. The cropped blots are used in the figure and full length blots are available in supplementary Figure 2.S8-S11: One-way ANOVA followed by LSD Fisher's test.*p<0.05; *** p<0.001. B, C, F, G and H: One tail t test. P<0.05. n:3-4. Mean ± SEM. D and E: Two-way ANOVA followed by LSD Fisher's test.n: 4. *p<0.05.Mean ± SEM

The impact of Wnt activation on downstream gene expression of intracellular transporters and intercellular junction proteins were examined (Fig. 2.5d and 2.5e). There was a consistent pattern showing that extrinsic activation of Wnt/β-catenin signaling strengthened the BBB phenotype of the hCMEC/D3 cells. The natural ligand, Wnt3a, produced the greatest increase in Axin-2 reporter gene expression and resulted in the highest up-regulation of BBB important transporters such as P-glycoprotein (Pgp), Glucose transporter 1 (Glut-1), breast cancer resistance protein (BCRP), multidrug resistance associated protein 4 (MRP-4) (Fig. 2.5d). Besides the changes in expression of various transporters important for BBB function, Wnt/β-catenin modulation altered expression of several intercellular junction proteins as well (Fig. 2.5e). The greatest alterations in BBB gene expression were exhibited by Wnt3a where 7-fold and 3-fold increases in Pgp and claudin-1 mRNA expression, respectively, were observed compared to control monolayers (Fig. 2.5d and 2.5e, $p<0.05$). Up-regulation of Pgp and claudin-1 expression was also observed with LiCl treatment although the magnitude was less than associated with Wnt3a (Fig 2.5d and 2.5e). In contrast to Wnt3a treated cells, LiCl did not significantly alter Glut-1 or MRP-4 transporter expression in hCMEC/D3 monolayers (Fig. 2.5d).

The increased expression of BBB relevant transporters and adhesion molecules observed at the mRNA level following extrinsic pharmacological activation of Wnt/β-catenin were also observed at the protein level. Both Wnt3a and LiCl treatments resulted in significant increases in cellular Pgp protein levels by ~2.0 and ~1.6 fold respectively ($p<0.05$, Fig. 2.5f). Similarly, a two-fold increase in claudin-1 protein expression was observed both in Wnt3a and LiCl treatment ($p<0.05$, Fig. 2.5g).

Exposure of hCMEC/D3 to Wnt3a increased monolayer electrical impedance suggesting improvement of the paracellular barrier (Fig. 2.3a). To identify if the changes in paracellular

barrier observed with electrical impedance corresponded to changes in solute permeability, a series of fluorescent markers were used. Treatment with Wnt3a reduced hCMEC/D3 permeabilty to the small molecule diffusion marker NaF (MW 376 g/mol), large molecular weight diffusion marker, IRdye 800CW PEG (MW 35 kDa) as well as the P-glycoprotein transport substrate, Rhodamine800 (Fig. 2.3b-d). As the intracellular levels of NaF and IR Dye 800CW PEG were not influenced by either Wnt3a or LiCl (see supplementary Fig. 2.S2d and 2.S2e), the reductions in monolayer permeability observed are likely attributable to reductions in paracellular diffusion of the fluorescent markers. In contrast, as intracellular levels of Rhodamine800 were reduced following Wnt3a treatment, the decreased permeability of Rhodamine800 observed following Wnt activation was likely a combination of decreased paracellular leak and enhanced intracellular drug efflux transport (see supplementary Fig. 2.S2f).

Additional investigation of transporter activity was done using cellular accumulation studies. As depicted in Fig. 2.3e, the cellular accumulation of the Pgp substrates Rhodamine 800[39], was decreased by ~20-25% under Wnt3a treatment ($p<0.05$). Inhibition of Pgp function by GF increased accumulation of Rhodamine 800 by ~30% ($p<0.01$). A similar increase in BCRP drug efflux transporter activity was observed following Wnt3a treatment. Wnt3a reduced mitoxantrone accumulation inside the cell by ~30% ($p<0.05$, Fig. 2.3f). Inhibition of BCRP function by GF120918 (GF) increased mitoxantrone accumulation by ~30% ($p<0.001$). Both studies suggested that BCRP and Pgp expression and function was partly regulated by Wnt/β-catenin signaling.

2.4.3.2 Inhibition of LiCl response using ICRT-3

To confirm that the changes in BBB phenotypes observed under LiCl treatment were associated with Wnt/β-catenin activity, a separated set of experiments was done by co-exposure of LiCl and ICRT-3 (Fig. 2.6). In this studies, combination of LiCl and ICRT-3 prevented the up-regulation of

Pgp, BCRP, claudin-1, and claudin-3 mRNA that was previously observed with LiCl treatment ($p<0.05$, Fig. 2.6a). In the electrical impedance studies, ICRT-3 was able to inhibit the improved barrier function initiated by LiCl (Fig. 2.6b).

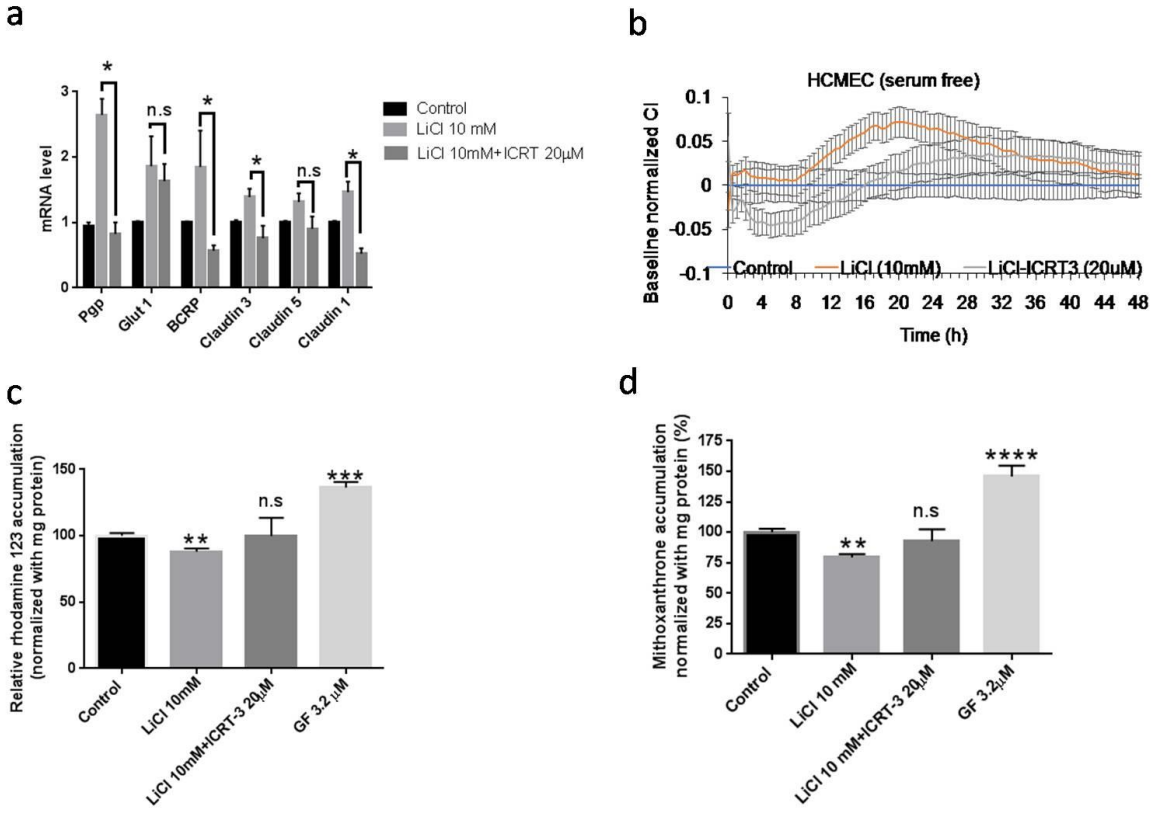

Figure 2.6: Wnt/β-catenin dependent responses to LiCl treatment in hCMEC/D3 monolayers. ICRT-3 inhibited the effects of LiCl (10mM) on gene expression (Panel A); paracellular permeability (Panel B) and drug efflux transporter activity (Panels C and D). Values represent the mean ± SEM of n of 4-6 monolayers per treatment group. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ as determined using two-way ANOVA and LSD Fisher's test for Panel A and one-way ANOVA and LSD Fisher's test for Panel C and D.

Treatment with LiCl reduced accumulation of Rhodamine123 and mitoxantrone by ~20% in the hCMEC/D3 suggesting improvement of Pgp and BCRP efflux function ($p<0.01$, Fig. 2.6c and 2.6d). The effects of LiCl on both Rhodamine123 and mitoxantrone accumulation were abolished by ICRT-3 (Fig. 2.6c and 2.6d). These studies suggested that improvements in paracellular barrier, P-glycoprotein and BCRP function observed with LiCl treatment were mediated by the binding of β-catenin to TCF-4.

2.4.4 Alteration of PLVAP expression by Wnt/β-catenin signaling

In addition to transporter and intercellular junction proteins, canonical Wnt signaling regulates the expression of plasmalemma vesicle-associated protein (PLVAP/Mecca 32/PV-1). This protein is associated with the ability of the brain endothelial cells to form stomatal caveolae or endothelial fenestrations[40,41], and increased expression of PLVAP is a marker for BBB dysfunction[42]. Activation of Wnt/β-catenin signaling by Wnt3a reduced PLVAP by ~40% while inhibition of β-catenin activity in nucleus by ICRT-3 increased PLVAP by ~50% ($p<0.05$ for both) (Fig. 2.7a). Functionally, reduction of PLVAP expression under Wnt3a treatment was associated with reduced accumulation of tetramethylrhodamine BSA by ~40% ($p<0.05$, Fig. 2.7b). A similar reduction was observed following treatment with the vesicular transport inhibitor, genestein, (200 µM), suggesting a role of Wnt/β-catenin signaling in regulating vesicular transport in the hCMEC/D3 brain endothelial cell model.

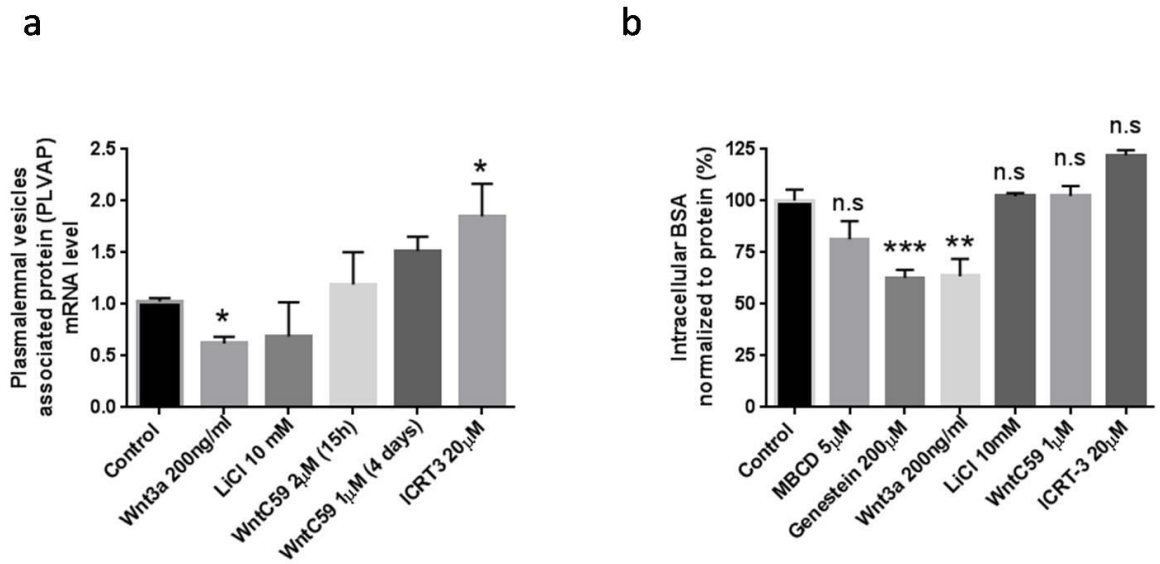

Figure 2.7: Modulation of Wnt/β-catenin in hCMEC/D3 cells alters endocytic activity. Altered PLVAP gene expression with pharmacological modulation of Wnt activity (Panel A). The reduction of PLVAP gene expression by Wnt3a is associated with less vesicular transport activity (Panel B). A and B:, n: 4-6; value was Mean ± SEM. *$p<0.05$; ** $p<0.005$ and *** $p<0.001$ determined by One-way ANOVA followed by LSD Fisher's test

2.5 Discussion

The importance of canonical Wnt signaling for BBB development has been widely studied[7,9,22,43]. Although the Wnt/β-catenin signaling activity in the brain microvasculature is significantly reduced following maturation, its role in BBB maintenance appears vital[8,9]. Evidence in support of this are the studies linking loss of endothelial β-catenin activity in an adult animal model with seizures and the resulting depletion of claudin-1 in the brain microvasculature[11]. Another study observed widespread sulfo-NHS-biotin leakage into the brain upon LRP-5 and LRP-6 conditional knockout in 24-day old mice[22].

It should be noted that public database analysis (www.genecards.org) confirms that many intracellular transporters and intercellular junction proteins important in maintaining a BBB phenotype have a binding site for TCF4, TCF7 and LEF-1, that are known transcription factors in the Wnt/β-catenin pathway. Genes for Pgp, BCRP, MRP4, Glut-1, claudin-1, claudin-3 and PLVAP have a regulatory element that interacts with TCF-4. In addition, studies using a TCF4 dominant negative mutant mouse demonstrated reduction in claudin-5 expression[22]. Together these studies suggest that Wnt/β-catenin signaling may play an important role in regulating gene expression important for maintaining the BBB phenotype.

Current understanding suggests that astrocytes are a source for Wnt ligand in the BBB[44]. There is also an emerging evidence to suggest that brain endothelial cells may be activated by Wnt proteins in an autocrine fashion in both *in vivo* and *in vitro* settings[16,45-47]. However, comprehensive studies looking at the functional impact of Wnt/β-catenin activation in human brain endothelial cell either through autocrine and exogenous activation have not been reported. The results of the present study suggest that 1) the contribution of Wnt ligand, produced by brain endothelial cells themselves, towards maintaining the BBB phenotype is likely minimal, 2) modulation of Wnt/β-

catenin signaling in brain endothelial cells from exogenous sources can significantly alter BBB function, and 3) the extent of BBB phenotype changes observed with Wnt activators correlated with the magnitude of Wnt/β-catenin produced.

The immortalized human cerebral microvessel endothelial cell line hCMEC/D3 is widely used as an *in-vitro* BBB model due to its ability to retain the brain endothelial phenotype from passage to passage compared human primary brain endothelial cells (HBMEC)[24-28]. While canonical Wnt signaling in the hCMEC/D3 cell model has been reported[29], the present study is the first to provide a comprehensive profiling of the effects of Wnt/β-catenin signaling, through both endogenous and exogenous activation routes, on the barrier properties of this cell. The current studies in the hCMEC/D3 culture model show the expression of multiple Wnt receptors, with eight frizzled receptors along with LRP-5 and LRP-6. The Wnt receptor profile observed in hCMEC/D3 was similar to previous reports in mouse brain microvessels[7]. Expression of frizzled-4 was particularly high in the hCMEC/D3 BBB culture model. Besides being a receptor for Wnt ligand, frizzled-4 is also important for Norrin, a non traditional ligand for Wnt canonical signaling[48,49]. Endothelial frizzled-4 deletion in the embryonic, postnatal and adulthood is associated with reduction of claudin-5 and increase expression of PLVAP in the cerebellum[22,48]. This suggested the important of Frizzled-4 not only for BBB development but also BBB maintenance.

In addition to Wnt receptors and co-receptors, the hCMEC/D3 also expressed various Wnt ligands including Wnt2b, Wnt3, Wnt4 and Wnt6. The hCMEC/D3 Wnt ligand profiling also showed moderate levels of Wnt7a and Wnt7b, Wnt ligands that were absent in various peripheral endothelial cell preparations[16,45]. In agreement with hCMEC/D3, our RT-PCR examination of primary human brain endothelial cells (HBMECs) also indicated expression of Wnt2b and Wnt3 as the major canonical Wnt ligand (see supplementary Fig. 2.S3). In general, HBMEC expressed

a similar Wnt receptor profile compared to hCMEC/D3 but more limited range of Wnt ligands and Wnt modulators compared to hCMEC/D3. Of note, Wnt7a was expressed in both primary cultured brain endothelial cells as well as hCMEC/D3. Wnt7a has an important role in regulating CNS angiogenesis and Glut 1, PLVAP as well as claudin-5 expression[43,50]. In the mouse embryo, Wnt7a and Wnt7b exhibited the broadest expression pattern in the developing CNS. Wnt7a and Wnt7b double knockout mice were non-viable due to CNS vessel malformation. *In vitro*, Wnt7a treatment was also reported to increase the expression of Glut-1 transporter in mouse brain endothelial cells[7].

The Wnt profiling of hCMEC/D3 also showed expression of several Wnt modulators including Dkk-1, Dkk-3, sFRP-1 and sFRP-3. Quantitative PCR studies suggested that hCMEC/D3 produced more Wnt modulators compared to Wnt ligands. These findings, together with the increased responses to exogenous Wnt activators versus endogenously released Wnt ligands in the hCMEC/D3, suggest that activation of Wnt/β-catenin in brain endothelial cells is more likely through a paracrine pathway involving Wnt agonists released from neighboring cells such as pericytes, neurons and astrocytes.

Additional evidence in favor of paracrine pathways for activation of Wnt/β-catenin processes in the brain endothelium are the studies using WntC59 to inhibit endogenous release of Wnt from the endothelial cells. Of the various BBB genes examined, only the expression of claudin-5 was significantly reduced following WntC59 treatment. Functionally, although there was a modest decrease in electric impedance of the hCMEC/D3, suggesting changes in tight junction formation, the permeability of both small and large permeability markers was unchanged following WntC59 treatment. Together these findings suggest that the brain endothelial cells have minimal Wnt activation through autocrine routes, but can greatly enhance their barrier properties following exogenous activation through Wnt receptor agonists or downstream pathway modulators.

While these studies showed minimal effect of inhibition of Wnt ligand release on barrier properties of hCMEC/D3, the brain microvessel endothelium has the capability to adjust the level of Wnt activation through autocrine pathways involving Wnt modulators or R-spondin (RSPO). Our studies and others found relatively high expression of RSPO in the human brain endothelial cell at the mRNA and protein level[51]. R-spondin (RSPO) is a non traditional Wnt agonist capable of regulating Wnt/β-signaling strength through altering frizzled receptor turnover[32,52]. Previous studies suggested that brain endothelial cells were the major producer of R-spondin compared to brain pericytes[51] and addition of RSPO together with Wnt3a increased Wnt/β-catenin activation by 10-fold compared to Wnt3a alone[33]. Thus, any small leak of Wnt release following WntC59 treatment could be potentiated by RSPO and result in some basal activity. In contrast, ICRT-3 blocks the TCF-4 transcription factor. Blocking at the level of the transcription factor would be expected to be more effective way of reducing the intrinsic Wnt activation as the pharmacological intervention occurs downstream of ligand interactions with the membrane receptors. This may explain why the effects observed with ICRT-3 appear to be more robust than the effect observed with WntC59.

While blocking endogenous Wnt ligand release and downstream transcription factor interactions had minimal impact on the BBB properties of the cell culture model, exogenous activation of Wnt/β-catenin using either Wnt3a or LiCl, significantly improved the BBB phenotype in hCMEC/D3 cells. Using Axin-2 expression as a reporter of Wnt/β-catenin activity, the highest activation of Wnt signaling in hCMEC/D3 cells was achieved through its natural ligand (Wnt3a). Activation of canonical Wnt pathways with Wnt3a also produced a robust effect on gene and protein expression involved in the BBB phenotype. This included selected transporters, such as

Pgp, Glut-1, BCRP, MRP-4, as well as tight junction proteins including claudin-1, claudin-3, claudin-5, and occludin.

Selection of Wnt3a, from the many Wnt ligands is based on the following. First, Wnt3a is not expressed in our hCMEC/D3 culture model of the BBB, nor in primary human brain microvessel endothelial cells. Studies in other peripheral endothelial cells also suggest that Wnt3a is not a ligand that would be produced by the endothelial cells themselves[16,45]. The source of Wnt3a in the brain is more likely from neuron, astrocytes and pericytes [53-55]. Thus the study with Wnt3a provided us with a way to examine the ability of the brain endothelial cells to respond to non-endothelial based Wnt ligands.

The effects observed with exogenous Wnt3a were more robust compared to the activation of Wnt/β-catenin through GSK inhibition. Similar results have also been observed in the bEnd-3, mouse endothelial cell line where Wnt3a conditioned media improved the BBB phenotype compared to LiCl treatment[21]. This could be explained by high potency of Wnt3a in activating Wnt/β-catenin signaling compared to other canonical Wnt ligands[38]. Unlike Wnt3a which activates the Wnt receptor, and triggers the binding of Axin and prevents the formation of the β-catenin destruction complex, LiCl activates Wnt indirectly through inhibition of GSK3[56]. As only a small pool of GSK3 is associated with Axin, it takes substantial inhibition of GSK3 to impact Wnt/β-catenin signaling. Indeed, it has been reported that GSK3 has to be inhibited by at least 80% before activation of Wnt/β-catenin is observed[57]. Thus there is a limit to activation of Wnt through GSK3 blockers that is not encountered with the Wnt receptor ligands.

In the present studies we also examined the effects of Wnt/β-catenin signaling on plasmalemma vesicle-associated protein PLVAP/Mecca 32 gene expression. This protein is responsible for

formation of stomatal fenestrae and caveolae involved in pore formation and vesicular transport, respectively, and is normally expressed at very low levels in brain endothelial cells[40,41]. Treatment with Wnt3a resulted in reductions in both PLVAP expression and fluorescently labeled albumin uptake in hCMEC/D3. As BSA is internalized through a caveoli-mediated process[58], the reductions in PLVAP expression observed with Wnt3a resulted in reduced endocytosis in the cells. Inhibition of autocrine activation of Wnt signaling with ICRT-3 resulted in an increase in PLVAP gene expression, although no significant alteration in caveoli-mediated endocytosis was observed. These finding are support the previous studies reporting that Wnt/β-catenin signaling influences PLVAP expression in brain endothelial cell[9,22,48,50].

The hCMEC/D3 monoculture model used in this study allowed examination of the intrinsic canonical Wnt signaling without interference from Wnt ligands produced by other brain cells. In the BBB neurovascular unit, astrocytes and perycites are known to release Wnt ligand for brain endothelial Wnt activity[44,53]. The present studies characterized the impact of Wnt/β-catenin signaling in the hCMEC/D3 cell culture model of the blood brain barrier, demonstrating changes in transporter, paracellular barrier and vesicular endocytosis activity through altered expression of genes important for the BBB phenotype (illustration in Fig. 2.S4). While the present studies showed limited contribution of autocrine Wnt signaling pathways in the hCMEC/D3 to the establishment of the BBB phenotype, a more robust response was observed from exogenous agents that activated the canonical Wnt signaling pathway. The improvement in barrier properties of the hCMEC/D3 following Wnt ligand and LiCl treatment suggests Wnt/β-catenin signaling may ameliorate BBB compromise under various pathophysiological conditions.

2.6 References

1 On, N. H. & Miller, D. W. Transporter-based delivery of anticancer drugs to the brain: improving brain penetration by minimizing drug efflux at the blood-brain barrier. *Current pharmaceutical design* **20**, 1499-1509 (2014).

2 Daneman, R. & Prat, A. The blood-brain barrier. *Cold Spring Harbor perspectives in biology* **7**, a020412, doi:10.1101/cshperspect.a020412 (2015).

3 Mahringer, A. & Fricker, G. ABC transporters at the blood-brain barrier. *Expert opinion on drug metabolism & toxicology* **12**, 499-508, doi:10.1517/17425255.2016.1168804 (2016).

4 Liebner, S. *et al.* Functional morphology of the blood-brain barrier in health and disease. *Acta neuropathologica* **135**, 311-336, doi:10.1007/s00401-018-1815-1 (2018).

5 Dyrna, F., Hanske, S., Krueger, M. & Bechmann, I. The blood-brain barrier. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology* **8**, 763-773, doi:10.1007/s11481-013-9473-5 (2013).

6 Abbott, N. J., Patabendige, A. A., Dolman, D. E., Yusof, S. R. & Begley, D. J. Structure and function of the blood-brain barrier. *Neurobiology of disease* **37**, 13-25, doi:10.1016/j.nbd.2009.07.030 (2010).

7 Daneman, R. *et al.* Wnt/beta-catenin signaling is required for CNS, but not non-CNS, angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 641-646, doi:10.1073/pnas.0805165106 (2009).

8 Harati, R., Benech, H., Villegier, A. S. & Mabondzo, A. P-glycoprotein, breast cancer resistance protein, Organic Anion Transporter 3, and Transporting Peptide 1a4 during blood-brain barrier maturation: involvement of Wnt/beta-catenin and endothelin-1 signaling. *Molecular pharmaceutics* **10**, 1566-1580, doi:10.1021/mp300334r (2013).

9 Liebner, S. *et al.* Wnt/beta-catenin signaling controls development of the blood-brain barrier. *The Journal of cell biology* **183**, 409-417, doi:10.1083/jcb.200806024 (2008).

10 Lengfeld, J. E. *et al.* Endothelial Wnt/beta-catenin signaling reduces immune cell infiltration in multiple sclerosis. *Proceedings of the National Academy of Sciences of the United States of America* **114**, E1168-E1177, doi:10.1073/pnas.1609905114 (2017).

11 Tran, K. A. *et al.* Endothelial beta-Catenin Signaling Is Required for Maintaining Adult Blood-Brain Barrier Integrity and Central Nervous System Homeostasis. *Circulation* **133**, 177-186, doi:10.1161/CIRCULATIONAHA.115.015982 (2016).

12 Liu, L., Wan, W., Xia, S., Kalionis, B. & Li, Y. Dysfunctional Wnt/beta-catenin signaling contributes to blood-brain barrier breakdown in Alzheimer's disease. *Neurochemistry international* **75**, 19-25, doi:10.1016/j.neuint.2014.05.004 (2014).

13 Lim, R. G. *et al.* Huntington's Disease iPSC-Derived Brain Microvascular Endothelial Cells Reveal WNT-Mediated Angiogenic and Blood-Brain Barrier Deficits. *Cell reports* **19**, 1365-1377, doi:10.1016/j.celrep.2017.04.021 (2017).

14 Inestrosa, N. C. & Arenas, E. Emerging roles of Wnts in the adult nervous system. *Nature reviews. Neuroscience* **11**, 77-86, doi:10.1038/nrn2755 (2010).

15 Oliva, C. A., Vargas, J. Y. & Inestrosa, N. C. Wnts in adult brain: from synaptic plasticity to cognitive deficiencies. *Frontiers in cellular neuroscience* **7**, 224, doi:10.3389/fncel.2013.00224 (2013).

16 Goodwin, A. M., Sullivan, K. M. & D'Amore, P. A. Cultured endothelial cells display endogenous activation of the canonical Wnt signaling pathway and express multiple ligands, receptors, and secreted modulators of Wnt signaling. *Developmental dynamics : an official publication of the American Association of Anatomists* **235**, 3110-3120, doi:10.1002/dvdy.20939 (2006).

17 Nelson, W. J. & Nusse, R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* **303**, 1483-1487, doi:10.1126/science.1094291 (2004).

18 Mikels, A. J. & Nusse, R. Wnts as ligands: processing, secretion and reception. *Oncogene* **25**, 7461-7468, doi:10.1038/sj.onc.1210053 (2006).

19 Gordon, M. D. & Nusse, R. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. *The Journal of biological chemistry* **281**, 22429-22433, doi:10.1074/jbc.R600015200 (2006).

20 Valkenburg, K. C., Graveel, C. R., Zylstra-Diegel, C. R., Zhong, Z. & Williams, B. O. Wnt/beta-catenin Signaling in Normal and Cancer Stem Cells. *Cancers* **3**, 2050-2079, doi:10.3390/cancers3022050 (2011).

21 Paolinelli, R. *et al.* Wnt activation of immortalized brain endothelial cells as a tool for generating a standardized model of the blood brain barrier in vitro. *PloS one* **8**, e70233, doi:10.1371/journal.pone.0070233 (2013).

22 Zhou, Y. *et al.* Canonical WNT signaling components in vascular development and barrier formation. *The Journal of clinical investigation* **124**, 3825-3846, doi:10.1172/JCI76431 (2014).

23 Weksler, B., Romero, I. A. & Couraud, P. O. The hCMEC/D3 cell line as a model of the human blood brain barrier. *Fluids and barriers of the CNS* **10**, 16, doi:10.1186/2045-8118-10-16 (2013).

24 Poller, B. *et al.* The human brain endothelial cell line hCMEC/D3 as a human blood-brain barrier model for drug transport studies. *Journal of neurochemistry* **107**, 1358-1368, doi:10.1111/j.1471-4159.2008.05730.x (2008).

25 Vu, K., Weksler, B., Romero, I., Couraud, P. O. & Gelli, A. Immortalized human brain endothelial cell line HCMEC/D3 as a model of the blood-brain barrier facilitates in vitro studies of central nervous system infection by Cryptococcus neoformans. *Eukaryotic cell* **8**, 1803-1807, doi:10.1128/EC.00240-09 (2009).

26 Helms, H. C. *et al.* In vitro models of the blood-brain barrier: An overview of commonly used brain endothelial cell culture models and guidelines for their use. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* **36**, 862-890, doi:10.1177/0271678X16630991 (2016).

27 Kulczar, C., Lubin, K. E., Lefebvre, S., Miller, D. W. & Knipp, G. T. Development of a direct contact astrocyte-human cerebral microvessel endothelial cells blood-brain barrier coculture model. *The Journal of pharmacy and pharmacology* **69**, 1684-1696, doi:10.1111/jphp.12803 (2017).

28 Nguyen, H. M. *et al.* Reduction in cardiolipin decreases mitochondrial spare respiratory capacity and increases glucose transport into and across human brain cerebral microvascular endothelial cells. *Journal of neurochemistry* **139**, 68-80, doi:10.1111/jnc.13753 (2016).

