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Glial derived neurotrophic factor: a sufficient essential molecular regulator