29 Lim, J. C. *et al.* Activation of beta-catenin signalling by GSK-3 inhibition increases p-glycoprotein expression in brain endothelial cells. *Journal of neurochemistry* **106**, 1855-1865, doi:10.1111/j.1471-4159.2008.05537.x (2008).

30 Pinzon-Daza, M. L. *et al.* The cross-talk between canonical and non-canonical Wnt-dependent pathways regulates P-glycoprotein expression in human blood-brain barrier cells. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* **34**, 1258-1269, doi:10.1038/jcbfm.2014.100 (2014).

31 Cruciat, C. M. & Niehrs, C. Secreted and transmembrane wnt inhibitors and activators. *Cold Spring Harbor perspectives in biology* **5**, a015081, doi:10.1101/cshperspect.a015081 (2013).

32 de Lau, W. B., Snel, B. & Clevers, H. C. The R-spondin protein family. *Genome biology* **13**, 242, doi:10.1186/gb-2012-13-3-242 (2012).

33 Janda, C. Y. *et al.* Surrogate Wnt agonists that phenocopy canonical Wnt and beta-catenin signalling. *Nature* **545**, 234-237, doi:10.1038/nature22306 (2017).

34 Proffitt, K. D. *et al.* Pharmacological inhibition of the Wnt acyltransferase PORCN prevents growth of WNT-driven mammary cancer. *Cancer research* **73**, 502-507, doi:10.1158/0008-5472.CAN-12-2258 (2013).

35 Takada, R. *et al.* Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion. *Developmental cell* **11**, 791-801, doi:10.1016/j.devcel.2006.10.003 (2006).

36 Teng, Z., Kuang, X., Wang, J. & Zhang, X. Real-time cell analysis--a new method for dynamic, quantitative measurement of infectious viruses and antiserum neutralizing activity. *Journal of virological methods* **193**, 364-370, doi:10.1016/j.jviromet.2013.06.034 (2013(Sun *et al.* 2012)).

37 Gonsalves, F. C. *et al.* An RNAi-based chemical genetic screen identifies three small-molecule inhibitors of the Wnt/wingless signaling pathway. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 5954-5963, doi:10.1073/pnas.1017496108 (2011).

38 Voloshanenko, O., Gmach, P., Winter, J., Kranz, D. & Boutros, M. Mapping of Wnt-Frizzled interactions by multiplex CRISPR targeting of receptor gene families. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **31**, 4832-4844, doi:10.1096/fj.201700144R (2017).

39 On, N. H., Chen, F., Hinton, M. & Miller, D. W. Assessment of P-glycoprotein activity in the Blood-Brain Barrier (BBB) using Near Infrared Fluorescence (NIRF) imaging techniques. *Pharmaceutical research* **28**, 2505-2515, doi:10.1007/s11095-011-0478-6 (2011).

40 Guo, L., Zhang, H., Hou, Y., Wei, T. & Liu, J. Plasmalemma vesicle-associated protein: A crucial component of vascular homeostasis. *Experimental and therapeutic medicine* **12**, 1639-1644, doi:10.3892/etm.2016.3557 (2016).

41 Herrnberger, L. *et al.* Lack of endothelial diaphragms in fenestrae and caveolae of mutant Plvap-deficient mice. *Histochemistry and cell biology* **138**, 709-724, doi:10.1007/s00418-012-0987-3 (2012).

42 Shue, E. H. *et al.* Plasmalemmal vesicle associated protein-1 (PV-1) is a marker of blood-brain barrier disruption in rodent models. *BMC neuroscience* **9**, 29, doi:10.1186/1471-2202-9-29 (2008).

43 Stenman, J. M. *et al.* Canonical Wnt signaling regulates organ-specific assembly and differentiation of CNS vasculature. *Science* **322**, 1247-1250, doi:10.1126/science.1164594 (2008).

44 Wevers, N. R. & de Vries, H. E. Morphogens and blood-brain barrier function in health and disease. *Tissue barriers* **4**, e1090524, doi:10.1080/21688370.2015.1090524 (2016).

45 Korn, C. *et al.* Endothelial cell-derived non-canonical Wnt ligands control vascular pruning in angiogenesis. *Development* **141**, 1757-1766, doi:10.1242/dev.104422 (2014).

46 Klein, D. *et al.* Wnt2 acts as a cell type-specific, autocrine growth factor in rat hepatic sinusoidal endothelial cells cross-stimulating the VEGF pathway. *Hepatology* **47**, 1018-1031, doi:10.1002/hep.22084 (2008).

47 Franco, C. A. *et al.* Non-canonical Wnt signalling modulates the endothelial shear stress flow sensor in vascular remodelling. *eLife* **5**, e07727, doi:10.7554/eLife.07727 (2016).

48 Wang, Y. *et al.* Norrin/Frizzled4 signaling in retinal vascular development and blood brain barrier plasticity. *Cell* **151**, 1332-1344, doi:10.1016/j.cell.2012.10.042 (2012).

49 Luhmann, U. F. *et al.* Vascular changes in the cerebellum of Norrin /Ndph knockout mice correlate with high expression of Norrin and Frizzled-4. *The European journal of neuroscience* **27**, 2619-2628, doi:10.1111/j.1460-9568.2008.06237.x (2008).

50 Wang, Y. *et al.* Interplay of the Norrin and Wnt7a/Wnt7b signaling systems in blood-brain barrier and blood-retina barrier development and maintenance. *Proceedings of the National Academy of Sciences of the United States of America* **115**, E11827-E11836, doi:10.1073/pnas.1813217115 (2018).

51 Scholz, B. *et al.* Endothelial RSPO3 Controls Vascular Stability and Pruning through Non-canonical WNT/Ca(2+)/NFAT Signaling. *Developmental cell* **36**, 79-93, doi:10.1016/j.devcel.2015.12.015 (2016).

52 de Lau, W., Peng, W. C., Gros, P. & Clevers, H. The R-spondin/Lgr5/Rnf43 module: regulator of Wnt signal strength. *Genes & development* **28**, 305-316, doi:10.1101/gad.235473.113 (2014).

53 Cecchelli, R. *et al.* A stable and reproducible human blood-brain barrier model derived from hematopoietic stem cells. *PloS one* **9**, e99733, doi:10.1371/journal.pone.0099733 (2014).

54 Okamoto, M. *et al.* Reduction in paracrine Wnt3 factors during aging causes impaired adult neurogenesis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **25**, 3570-3582, doi:10.1096/fj.11-184697 (2011).

55 Chen, J., Park, C. S. & Tang, S. J. Activity-dependent synaptic Wnt release regulates hippocampal long term potentiation. *The Journal of biological chemistry* **281**, 11910-11916, doi:10.1074/jbc.M511920200 (2006).

56 Stambolic, V., Ruel, L. & Woodgett, J. R. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Current biology : CB* **6**, 1664-1668 (1996).

57 Doble, B. W., Patel, S., Wood, G. A., Kockeritz, L. K. & Woodgett, J. R. Functional redundancy of GSK-3alpha and GSK-3beta in Wnt/beta-catenin signaling shown by using an allelic series of embryonic stem cell lines. *Developmental cell* **12**, 957-971, doi:10.1016/j.devcel.2007.04.001 (2007).

58 Sun, Z. Z. *et al.* Differential internalization of brick shaped iron oxide nanoparticles by endothelial cells. *J Mater Chem B* **4**, 5913-5920, doi:10.1039/c6tb01480a (2016).

59 Weksler, B. B. *et al.* Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **19**, 1872-1874, doi:10.1096/fj.04-3458fje (2005).

60 Bardelmeijer, H. A. *et al.* Increased oral bioavailability of paclitaxel by GF120918 in mice through selective modulation of P-glycoprotein. *Clinical cancer research : an official journal of the American Association for Cancer Research* **6**, 4416-4421 (2000).

61 Fontaine, M., Elmquist, W. F. & Miller, D. W. Use of rhodamine 123 to examine the functional activity of P-glycoprotein in primary cultured brain microvessel endothelial cell monolayers. *Life sciences* **59**, 1521-1531 (1996).

62 Pan, G., Giri, N. & Elmquist, W. F. Abcg2/Bcrp1 mediates the polarized transport of antiretroviral nucleosides abacavir and zidovudine. *Drug metabolism and disposition: the biological fate of chemicals* **35**, 1165-1173, doi:10.1124/dmd.106.014274 (2007).

63 Vercauteren, D. *et al.* The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls. *Molecular therapy : the journal of the American Society of Gene Therapy* **18**, 561-569, doi:10.1038/mt.2009.281 (2010).

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AUTHOR CONTRIBUTION

DWM and ML designed the study. ML performed most of the experiment, analyzed the data and prepared the manuscript. VY performed RTCA. WX assisted ML with permeability studies. SHK provide proteomic data of hCMEC/D3.

2.7 SUPPLEMENTARY INFORMATON CHAPTER 2

Supplemental information for Modulation of Wnt/β-catenin signaling promotes blood-brain barrier phenotype in cultured brain endothelial cells

Marlyn Laksitorini[1,4], Vinith Yathindranath[1], Wei Xiong[1], Sabine Hombach-Klonisch[3], Donald W. Miller[1,2]
[1]*Department of Pharmacology and Theurapetics, Max Rady College of Medicine, University of Manitoba, R3E 0T6, Canada.*
[2.] *Kleysen Institute of Advanced Medicine, Health Sciences Center, Winnipeg, Manitoba, R3E 0T6, Canada.*
[3.]*Department of Human Anatomy and Cell Science, Max Rady College of Medicine, University of Manitoba, R3E 0J9, Canada.*
[4]*Department of Pharmaceutics, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, 55281, Indonesia.*
**corresponding author: Donald.Miller@umanitoba.ca*

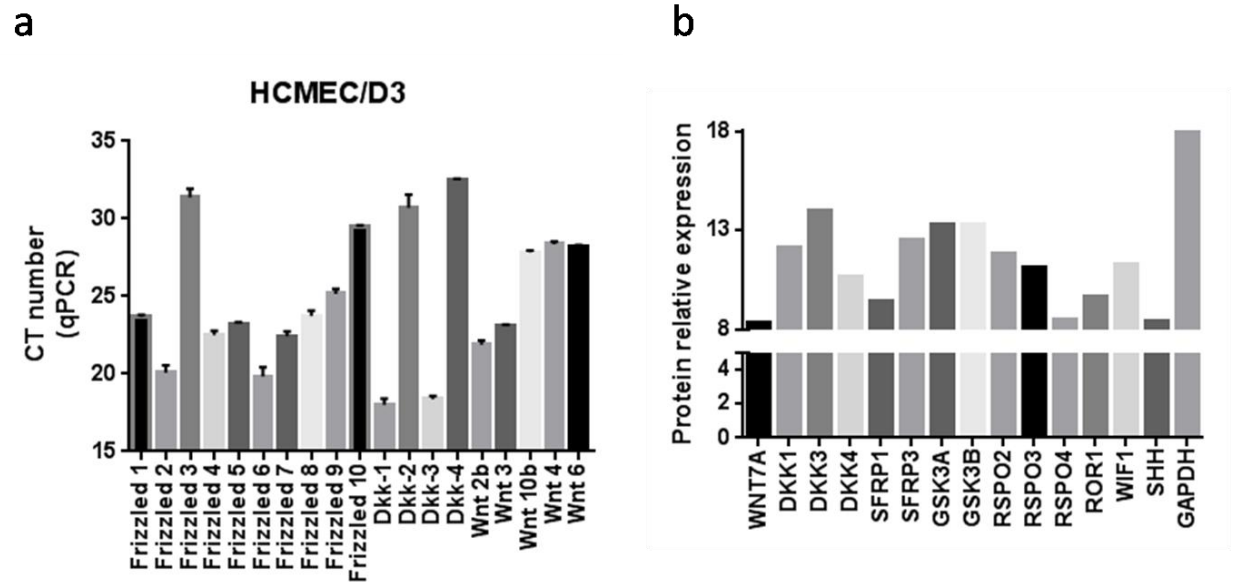

Figure 2.S1: Relative quantification of Wnt component at hCMEC/D3. a) Comparison of major Wnt component expression level at hCMEC/D3 examined using qPCR. RNA sample was isolated from three separated cultured. CT number expressed the abundance of the RNA transcript in the cell. CT number for β-actin was approximately 11.9. b) Relative expression of Wnt component protein expression examined by Somologic Inc. Cell were grown in T75 cell under normal condition until confluence before sent to Somologic Inc for proteomic analysis.

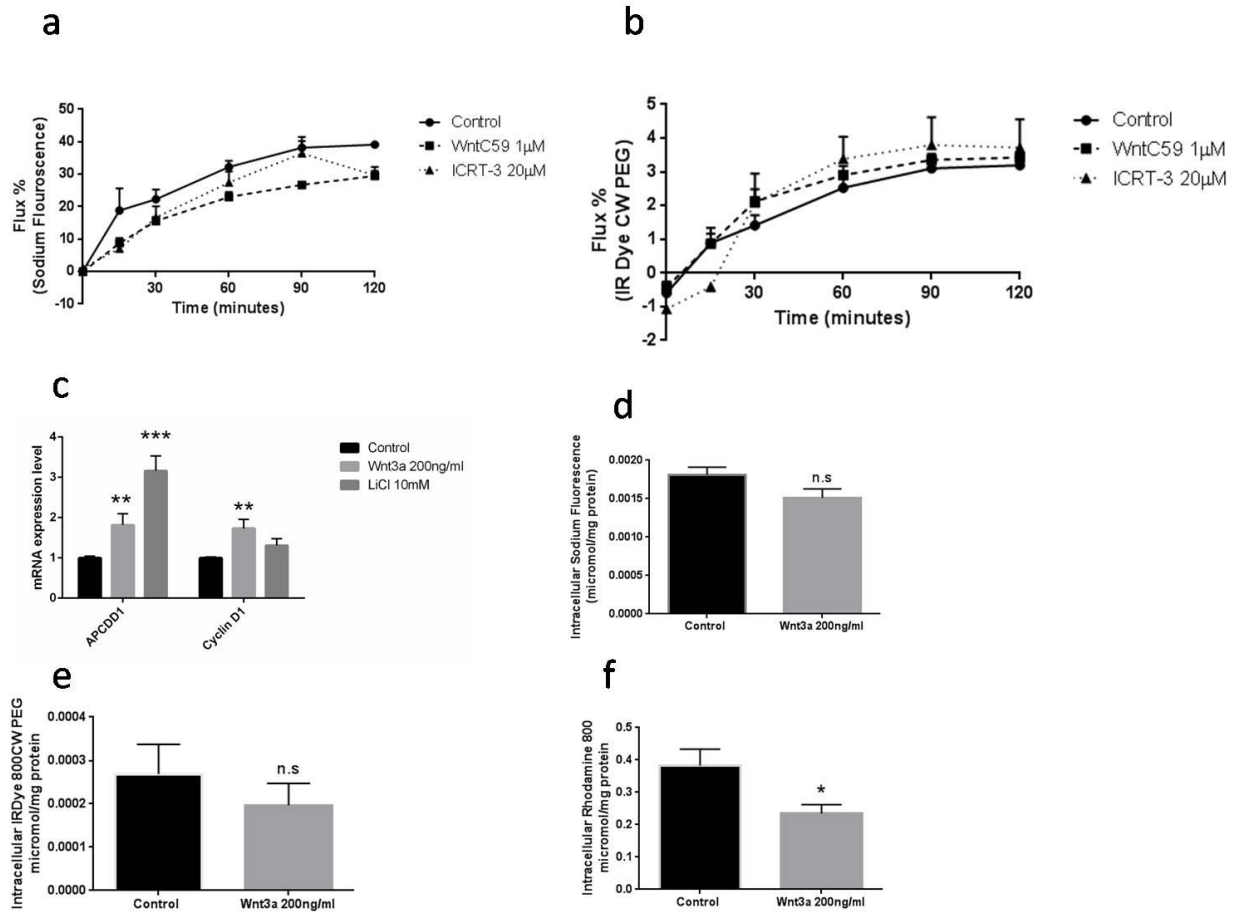

Figure 2.S2: Permeability studies on Wnt inhibitior. a) Permeability of sodium fluorescein across hCMEC/D3 monolayer upon WntC59 or ICRT-3 treatment. b) Permeability of IR Dye 800CW PEG across hCMEC/D3 monolayer upon WntC59 or ICRT-3 treatment. c) Upregulation of APCDD1 and Cyclin D1 under Wnt3a and LiCl treatment. d, e and f) Intracellular accumulation of sodium fluorescein, IR Dye 800CW PEG and Rhodamine 800 post permeability study on cell that been treated with Wnt3a. a and b: n of 6-9, mean±SEM; c: n of 4, two-way ANOVA followed by LSD Fisher's test, d, e and f: One tail t-test, n of 3. *p<0.05; *p<0.01; ** p<0.001. mean±SEM.

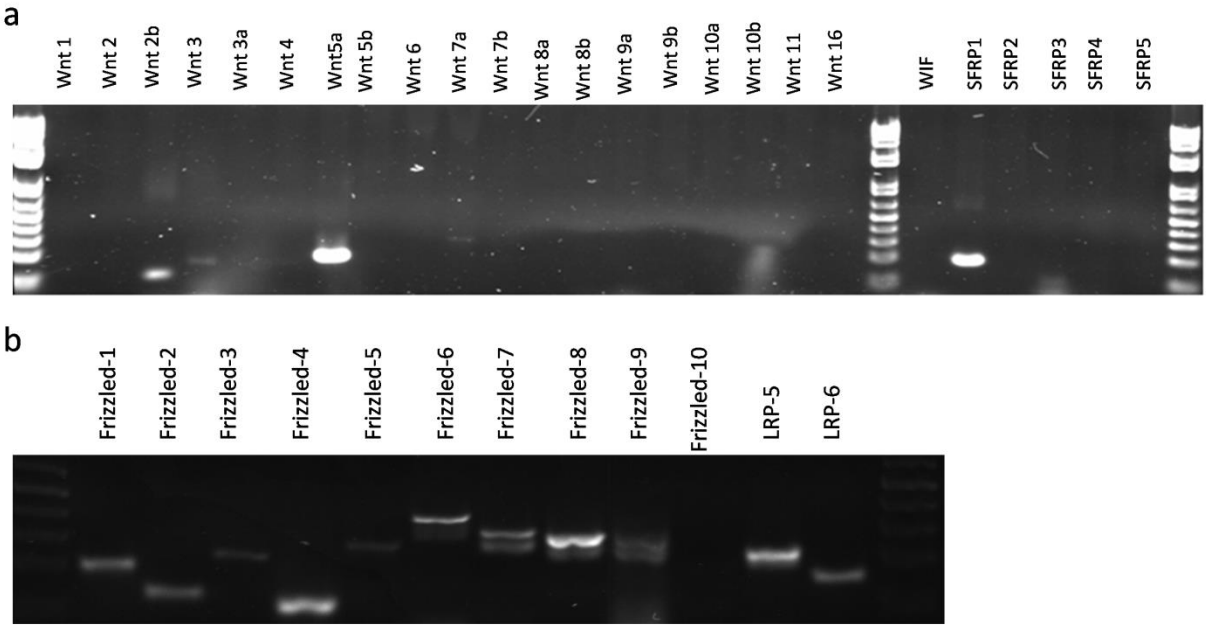

Figure 2.S3: Expression of Wnt ligand, Wnt modulator (a) and Wnt receptor (b) at primary brain endothelial cell (HBMEC). HBMEC was cultured in completed EBM-2 media. Cell was harvested when reaching confluence

2.Modulation of Wnt/β-catenin signaling promotes BBB phenotypes

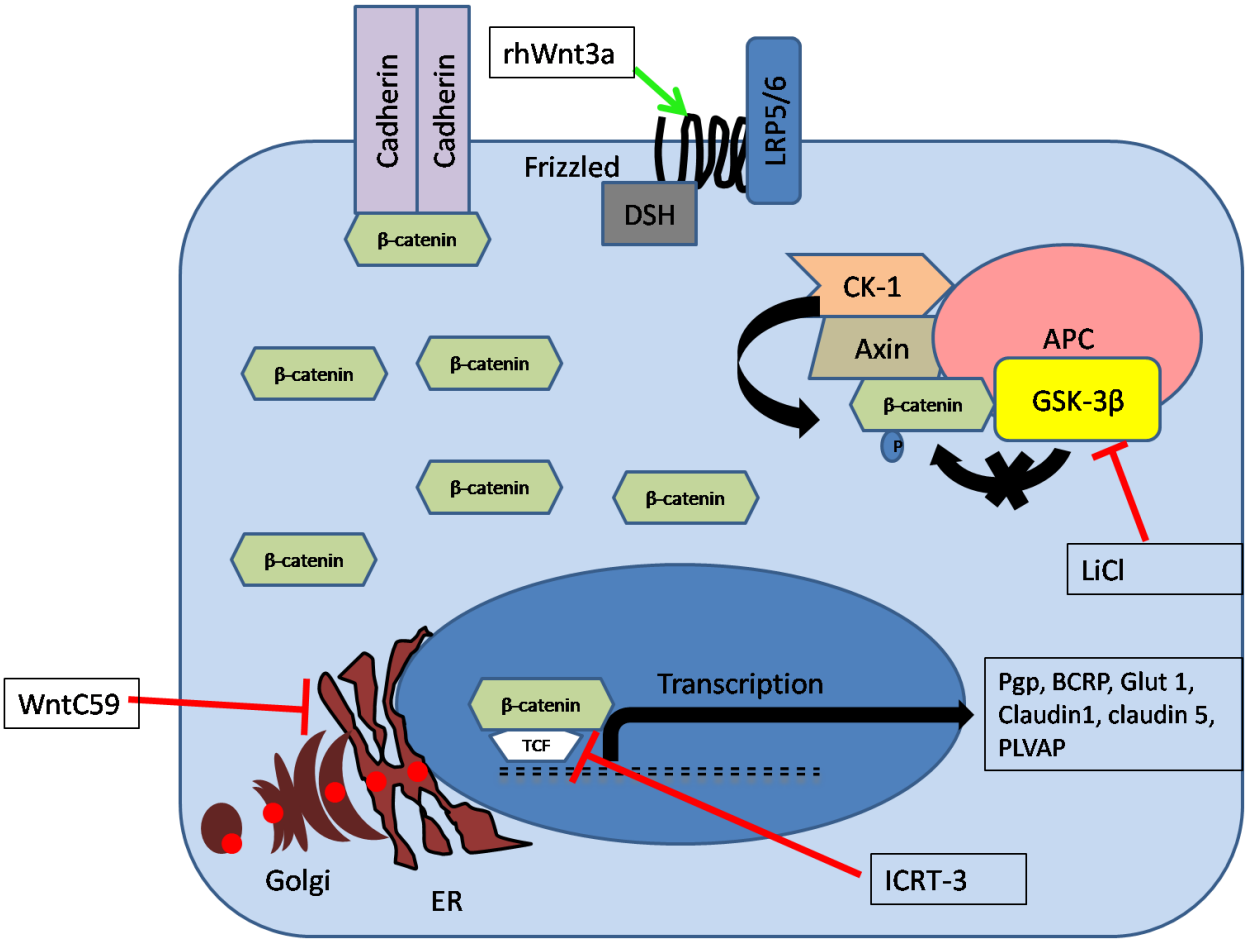

Figure 2.S4: Modulation of Wnt/β-catenin signaling at immortalized human brain endothelial cell (hCMEC/D3). Both natural ligand (Wnt3a) and GSK inhibition (LiCl) activated Wnt/β-catenin signaling although Wnt3a produced more robust improvement in blood brain barrier function. Inhibition of Wnt released from hCMEC/D3 using WntC59 produced minimal changed on BBB function. More downstream Wnt inhibition by inhibiting β-catenin binding to TCF-4 using ICRT-3 resulted more dramatic changes in BBB function. Activation of Wnt/β-catenin upregulated P-glycoprotein, BCRP, claudin-1, claudin-5 and PLVAP.

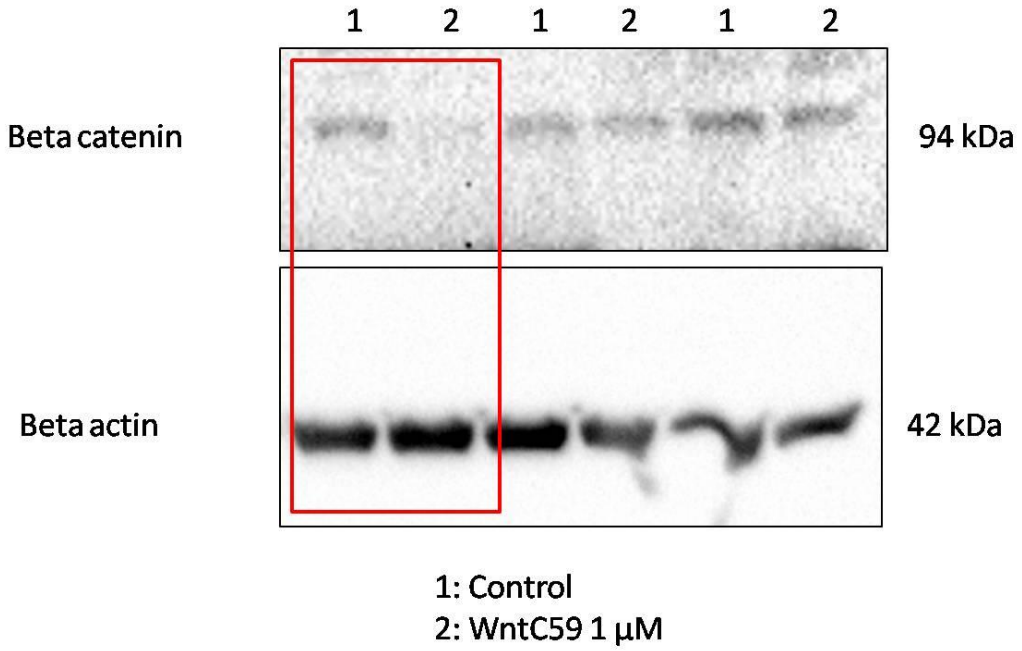

Figure 2.S5: Original full lenght blot of Fig. 2. 2a. Boxes indicated areas shown in the figure.

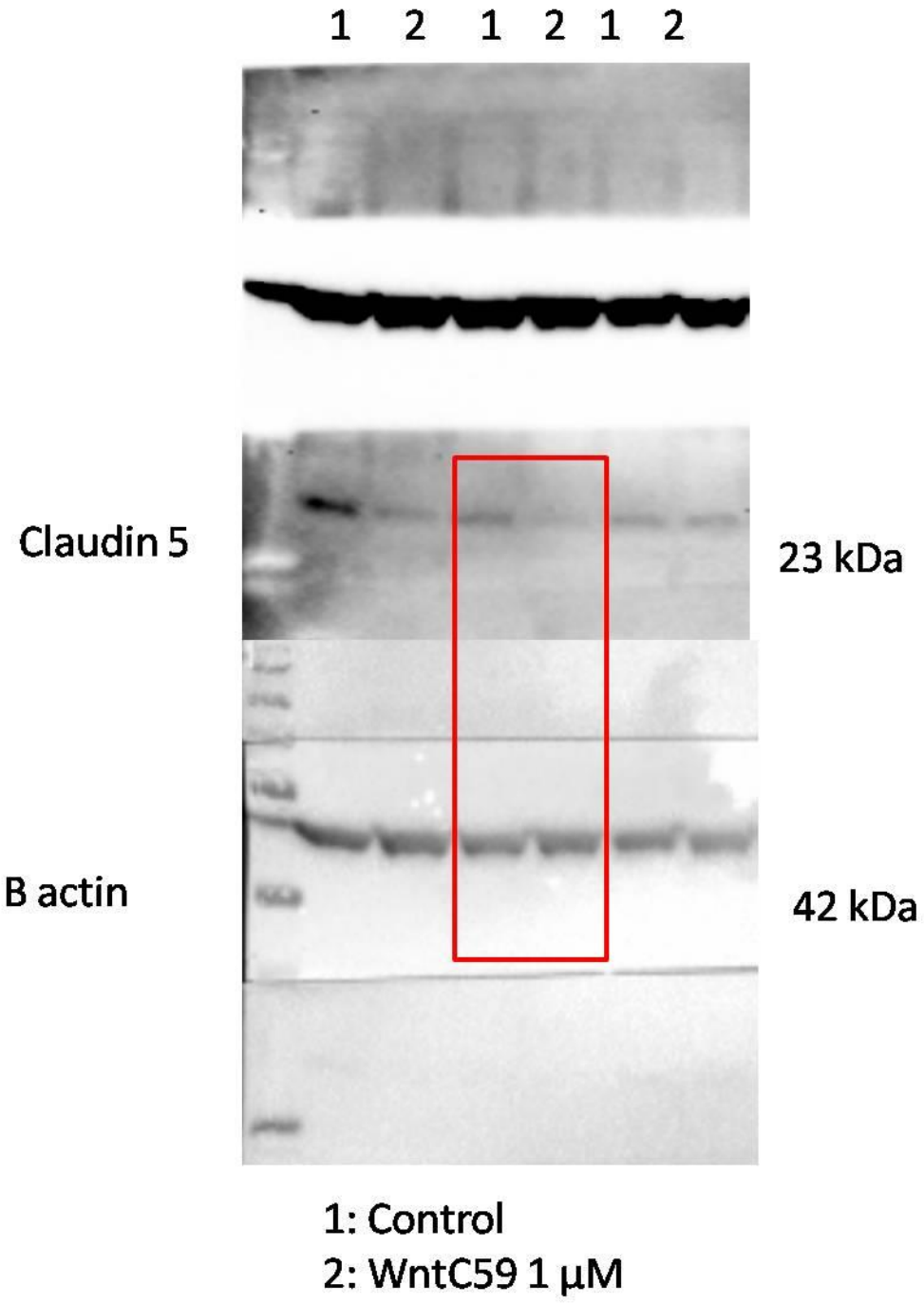

Figure 2.S6: Original full lenght blot of Fig. 2.2d. Boxes indicated areas shown in the figure.

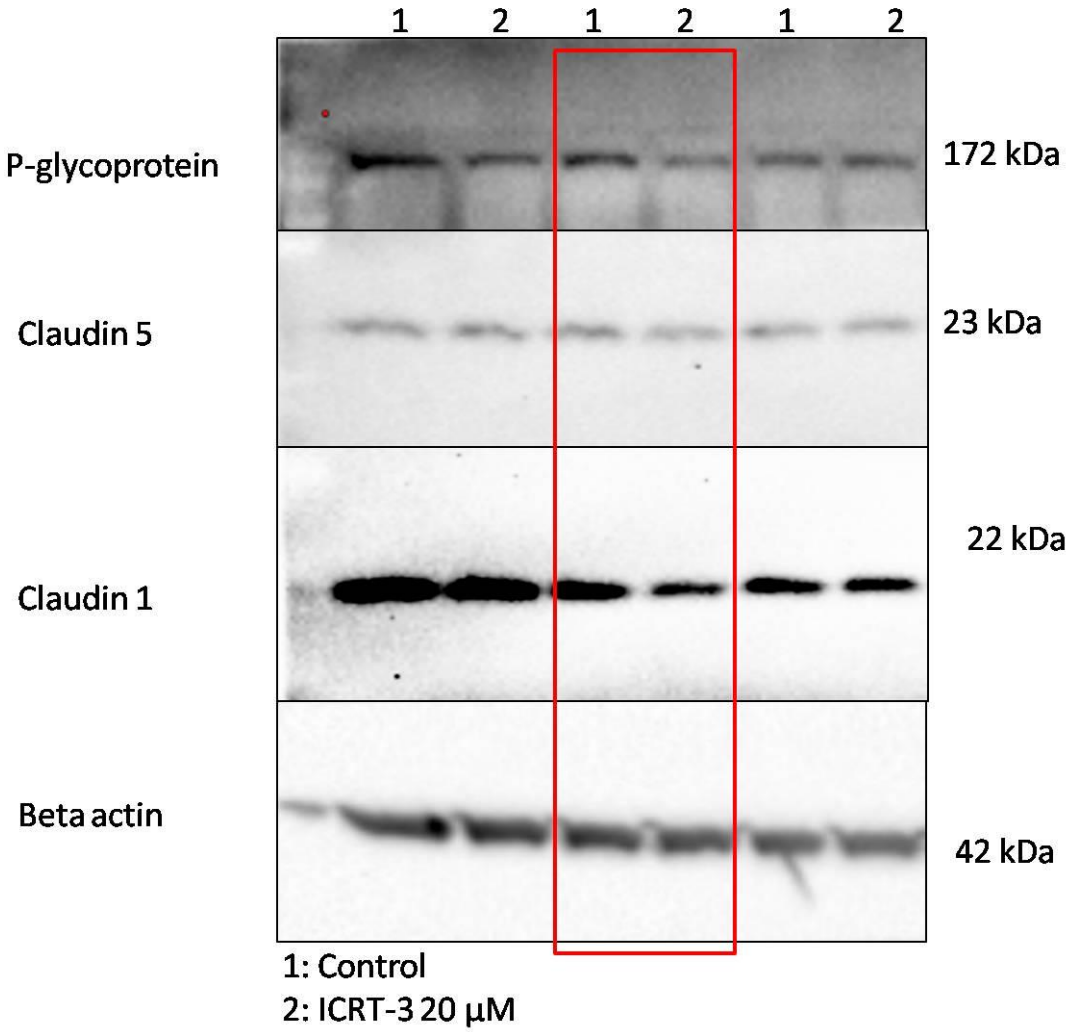

Figure 2.S7: Original full lenght blot of Fig. 2.3b, 2.3c and 2.3d. Boxes indicated areas shown in the figure.

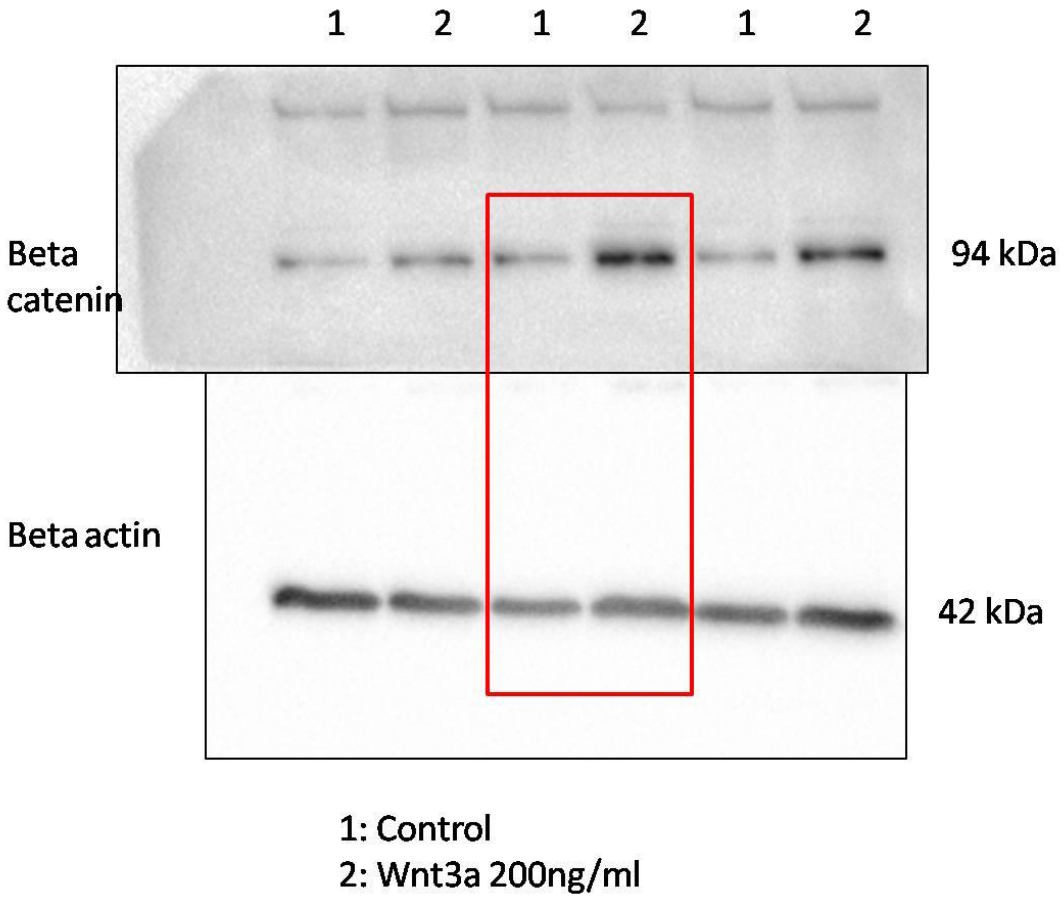

Figure 2.S8: Original full lenght blot of Fig. 2.4a. Boxes indicated areas shown in the figure.

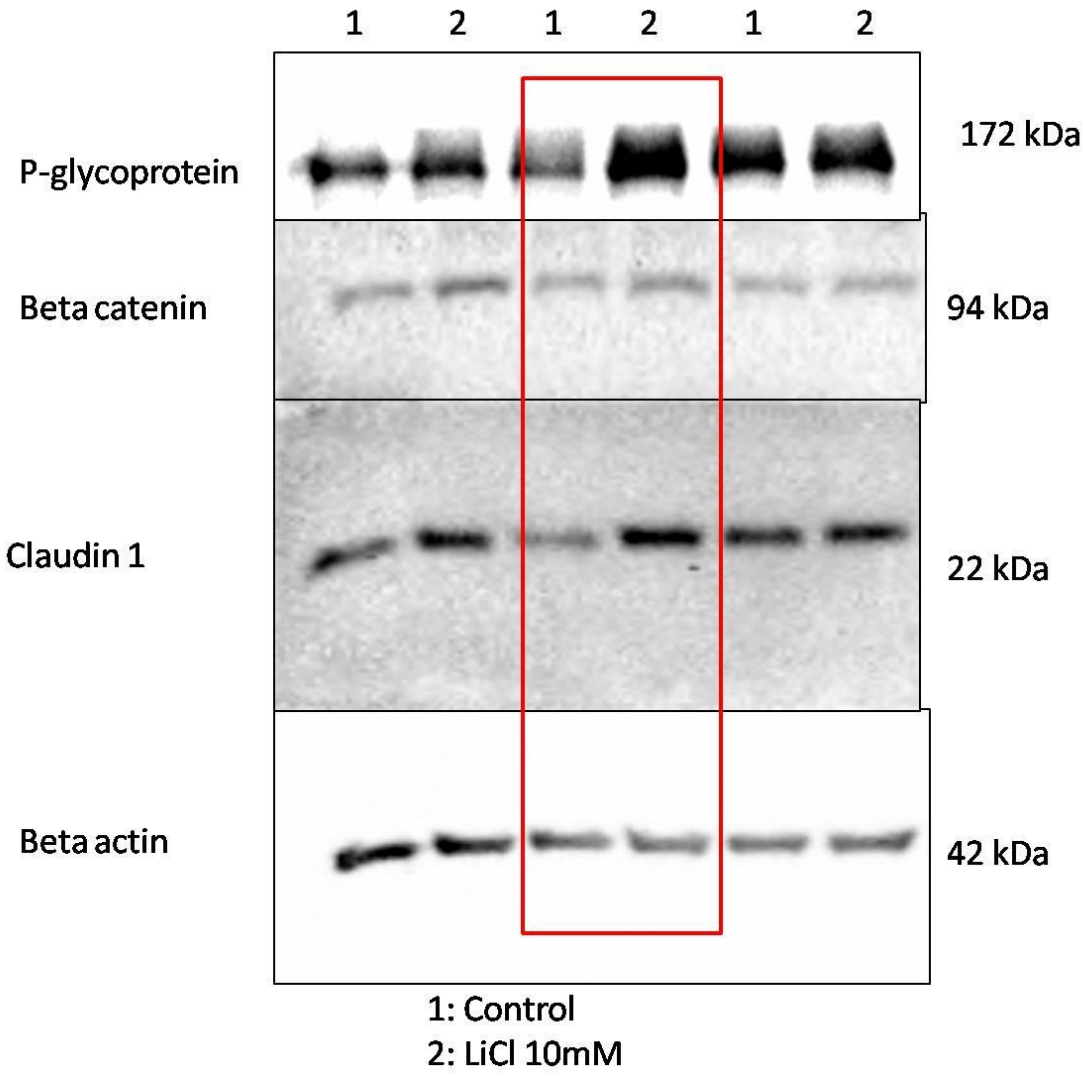

Figure 2.S9: Original full lenght blot of Fig. 2.4b, 2.4f and 2.4g for LiCl. Boxes indicated areas shown in the figure.

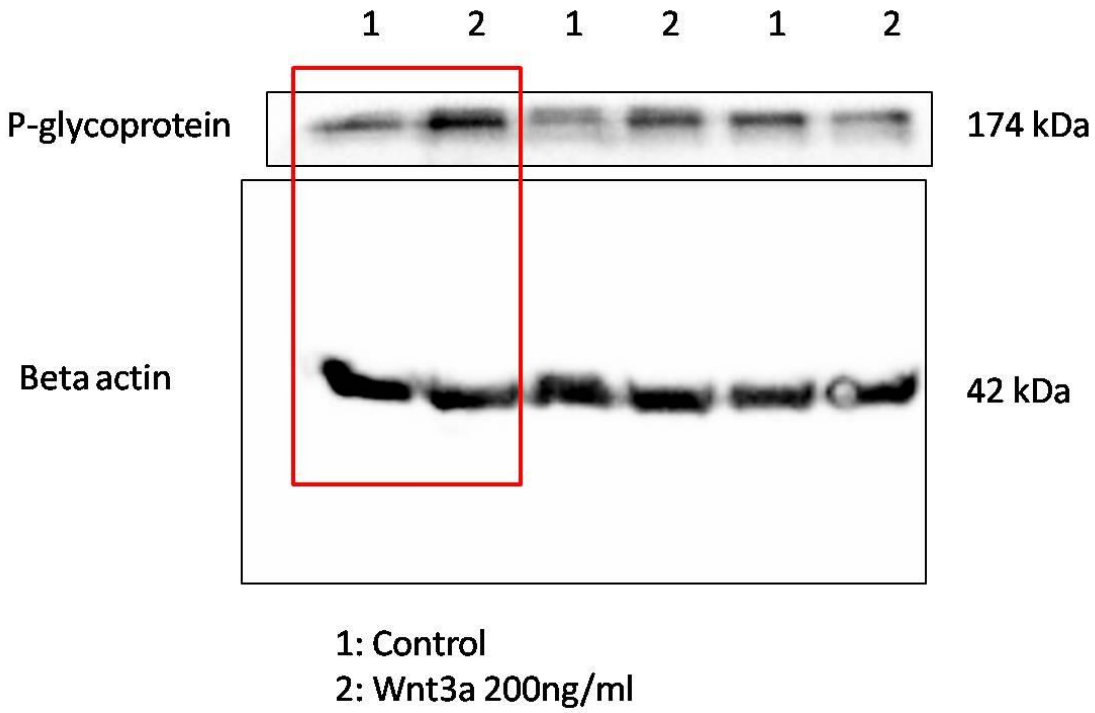

Figure 2. S10: Original full lenght blot of Fig. 2.4f for Wnt3a. Boxes indicated areas shown in the figure.

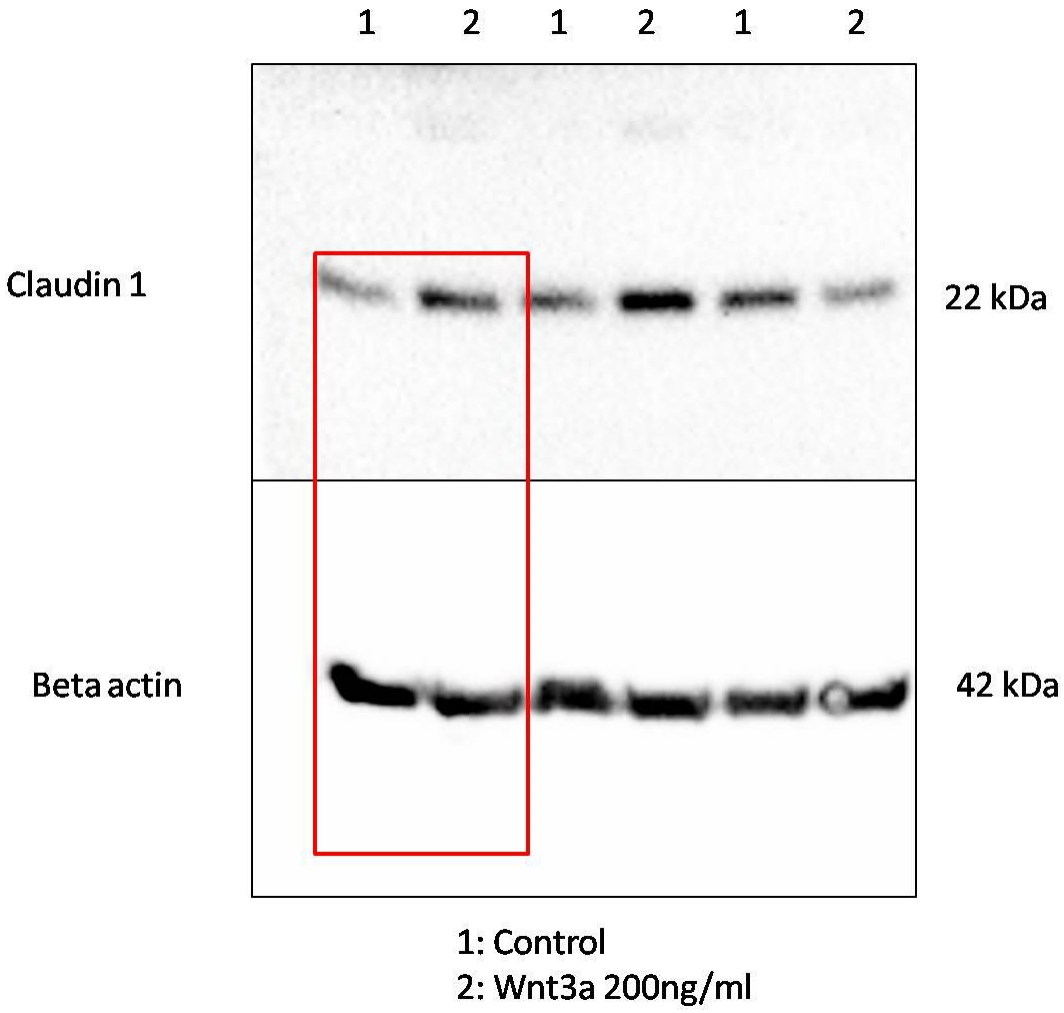

Figure 2.S11: Original full lenght blot of Fig. 2.4g for Wnt3a. Boxes indicated areas shown in the figure.

Table 2.1. Primer sequence for Human Wnt machineries and BBB phenotypes.

No	Primer	Sequence
1	hWnt1-Forward	CAAGATCGTCAACCGAGGCT
2	hWnt1-Reverse	TCACACGTGCAGGATTCGAT
3	hWnt2-Forward	CGTGTGTGCAACCTGACTTC
4	hWnt2-Reverse	TGTGTGCACATCCAGAGCTT
5	hWnt2b-Forward	GATCCGAGAGTGTCAGCACC
6	hWnt2b-Reverse	CCTCTCGGCTACTTCTGAGC
7	hWnt3-Forward	TGACTCGCATCATAAGGGGC
8	hWnt3-Reverse	GTGGTCCAGGATAGTCGTGC
9	hWnt3a-Forward	AGCAGGACTCCCACCTAAAC
10	hWnt3a-Reverse	AGAGGAGACACTAGCTCCAGG
11	hWnt4-Forward	TCTTCGCCGTCTTCTCAGCC
12	hWnt4-Reverse	GCACCGAGTCCATGACTTCC
13	hWnt5a-Forward	TGTTGCTCGGCCCAGAAGTC
14	hWnt5a-Reverse	GCTTCAATTACAACCTGGGCG
15	hWnt5b-Forward	GCGAGAAGACTGGAATCAGGG
16	hWnt5b-Reverse	TAATGACCACCAGGAGTTGGC
17	hWnt6-Forward	CGGGGAGCGTTTAAAGGACA
18	hWnt6-Reverse	TTATTGATACTAACCTCACCCACC
19	hWnt7a-F-Forward	AGTACAACGAGGCCGTTCAC
20	hWnt7a-Reverse	GCACGTGTTGCACTTGACAT
21	hWnt7b-Forward	TACGTGAAGCTCGGAGCACT
22	hWnt7b-Reverse	CGGAACTGGTACTGGCACTC
23	hWnt8a-F-Forward	CTGGTCAGTGAACAATTTCC
24	hWnt8a-Reverse	GTAGCACTTCTCAGCCTGTT
25	hWnt8b-Forward	TATCAGTTTGCCTGGGACCG
26	hWnt8b-Reverse	CTGTCTCCCGATTGGCACTG
27	hWnt10a-Forward	CTGTTCTTCCTACTGCTGCT
28	hWnt10a-Reverse	ACACACACCTCCATCTGC
29	hWnt10b-Forward	GTCTCCCCACGGTTTAAGCA
30	hWnt10b-Reverse	TCAGGACCTCCAGTGGTTTG
31	hWnt11-Forward	TCTTTGGGGTGGCACTTCTC
32	hWnt11-Reverse	TCTGCCGAGTTCACTTGACG
33	hWnt9a-Forward	GACGGTCAAGCAAGGATCTG
34	hWnt9a-Reverse	TGCTCTCGCAGTTCTTCTCA
35	hWnt9b-Forward	GTGTCTTGCCATAGCAGGCTT
36	hWnt9b-Reverse	AATAAGGAGGCCGTGTGTCAG
37	hWnt16-Forward	TCAGGGAGACCCTCTTCACAG
38	hWnt16-Reverse	AGCAGGTACGGTTTCCTCTTG

39	hFrizzled1-Forwad	GTGAGCCGACCAAGGTGTAT
40	hFrizzled1-Reverse	CAGCCGGACAAGAAGATGAT
41	hFrizzled2-Forward	GCGAAGCCCTCATGAACAAG
42	hFrizzled2-Reverse	TCCGTCCTCGGAGTGGTTCT
43	hFrizzled3-Forward	TGAGTGTTCGAAGCTCTATGG
44	hFrizzled3-Reverse	ATCACGCACATGCAGAAAAG
45	hFrizzled4-Forward	CAGTGAGGCATGGAGGTGTT
46	hFrizzled4-Reverse	AAAGAGCTCAAGGGGCCATC
47	hFrizzled5-Forward	TACCCAGCCTGTCGCTAAAC
48	hFrizzled5-Reverse	AAAACCGTCCAAAGATAAACTGC
49	hFrizzled6-Forward	TGGCCTGAGGAGCTTGAATGTGAC
50	hFrizzled6-Reverse	TATCGCCCAGCAAAAATCCAATGA
51	hFrizzled7-Forward	GTTTGGATGAAAAGATTTCAGGC
52	hFrizzled7-Reverse	GACCACTGCTTGACAAGCACAC
53	hFrizzled8-Forward	ACAGTGTTGATTGCTATTAGCATG
54	hFrizzled8-Reverse	GTGAAATCTGTGTATCTGACTGC
55	hFrizzled9-Forward	CCCTAGAGACAGCTGACTAGCAG
56	hFrizzled9-Reverse	CGGGGGTTTATTCCAGTCACAGC
57	hFrizzled10-Forward	ACACGTCCAACGCCAGCATG
58	hFrizzled10-Reverse	ACGAGTCATGTTGTAGCCGATG
59	hsFRP1-Forward	TGGCCCGAGATGCTTAAGTG
60	hsFRP1-Reverse	CCTCAGTGCAAACTCGCTGG
61	hsFRP2-Forward	CTCGCTGCTGCTGCTCTTC
62	hsFRP2-Reverse	GGCTTCACATACCTTTGGAG
63	hsFRP-3-Forward	ATGGTCTGCGGCAGCCCGG
64	hsFRP-3-Reverse	CTGTCGTACACTGGCAGCTC
65	hsFRP-4-Forward	GTTCCTCTCCATCCTAGTGG
66	hsFRP-4-Reverse	GCTGAGATACGTTGCCAAAG
67	hsFRP5-Forward	CTACTGGAGGGTGTTTTCAC
68	hsFRP5-Reverse	CTTTCCCTTACCCTCTCCT
69	hWIF1-Forward	CACCTGGATTCTATGGAGTG
70	hWIF1-Reverse	ACAGAGGTCTCCCTGGTAAC
71	hDKK-1-Forward	CAGGATTGTGTTGTGCTAGA
72	hDKK1-Reverse	TGACAAGTGTGAAGCCTAGA
73	hDKK-2-Forward	CTCAACTCCATCAAGTCCTC
74	hDKK-2-Reverse	TACCTCCCAACTTCACACTC
75	hDKK3-Forward	GAGGTTGAGGAACTGATGG
76	hDKK3-Reverse	CCAGTCTGGTTGTTGGTTAT
77	hDKK4-Forward	GTCCTGGACTTCAACAACAT
78	hDKK4-Reverse	GTTGCATCTTCCATCGTAGT
79	hPVLAP-Forward	CTGCGATGCCTTGCTCTTCAT

80	hPVLAP-Reverse	AGTCCCTCCACAGGTTACGA
81	hOccludin-Forward	AAG CAA GTG AAG GGA TCT GC
82	hOccludin-Reverse	GGG GTT ATG GTC CAA AGT CA
83	hGlut-1-Forward	AAT ACA CCA CCT CAC TCC TG
84	hGut-1-Reverse	GAG GTA CGT GTA AGG GAC TG
85	hBCRP-Forward	CAG TCT TCA AGG AGA TCA GC
86	hBCRP-Reverse	CCA GTA CGA CTG TGA CAA TG
87	hPgp-Forward	ATA TCA GCA GCC CAC ATC AT
88	hPgp-Reverse	GAA GCA CTG GGA TGT CCG GT
89	hVE-cadherin-Forward	GTT CGG CTG ACA GGT CCA CA
70	hVE-cadherin-Reverse	CGA TGT GGC GAG GAG CAT CA
71	hZO-1-Forward	ATC TCG GAA AAG TGC CAG GA
72	hZO-1-Reverse	TTT CAG CGC ACC ATA CCA AC
73	hClaudin-5-Forward	AGGCGTGCTCTACCTGTTTTG
74	hClaudin-5-Reverse	AACTCGCGGACGACAATGTT
75	hClaudin-3-Forw	GCCACCAAGGTCGTCTACTC
76	hClaudin-3-Reverse	CGTAGTCCTTGCGGTCGTAG
77	hClaudin-1-Forward	TTTACTCCTATGCCGGCGAC
78	hClaudin-1-Reverse	GAGGATGCCAACCACCATCA
79	hCyclin-D-Forward	GTCCCACTCCTACGATACGC
80	hCyclin D-Reverse	CAGGGCCGTTGGGTAGAAAA
81	hAPCDD1-Forward	AAGGAGTCACAGTGCCATCA
82	hAPCDD1-Reverse	TTGTGATGAACTCTGGGCCT
83	hAxIn-2-Forward	GACAGGAATCATTCGGCCAC
84	hAxin-2-Reverse	CCTTCAGCATCCTCCGGTAT

CHAPTER III: Impact of Wnt/β-catenin signaling on ethanol-induced changes in brain endothelial cell permeability

This chapter was published by Journal of Neurochemistry (Sep 30th 2020) with the tittle: Impact of Wnt/β-catenin signaling on ethanol-induced changes in brain endothelial cell permeability (doi: 10.1111/jnc. 15203). Marlyn Laksitorini designed the experiment, performed cell cultures, isolated RNA from the cells and brain samples, isolated brain microvessels, performed qPCR, performed ethanol evaporation studies, performed permeability studies, performed Pgp functional assay studies, analyzed the data and prepared the manuscript. Vinith Yathindranath performed cell impedance assay. Wei Xiong performed toxicity studies, animal studies and assisted Marlyn with permeability assay on fluoxetine. James A Thliveris performed electron microscopy analysis.

Impact of Wnt/β-catenin signaling on ethanol-induced changes in brain endothelial cell permeability

Marlyn D. Laksitorini[1,4], Vinith Yathindranath[1], Wei Xiong[1], Fiona E. Parkinson[1,5], James A. Thliveris[3], Donald W. Miller[1,2]*
[1]*Department of Pharmacology and Therapeutics, Max Rady College of Medicine, University of Manitoba, Winnipeg, R3E 0T6, Canada.*
[2.] *Kleysen Institute of Advanced Medicine, Health Sciences Center, Winnipeg, R3E 0T6, Canada.*
[3] *Department of Human Anatomy and Cell Science, University of Manitoba, Winnipeg,, R3E 0J9, Canada.*
[4]*Department of Pharmaceutics, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, 55281, Indonesia*
[5]*Present address: Ross University School of Medicine, Miramar, Florida, 33027, USA*

**corresponding author: Donald.Miller@umanitoba.ca*

3.1 Abstract

Chronic exposure to ethanol is associated with enhanced leakiness in the brain microvessel endothelial cells that form the blood-brain barrier (BBB). As previous studies suggested Wnt/β-catenin signaling could improve the BBB phenotype of brain endothelial cells, we examined the extent to which Wnt signaling is altered following ethanol exposure, using both a cell culture model of the BBB and mice exposed to ethanol, and the ability of Wnt activation to reverse the permeability effects of ethanol. The human brain endothelial cells, hCMEC/D3, were exposed to ethanol (17-200 mM) for various periods of time (0-96 hours) and Wnt signaling, as well as expression of downstream genes influencing BBB integrity in the cell monolayers were monitored. Determination of Wnt signaling in both brain homogenates and brain microvessels from mice exposed to ethanol was also performed. The effects of ethanol on the permeability of the hCMEC/D3 monolayers were examined using both small molecular weight (sodium fluorescein) and large molecular weight (IRdye 800CW PEG) fluorescent markers. Exposure of hCMEC/D3 to ethanol (50 mM) caused a downregulation of Wnt/β-catenin signaling, a reduction of tight junction protein expression and upregulation of plasmalemma vesicle associated protein (PLVAP). A similar reduction in Wnt/β-catenin activity in both cortical brain homogenates and isolated cortical cerebral microvessels were observed in mice. Other areas such as cerebellum and striatum displayed as much as 3-6 fold increases in Dkk-1, an endogenous Wnt inhibitor. Ethanol exposure caused significant changes in both sodium fluorescein and IRdye 800CW PEG permeability (2-fold compared to control). The ethanol-induced increases in permeability were attenuated by treatment with known Wnt activators (i.e. LiCl or Wnt3a). Additional screens of CNS active agents with possible Wnt activity indicated fluoxetine could also prevent the permeability effects of ethanol. These studies suggest that ethanol-induced changes in brain microvessel permeability can be reversed through activation of Wnt signaling.

Keywords: Wnt/β-catenin signaling, chronic ethanol, blood-brain barrier, fluoxetine, permeability

3.2. Introduction

Ethanol is the most frequently abused substance worldwide surpassing even cannabis, amphetamine, cocaine and opioids[1]. Long-term consumption of as little as 100 g of alcohol per week has been attributed to an increased risk of several cardiovascular diseases, including stroke, heart failure, hypertension, and coronary heart disease[2]. In addition, ethanol consumption has been associated with neurological changes such as reduced adult neurogenesis, hippocampal atrophy, cognitive decline, and ataxia[3]. An intact BBB protects the brain from xenobiotics, maintains brain homeostasis and provides proper nutrient, oxygen and neurotransmitter levels to support proper brain function[4]. This is accomplished through the presence of complex intercellular junctions, formed by a variety of tight junction proteins (i.e. claudin-1, claudin-5 and occludin), that prevent the paracellular diffusion of solutes to the brain[5-7]. In addition, the brain microvessel endothelial cells express various efflux transporters that restrict intercellular passage of solutes[8]. Due to its size and lipophilicity, ethanol readily crosses the BBB, where it induces brain oxidative stress, neuroinflammation and BBB dysfunction[9,10].

Wnt/β-catenin signaling is essential for BBB development and maintenance[11,12]. Wnt/β-catenin signaling influences the expression of transporters, tight junction proteins and brain endothelial fenestrations[11,13-15], all of which contribute to the barrier properties of these cells. Signaling is initiated through Wnt binding to Frizzled and LRP-5/6 in the plasma membrane that transduces to Dishevelled (Dvl). Subsequently, GSK3 and CK-1 are recruited to phosphorylate LRP5/6, and the resulting Frizzled-LRP5/6-GSK3-CK-1 complex is internalized and sequestered in multivesicular bodies (MVB). The sequestered complex inhibits GSK3 phosphorylation of β-catenin resulting in more available β-catenin for translocation to the nucleus where it can bind to TCF/LEF and activate transcription of downstream Wnt/β-catenin target genes. In the absence of Wnt ligand, the β-

catenin destruction complex (Axin, GSK3, CK-1 and APC) phosphorylates β-catenin in the cytosol and tags it for proteosomal degradation thereby maintaining low cytosolic β-catenin and reduced Wnt/β-catenin signaling[16,17]. Impaired Wnt/β-catenin signaling has been implicated in the BBB dysfunction observed under pathological conditions such as multiple sclerosis, brain tumors, Alzheimer's disease and Parkinson's disease[18-20].

Recently we reported, activation of Wnt/β-catenin by LiCl or Wnt3a improved brain endothelial barrier function in an *in vitro* cell culture model of the BBB[21]. Given the improved BBB phenotype produced by Wnt/β-catenin activation, we hypothesized that activation of Wnt/β-catenin signaling could reverse the detrimental effects of ethanol on brain endothelial cell function. In the current study, we examined Wnt/β-catenin activity in the brain following ethanol exposure in adult mice, to identify potential alterations in this signaling pathway. In addition, using cultured human cerebral microvessel endothelial cells (hCMEC/D3) as an *in vitro* BBB model, we tested if external Wnt/β-catenin activation could offset the BBB dysfunction induced by prolonged ethanol exposure. The hCMEC/D3 culture model was also used to screen CNS drugs for their ability to activate Wnt/β-catenin signaling and reduce ethanol induced barrier breakdown.

3.3. Materials and Methods

3.3.1. Materials

The information on the materials used in this study is summarized in Table 3.1. Primers were obtained from Invitrogen (CA, USA) and primer sequence information is provided in Supplemental Table 3.S3.

Table 3.1: List of materials

Reagent or resource	Source	Identifier	Working solution
C57BL6 mouse	University of Manitoba	RRID: MGI:2159769	
hCMEC/D3 cells	Dr.Pierre-Oliver Coroud	RRID:CVCL_U985 (This cell line is not listed in the list of commonly misidentified cell line by the International Cell Line Authentication Committee	
Human recombinant Wn3a	R&D Systems	Cat no. 5036-WN-010	
IRdye 800CW PEG	LICOR	Cat. no926-50401	
EBM-2 media	Lonza	Cat no. 00190860	
Transwell® polycarbonate membrane with 0.4μm pore	Corning	Cat no. 3412	
Trizol™	Life Technologies	Cat no. 15596018	
Claudin-1 antibody	Abcam	RRID:AB_301644; Cat no. ab15098	0.35 μg/ml
Claudin-5 antibody	Sigma	RRID:AB_10753223 ; Cat no. SAB4502981-100UG	1μg/ml
β-actin antibodies	Sigma	RRID:AB_476697; Cat. no.A2228-100UL	0.25 μg/ml
Horse anti mouse IgG secondary antibody	Cell Signaling Inc.	RRID:AB_330924; Cat no. 7076S	0.02 μg/ml
Goat anti rabbit IgG secondary antibody	Novus biologicals	RRID:AB_524669;Cat. no. NB7160	0.03 μg/ml
QuantiChrom Ethanol Assay Kit	Bioassay systems	DIET-500	

3.3.2. Animal Studies

The animal studies described in this manuscript were not pre-registered studies. Brain tissue was obtained from an approved animal study examining the effect of ethanol on the expression of various adenosine metabolism and transport pathways [22]. The animal protocol (animal protocol

no. 14-009) adhered to Canadian Council on Animal Care and was approved by University of Manitoba Animal Protocol and Management Committee. Briefly, adult C57BL/6 male mice (7-8 weeks old) were obtained from University of Manitoba breeding colony and housed in the University of Manitoba animal facility with three mice per cage, free access to food and water, and a 12-hour light cycle. Mice received standard cage enrichment. Simple randomization, (i.e. coin toss) was used to assign mice to control or ethanol treatment groups. Ethanol stock solution was prepared fresh daily in phosphate buffered saline (PBS) at a concentration 20% v/v. Mice in the ethanol treatment groups were administered 2 g/kg ethanol via intra-peritoneal injection on a daily basis for 7 consecutive days. The control group received phosphate buffered saline injections (1 ml/kg). Ethanol mice were injected first, followed by the PBS treatment group. Tissues were collected at the end of the experiment in the same order. All treatments and tissue collections occurred during the light cycle. Animals were heavily anesthetized with isoflurane (4%), have been tested for absence of pain response prior to decapitation. In terms of outcomes, the study was exploratory, with no pre-determined exclusion criteria. All of the mice completed the entire 7-day treatment protocol and no mice were excluded from the study.

A total of 5 brains from both the ethanol and control groups were used for determining Wnt pathway activity in the various brain regions. An additional 3 brains from each treatment group were available for the capillary depletion studies. The capillary depletion was carried out on brain homogenates as previously described[23]. The brain cortex from control and ethanol treated mice were placed in 20 ml DMEM media (serum free) and minced into small pieces. Cortex tissue samples were manually homogenized and aliquoted into two tubes with 10 ml each. The brain homogenates were centrifuged for 10 minutes 1000xg at 4^{o}C. The homogenized pellet from each tube was re-suspended with 10 ml 17% dextran in DMEM and further centrifuged at 10,000 g for

30 minutes at a temperature of 4°C. The capillary enriched fraction residing in the pellet at the bottom of the tube was collected and assayed for Wnt activity as described below. As the studies were performed on the remaining unused brain tissue available from a previously approved study, no sample size determination was performed and blinding of investigator was not possible.

3.3.4. Cell Culture

For routine culture, hCMEC/D3 (passage 29-35) were seeded at 10,000 cells/cm^2 onto T75 flasks coated with rat tail collagen (0.1 mg/ml). Cells were cultured at 37°C and 5% CO_2 in EBM-2 media supplemented with 5% FBS, 1% penicillin streptomycin, 1 ng/ml bFGF, 10 mM HEPES, 5 µg/ml ascorbic acid, 1/100 CD lipid concentrate, and 1.4 µM hydrocortisone. For expression and functional studies, cells were seeded onto culture plates coated with rat tail collagen at a density of 25,000 cells/cm^2 and used upon reaching confluency. Neuroblastoma cells, SH-SY5Y (RRID:CVCL_0019) were culture in EBM-2 complete media and maintained in 37°C and 5% CO_2.

3.3.5. Immunoblotting

Cells were solubilized using RIPA buffer (Sigma) supplemented with protease and phosphatase inhibitors (Thermo Scientific). The lysates were subsequently centrifuged at 12,000 rpm for 8 minutes. The protein concentration of whole cell lysate was determined by Pierce BCA protein analysis (Thermo Scientific). Thirty micrograms of protein extract was subjected to SDS-polyacrylamide gel electrophoresis using 12% gel. The proteins were then transferred to PVDF membrane (BioRad) for 2 hours at 200 mA. The membrane was subsequently blocked in 5% non-fat milk with TBST buffer for an hour at room temperature. The membrane was incubated with the primary antibodies dissolved in 5% non-fat milk TBST buffer at 4°C on rocking rack overnight. Membranes were washed thrice with TBST buffer for ten minutes each before being incubated with secondary antibodies for 1 hour at room temperature. Bands were visualized using

chemiluminescence with ChemiDoc Imager (Biorad) and analyzed using ImageLab software (Biorad).

3.3.6. RT-qPCR

Total RNA was isolated using Trizol®. RNA concentration was measured using a Nanodrop UV Vis Spectrometer (Fisher Scientific). Total RNA was subjected to qPCR using ITaq Universal SYBR Green One-Step Kit according to manufacturer protocol using QuantStudio 5 (Thermo Fisher). Briefly, reverse transcription reaction for 10 minutes at 50^{o}C followed by polymerase activation and DNA denaturation for 1 minute at 95^{o}C. Subsequently, the amplification steps were done in two stages; denaturation for 10 seconds at 95^{o}C, followed by 30 seconds for annealing/extension at 60^{o}C, and repeated for 40 cycles. Melting curve analysis was done over a 65-95^{o}C temperature range with 0.5^{o}C increment of 2-5 seconds per step.

3.3.7. Permeability Studies

Permeability studies were performed using 6-well Transwell polycarbonate membranes (0.4 µm pore; 24 mm diameter) coated with rat-tail collagen 0.1mg/ml. The hCMEC/D3 cells were seeded onto the inserts at a density of 150,000 cells/well. Apical and basolateral compartments were filled with 1.5 ml and 2.5 ml of complete EBM-2 media, respectively. The hCMEC/D3 monolayers were used upon reaching confluency, typically 5 days after seeding. Monolayers were treated with ethanol (17-200 mM) alone or in combination with either LiCl 10 mM or Wnt3a 200 ng/ml for at least 48 hours prior to permeability studies. The graphical timeline of each experiment are available at supplemental Fig. 3.S6. On the day of the permeability experiment, the media was removed from both apical and basolateral compartments and replaced with assay buffer. The assay buffer in the apical compartment contained 0.1 µM IRdye 800CW PEG and 1.0 µM sodium fluorescein to assess barrier integrity. A one hundred microliter sample was removed from the basolateral compartment at various times (0, 15, 30, 60, 90 and 120 minutes) and replaced with an

equal volume of fresh assay buffer. Samples (20 µl) were also taken from the apical compartment at the start and conclusion of permeability study. The samples from the apical and basolateral compartments were placed in black 96-well plates. Quantitative analysis of IRdye 800CW PEG was performed using an Odyssey Near-Infrared Imager (Licor) at 800 nm channel. Sodium fluorescein was analyzed using Biotek Synergy HT Microplate Reader at excitation 485 nm and emission 528 nm. The amount of fluorescence activity was quantitated using standard curves for each fluorescent compound. Permeability data were presented as either apparent permeability (P_{app}) coefficient or converted to % flux.

Apparent permeability was calculated based on the equation:

$$Papp = \frac{dC/dt}{A.Co}$$

dC/dt is the transport rate (µM/second) of the permeability marker, defined as the slope obtained from linear regression of the marker concentration at basolateral over time. C_0 is the initial concentration of permeability marker on the donor side (µM). A is the surface area of the permeable support (4.67 cm^2).

3.3.8. Electron Microscopy

The effects of ethanol exposure on cell morphology were examined using electron microscopy. For these studies, hCMEC/D3 cells were incubated with ethanol (17-200 mM) for 48 and 96 hours. At the end of the exposure period, cells were washed thrice and collected using 0.25% trypsin. Cells were subsequently fixed using 3% glutaraldehyde in 0.1 M of phosphate buffer (pH 7.3), followed by post-fixation in 1% tetraoxide in 0.1 M phosphate buffer (pH 7.3). Cells were then dehydrated and embedded in Epon 812 as previously described[24]. Sections were stained using

uranyl acetate and lead citrate and viewed using a Philips CM 10 electron microscope. In order to eliminate the observer bias, sections were examined without knowledge of treatment.

3.3.8. Statistical analysis

All numerical data are expressed as the mean ± SEM, unless otherwise indicated. The statistical analysis of data sets was performed using GraphPad Prism version 6.0 software. To compare between two groups, unpaired t-test was used. One-way ANOVA followed by Fisher's LSD test was used for comparisons involving more than two different treatment groups. To analyze two independent factors, two-way ANOVA followed by Fisher's LSD test were employed. For all statistical testing, $p<0.05$ was considered statistically significant. Data were not assessed for normality and all data points were used for statistical analysis.

3.4. Results

3.4.1. Wnt signaling following ethanol exposure in hCMEC/D3 cells.

To test the effect of prolonged ethanol exposure on Wnt/β-catenin signaling, hCMEC/D3 cells were treated with 17 mM, 50 mM and 200 mM ethanol for 2, 4 and 7-days. As there was substantial loss of ethanol from the media due to evaporation (supplemental Fig. 3.S1a), media was replaced and replenished every 24 hours. Under these conditions, cell viability was not significantly changed in any of the ethanol treatment groups examined (supplemental Fig. 3.S1b). Furthermore, evaluation of the morphology of the cells using electron microscopy (EM) showed no apparent change in cells exposed to 17 mM ethanol compared to cells receiving media alone (Supplemental Fig. 3.S2). The cells receiving higher concentrations of 50 and 200 mM ethanol showed an increased appearance of multivesicular organelles (Supplemental Fig. 3.S2). The number and extent of formation of the multivesicular organelles was correlated with ethanol concentration, with 200 mM producing more of the organelles than 50 mM ethanol (Supplemental Fig. 3.S2).

However, apoptotic and necrotic cell numbers under all ethanol exposure conditions examined were similar with that observed in control cells exposed to media alone.

Exposure of hCMEC/D3 cells to ethanol produced changes in Wnt activation that were both concentration and exposure time dependent. While 17 mM ethanol had no effect on Axin-2 expression, 50 mM ethanol produced a significant reduction in Axin-2 expression which was apparent after 4-days and reached a maximal inhibition (50%) at 7-days (Fig. 3.1a). Exposure to even higher concentrations of ethanol (200 mM) produced similar reductions in Axin-2 (Fig. 3.1a). Shorter ethanol exposure (i.e. two days; Fig.3.1a), did not alter Axin-2 expression. As Axin-2 is a reporter gene used to assess Wnt/β-catenin activity [25], such reductions in expression observed in the present study suggest prolonged ethanol exposure can reduce Wnt/β-catenin signaling. In addition to Axin-2 expression, changes in the expression of Wnt co-receptors (LRP-5 and LRP-6) and Wnt ligands (Wnt2b) were also observed following exposure of hCMEC/D3 cells to 50 mM ethanol (Fig. 3.1c-f). In the case of the Wnt receptors and Wnt ligands, a significant decrease in expression was observed following 50 mM ethanol exposure. Interestingly, both GPR124 and Reck expression was upregulated following exposure to 50 mM ethanol at both 4- and 7-days (Fig. 3.1g-h). An even more dramatic increase in GPR124 and Reck expression was observed following 200 mM ethanol exposure (Supplemental Figure 3.S3a and 3.S3b). GPR-124 and Reck are co-receptor for Wnt7a/7b-triggered β-catenin signaling required for BBB development and maintenance[12,26-28].

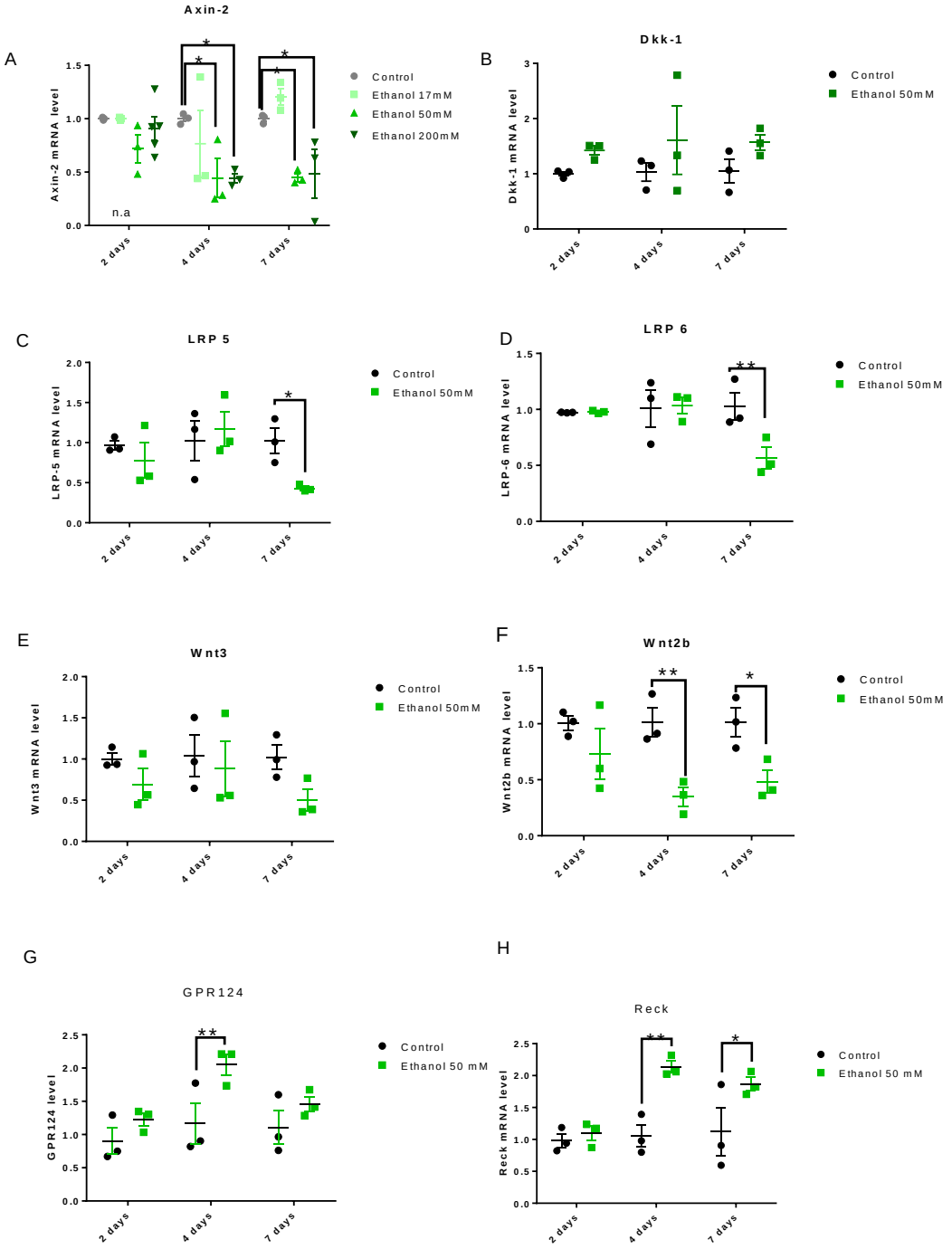

Figure 3.1: Effects of ethanol exposure on Wnt/β-catenin activity in human cerebral microvessel endothelial cells (hCMEC/D3). Wnt/β-catenin activity was assessed using Axin-2 reporter gene expression in hCMEC/D3 cells following different durations and concentrations of ethanol exposure (A). The effect of ethanol on the expression of Wnt modulators Dkk-1 (B), receptors LRP-5 (C), LRP-6 (D), and ligands Wnt3 (E), Wnt2b (F), GPR124 (G), and Reck (H) was also examined. All values represent mean±SEM of three samples. Each data point represents independent cell monolayer. Statistical significance was determined using one-way ANOVA followed by LSD Fisher's test for multiple comparison of the means; *$p<0.05$; **$p<0.01$.

3.4.2. Effects of ethanol exposure on Wnt/β-catenin activity in mouse brain cortex

To evaluate the effect of chronic ethanol exposure on Wnt/β-catenin activity in the brain, we obtained the brains from mice that were administered ethanol (2 g/kg body weight; i.p.) every 24 hours for 7 days. This treatment resulted in maximal blood ethanol concentrations of 50 mM within 30 minutes following i.p injection, declined to approximately 20 mM within 2 hours following ethanol injections[22]. Examination of the brains from ethanol treated mice indicated a significant increase in the expression of the endogenous Wnt inhibitor, Dkk-1, in several regions including the cerebellum, striatum, and to a lesser extent, the hippocampus (Fig. 3.2b-d). While Dkk-1 expression was unaltered in the cortex following ethanol treatment, significant reductions ($p<0.05$) in Axin-2 reporter gene expression were observed (Fig. 3.2e). The brain cortex from ethanol treated mice also displayed an increase in GPR124 expression. In contrast, expression of both Axin-2 and GPR124 in the hippocampus and striatum were unchanged (Supplemental Fig 3.S4). These studies suggested that the effects of ethanol exposure on Wnt/β-catenin signaling varied depending on the region of brain examined.

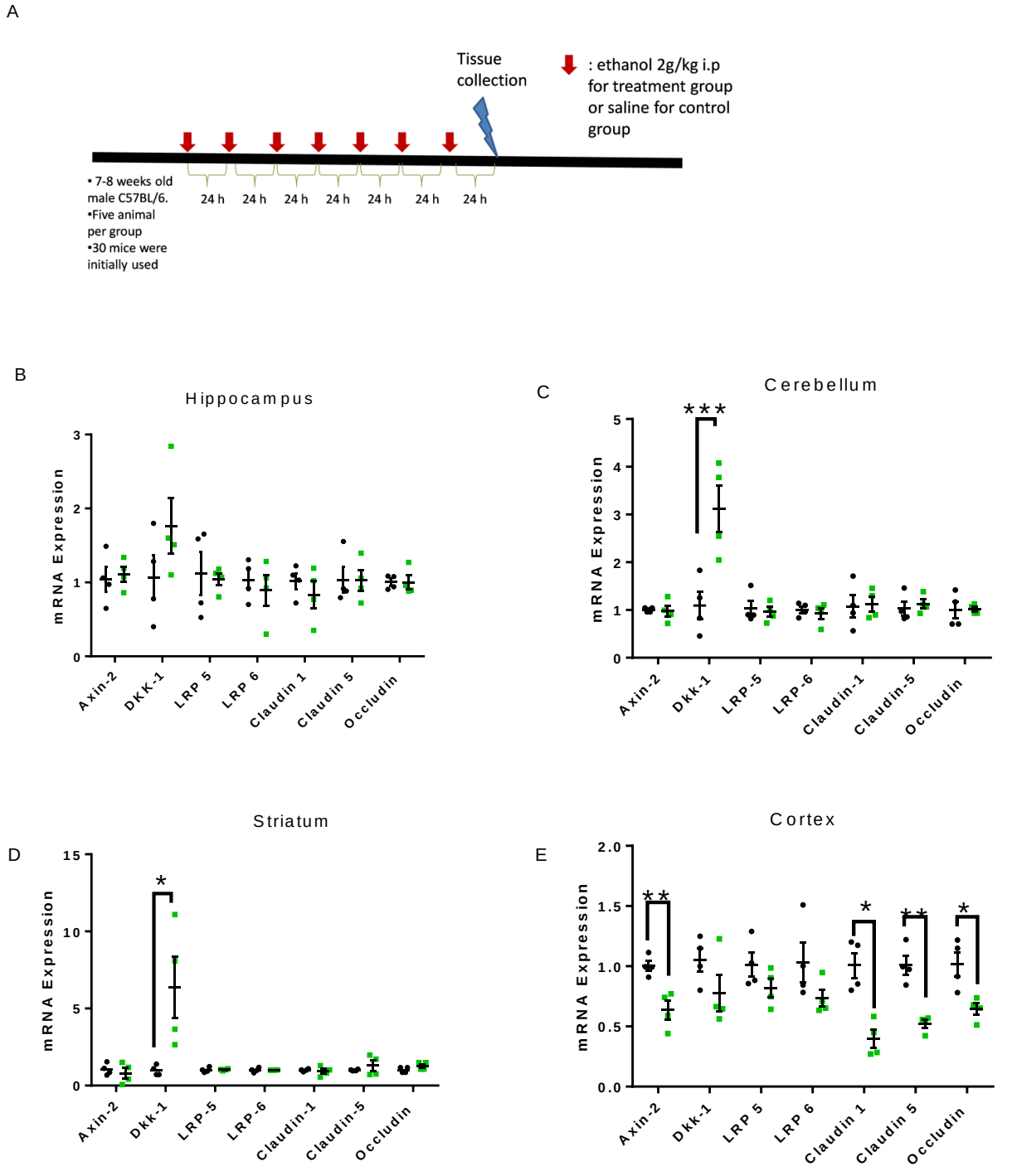

Figure 3.2: Effects of ethanol exposure on Wnt/β-catenin signaling in various brain regions. Mice were treated with i.p injections of saline (black) or ethanol 2g/kg (green) every 24 hours for a total of 7 days (A). Expression of the Wnt reporter gene, Axin-2 and various Wnt/β-catenin components and downstream tight junction molecules in tissue homogenate from the hippocampus (B), cerebellum (C), striatum (D), and cortex (E) were examined. Each data point represents RNA preparation from different individual mice within the same region. Values represent mean ± SEM. *p<0.05; **p<0.01;***p<0.001 based on t-test comparing control and ethanol treatment conditions

3.4.3. Wnt/β-catenin activity in isolated brain microvessels following ethanol exposure.

As the cortex showed the greatest reductions in Wnt/β-catenin activity following ethanol exposure, further analysis was undertaken to examine the state of Wnt/β-catenin activity in the cerebral microvasculature upon chronic ethanol exposure. For these studies, the microvessels from the brain cortex were isolated using the capillary depletion method. The ability to successfully isolate the brain microvessels was demonstrated by comparing the expression of endothelial specific marker genes such as claudin-5 and VE-cadherin in the collected capillary fractions to expression in the whole cortex homogenates. Claudin-5 and VE-cadherin are specific markers for endothelial cells and their expression in other adult brain cells is considered negligible[29,30]. The expression of claudin-5 and VE-cadherin in the capillary enriched fraction was more abundant compared to whole cortex homogenates, with 36-fold and 16-fold enrichment in mRNA expression observed, respectively (Supplemental Table 3.S1). A 21-fold enrichment of Pgp was also seen in the capillary enriched fraction. While Pgp expression has been reported in astrocytes[31], this efflux transporter is abundant in brain microvessels[32]. These data suggested that the fraction collected was enriched with brain microvessels.

Similar to the brain cortex homogenates, a significant downregulation of Axin-2 expression was observed in the microvessel fractions isolated from the cortex (Table 3.2). The decreased Axin-2 expression observed in the microvessel fractions from ethanol treated mice was also correlated with a significant decrease in Claudin-5 expression (Table 3.2). In contrast to other brain regions examined, Dkk-1 expression in both cortical homogenate and brain microvessels were lower (Fig. 3.2e and Table 3.2).

Table 3.2: Expression of selected genes involved in Wnt signaling and BBB integrity in isolated microvessels from brain cortex

	Gene	Control*	Ethanol treated*	p-value
Wnt-related gene	Axin-2	100 ± 8.4	46.5 ± 2.3	0.02
	Dkk-1	100 ± 14.9	22.4 ± 2.5	0.03
	LRP-5	100 ± 23.8	86.4 ± 28.6	n.s
	LRP-6	100 ± 4.6	95.3 ± 13.7	n.s
BBB-related gene	Claudin-5	100 ± 4.3	26.6± 9.6	0.02
	VE-cadherin	100 ± 32.6	108.6 ± 29.3	n.s
	ZO-1	100 ± 3.5	123.1 ± 15.0	n.s
	Occludin	100 ± 15.6	131±18.1	n.s
	P-glycoprotein	100 ± 6.7	161 ± 56.5	n.s

*Microvessels were isolated from the frontal cortex collected from 3 mice in each treatment group.

3.4.4. The effect of prolonged exposure of ethanol on BBB phenotype

Our previous studies and others suggested that Wnt/β-catenin signaling regulated tight junction and PLVAP expression in brain endothelial cells[11,14,21]. In the present study, exposure of cultured hCMEC/D3 cells to ethanol was associated with an upregulation of PLVAP plasmalemma vesicle-associated protein ($p<0.01$) and a trend toward reductions in claudin-1 (Supplemental Fig. 3.S5a-c). The increase in PLVAP expression in response to ethanol exposure is important as PLVAP is associated with the formation of endothelial cell fenestrations, transendothelial channels and caveolae[33] and increased expression of PLVAP in brain endothelial cells is a hallmark of BBB dysfunction[34]. Reductions in claudin-1, claudin-5, and occludin were observed in the mouse brain cortex and reduction in claudin-5 were observed in microvessels isolated from the brain cortex following 7 days of ethanol exposure (Fig. 3.2d and Table 3.2). Together these studies suggest that

reductions in Wnt/β-catenin signaling during chronic ethanol exposure coincides with downregulation of tight junction proteins and upregulation of PLVAP expression in brain endothelial cells.

3.4.5. Effects of ethanol on brain microvessel endothelial cell permeability

The effects of ethanol on hCMEC/D3 barrier function were assessed using both electrical impedance and solute permeability studies. Electrical impedance measurements provided a real-time assessment of tight junction integrity following ethanol exposure. As shown in Figure 3.3a, the effects of ethanol on electrical impedance were bi-phasic and concentration-dependent with both 50 and 200 mM ethanol causing a rapid decline in impedance followed by a partial restoration towards baseline. In contrast, 17 mM ethanol had minimal effects on electrical impedance compared to the control group receiving media alone. While there was a partial restoration of impedance the recovery was incomplete. To quantitatively assess the loss of barrier function following prolonged ethanol exposure, permeability studies were performed with the large molecule paracellular diffusion tracer, IRdye 800CW PEG (35kDa) and the small molecule paracellular diffusion permeability marker, sodium fluorescein (376.3 g/mol) (Fig. 3.3b and 3.3c). Similar to electrical impedance, exposure of hCMEC/D3 cell monolayers to 17 mM ethanol for 48 hours had no impact on paracellular permeability in hCMEC/D3 cell monolayers as neither sodium fluorescein nor IRdye 800CW PEG P_{app} were altered compared to control monolayers (Fig 3.3b and 3.3c). In contrast, significant increases in paracellular diffusion were observed following 50 mM and 200 mM ethanol (Fig. 3.3b and 3.3c).

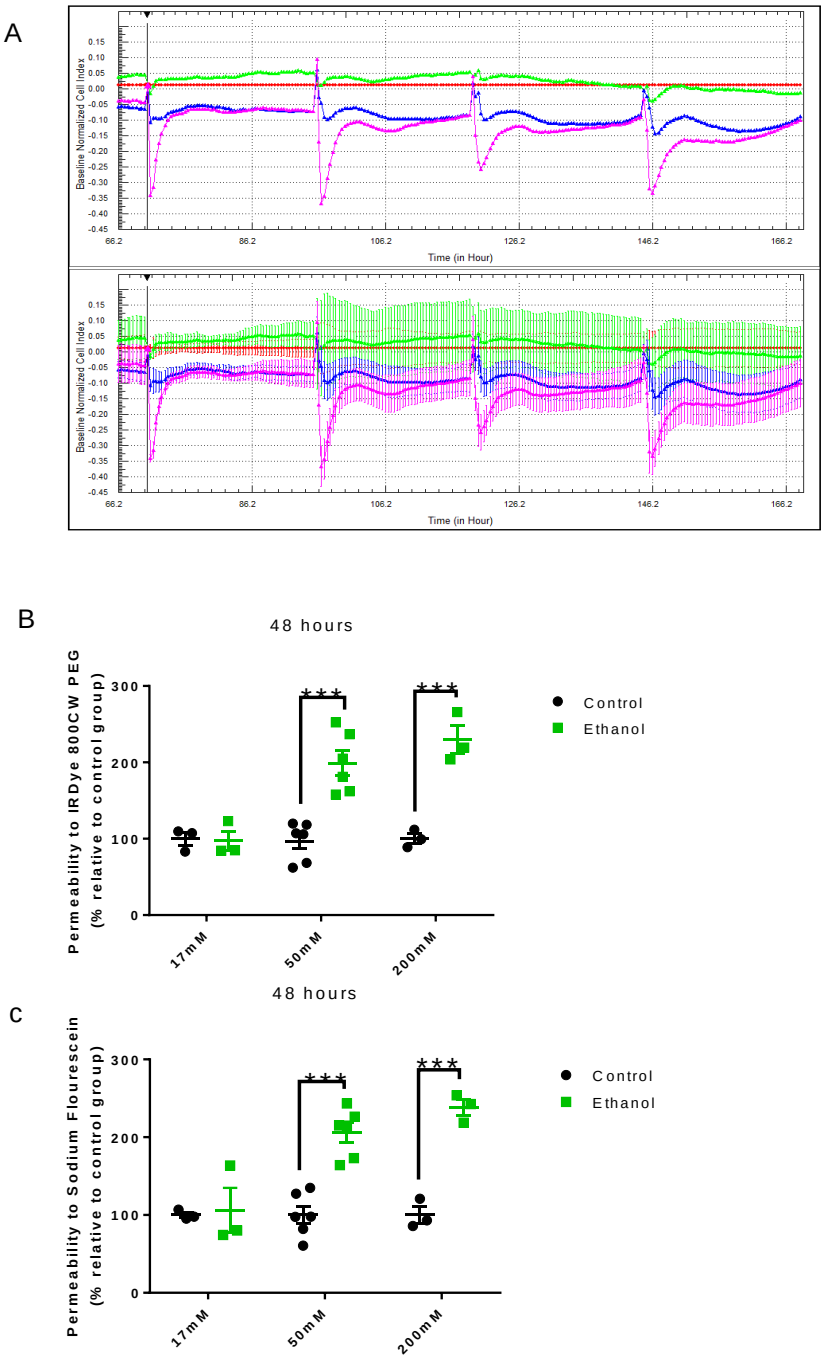

Figure 3.3: Permeability was determined using electrical impedance and permeability assay. The effect of different ethanol concentrations on electrical impedance (A). Apparent permeability (P_{app}) of IRDye 800CW PEG (B) and sodium fluorescein (C) following different durations and concentrations of ethanol exposure. The values were normalized to control group where the P_{app} of IRdye 800CW PEG was 2.3±0.2x 10^{-6} cm/s and P_{app} of sodium fluorescein was 18.95 ± 1.0 x 10^{-6} cm/s. Each data point represents independent monolayers preparation. Values in A represent mean (upper panel) and mean±SD (lower panel). Values in B and C represent mean±SEM. B and C were analyzed using two-way ANOVA followed by LSD Fisher's test; *p<0.05; **p<0.01; ***p<0.001.

3.4.6. External Wnt/β-catenin signaling activation reverses ethanol-induced barrier dysfunction.

In our previous report, LiCl 10 mM and Wnt3a 200 ng/ml activated Wnt/β-catenin signaling and improved paracellular barrier function in the *in vitro* model of the BBB[21]. In the current studies we examined whether LiCl and natural Wnt ligand, Wnt3a, could be employed to mitigate the barrier leakiness induced by ethanol. LiCl (10 mM) or Wnt3a (200 ng/ml) were co-treated with ethanol for 48 hours and the permeability to both small molecule and macromolecule markers of paracellular diffusion were examined. As shown in Fig. 3.4a, addition of LiCl or Wnt3a significantly reduced the P_{app} for sodium fluorescein from 36.1±2.7 x10^{-6} cm/s for ethanol alone to 27.2±0.9 x 10^{-6} cm/s for ethanol+LiCl and 27.2±2.2 x 10^{-6} cm/s for ethanol+Wnt3a (Fig. 3.4a, $p<0.05$). In terms of macromolecule permeability, activation of Wnt/β-catenin with either LiCl or Wnt3a also reduced P_{app} of IRdye 800CW PEG from 3.8±0.5 x 10^{-6} cm/s in the 50 mM ethanol treatment group to 0.98±0.1 x 10^{-6} cm/s for ethanol+LiCl group and 1.8±0.3 x 10^{-6} cm/s for ethanol+Wnt3a (Fig. 3.4b, $p<0.001$). In the case of LiCl treatment, barrier function for the large molecular weight permeability marker was restored to control levels (1.0±0.2 10^{-6}cm/s). Examination of Axin-2 expression in cells receiving 50 mM ethanol in combination with LiCl and Wnt3a confirmed the activation of Wnt/β-catenin signaling in the brain endothelial cells (Fig. 3.4c). The increased Wnt/β-catenin activity observed with LiCl and Wnt3a following ethanol exposure were also associated with an up regulation of tight junction molecules with LiCl increasing both claudin-1 and claudin-5, by 2.3-fold ($p<0.01$) and 1.7-fold ($p<0.05$) respectively (Fig. 3.4c) and Wnt3a significantly increasing claudin-1 ($p<0.05$) expression. These studies suggested that activation of Wnt signaling either by LiCl or Wnt3a could restore brain endothelial barrier function (Fig. 3.4a and 3.4b).

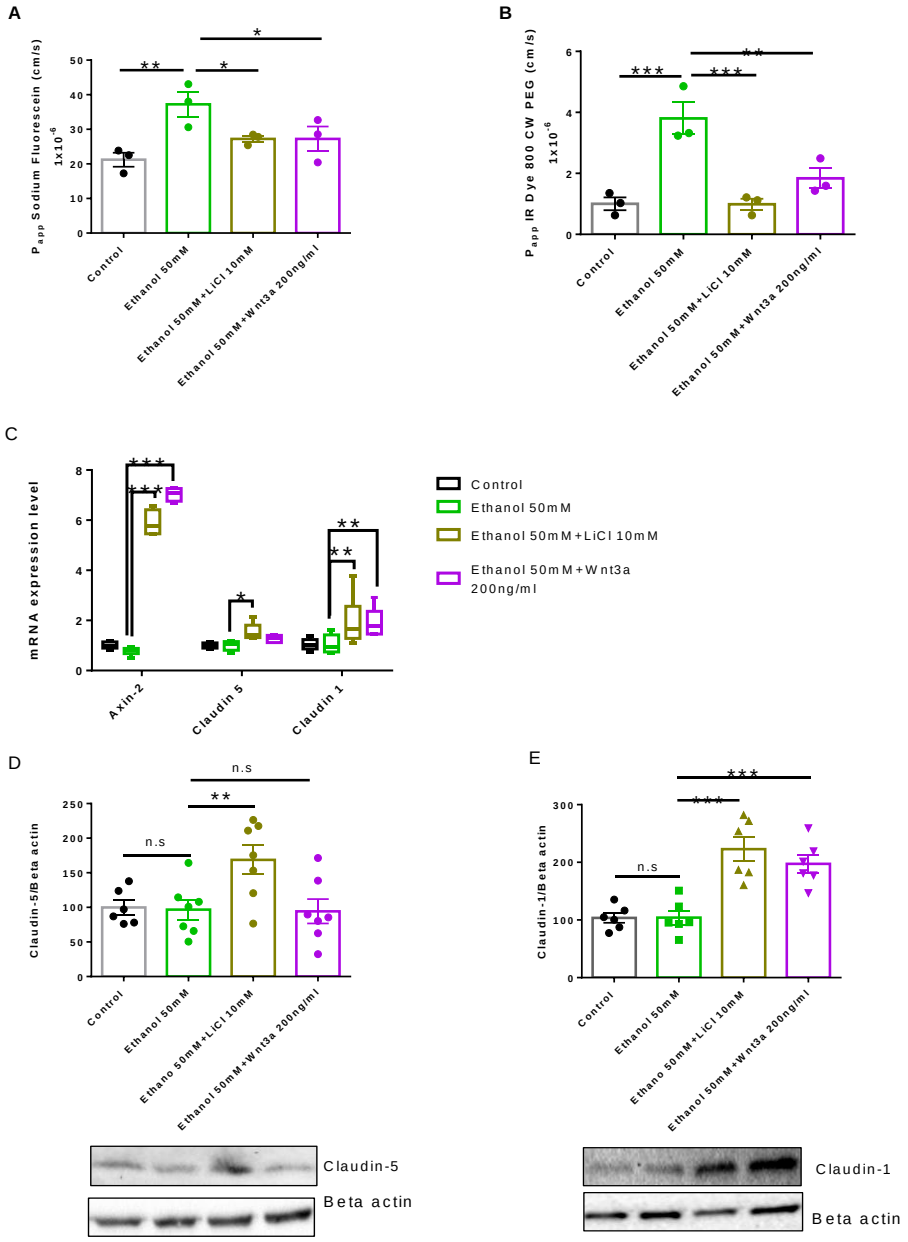

Figure 3.4: Effects of Wnt/β-catenin activation on paracellular barrier dysfunction induced by ethanol 50 mM in hCMEC/D3 monolayers. Permeability was assessed using low molecular weight sodium fluorescein permeability marker (A) and high molecular weight IRdye 800CW PEG fluorescent permeability marker (B) under control conditions and following 48 hour exposure to 50 mM ethanol in the presence and absence of LiCl (10 mM) or Wnt3a (200 ng/ml). Permeability coefficients were calculated as described in methods. Expression of Axin-2, claudin-5 and claudin-1 following ethanol co-treatment with LiCl or Wnt3a in hCMEC/D3 (C). Protein expression of claudin-5 and claudin-1 following ethanol and Wnt activation with LiCl or Wnt3a (D and E). Values in A, B, D, and E represent mean±SEM of three monolayers per treatment group. Values in C represent median with minimum and maximum value of 5-6 samples. Statistical significance was determined using one-way ANOVA followed by LSD Fisher's test; *p<0.05; **p<0.01;***p<0.001. The graphical timeline of the experimental procedure for these data is available at Supplemental Figure 3.S6a

The ability of exogenous Wnt/β-catenin activation by LiCl and Wnt3a to restore the barrier properties of the brain microvessel endothelial cells was less effective following exposure to a supra-physiological ethanol concentration of 200 mM (Fig. 3.5a and 3.5b). The P_{app} value of small and large molecule tracer is provided in supplemental material, Table 3.S2. Of note, while LiCl partially restored barrier function, Wnt3a treatment was completely ineffective at restoring hCMEC/D3 monolayer barrier integrity following exposure to 200 mM ethanol (Fig. 3.5a and 3.5b).

To understand the extent to which Wnt/β-catenin could restore the barrier tightness, we tested if a delay in Wnt activation could still mitigate the effect of ethanol on barrier breakdown. For these studies, cells were pretreated with 50 mM ethanol for 24 hours. Subsequently, LiCl or Wnt3a were co-treated with 50 mM ethanol for another 24 hours (Fig. 3.5c and 3.5d). As shown in Fig. 3.5c and 3.5d, ethanol increased sodium fluorescein permeability by 2-fold ($18.1\pm1.8 \times 10^{-6}$ cm/s for untreated group and $36.2\pm2.8 \times 10^{-6}$ cm/s for ethanol group). LiCl treatment led to a P_{app} of sodium fluorescein of $17.1\pm1.7 \times 10^{-6}$ cm/s, which was similar to the control group that did not receive ethanol. In contrast, 24-hour exposure of Wnt3a was insufficient to repair ethanol-induced barrier breakdown (Fig. 3.5d). A similar trend was also observed with the large molecular weight permeability marker where LiCl restored the P_{app} of IRdye 800CW PEG to values similar to control group receiving no ethanol ($1.9\pm0.3 \times 10^{-6}$ cm/s for ethanol+LiCl and $1.8\pm0.2 \times 10^{-6}$ cm/s for ethanol group) while Wnt3a had no effect on permeability in ethanol treated cells (Fig. 3.5c). Collectively, these experiments suggested that LiCl was more robust in mitigating barrier breakdown induced by ethanol compared to Wnt3a.

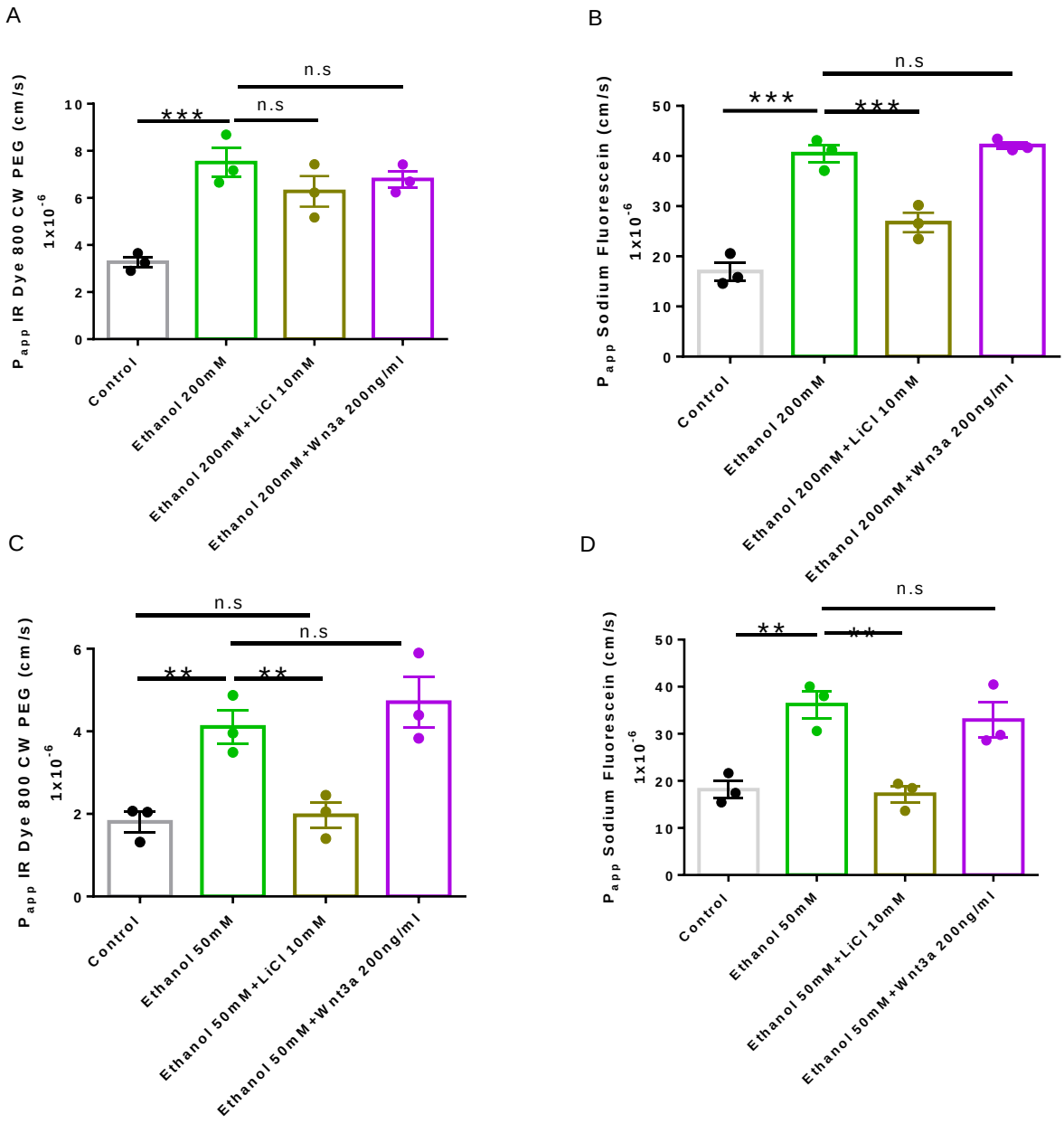

Figure 3.5: The effect of Wnt/β-catenin activation on the paracellular barrier of hCMEC/D3 monolayer at supra-physiological concentration of ethanol (A and B) and in the studies where Wnt activation were occurred after ethanol pre-treatment (C and D). A and B: hCMEC/D3 cells were treated with ethanol 200 mM alone or co-treated with either LiCl or Wnt3a for 48 hours. C and D: hCMEC/D3 cells were pre-treated with ethanol 50 mM for 24-hour before LiCl or Wnt3a were applied to the culture to activate Wnt for an additional 24 hours. Each data point represents independent monolayer preparations. The values represent mean±SEM. A-D were analyzed using one- way ANOVA followed by LSD Fisher's test; *p<0.05; **p<0.01; ***p<0.001. The graphical timeline of the experimental procedure for A and B is available at Supplemental Figure 3.S6b while for C and D is available at Supplemental Figure 3.S6c.

To evaluate the reduced effectiveness of Wnt3a in reversing the permeability effects following ethanol exposure, studies were performed to determine whether Wnt3a activation was blunted in ethanol exposed cells. For these studies, the hCMEC/D3 cells were pretreated with 50 mM ethanol for 48-hours and then exposed to Wnt3a. While Wnt3a upregulated Axin-2 by 40-fold in the control group receiving no ethanol, Axin-2 expression was significantly diminished in ethanol treated cells (Supplemental Fig. 3.S7).

3.4.7. Screening of other drugs for Wnt/β-catenin activation and ethanol-induced barrier disruption

Although 10mM LiCl rescued the barrier function of hCMEC/D3 upon prolonged ethanol exposure, this concentration is out of the therapeutic range for LiCl. Therefore, other clinically approved CNS drugs with known Wnt activity were considered. Before screening, the hCMEC/D3 model was examined for the expression of dopamine-2 receptor (D2R), serotonin transporter (SERT), serotonin receptor (5HT1A and 5HT2A), the intended pharmacological targets for the antipsychotics and selective serotonin re-uptake inhibitors (SSRI). All the receptors/transporters examined were detected in the hCMEC/D3 cells although compared to the SH-SY5Y monoaminergic neuronal cell line, D2R receptor expression was significantly lower (Supplemental Fig 3.S8). Based on the expression of D2R, SERT, 5HT1A and 5HT2A in the hCMEC/D3 and potential Wnt activity, clozapine, risperidone and fluoxetine were examined, along with LiCl (1 mM). Experiments were focused on using concentrations that were therapeutically relevant based on plasma concentrations previously reported[35-37].

Of the CNS drugs screened, LiCl, fluoxetine and risperidone activated Wnt/β-catenin signaling in hCMEC/D3 (Fig. 3.6a and 3.6b). Treatment of hCMEC/D3 monolayers with LiCl and to lesser extent risperidone, increased Wnt/β-catenin activity by 30-40% following 7-day treatment, but not after 24 hour treatment (Fig. 3.6a, $p<0.05$). Clozapine did not change Wnt/β-catenin activity in

hCMEC/D3 cells at any time point examined. Fluoxetine increased Wnt/β-catenin activity as early as 24-hours with the greatest increase occurring following 96-hours of exposure (Fig. 3.6b, $p<0.01$). Increased Wnt/β-catenin activity was also observed in neuroblastoma SH-SY5Y cells following 24 hours treatment with fluoxetine (supplemental Fig. 3.S9, $p<0.01$).Together, this suggested that clinically relevant concentrations of LiCl, fluoxetine and risperidone were able to activate Wnt/β-catenin signaling in the hCMEC/D3 cells.

Examination of the downstream effect of Wnt/β-catenin signaling in brain endothelial cells showed that fluoxetine treatment for 96 hours caused the up regulation of claudin-5 by 6-fold (Fig 3.6b, $p<0.01$). Increased Wnt/β-catenin initiated by LiCl or risperidone was not sufficient to improve BBB phenotype in the hCMEC/D3 monolayers.

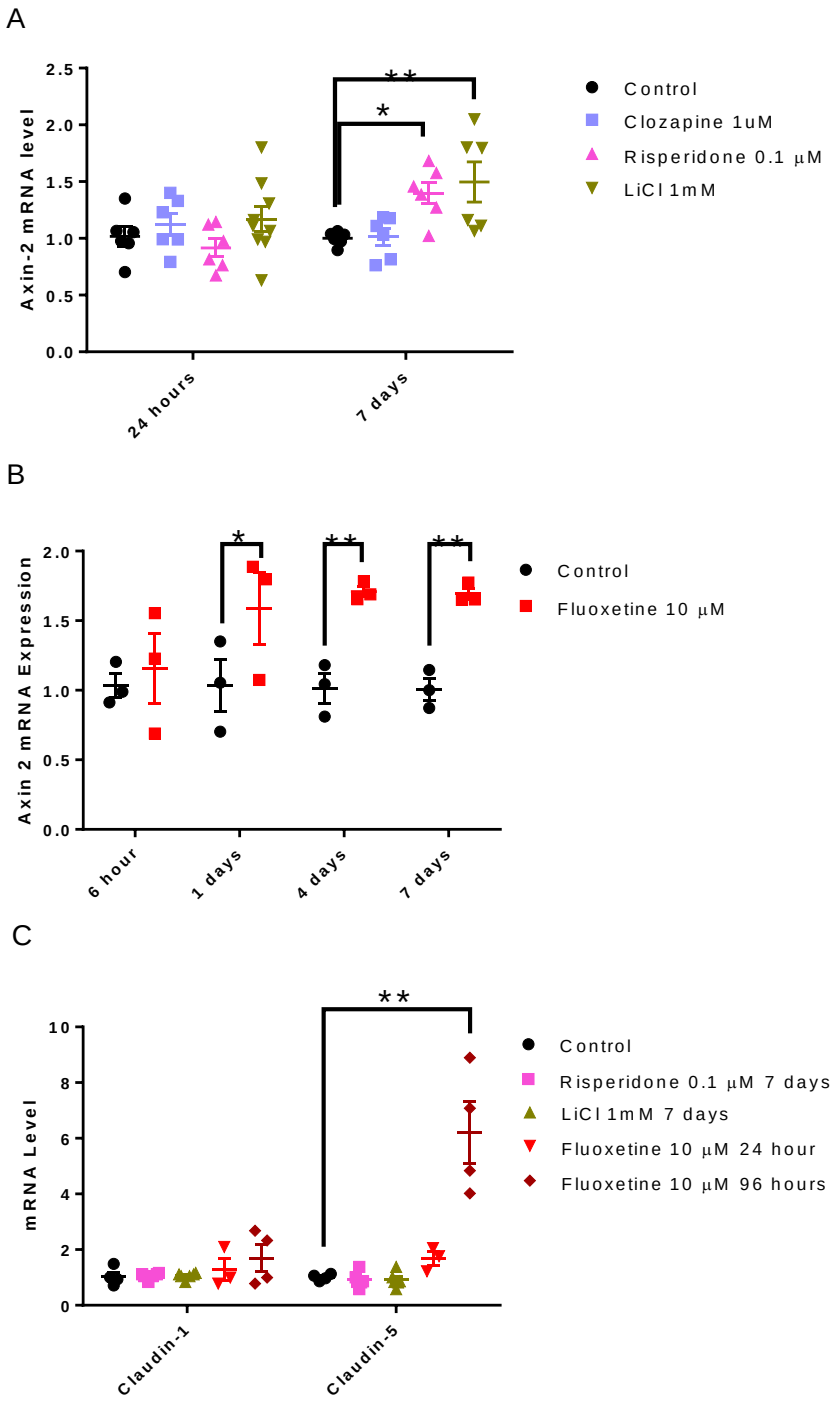

Figure 3.6: The effect of different CNS active drugs, at therapeutically relevant concentrations, to Wnt/β-catenin activity in the hCMEC/D3 cells (A and B) and selected BBB markers regulated by Wnt/β-catenin signaling (C). A and C: data were analyzed using one-way ANOVA followed by LSD Fisher's test. B: data were analyzed using two-way ANOVA followed by LSD Fisher's test. Each data point represents RNA isolated from independent monolayer preparations. The values represent mean±SEM; *p<0.05; **p<0.01.

To examine the functional impact of Wnt/β-catenin activation, the effect of fluoxetine on ethanol disruption of barrier function (50 mM for 96 hour) was assessed. Fluoxetine attenuated the increase in sodium fluorescein P_{app} induced by 50 mM ethanol. Sodium fluorescein P_{app} of ethanol treated cells (47.8.1±2.8 x 10^{-6} cm/s) was reduced to 24.5±8.7 x 10^{-6} cm/s in ethanol+fluoxetine treatment cells (Fig. 3.7a, $p<0.01$). LiCl (10mM), as a positive control, showed similar potency reversing the sodium fluorescein P_{app} to a value similar to the control group, 20.6±1.8 x 10^{-6} cm/s and 22.3±6.5 x 10^{-6} cm/s, respectively. The barrier preserving properties of fluoxetine were associated with an increased expression of claudin-5 (Fig. 3.7b, $p<0.05$). Together, these results suggest that fluoxetine activated Wnt/β-catenin signaling and preserved barrier integrity despite prolonged ethanol exposure.

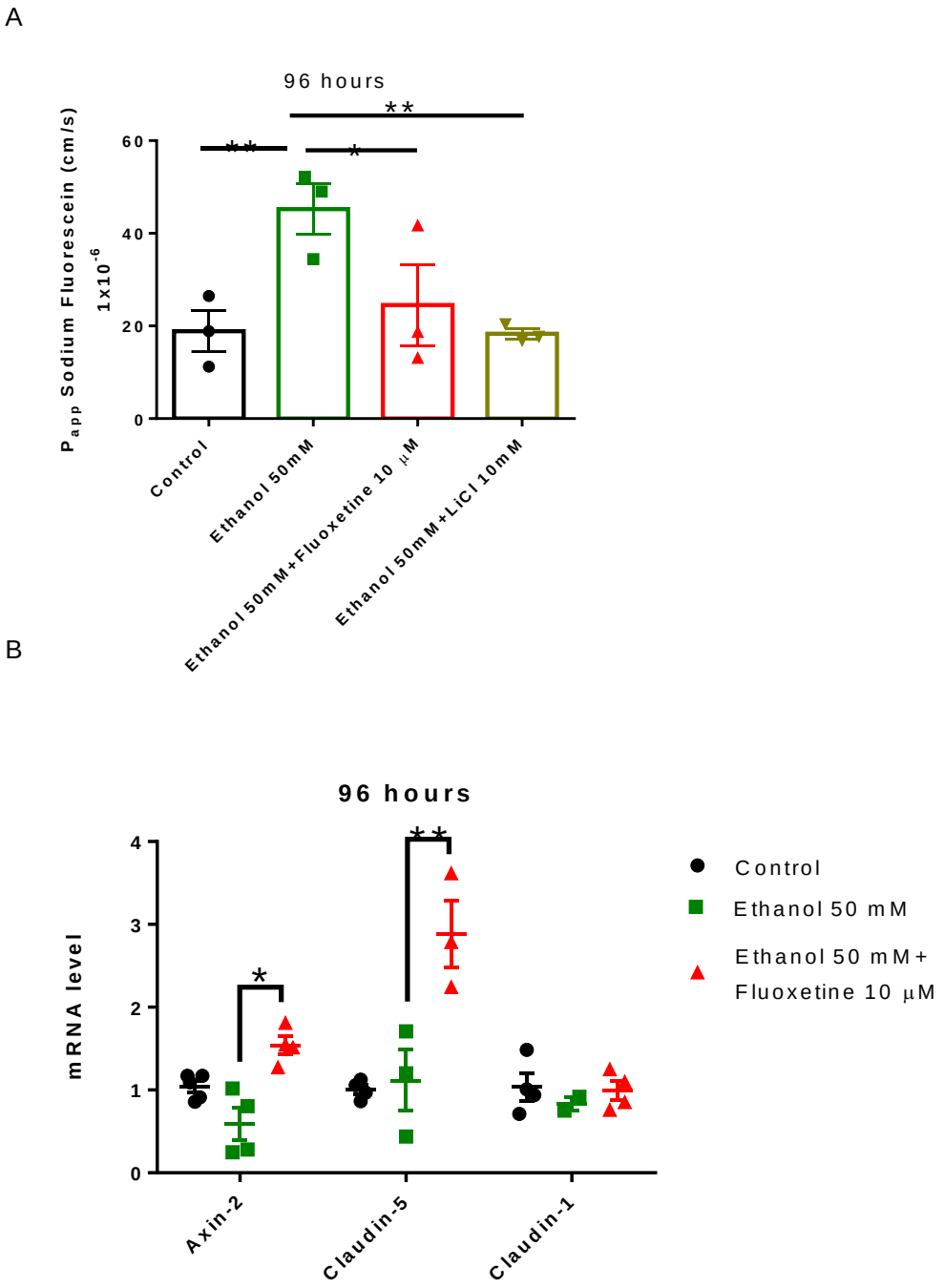

Figure 3.7: Effects of fluoxetine on the barrier properties of hCMEC/D3 following ethanol exposure. Permeability of sodium fluorescein was assessed in confluent hCMEC/D3 monolayers under control conditions and following ethanol exposure in the presence or absence of fluoxetine (10 µM) or LiCl (10 mM) (A). The effects of fluoxetine on Axin-2, Claudin-1 and Claudin-5 mRNA expression in hCMEC/D3 following ethanol exposure (B). Each data point represents apparent permeability value (A) and RNA (B) isolated from independent monolayer preparations. A and B: data were analyzed with one-way ANOVA followed by LSD Fisher's test. *p<0.05; **p<0.01; ***p<0.001. The graphical timeline of the experimental procedure for these data is available at Supplemental Figure 3.S6d

3.5. DISCUSSION

The current studies aimed to identify the effect of ethanol exposure on Wnt/β-catenin signaling activity in the brain endothelial cells and to determine if treatments directed at Wnt/β-catenin activation could restore the barrier properties to these cells following ethanol exposure. The main findings in this study were that ethanol exposure, at concentrations expected during binge alcohol drinking, downregulated Wnt/β-catenin signaling in the blood-brain barrier, and activation of Wnt/β-catenin with agents such as LiCl (10 mM), Wnt3a (200 ng/ml) or fluoxetine restored barrier function in hCMEC/D3 monolayers

Previous studies provide evidence that ethanol exposure impairs Wnt/β-catenin activity in the developing brain with *intra-utero* exposure to ethanol reducing cerebellar Wnt/β-catenin activity in developing rat pups[38]. In addition, cultured neural progenitor cells (NPCs) exposed to four days of ethanol (20 μM and 100 μM) displayed reductions in Wnt3a, Dishevelled-2, LRP-6 and β-catenin expression[39]. In separate studies, hippocampal NPC isolated from pups prenatally exposed to ethanol also exhibited decreased Wnt3a expression and reduced Wnt transcriptional activity compared to hippocampal NPC isolated from pups with mothers that did not receive ethanol[40]. Together, these studies demonstrate that ethanol diminished Wnt/β-catenin activity in the developing brain.

The evidence for diminished Wnt activity following ethanol exposure in adolescent and adult central nervous system is less clear. However, numerous studies have demonstrated the negative effects of ethanol on adult neurogenesis in both rats and monkeys[41,42], and this process is known to be partly regulated by Wnt/β-catenin signaling[43]. The current study is the first to provide evidence that ethanol exposure in adult mice impairs Wnt/β-catenin signaling in the brain in general and BBB in specific. In the present study, mice exposed to ethanol over a one-week period

showed reductions in Axin-2 expression in both cortical brain homogenates as well as isolated cortical brain microvessels. As Axin-2 is a reporter gene for Wnt/β-catenin activity[25] these studies demonstrate reduced activation of the Wnt pathway in the cortex. Isolation of the brain microvessels from the cortex allowed for the examination of Wnt signaling within the vasculature. These studies showed reductions in Axin-2 as well as reductions in downstream Wnt activated gene targets known to contribute to a BBB phenotype.

While the other brain areas examined did not show reduced Axin-2 activity, substantial increases in the expression of Dkk-1, an endogenous Wnt inhibitor, were observed. These findings suggest alterations in Wnt/β-catenin signaling do occur in the adult brain as well as within the mature cerebral microvasculature and that the effects on Wnt/β-catenin vary depending on the area of the brain examined.

Most of the changes in Wnt/β-catenin following ethanol exposure observed in the present study reflected reductions in this signaling pathway, either directly through decreased activity, or indirectly through increased expression of inhibitory Wnt modulators such as Dkk-1. In this regard, it is important to note the increased expression of GPR 124 and Reck in both the cultured brain endothelial cells and brain homogenates. In the case of Wnt7a/7b, non-endothelial Wnt ligands, GPR124 and Reck increase the stability of ligand and facilitate interactions with the Wnt receptors[44,45]. Under normal conditions GPR124 knockout in adult mice have no affect on BBB integrity[46]. However, in pathological conditions such as glioblastoma or brain ischemia, the absence of GPR124 aggravated BBB dysfunction[46]. Thus the increased expression of GPR124 and Reck observed in the present study may be a compensatory response to the decreased Wnt/β-catenin activity caused by ethanol exposure.

To further examine the effects of ethanol exposure in the human cortical microvessels, cultured hCMEC/D3 were used as an *in vitro* model. The hCMEC/D3 cell line was derived from the temporal lobe of the donor patient[47], and has been used previously to characterize Wnt/β-catenin response[21]. Consistent with the data from the cortical microvessels isolated from ethanol exposed mice, hCMEC/D3 cells showed a downregulation of Wnt/β-catenin activity following prolonged exposure to similar concentrations (50 mM) of ethanol. Ethanol exposure in hCMEC/D3cells caused a reduction in Axin-2, Wnt2b, LRP-5 and LRP-6 expression, as well as a trend towards increased expression of Dkk-1. Both LRP-5 and LRP-6 are essential co-receptors required for Wnt/β-catenin signaling. Conditional LRP-5 and LRP-6 deletion in postnatal mice is associated with a widespread compromised BBB, reduced claudin-5 expression and increased PLVAP expression in retinal and cerebral microvessels[11]. As downregulation of Wnt/β-catenin signaling in both hCMEC/D3 cells and isolated microvessels from the cortex of mice following ethanol exposure coincides with reduced claudin-5 expression and upregulation of PLVAP, this could contribute, at least in part, to the permeability effects observed in the present study.

In contrast to most studies, the effects of ethanol on hCMEC/D3 cell permeability focused on longer-term exposures of 2 and 4 days as these would more closely replicate the types of ethanol exposures observed in binge and chronic alcohol consumption. According to the National Institute of Alcohol Abuse and Addiction (NIAAA) binge drinking is defined as the consumption of 4 or more drinks over a period of around 2 hours to produce blood alcohol concentrations (BACs) of 0.08 grams/dL or higher[48]. Further the NIAAA defines heavy drinking as being a binge drinking for more than five days a month. Both binge and heavy alcohol consumption leads to increased risk of alcohol disorders. Studies by Substance Abuse and Mental Health Services Administration

(SAMHSA) in 2010 on alcohol consumption among college students (age 18-25), revealed that 41.7% were binge drinkers and 13.7% were heavy drinkers.

The BACs associated with binge and heavy drinking can vary dramatically. While 0.08 g/dL is the minimal level, exposures resulting in BACs greater than 0.42 g/dL are not uncommon. This is illustrated in the studies of Olson and colleagues (2013) that examined BACs among 374 patients presenting to the emergency department with alcohol intoxication symptoms. Of the 374 individuals, less than 10% had BACs<0.099 g/dL while 34% had BACs between 0.10-0.199 g/dL, 56% had BAC 0.20-0.39 g/dL and 0.7% had BAC>0.40 g/dL[49]. The alcohol concentrations used in the *in vitro* culture model of the BBB in the present study ranged from 17-200 mM (0.08-0.922 g/dL), while the ethanol doses administered to the mice (2g/kg) resulted in peak BACs of around 50 mM (0.23 g/dL). Aside from the 200 mM concentration used to examine the effects of extreme levels of alcohol, the concentrations used represent ranges of exposure observed in binge and heavy drinkers[49,50].

The present study shows ethanol exposure can lead to loss of barrier function in brain endothelial cells. The barrier disrupting effects of ethanol were concentration dependent with 17 mM having minimal impact on barrier properties and concentrations of 50 mM and higher producing a disruption of permeability to both small and large hydrophilic markers. This is consistent with previous studies examining ethanol exposure and BBB function in developing rat pups[51,52]. In these studies, pups from the mother's administered ethanol (BAC 0.14 g/dL) showed no consistent evidence of BBB dysfunction at gestational day 16 to postnatal day 4[51]. In contrast, rat pups P10 feed with ethanol for 5 days (BAC 0.3 g/dL) showed damage to brain capillaries in the cortex[52]. Together these findings suggest that BBB dysfunction is most likely to occur with binge and/or heavy alcohol consumption.

While the present study showed ethanol-induced alterations in Wnt/β-catenin, mechanistically, the involvement of Wnt in ethanol-induced BBB dysfunction is likely more important for chronic ethanol exposure. This is based on the observation that ethanol-induced alterations in permeability in the current study could be observed as early as 2 days, while attenuation of Wnt signaling in the endothelial cell model required prolonged exposure for 4 or 7 days. Several different factors have been identified as potential components important for regulating tight junction protein expression in the BBB during acute ethanol exposure. Previous studies have linked the barrier impairment observed in brain microvessel endothelial cells following acute ethanol exposure with cytoskeleton re-organization and translocation of tight junction proteins from plasma membrane to the cytoplasm via ROS/IP_3/Ca^{2+}/MLCK[53]. Due to the rapidity of response, these acute barrier-altering effects of ethanol are unlikely to be associated with Wnt/β-catenin downregulation.

While much is known about the acute barrier disrupting effects of ethanol, cellular factors involved in BBB functional changes in response to chronic ethanol exposure have been more difficult to assess. Multiple studies in various cell models have shown extended ethanol exposure induces dephosphorylation of GSK3α/β resulting in increased β-catenin phosphorylation and β-catenin degradation[39,54]. Such findings are in agreement with the present study that showed reduced Wnt/β-catenin signaling following ethanol exposure and the subsequent decreased expression of claudin-1 and an increased expression of PLVAP that are important cellular determinants of brain endothelial barrier properties. Additional studies have linked BBB dysfunction following chronic ethanol exposure, at least in part, to an increase in MMP-9 and the degradation of tight junction molecules, extracellular matrix and VEGF receptor[9,10]. Other studies reported that chronic ethanol-induced BBB dysfunction were associated with alterations in ERK/MAPK signaling pathways and the phosphorylation of tight junction proteins[10,55].

Another consideration in the present study is the potential contributions of osmotic stress to ethanol-induced barrier disruption. Previous studies reported that ethanol increases media osmolarity[56,57]. Fifty milimolar ethanol could increase media osmolarity by 50 mOsm/Kg H_2O[57]. However, ethanol is freely permeable and rapidly equilibrates within the intracellular fluid[58,59]. Thus, it is unlikely that the concentrations of ethanol would be sufficient to cause increased permeability through hyperosmotic effects such as observed with 1.4 M mannitol[60,61]. It is likely that the changes in BBB integrity in response to longer-term exposure to ethanol involve multiple pathways and mechanisms.

Regardless of the extent to which alterations in Wnt/β-catenin signaling are mechanistically contributing to ethanol-induced barrier dysfunction in brain microvessel endothelial cells, the results of the present study provides clear evidence that activation of Wnt/β-catenin can restore BBB integrity upon ethanol insult. Activation of Wnt/β-catenin with pharmacological agents such as LiCl (10 mM) or Wnt3a (200 ng/ml) alleviated the BBB breakdown induced by ethanol. Although LiCl and Wnt3a were both able to restore the barrier function upon 50 mM ethanol exposure, LiCl was generally more effective in mitigating the barrier breakdown. The fact that LiCl was more effective compared to Wnt3a was interesting as our previous studies suggested that natural ligand Wnt3a was a stronger Wnt activator in hCMEC/D3 cells compared to LiCl[21]. Comparison of Wnt3a effects in hCMEC/D3 monolayers under normal culture conditions and following ethanol exposure indicated that there was a dramatic reduction in response to Wnt3a, suggesting ethanol may impact Wnt ligand interactions with its receptors. In support of this, the present study and others[39,62,63] have shown ethanol exposure can decrease expression of important co-receptors (LRP-5/6) and modulators (Dkk-1 and sFRP2) that would impact on Wnt ligand signaling and result in a reduced cell response to Wnt ligands[64]. Further studies are needed to

examine if the combination of increased expression of Wnt modulators (such as Dkk-1) and reduced expression of Wnt receptors (i.e. LRP5/6) following chronic ethanol exposure will reduce Wnt activity in the BBB.

Inhibition of GSK3 activity using LiCl has been previously reported to reverse the deleterious effect of ethanol in both neuronal and non-neuronal cells[54,65]. However, the present study is the first to report that LiCl can reverse the permeability effects of ethanol on brain endothelial cells. While such observations were encouraging, the concentration of LiCl required to impact Wnt activation would result in clinical toxicity. Thus, an additional objective of these studies was to identify other central nervous system (CNS) drugs capable of activating Wnt/β-catenin signaling at clinically relevant concentrations. Based on reported GSK3 inhibitor activity, clozapine, risperidone and fluoxetine were examined[66], along with a more clinically relevant concentration of LiCl (1 mM). Using Axin-2 expression as a reporter gene for Wnt/β-catenin activation in hCMEC/D3 monolayers, only lithium and fluoxetine activated Wnt/β-catenin at therapeutically relevant concentrations. The inability of clozapine and risperidone to activate Wnt/β-catenin signaling and improved BBB phenotypes could be due to the transient nature of inhibition of GSK3 produced by these agents, compared to LiCl that exhibits a stable GSK3 inhibition[66]. Furthermore, as the expression of receptor targets for these agents may be lower in brain endothelial cells compared to neurons the level of downstream GSK3 inhibition may be reduced in the brain endothelial cells.

Of the two pharmacological “hits” for Wnt activation, only fluoxetine produced a significant increase in the expression of claudin-5 expression and was able to reverse the barrier breakdown induced by ethanol exposure in the hCMEC/D3 cell culture model of the BBB. In addition to BBB protection, fluoxetine has been shown to promote adult neurogenesis[67], a process that is partly

driven by Wnt/β-catenin signaling[43]. Thus the effects of fluoxetine on Wnt signaling could be beneficial in both restoring BBB function and promoting neurogenesis. Although the fluoxetine-mediated BBB protection in the present studies has been associated with upregulation of Wnt/β-catenin signaling in the present study, we cannot exclude other possible mechanisms accounting for fluoxetine's effect, for example by preventing MMP activity during BBB insult[68].

The ability of fluoxetine to alleviate ethanol-induced barrier breakdown in brain endothelial cells is an encouraging finding. Clinical and pre-clinical studies suggest that serotonin deficiency is associated with higher alcohol consumption and reduced sedative-like effects of alcohol[69], and drugs that increased extracellular serotonin such as fluoxetine, reduced alcohol consumption[70,71]. Thus potential protective effects on BBB integrity with fluoxetine could provide an additional advantage for this drug in individuals with heavy alcohol use.

In summary, we have shown downregulation of Wnt/β-catenin activity in the BBB following ethanol exposure that potentially can be reversed by exogenous activation of Wnt/β-catenin signaling (illustrated at supplemental Fig 3.S10). In ethanol-induced BBB dysfunction, inhibition of GSK3 provided more robust activation of Wnt/β-catenin than the natural Wnt ligand and was more effective in restoring paracellular barrier function. While further *in vivo* studies are necessary, the results of the present study suggest that modulation of Wnt/β-catenin may be beneficial in reversing chronic ethanol-induced BBB dysfunction

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CONFLICT OF INTEREST DISCLOSURE

The author(s) declared no competing interest

3.6. REFERENCES

1 Alcohol, G. B. D. & Drug Use, C. The global burden of disease attributable to alcohol and drug use in 195 countries and territories, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *The lancet. Psychiatry* **5**, 987-1012, doi:10.1016/S2215-0366(18)30337-7 (2018).

2 Wood, A. M. *et al.* Risk thresholds for alcohol consumption: combined analysis of individual-participant data for 599 912 current drinkers in 83 prospective studies. *Lancet* **391**, 1513-1523, doi:10.1016/S0140-6736(18)30134-X (2018).

3 Sullivan, E. V., Harris, R. A. & Pfefferbaum, A. Alcohol's effects on brain and behavior. *Alcohol research & health : the journal of the National Institute on Alcohol Abuse and Alcoholism* **33**, 127-143 (2010).

4 Abbott, N. J., Ronnback, L. & Hansson, E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nature reviews. Neuroscience* **7**, 41-53, doi:10.1038/nrn1824 (2006).

5 Laksitorini, M., Prasasty, V. D., Kiptoo, P. K. & Siahaan, T. J. Pathways and progress in improving drug delivery through the intestinal mucosa and blood-brain barriers. *Therapeutic delivery* **5**, 1143-1163, doi:10.4155/tde.14.67 (2014).

6 Liebner, S., Kniesel, U., Kalbacher, H. & Wolburg, H. Correlation of tight junction morphology with the expression of tight junction proteins in blood-brain barrier endothelial cells. *European journal of cell biology* **79**, 707-717, doi:10.1078/0171-9335-00101 (2000).

7 Liebner, S. *et al.* Claudin-1 and claudin-5 expression and tight junction morphology are altered in blood vessels of human glioblastoma multiforme. *Acta neuropathologica* **100**, 323-331, doi:10.1007/s004010000180 (2000).

8 On, N. H. & Miller, D. W. Transporter-based delivery of anticancer drugs to the brain: improving brain penetration by minimizing drug efflux at the blood-brain barrier. *Current pharmaceutical design* **20**, 1499-1509 (2014).

9 Haorah, J., Schall, K., Ramirez, S. H. & Persidsky, Y. Activation of protein tyrosine kinases and matrix metalloproteinases causes blood-brain barrier injury: Novel mechanism for neurodegeneration associated with alcohol abuse. *Glia* **56**, 78-88, doi:10.1002/glia.20596 (2008).

10 Rubio-Araiz, A. *et al.* Disruption of blood-brain barrier integrity in postmortem alcoholic brain: preclinical evidence of TLR4 involvement from a binge-like drinking model. *Addiction biology* **22**, 1103-1116, doi:10.1111/adb.12376 (2017).

11 Zhou, Y. *et al.* Canonical WNT signaling components in vascular development and barrier formation. *The Journal of clinical investigation* **124**, 3825-3846, doi:10.1172/JCI76431 (2014).

12 Wang, Y. *et al.* Interplay of the Norrin and Wnt7a/Wnt7b signaling systems in blood-brain barrier and blood-retina barrier development and maintenance. *Proceedings of the National Academy of*

Sciences of the United States of America **115**, E11827-E11836, doi:10.1073/pnas.1813217115 (2018).

13 Stenman, J. M. *et al.* Canonical Wnt signaling regulates organ-specific assembly and differentiation of CNS vasculature. *Science* **322**, 1247-1250, doi:10.1126/science.1164594 (2008).

14 Liebner, S. *et al.* Wnt/beta-catenin signaling controls development of the blood-brain barrier. *The Journal of cell biology* **183**, 409-417, doi:10.1083/jcb.200806024 (2008).

15 Daneman, R. *et al.* Wnt/beta-catenin signaling is required for CNS, but not non-CNS, angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 641-646, doi:10.1073/pnas.0805165106 (2009).

16 Niehrs, C. The complex world of WNT receptor signalling. *Nature reviews. Molecular cell biology* **13**, 767-779, doi:10.1038/nrm3470 (2012).

17 Logan, C. Y. & Nusse, R. The Wnt signaling pathway in development and disease. *Annual review of cell and developmental biology* **20**, 781-810, doi:10.1146/annurev.cellbio.20.010403.113126 (2004).

18 Lengfeld, J. E. *et al.* Endothelial Wnt/beta-catenin signaling reduces immune cell infiltration in multiple sclerosis. *Proceedings of the National Academy of Sciences of the United States of America* **114**, E1168-E1177, doi:10.1073/pnas.1609905114 (2017).

19 Liu, L., Wan, W., Xia, S., Kalionis, B. & Li, Y. Dysfunctional Wnt/beta-catenin signaling contributes to blood-brain barrier breakdown in Alzheimer's disease. *Neurochemistry international* **75**, 19-25, doi:10.1016/j.neuint.2014.05.004 (2014).

20 Phoenix, T. N. *et al.* Medulloblastoma Genotype Dictates Blood Brain Barrier Phenotype. *Cancer cell* **29**, 508-522, doi:10.1016/j.ccell.2016.03.002 (2016).

21 Laksitorini, M. D., Yathindranath, V., Xiong, W., Hombach-Klonisch, S. & Miller, D. W. Modulation of Wnt/beta-catenin signaling promotes blood-brain barrier phenotype in cultured brain endothelial cells. *Scientific reports* **9**, 19718, doi:10.1038/s41598-019-56075-w (2019).

22 Zhang, D., Xiong, W., Jackson, M. F. & Parkinson, F. E. Ethanol Tolerance Affects Endogenous Adenosine Signaling in Mouse Hippocampus. *The Journal of pharmacology and experimental therapeutics* **358**, 31-38, doi:10.1124/jpet.116.232231 (2016).

23 On, N. H., Savant, S., Toews, M. & Miller, D. W. Rapid and reversible enhancement of blood-brain barrier permeability using lysophosphatidic acid. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* **33**, 1944-1954, doi:10.1038/jcbfm.2013.154 (2013).

24 Sun, Z. Z. *et al.* Differential internalization of brick shaped iron oxide nanoparticles by endothelial cells. *J Mater Chem B* **4**, 5913-5920, doi:10.1039/c6tb01480a (2016).

25 Jho, E. H. *et al.* Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Molecular and cellular biology* **22**, 1172-1183 (2002).

26 Vanhollebeke, B. *et al.* Tip cell-specific requirement for an atypical Gpr124- and Reck-dependent Wnt/beta-catenin pathway during brain angiogenesis. *eLife* **4**, doi:10.7554/eLife.06489 (2015).

27 Cho, C., Smallwood, P. M. & Nathans, J. Reck and Gpr124 Are Essential Receptor Cofactors for Wnt7a/Wnt7b-Specific Signaling in Mammalian CNS Angiogenesis and Blood-Brain Barrier Regulation. *Neuron* **95**, 1221-1225, doi:10.1016/j.neuron.2017.08.032 (2017).

28 Eubelen, M. *et al.* A molecular mechanism for Wnt ligand-specific signaling. *Science* **361**, doi:10.1126/science.aat1178 (2018).

29 Vestweber, D. VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. *Arteriosclerosis, thrombosis, and vascular biology* **28**, 223-232, doi:10.1161/ATVBAHA.107.158014 (2008).

30 Greene, C., Hanley, N. & Campbell, M. Claudin-5: gatekeeper of neurological function. *Fluids and barriers of the CNS* **16**, 3, doi:10.1186/s12987-019-0123-z (2019).

31 Ronaldson, P. T., Bendayan, M., Gingras, D., Piquette-Miller, M. & Bendayan, R. Cellular localization and functional expression of P-glycoprotein in rat astrocyte cultures. *Journal of neurochemistry* **89**, 788-800, doi:10.1111/j.1471-4159.2004.02417.x (2004).

32 Pardridge, W. M., Golden, P. L., Kang, Y. S. & Bickel, U. Brain microvascular and astrocyte localization of P-glycoprotein. *Journal of neurochemistry* **68**, 1278-1285, doi:10.1046/j.1471-4159.1997.68031278.x (1997).

33 Bosma, E. K., van Noorden, C. J. F., Schlingemann, R. O. & Klaassen, I. The role of plasmalemma vesicle-associated protein in pathological breakdown of blood-brain and blood-retinal barriers: potential novel therapeutic target for cerebral edema and diabetic macular edema. *Fluids and barriers of the CNS* **15**, 24, doi:10.1186/s12987-018-0109-2 (2018).

34 Shue, E. H. *et al.* Plasmalemmal vesicle associated protein-1 (PV-1) is a marker of blood-brain barrier disruption in rodent models. *BMC neuroscience* **9**, 29, doi:10.1186/1471-2202-9-29 (2008).

35 Mauri, M. C. *et al.* Lithium safety in the prophylaxis of bipolar disorders: a study with plasma levels. *European review for medical and pharmacological sciences* **3**, 63-69 (1999).

36 Hiemke, C. *et al.* AGNP Consensus Guidelines for Therapeutic Drug Monitoring in Psychiatry: Update 2011. *Pharmacopsychiatry* **44**, 195-235, doi:10.1055/s-0031-1286287 (2011).

37 Karson, C. N. *et al.* Human brain fluoxetine concentrations. *The Journal of neuropsychiatry and clinical neurosciences* **5**, 322-329, doi:10.1176/jnp.5.3.322 (1993).

38 Tong, M. *et al.* Motor Function Deficits Following Chronic Prenatal Ethanol Exposure are Linked to Impairments in Insulin/IGF, Notch and Wnt Signaling in the Cerebellum. *Journal of diabetes & metabolism* **4**, 238 (2013).

39 Vangipuram, S. D. & Lyman, W. D. Ethanol affects differentiation-related pathways and suppresses Wnt signaling protein expression in human neural stem cells. *Alcoholism, clinical and experimental research* **36**, 788-797, doi:10.1111/j.1530-0277.2011.01682.x (2012).

40 Singh, A. K., Gupta, S., Jiang, Y., Younus, M. & Ramzan, M. In vitro neurogenesis from neural progenitor cells isolated from the hippocampus region of the brain of adult rats exposed to ethanol during early development through their alcohol-drinking mothers. *Alcohol and alcoholism* **44**, 185-198, doi:10.1093/alcalc/agn109 (2009).

41 Taffe, M. A. *et al.* Long-lasting reduction in hippocampal neurogenesis by alcohol consumption in adolescent nonhuman primates. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 11104-11109, doi:10.1073/pnas.0912810107 (2010).

42 Morris, S. A., Eaves, D. W., Smith, A. R. & Nixon, K. Alcohol inhibition of neurogenesis: a mechanism of hippocampal neurodegeneration in an adolescent alcohol abuse model. *Hippocampus* **20**, 596-607, doi:10.1002/hipo.20665 (2010).

43 Lie, D. C. *et al.* Wnt signalling regulates adult hippocampal neurogenesis. *Nature* **437**, 1370-1375, doi:10.1038/nature04108 (2005).

44 Cho, C., Wang, Y., Smallwood, P. M., Williams, J. & Nathans, J. Molecular determinants in Frizzled, Reck, and Wnt7a for ligand-specific signaling in neurovascular development. *eLife* **8**, doi:10.7554/eLife.47300 (2019).

45 Vallon, M. *et al.* A RECK-WNT7 Receptor-Ligand Interaction Enables Isoform-Specific Regulation of Wnt Bioavailability. *Cell reports* **25**, 339-349 e339, doi:10.1016/j.celrep.2018.09.045 (2018).

46 Chang, J. *et al.* Gpr124 is essential for blood-brain barrier integrity in central nervous system disease. *Nature medicine* **23**, 450-460, doi:10.1038/nm.4309 (2017).

47 Weksler, B. B. *et al.* Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **19**, 1872-1874, doi:10.1096/fj.04-3458fje (2005).

48 Wechsler, H. & Nelson, T. F. Binge drinking and the American college student: what's five drinks? *Psychology of addictive behaviors : journal of the Society of Psychologists in Addictive Behaviors* **15**, 287-291, doi:10.1037//0893-164x.15.4.287 (2001).

49 Olson, K. N., Smith, S. W., Kloss, J. S., Ho, J. D. & Apple, F. S. Relationship between blood alcohol concentration and observable symptoms of intoxication in patients presenting to an emergency department. *Alcohol and alcoholism* **48**, 386-389, doi:10.1093/alcalc/agt042 (2013).

50 Pelissier, F., Lauque, D., Charpentier, S. & Franchitto, N. Blood alcohol concentration in intoxicated patients seen in the emergency department: does it influence discharge decisions? *Journal of studies on alcohol and drugs* **75**, 937-944, doi:10.15288/jsad.2014.75.937 (2014).

51 Phillips, D. E., Krueger, S. K., Wall, K. A., Smoyer-Dearing, L. H. & Sikora, A. K. The development of the blood-brain barrier in alcohol-exposed rats. *Alcohol* **14**, 333-343, doi:10.1016/s0741-8329(96)00180-2 (1997).

52 West, J. R. & Goodlett, C. R. Teratogenic effects of alcohol on brain development. *Annals of medicine* **22**, 319-325, doi:10.3109/07853899009147914 (1990).

53 Haorah, J. *et al.* Ethanol-induced activation of myosin light chain kinase leads to dysfunction of tight junctions and blood-brain barrier compromise. *Alcoholism, clinical and experimental research* **29**, 999-1009 (2005).

54 Lauing, K. L., Sundaramurthy, S., Nauer, R. K. & Callaci, J. J. Exogenous activation of Wnt/beta-catenin signaling attenuates binge alcohol-induced deficient bone fracture healing. *Alcohol and alcoholism* **49**, 399-408, doi:10.1093/alcalc/agu006 (2014).

55 Singh, A. K., Jiang, Y., Gupta, S. & Benlhabib, E. Effects of chronic ethanol drinking on the blood brain barrier and ensuing neuronal toxicity in alcohol-preferring rats subjected to intraperitoneal LPS injection. *Alcohol and alcoholism* **42**, 385-399, doi:10.1093/alcalc/agl120 (2007).

56 Purssell, R. A., Pudek, M., Brubacher, J. & Abu-Laban, R. B. Derivation and validation of a formula to calculate the contribution of ethanol to the osmolal gap. *Annals of emergency medicine* **38**, 653-659, doi:10.1067/mem.2001.119455 (2001).

57 Nguyen, M. K. *et al.* Is the Osmolal Concentration of Ethanol Greater Than Its Molar Concentration? *Frontiers in medicine* **6**, 306, doi:10.3389/fmed.2019.00306 (2019).

58 Oster, J. R. & Singer, I. Hyponatremia, hyposmolality, and hypotonicity: tables and fables. *Archives of internal medicine* **159**, 333-336, doi:10.1001/archinte.159.4.333 (1999).

59 Gennari, F. J. Current concepts. Serum osmolality. Uses and limitations. *The New England journal of medicine* **310**, 102-105, doi:10.1056/NEJM198401123100207 (1984).

60 Sun, Z. *et al.* Magnetic field enhanced convective diffusion of iron oxide nanoparticles in an osmotically disrupted cell culture model of the blood-brain barrier. *International journal of nanomedicine* **9**, 3013-3026, doi:10.2147/IJN.S62260 (2014).

61 Norouzi, M., Yathindranath, V., Thliveris, J. A. & Miller, D. W. Salinomycin-Loaded Iron Oxide Nanoparticles for Glioblastoma Therapy. *Nanomaterials* **10**, doi:10.3390/nano10030477 (2020).

62 Xu, C. Q., de la Monte, S. M., Tong, M., Huang, C. K. & Kim, M. Chronic Ethanol-Induced Impairment of Wnt/beta-Catenin Signaling is Attenuated by PPAR-delta Agonist. *Alcoholism, clinical and experimental research* **39**, 969-979, doi:10.1111/acer.12727 (2015).

63 Chen, J. R. *et al.* A role for ethanol-induced oxidative stress in controlling lineage commitment of mesenchymal stromal cells through inhibition of Wnt/beta-catenin signaling. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* **25**, 1117-1127, doi:10.1002/jbmr.7 (2010).

64 Sakane, H., Yamamoto, H. & Kikuchi, A. LRP6 is internalized by Dkk1 to suppress its phosphorylation in the lipid raft and is recycled for reuse. *Journal of cell science* **123**, 360-368, doi:10.1242/jcs.058008 (2010).

65 Liu, Y. *et al.* Overexpression of glycogen synthase kinase 3beta sensitizes neuronal cells to ethanol toxicity. *Journal of neuroscience research* **87**, 2793-2802, doi:10.1002/jnr.22098 (2009).

66 Li, X., Rosborough, K. M., Friedman, A. B., Zhu, W. & Roth, K. A. Regulation of mouse brain glycogen synthase kinase-3 by atypical antipsychotics. *The international journal of neuropsychopharmacology* **10**, 7-19, doi:10.1017/S1461145706006547 (2007).

67 Hui, J. *et al.* Fluoxetine regulates neurogenesis in vitro through modulation of GSK-3beta/beta-catenin signaling. *The international journal of neuropsychopharmacology* **18**, doi:10.1093/ijnp/pyu099 (2014).

68 Lee, J. Y., Kim, H. S., Choi, H. Y., Oh, T. H. & Yune, T. Y. Fluoxetine inhibits matrix metalloprotease activation and prevents disruption of blood-spinal cord barrier after spinal cord injury. *Brain : a journal of neurology* **135**, 2375-2389, doi:10.1093/brain/aws171 (2012).

69 Sachs, B. D., Salahi, A. A. & Caron, M. G. Congenital brain serotonin deficiency leads to reduced ethanol sensitivity and increased ethanol consumption in mice. *Neuropharmacology* **77**, 177-184, doi:10.1016/j.neuropharm.2013.09.010 (2014).

70 Naranjo, C. A., Poulos, C. X., Bremner, K. E. & Lanctot, K. L. Fluoxetine attenuates alcohol intake and desire to drink. *International clinical psychopharmacology* **9**, 163-172, doi:10.1097/00004850-199409000-00004 (1994).

71 Maurel, S., De Vry, J. & Schreiber, R. Comparison of the effects of the selective serotonin-reuptake inhibitors fluoxetine, paroxetine, citalopram and fluvoxamine in alcohol-preferring cAA rats. *Alcohol* **17**, 195-201, doi:10.1016/s0741-8329(98)00046-9 (1999).

3.7. SUPPLEMENTARY INFORMATION CHAPTER 3: Impact of Wnt/β-catenin signaling on ethanol-induced changes in brain endothelial cell permeability.

Supplemental Information for Impact of Wnt/β-catenin signaling on ethanol-induced changes in brain endothelial cell permeability.

Marlyn D. Laksitorini[1,4], Vinith Yathindranath[1], Wei Xiong[1], Fiona E. Parkinson[1,5], James A. Thliveris[3], Donald W. Miller[1,2]*
[1]*Department of Pharmacology and Therapeutics, Max Rady College of Medicine, University of Manitoba, Winnipeg, R3E 0T6, Canada.*
[2.] *Kleysen Institute of Advanced Medicine, Health Sciences Center, Winnipeg, R3E 0T6, Canada.*
[3] *Department of Human Anatomy and Cell Science, University of Manitoba, Winnipeg,, R3E 0J9, Canada.*
[4]*Department of Pharmaceutics, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, 55281, Indonesia*
[5]*Present address: Ross University School of Medicine, Miramar, Florida, 33027, USA*

**corresponding author: Donald.Miller@umanitoba.ca*

3.7.1. Extended method

3.7.1.1. MTT Assay

hCMEC/D3 cells (1000 cells/well) were seeded on 96 well plate that previously been coated with rat tail collagen. Twenty hours after seeding, cells were treated with different concentrations of ethanol for 7 days with media replenishment every 24 hours. Following ethanol treatment, media was removed, and replenished with 100 µl of new EBM-2 media. A 25 µl aliquot of thiazolyl blue tetrazolium bromide (5 mg/ml) was added to each well and cells were incubated for an additional two hours, after which the media was removed and 100 µl DMSO was added to each well. After an additional 30 minutes, the absorbance of dissolved formazan crystal were measured using a Synergy HT plate reader at wavelength 570 nm. The absorbance readings were expressed as a percentage of control cells exposed to media alone.

3.7.1.2.Cell impedance assay

Monolayer electrical impedance was measured using the xCELLigence RTCA system. Briefly, the cells were seeded on 16 well E-plate (ACEA Biosciences) coated with rat tail collagen at a density of 20,000 cells/well. The microelectrodes implanted in the E-plates measure the electrical impedance of the cells resulting a dimensionless value called Cell Index (CI). For barrier cells, electrical impedance can be used to monitor tight junction formation [1]. Electrical impedance was monitored in real time and once the impedance reached a plateau for at least 24 hrs, the media was removed and the various treatments were added in completed EBM-2 media. Impedance monitoring was continued for an additional 24-96 hrs post-treatment.

3.7.1.3.Examination on ethanol evaporation

The concentration of ethanol in the media was measured using QuantiChrom™ Ethanol Assay Kit (DIET-500). Briefly, 2 ml of media containing various amount of ethanol (17-200 mM) were place in 6 well culture plates and incubated at 37ºC and 5% CO_2 for various periods of time. To determine ethanol concentration in the media, 500 µL of 10% TCA was added to a 1 ml sample of media. The sample were centrifuged at 14,000 rpm for 5 minutes and 100 µL samples the supernatant or standard solution were transferred to a 96 well plate. A 100 µL aliquot of reagent A was mixed into each well and incubated for 25 minutes at room temperature until the media color was changed from yellow to visibly bluish. At this time, 100 µL reagent B was added to stop the reaction and the absorbance was measured in Synergy HT plate reader at 580 nm. Quantitaitve measurement of ethanol concentration in the media was determined by comparison of absorbance value to an ethanol standard curve.

3.7.2. Extended discussion

3.7.2.1.Contributions of cellular transport to ethanol-induced changes in hCMEC/D3 monolayer permeability.

Ethanol increased both the large molecular weight marker (IRDye 800CW PEG) and the small molecular weight marker (sodium fluorescein). As sodium fluorescein has been reported to be a substrate for organic anion transporters, the cellular accumulation of the fluorescent permeability markers were also assessed (Supplemental Fig. 3.S11). While the P_{app} of sodium fluorescein was increased in ethanol treatment group and activation of Wnt signaling reduced the permeability observed in the ethanol group, intracellular accumulation of fluorescein was unchanged compared to control (Figure 3.4, 3.5 and Supplemental Fig. 3.S11d). A similar finding was observed for IRDye 800CW PEG, with substantial increases in permeability following ethanol that was mitigated with Wnt activators despite minimal effects on cellular accumulation (Fig 3.4, supplemental Fig. 3.S11c).

While activation of Wnt/β-catenin does alter some transporters in brain endothelial cells, most notably the efflux transporters, it is difficult to interpret the extent to which transcellular transport pathways contribute to the permeability responses to ethanol observed. This is due to a generalized increase in paracellular diffusion that would make determination of the contributions of transcellular pathways hard to quantitatively assess. However based on the cellular accumulation of fluorescein and IRDye 800 PEG observed following ethanol exposure, the increases in

permeability are likely due to enhance paracellular diffusion as opposed to transcellular transport pathways.

3.7.2.2.Contribution of osmotic stress to permeability responses to ethanol in hCMEC/D3 monolayers

The concentrations of ethanol used in the current study could impact on the osmolality of the media. However, in terms of osmolality, ethanol has been recognized as ineffective solute [2-4]. This is due to the ability of ethanol to rapidly cross cell membranes. To examine potential effect of increased osmotic pressure with ethanol treatment and whether osmotic stress could be involved in ethanol effects, the electrical impedance of hCMEC/D3 monolayers were examined over a 4-day period following treatment with various concentrations of ethanol or mannitol (Supplemental Fig. 3.S12). Ethanol exposure caused a concentration dependent decrease in impedance in hCMEC/D3. The effects of ethanol were characterized by a rapid decrease in impedance followed by a slow return towards baseline levels (Supplemental Fig. 3.S12). These effects were substantially different from impedance changes associated with mannitol (Supplemental Fig. 3.S12). Exposure to 1.4 M mannitol, a concentration that has been used *in vitro*, *in vivo* and clinically to open the BBB, caused a rapid and sustained decrease in electrical impedance. In contrast, impedance changes in response to 200 mM mannitol showed a slight increase in impedance compared to control. As electrical impedance is a surrogate marker for tight junction integrity and paracellular leakage[1], these studies suggest the osmotic effects of ethanol are unlikely contributing to the permeability changes observed with ethanol.

3.7.3. Supplemental data

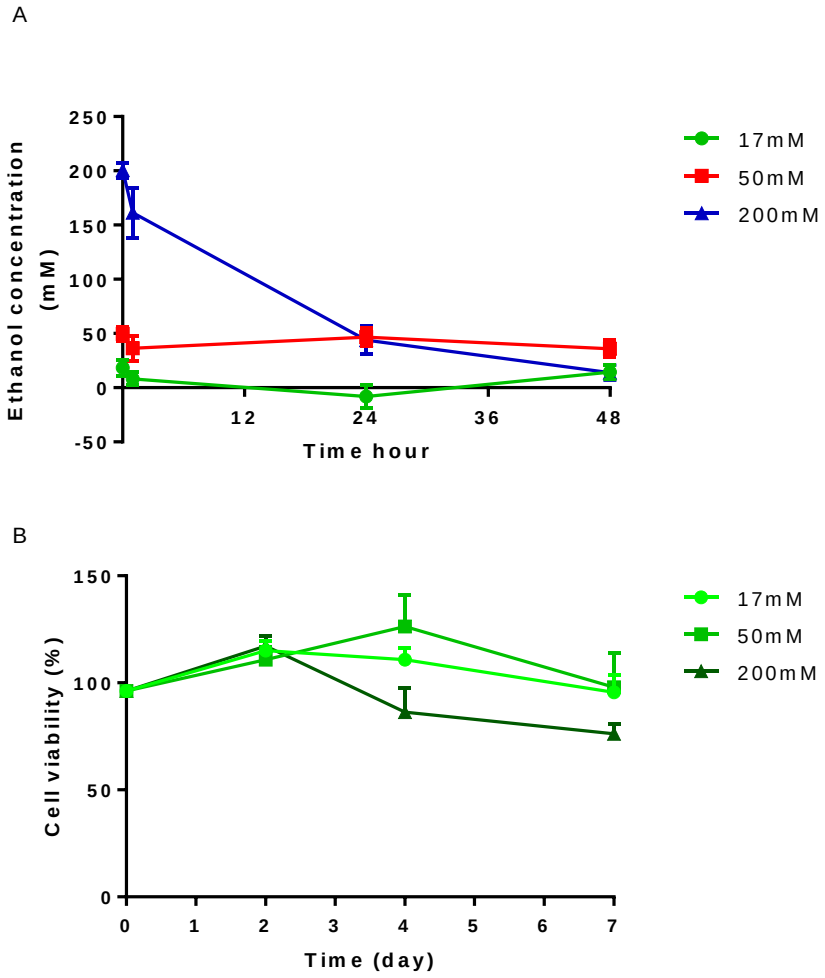

Figure 3.S1: A: Ethanol evaporation over time measured using QuantiChrom Ethanol Assay kit (A). Two milliliters of ethanol containing media were placed in 6 well plates and incubated for 1 hour, 24 hours and 48 hours. Experiments were done in three different wells for each time points. B: Viability of hCMEC/D3 cells following exposure of ethanol at different concentrations and durations of exposure. All values represent mean±SEM, N: 8 wells. Data were analyzed with two-way ANOVA followed with Fisher's LSD test. *$p<0.05$

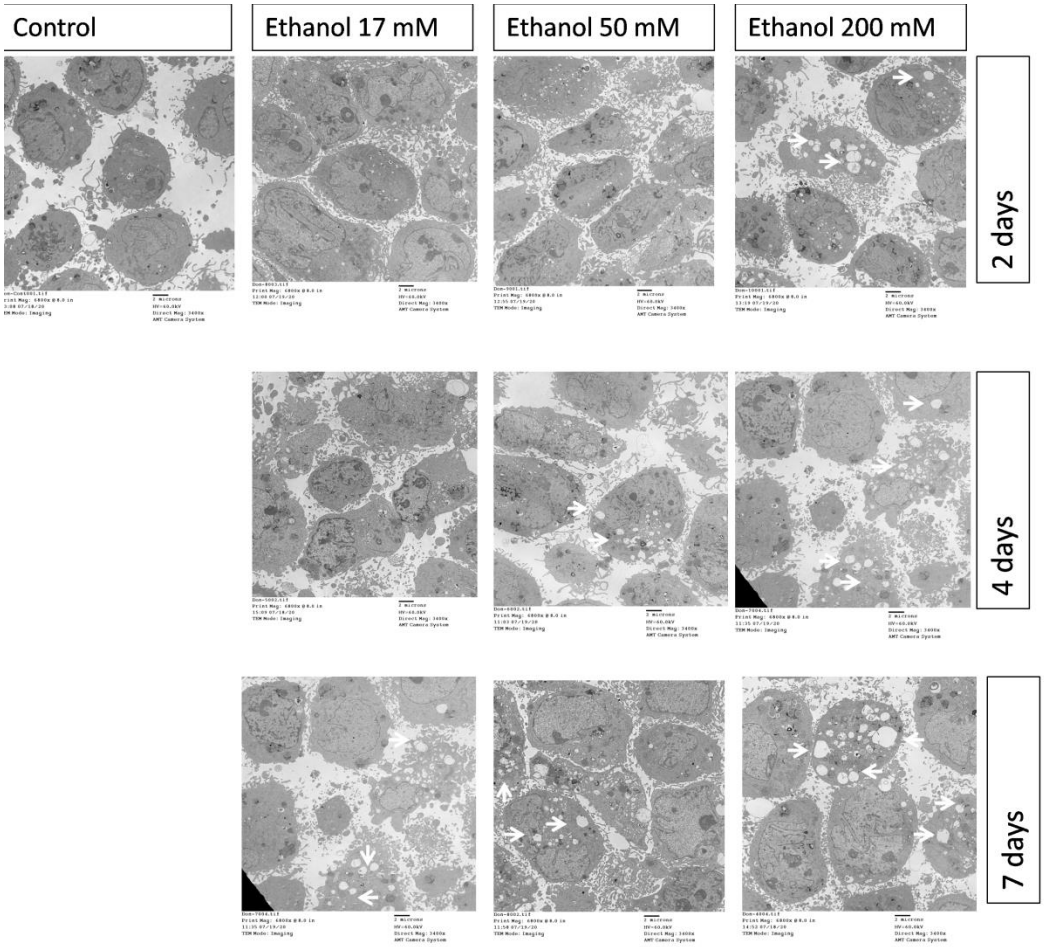

Figure 3.S2: Morphology of hCMEC/D3 cells following ethanol exposure at different condition. Cells were grown in T25 cells in EBM-2 complete media and treated with different ethanol concentration (17, 50, and 200 mM) and time exposure (2 days, 4 days, and 7 days). All of the treatment groups including control (media alone) were harvested 8 days after initial seeding. White arrows showed more abundance vesicles in hCMEC/D3 cells following ethanol exposure.

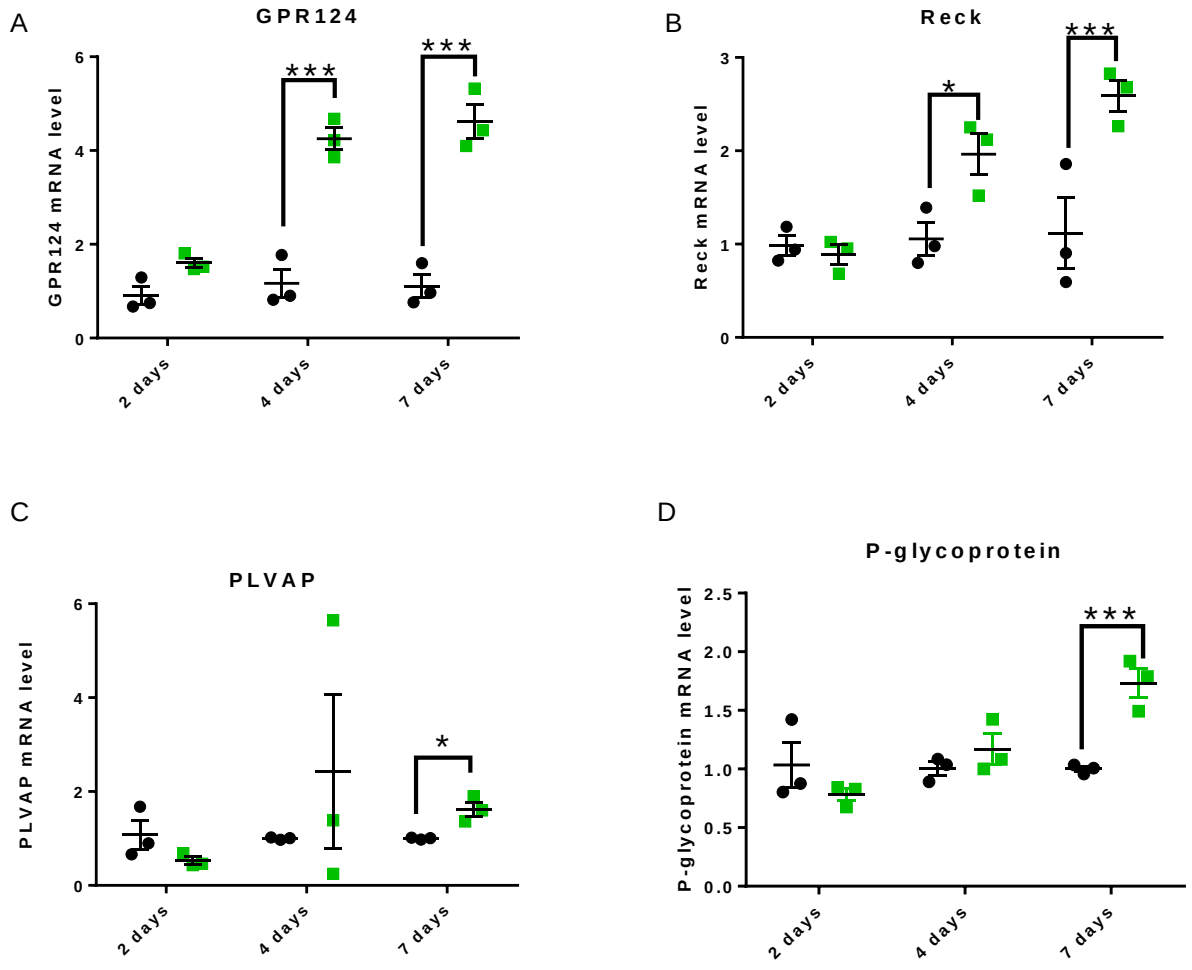

Figure 3.S3: Expression of GPR124 (A), Reck (B), PLVAP (C) and P-glycoprotein (D) in hCMEC/D3 cells following exposure to ethanol 200 mM. Black dots represent control group and green squares represent ethanol 200 mM group. All values represent mean±SEM. Each data point represents one cell monolayer. Data were analyzed using two-way ANOVA followed by LSD Fisher's test; *p<0.05; ***p<0.001.

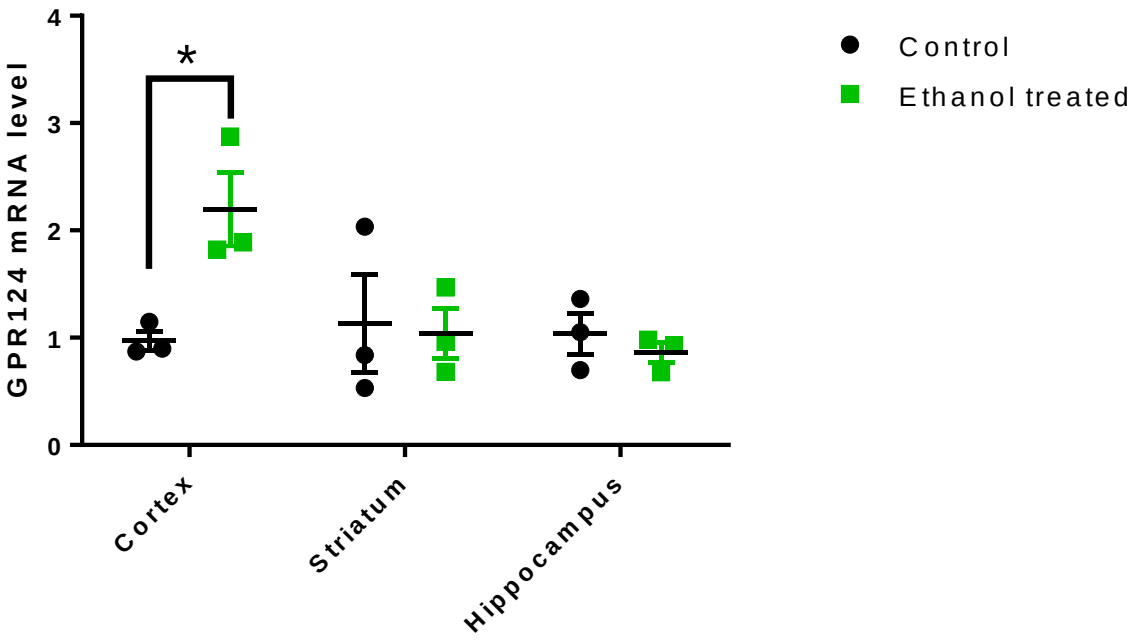

Figure 3.S4: Effects of ethanol exposure on GPR124 expression in various brain regions. Mice were treated with i.p injections of saline (control) or ethanol (2 g/kg) every 24 hours for a total of 7 days (A). Each data point represents regional expression data from different individual mice. Values represent the mean ± SEM. *$p<0.05$; based on t-test comparing control and ethanol treatment conditions.

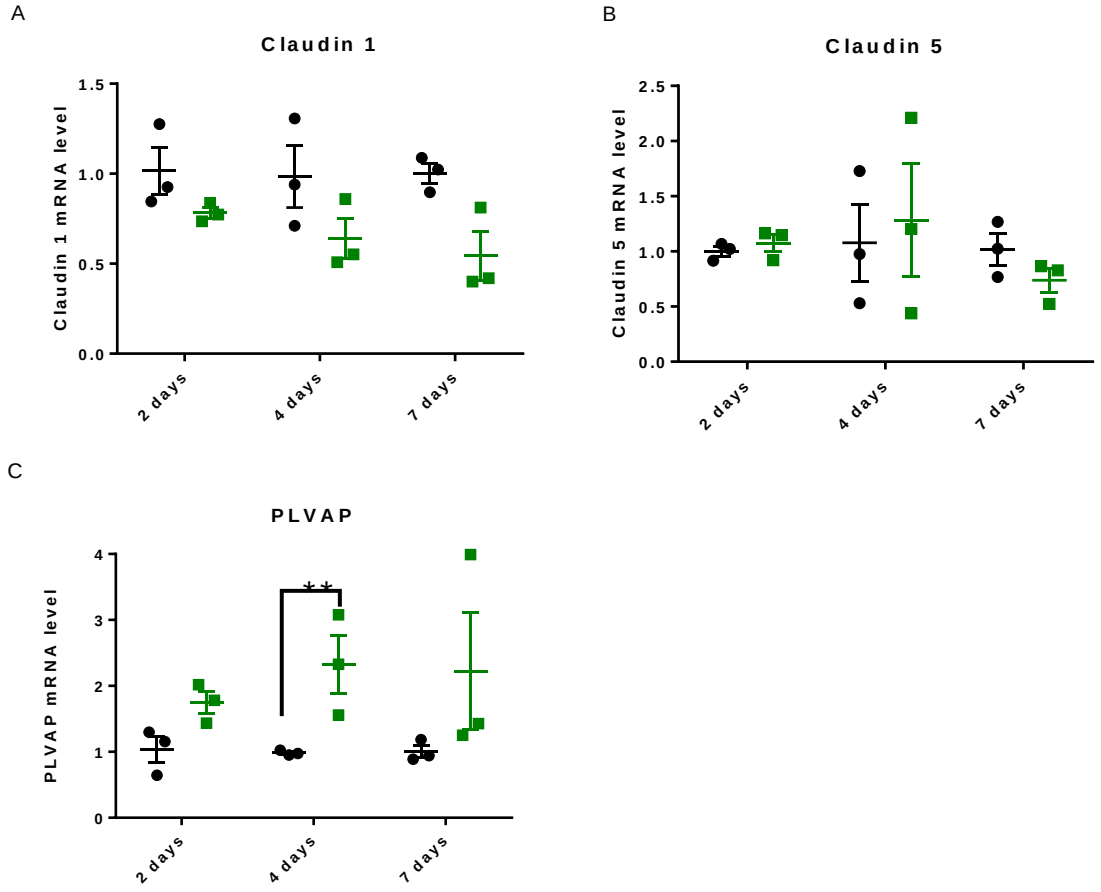

Figure 3.S5: Examination of BBB phenotypes claudin-1 (A), claudin-5 (B) and PLVAP (C) at hCMEC/D3 monolayer following ethanol 50 mM exposure. The mRNA levels were examined using qPCR. Black dots represent control group and green squares represent ethanol 50 mM group. All values represent mean±SEM. Each data point represents one cell monolayer Data were analyzed with two-way ANOVA followed by LSD Fisher's test. * $p<0.05$. PLVAP is plasmalemma vesicle-associated protein.

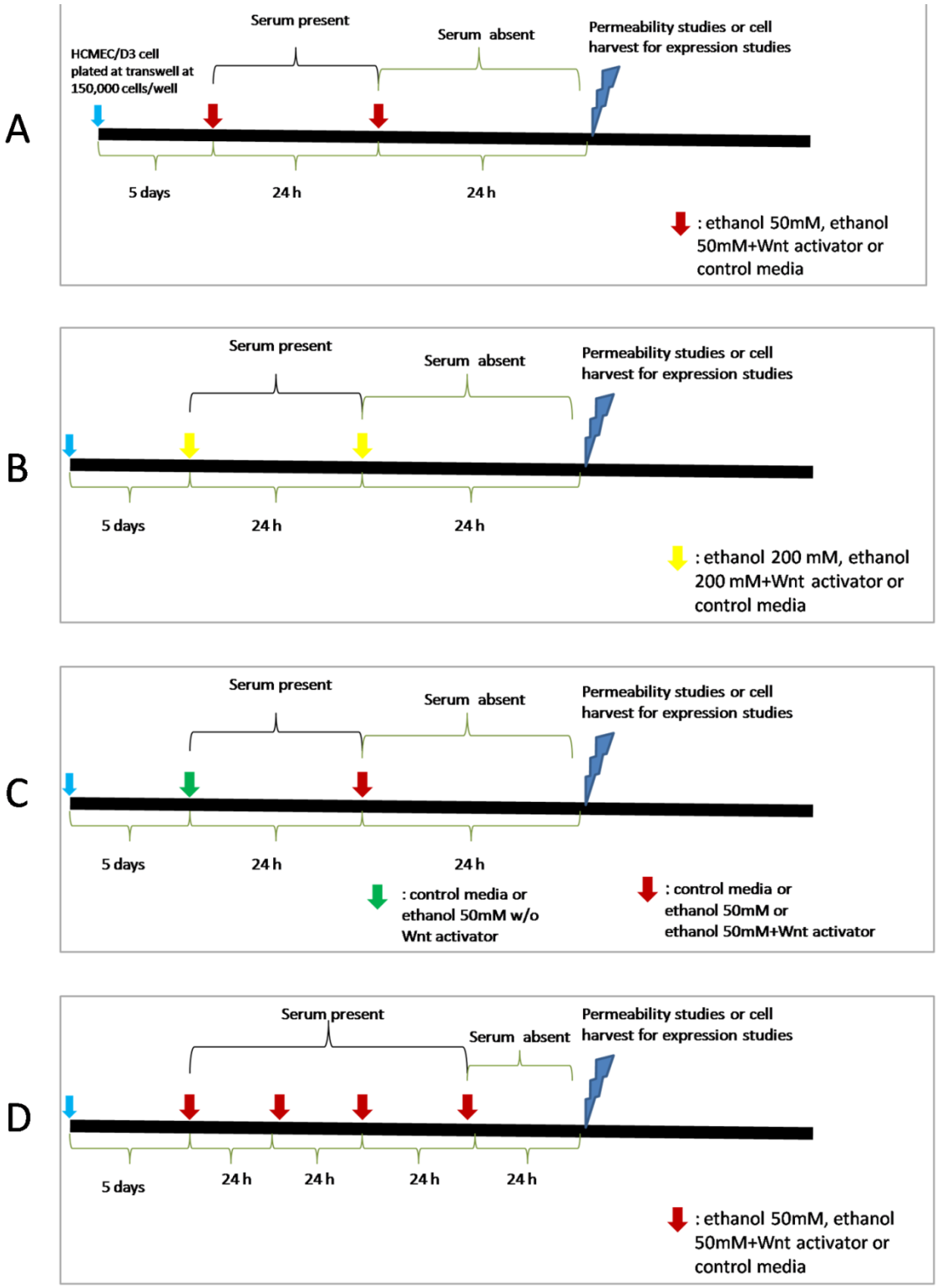

Figure 3.S6: The graphical timeline of the experimental procedure for the effect of ethanol exposure in cell culture model of the BBB. A) Two-day ethanol exposure 50 mM; B) Two-day ethanol exposure 200 mM; C) Two-day ethanol exposure but the Wnt activator were applied in the last 24-hour before the permeability studies; D) Four-day ethanol exposure.

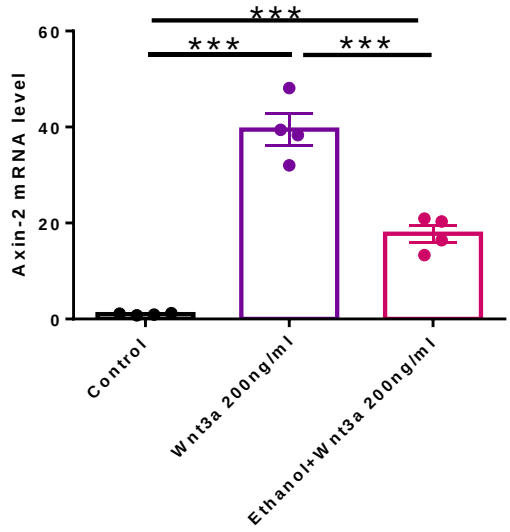

Figure273.S7: The effect of ethanol to Wnt/β-catenin elicited by Wnt3a in hCMEC/D3 cells. Control is media alone, Wnt3a group received Wnt3a 200 ng/ml for 6 hour and Ethanol+Wnt3a group was pretreated with ethanol 50 mM for 48-hour before Wnt3a was added for additional 6 hour. All values represent mean±SEM. Each data point represents one cell monolayer. Data were analyzed using one-way ANOVA followed by LSD Fisher's test; ***p<0.001.

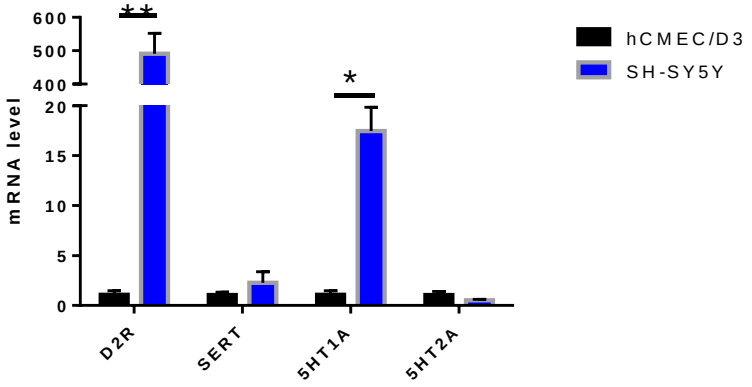

Figure 3.S8: The expression of targets of some neurotropic drugs in the hCMEC/D3 cells compared to neuroblastoma SH-SY5Y cells. All values represent mean±SEM. Data were analyzed using multiple t-test. *p<0.05; **p<0.01.

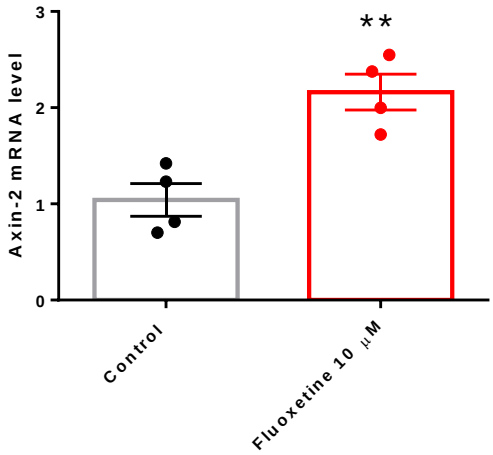

Figure 3.S9: Activation of Wnt/β-catenin signaling in SH-SY5Y cell following 24-hour treatment with fluoxetine 10 μM. All values represent mean±SEM. Each data point represents one cell monolayer. Data were analyzed using t-test. **p<0.01.

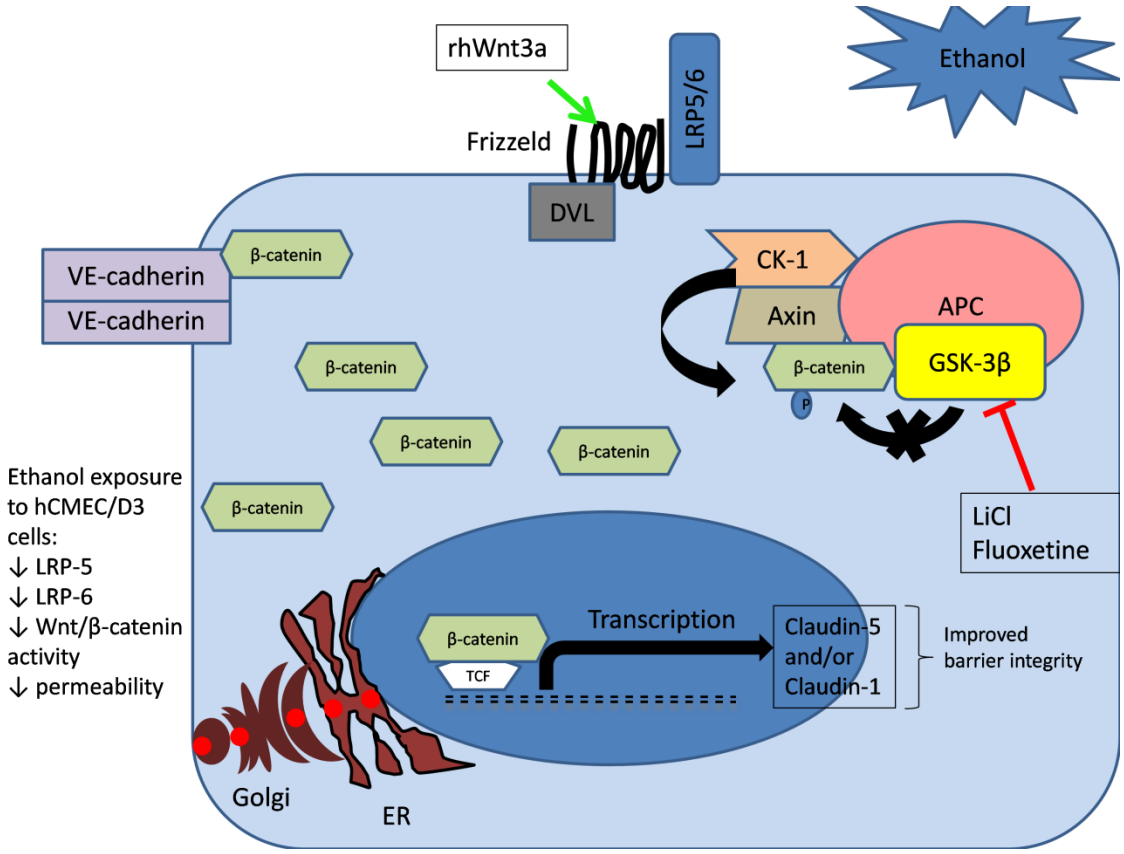

Figure 3. S10: Illustration of the effect of ethanol exposure to Wnt/β-catenin signaling at brain endothelial cells hCME/D3. Wnt/β-catenin signaling is downregulated in the BBB following prolonged ethanol exposure. Activation of Wnt/β-catenin signaling reversed the detrimental effects of ethanol on brain endothelial cell function. Examination of various CNS drugs for Wnt activation suggested that fluoxetine activated Wnt/β-catenin signaling and mitigated ethanol-induced barrier impairment.

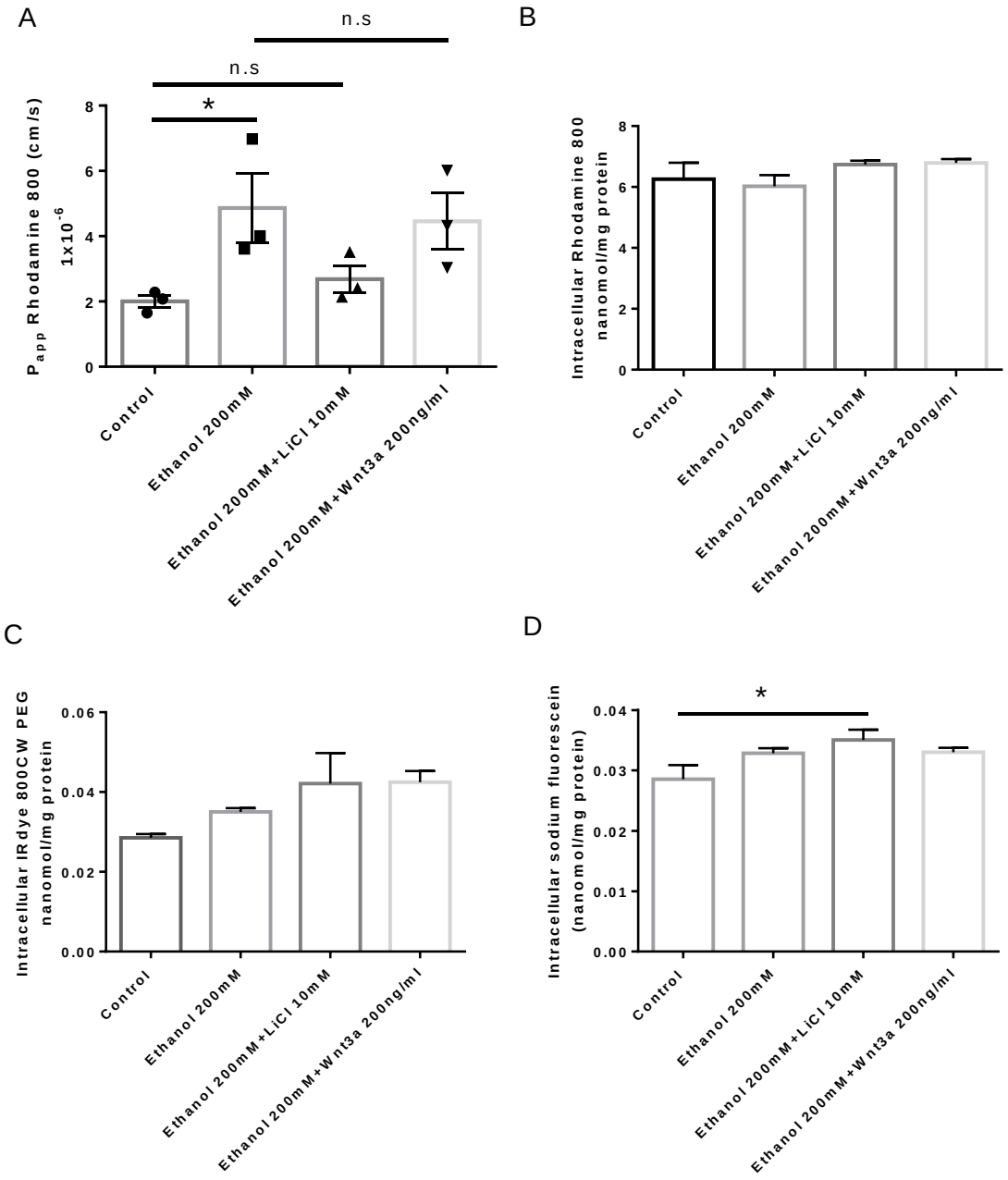

Figure 3.S11: Examination on the contribution of transcellular route to the permeability some markers on hCMEC/D3 following ethanol exposure. Permeability of Pgp substrate Rhodamine800 following ethanol exposure (A). Intracellular accumulation of rhodamine800 (B), IR Dye800CW PEG (C), and sodium fluorescein (D). hCMEC/D3 cells were treated with ethanol 200 mM alone or co-treated with either LiCl or Wnt3a for 48 hours. Each data point represents independent monolayer preparations. The values represent mean±SEM. A-D were analyzed using one-way ANOVA followed by LSD Fisher's test; *p<0.05; **p<0.01;***p<0.001. The graphical timeline of the experimental procedure for these data is available at Supplemental Figure 3.S6b.

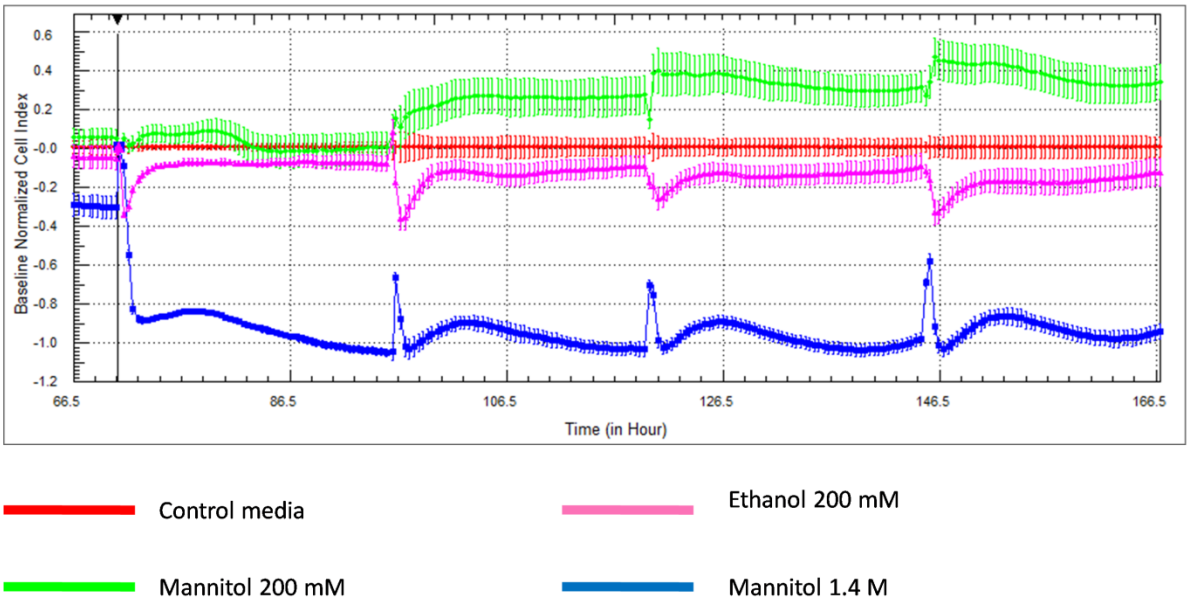

Figure 3.S12: Examination of hCMEC/D3 electrical impedance, a surrogate assay to assess paracellular barrier integrity, following exposure to ethanol 200 mM, mannitol 200 mM or mannitol 1.4 M. Values represent mean±SD. Ethanol or mannitol containing media were refreshed every 24 hours. Each group represents 4 monolayers

Table 3.S1: Enrichment of some brain endothelial marker in the isolated microvessels

Gene	Cortex homogenates	Isolated microvessels from cortex	Enrichment
P-glycoprotein	1.0 ± 0.1	21.5 ± 0.63	21-fold
Claudin-5	1.0 ± 0.01	36.3.8 ± 6.30	36-fold
VE-cadherin	1.0± 0.09	16.0 ± 1.17	16-fold

Microvessels enriched fractions were isolated from 3 mice cortex for each group.

Table 3.S2: P_{app} of permeability marker (x 10^{-6} cm/s)

Ethanol concentration	Time	Marker	Control	Ethanol	Ethanol+LiCl 10mM	Ethanol+Wnt3a
200mM	48 hour co-exposure	Sodium fluorescein	16.9±1.8	40.5±1.7	26.7±1.9*	42±0.6$^{(n.s)}$
200mM	48 hour co-exposure	IRdye 800CW PEG	3.2±0.2	7.5±0.6	6.9±0.6 $^{(n.s)}$	7.1±0.3$^{(n.s)}$

(n.s): not significantly different compared to ethanol treated group
*: significantly different from ethanol treated group with $p<0.001$

Table 3.S3. List of primer sequence

No	Primer	Sequence
1	mAxin-2- Forward	CGTAGGTTCCGGCTATGTCT
2	mAxin-2-Reverse	TTTCTTACTCCCCATGCGGT
3	mLRP-5-Forward	CCATTGTGTTGCACCCTGTG
4	mLRP-5-Reverse	CAGGACATGCCGATCTCTC
5	mLRP-6-Forward	TGCAAACAGACGGGACTTGA
6	mLRP-6-Reverse	CCAAACACAAAGTCCACCGC
7	mClaudin-1-Forward	GCAGAAGATGTGGATGGCTG
8	mClaudin-1-Reverse	GGGGTCAAGGGGTCATAGAA
9	mOccludin-Forward	TGG CAA GCG ATC ATA CCC AG
10	mOccludin-Reverse	CCT TCG TGG GAG CCC TTT TA
11	mVE-cadherin-Forward	GCT CAC GGA CAA GAT CAG CTC
12	mVE-cadherin-Reverse	GTG GGC AGG TAG CAT GTT GG
13	mZO-1-Forward	GTG CAA AGA GAT GAG CGG GC
14	mZO-1-Reverse	AGG AGA TCG TGA CTG GCT GC
15	mPgp-Forward	GTG GGG GAC AGA AAC AGA GA
16	mPgp-Reverse	TCT AGC CTT ATC CAG TGC GG
17	mBCRP-Forward	TCG CAG AAG GAG ATG TGT TG
18	mBCRP-Reverse	TCT AGC AAC GAA GAC TTG CC
19	hWnt2b-Forward	GATCCGAGAGTGTCAGCACC
20	hWnt2b-Reverse	CCTCTCGGCTACTTCTGAGC
21	hWnt3-Forward	TGACTCGCATCATAAGGGGC
22	hWnt3-Reverse	GTGGTCCAGGATAGTCGTGC
23	hDKK-1-Forward	CAGGATTGTGTTGTGCTAGA
24	hDKK1-Reverse	TGACAAGTGTGAAGCCTAGA
25	hPVLAP-Forward	CTGCGATGCCTTGCTCTTCAT
26	hPVLAP-Reverse	AGTCCCTCCACAGGTTACGA
27	hPgp-Forward	ATA TCA GCA GCC CAC ATC AT
28	hPgp-Reverse	GAA GCA CTG GGA TGT CCG GT
29	hVE-cadherin-Forward	GTT CGG CTG ACA GGT CCA CA
30	hVE-cadherin-Reverse	CGA TGT GGC GAG GAG CAT CA
31	hZO-1-Forward	ATC TCG GAA AAG TGC CAG GA
32	hZO-1-Reverse	TTT CAG CGC ACC ATA CCA AC
33	hClaudin-5-Forward	AGGCGTGCTCTACCTGTTTTG
34	hClaudin-5-Reverse	AACTCGCGGACGACAATGTT
35	hClaudin-1-Forward	TTTACTCCTATGCCGGCGAC
36	hClaudin-1-Reverse	GAGGATGCCAACCACCATCA
37	hAxin-2-Forward	GACAGGAATCATTCGGCCAC
38	hAxin-2-Reverse	CCTTCAGCATCCTCCGGTAT
39	hSERT-forward	TTGGACGTGTGAGGATGTGG

40	hSERT-reverse	TCCCTGTTCTCTCCTACGCA
41	hD2R-forward	CCCTATGGCTTGAAGAGCCT
42	hD2R-reverse	GGTGAGCAGTGTGGCATAGT
43	h5HT1A-forward	CGCTCACTTGGCTTATTGGC
44	h5HT1A-reverse	CGCCCATAGAGAACCAGCAT
45	h5HT2A-forward	GAGAGATGCAGCGAGTCACA
46	h5HT2A-reverse	TTCTCACCAAACCGAGGACA
47	hGPR124-forward	GGCACTGAGGTGAAGGGATA
48	hGPR124-reverse	AGAAGGTGGAGATCGTGGTG
49	hReck-forward	TGCAAGCAGGCATCTTCAAA
50	hReck-reverse	ACCGAGCCCATTTCATTTCTG
51	hCyp2E1-forward	CCTACATGGATGCTGTGGTG
52	hCyp2E1-Revese	TGGGGATGAGGTATCCTCTG
53	hADH1A-forward	CCCAAAACTTGTGGCTGATT
54	hADH1A-reverse	TTTCCCAGAGTGAAGCAGGT
55	hADH1B-forward	TACACGGTGGTGGATGAGAA
56	hADH1B-reverse	TGGCAACGTTAACTGCAGAC
57	mGPR124-forward	TCAACATCCACAACTACCGGG
58	mGPR124-reverse	TGGCTCCAGAGAGATCCTGTTA

3.7.5. References

1 Sun, M. *et al.* A dynamic real-time method for monitoring epithelial barrier function in vitro. *Analytical biochemistry* **425**, 96-103, doi:10.1016/j.ab.2012.03.010 (2012).

2 Oster, J. R. & Singer, I. Hyponatremia, hyposmolality, and hypotonicity: tables and fables. *Archives of internal medicine* **159**, 333-336, doi:10.1001/archinte.159.4.333 (1999).

3 Gennari, F. J. Current concepts. Serum osmolality. Uses and limitations. *The New England journal of medicine* **310**, 102-105, doi:10.1056/NEJM198401123100207 (1984).

4 Penney, M. D. & Walters, G. Are osmolality measurements clinically useful? *Annals of clinical biochemistry* **24 (Pt 6)**, 566-571, doi:10.1177/000456328702400603 (1987).

4.CHAPTER IV: Concluding remark

CHAPTER 4: CONCLUDING REMARKS

4.1 Summary

Wnt/β-catenin signaling is essential for BBB development and maintenance. Wnt/β-catenin signaling and the downstream effects on gene expression help establish the BBB phenotype that distinguishes CNS microvessels from their more permeable peripheral counterparts. Several studies have identified the importance of Wnt/β-catenin signaling in development of BBB at the embryonic stage as well as in the maintenance state. Regional differences in BBB permeability may also be tied to different levels of Wnt/β-catenin activity. An example is the choroid plexus, which has a leaky BBB, and displays significantly less endothelial Wnt/β-catenin activity. Modulation of canonical Wnt signaling using genetically engineered mice, was capable of converting the leaky barrier in the choroid plexus to a BBB competent state. Aberrant Wnt/β-catenin signaling is frequently found in pathophysiological conditions involving BBB dysfunction. On the pathophysiological research front, several reports have identified changes in various Wnt/β-catenin components in the brain following ethanol expsoure. However, these studies regarding the impact of ethanol exposure to Wnt/β-catenin signaling in the brain endothelial cells have not critically examined.

Chapter 2 presented the characterization of Wnt/β-catenin signaling at human cerebral microvessels endothelical cell (hCMEC/D3), a commonly used *in vitro* human BBB model. Examination of both primary human brain endothelial cells, as well as hCMEC/D3 indicated most of the Frizzled receptor isoforms and co-receptors were expressed. Interestingly, while the hCMEC/D3 expressed several canonical Wnt ligands, their contribution towards establishment of a BBB phenotype appeared to be minimal, as pharmacological inhibition of autocrine activation of Wnt/β-catenin had minor effects on permeability. These findings suggest that brain endothelial cells are more likely to rely on Wnt ligands secreted from other brain cells for activation of the

canonical Wnt signaling and maintenance of a BBB phenotype. In support of this, treatment of hCMEC/D3 monolayers with Wnt3a not only improved paracellular barrier properties but also increased Pgp and BCRP activity and reduced plasmalemma vesicle associated protein consistent with reduced transcellular solute permeability as well. As inhibition of β-catenin binding to TCF-4 reduced Pgp, BCRP and claudin-5 expression, this confirms that these BBB phenotypes were partly regulated by Wnt/β-catenin signaling. The studies in chapter 2 suggest that brain endothelial cells can utilize Wnt/β-catenin signaling to maintain a barrier phenotype but likely through a paracrine mechanism.

The studies contained in chapter 3 confirmed that downregulation of Wnt/β-catenin signaling observed in several brain regions following prolonged ethanol exposure. Some brain region exhibited upregulation of Dkk-1 and downregultion of Axin-2. Further, this report is the first to identify that the downregulation Wnt/β-catenin signaling not only present in the brain parenchyma but also at the blood-brain barrier *in vivo*. In support of this, the *in vitro* studies on the human brain endothelial cell (hCMEC/D3) showed similar responses where prolonged exposure to ethanol at concentrations of 50 mM and higher, downregulated Wnt/β-catenin signaling. Downregulation of Axin-2 was correlated with reduced expression of Wnt co-receptor, LRP5/6 and reduced Wnt2b expression. The downregulation of LRP-5/6 following prolonged ethanol exposure might explain the reduced potency of Wnt3a in activating Wnt signaling and mitigating ethanol-induced barrier breakdown. The observation that the GSK3 inhibitor, LiCl, was more robust in mitigating the barrier breakdown following ethanol exposure supports this possibility as LiCl bypasses Wnt receptors and co-receptors by acting downstream in the Wnt/β-catenin signaling process.

Since the concentration of LiCl required to improve the barrier integrity of the brain endothelial cells upon ethanol insult was supratherapeutic, other drugs known to act as GSK inhibitors were

examined. While these drugs did increase Wnt/β-catenin signaling in the hCMEC/D3, the level of activation was not sufficient to improve the BBB phenotype except for fluoxetine. Fluoxetine, at clinically relevant concentrations, activated Wnt/β-catenin signaling in the hCMEC/D3 and prevented the paracellular barrier dysfunction caused by prolonged ethanol exposure. Based on the present findings, the most likely situations leading to BBB alterations with ethanol following higher levels of exposure such as associated with binge alcohol drinking. Furthermore, canonical Wnt signaling is influenced by ethanol, and activation of Wnt signaling was able to reverse the permeability effects of ethanol in the BBB culture model.

4.2. Limitations

In this study, Wnt ligand secretion by hCMEC/D3 was not directly measured. The evidence for autocrine Wnt signaling relied on responses to pharmacological inhibitor of Wnt release (WntC59) or blocking of Wnt transcription factors (ICRT-3) The result of the current studies are in agreement with previous findings reporting autocrine Wnt signaling in hCMEC/D3[1-3]. Interstingly, these papers from three different lab groups, demonstrated a reduction in nuclear β-catenin following addition of Dkk-1 to the media. While the detection of various Wnt ligands released into the media would provide further confirmation of the autocrine capabilities of the hCMEC/D3, the question becomes which ligands to look for. Based on the current studies, hCMEC/D3 express Wnt2b and Wnt3 as well as Wnt7a and Wnt7b. Based on our unsuccessful attempt to measure Wnt7b in culture media using immunoblotting, future studies would need to employ more sensitive analytical methods such as ELISA. Further studies need to examine the concentration of these Wnt isoforms in the cell lysates as well as in media samples. Alternately, one could use an eGFAP tagged Wnt ligand to examine Wnt trafficking from Golgi apparatus to plasma membrane.

ICRT-3 is an effective Wnt/β-catenin signaling inhibitior yet its use does have some limitations. Of note, ICRT-3 is designed to inhibited TCF-4, one of the four transcription factors involved in Wnt-triggered or Norrin-triggered β-catenin signaling along with LEF-1, TCF-1 and TCF-3. Although TCF-4 is considered the major transcription factor in Wnt/β-catenin signaling (see Table 1.2 for list of downstream genes of importance to BBB), other transcription factor such as LEF-1, TCF-1 and TCF-3 may not be sensitive to ICRT-3 inhibition. To overcome this issue, it would be advantageous to inhibit canonical Wnt signaling in the middle of the signaling cascade for example by promoting the formation of β-catenin destruction complex using XAV939. In theory, this compound should not only inhibit the upstream pathways (β-catenin signaling triggered by both Norrin and Wnt) but also prevent all the downstream pathways including β-catenin binding to TCF-1, TCF-3, TCF-4 and LEF-1. This pharmacological inhibitor might be valuable to further confirm the Wnt target genes in brain endothelial cells that may not be affected by ICRT-3 inhibtions of TCF-4.

In the future, the effect of Wnt/β-catenin activation on BBB permeability as well as the impact on cognitive function in an animal model would be of interest. The *in vivo* studies in this thesis were originally performed to examine the changes in adenosine pathways, receptors, enzymes and response, following ethanol. We were able to obtain brain material from this study for analysis of Wnt signaling. Based on the similar decline in Wnt activation observed from the *in vivo* studies and the cell culture with hCMEC/D3 model, changes in permeability following chronic ethanol exposure are likely. As chronic ethanol exposure in various animal models produce a decline in cognitive ability, it would be interesting to assess whether pharmacological intervention directed towards Wnt activation could strengthen BBB integrity and cognitive functions.

4.2 Future Outlook

The present studies underscore the ability of Wnt/β-catenin signaling to improve BBB properties under both normal and pathophysiological conditions. From a pharmacological standpoint, it is important to identify the apical-basolateral distribution of frizzled receptors on brain endothelial cells. Although it has been speculated that frizzled receptor expression is primarily on the basolateral side of the BBB, definitive studies of the cellular localization of canonical frizzled receptors in brain endothelial cells have not been done. This information will be useful to design Wnt receptor agonists as the physicochemical properties are dramatically different for Wnt targets located at blood interface versus brain interface of the BBB.

The hypothesis that brain endothelial cells utilize Wnt ligand secreted by other brain cells to regulate its BBB phenotypes should also be followed up more closely. In this regard a co-culture model to compare the contribution of astrocytes vs neuron vs pericytes in providing Wnt ligand for brain endothelial canonical Wnt activity would be helpful. Identifying which cells are the source of Wnt ligand for the brain endothelial cells could be helpful in understanding alterations in BBB integrity under normal and pathophysiological conditions. As an example, studies have reported that the isolated microvessels that have more astrocytes coverage have tighter barrier properties. A similar effect has been observed with pericytes. Whether such phenomenon is tied to paracrine Wnt activity is still unexplored.

Although activation of Wnt/β-catenin signaling could mitigate the BBB breakdown observed under various pathophysiological conditions, translational research to develop Wnt activators is less developed compared to the search for Wnt inhibitors. This is understandable as activation of Wnt/β-catenin signaling might lead to cancer promoting conditions in the brain. In this regard,

understanding the limit to which activation of Wnt/β-catenin signaling within the BBB can be achieved without eliciting uncontrolled cell proliferation within other cells of the CNS is crucial.

The present studies were also the first to show expression of R-spondin in hCMEC/D3 cell. Follow up studies are required to examine to what extend R-spondin could potentiate Wnt ligand activation of Wnt/β-catenin signaling in brain endothelial cells. Studies showed that R-spondin expression in brain endothelial cell was 35-fold higher compared to pericytes suggests this may be an important amplification mechanism for brain endothelium. Our current studies shown that inhibition of β-catenin binding to TCF-4 transcription factor is more effective compared to inhibition of Wnt ligand release from the cell. In this regard, the potential contribution of R-spondin release toward autocrine and paracrine Wnt/β-catenin signaling of the brain endothelial cells deserves further study.

Due to limited sample material available, the current studies could not isolate microvessels from hippocampus, striatum or cerebellum. Other approaches in addition to capillary depletion are needed to understand the state of Wnt/β-catenin signaling within specific brain regions following prolonged ethanol exposure. The BAT-GAL mice (β-catenin/TCF/LEF reporter transgenic mice) that are available commercially, could be another approach to help determine the state of Wnt/β-catenin activity within distinct brain regions.

Chronic ethanol exposure has been associated with reduced cognitive function. To date, the studies to examine if therapeutic intervention that strengthening BBB function upon ethanol exposure would improve the cognitive function has not been performed. Wnt/β-catenin signaling potentially can be employed as this pathway not only improved the BBB function but also support the adult neurogenesis that also repressed by ethanol exposure.

4.4. References

1 Pinzon-Daza, M. L. *et al.* The cross-talk between canonical and non-canonical Wnt-dependent pathways regulates P-glycoprotein expression in human blood-brain barrier cells. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* **34**, 1258-1269, doi:10.1038/jcbfm.2014.100 (2014).

2 Lim, J. C. *et al.* Activation of beta-catenin signalling by GSK-3 inhibition increases p-glycoprotein expression in brain endothelial cells. *Journal of neurochemistry* **106**, 1855-1865, doi:10.1111/j.1471-4159.2008.05537.x (2008).

3 Riganti, C. *et al.* Temozolomide down-regulates P-glycoprotein in human blood-brain barrier cells by disrupting Wnt3 signaling. *Cellular and molecular life sciences : CMLS* **71**, 499-516, doi:10.1007/s00018-013-1397-y (2014).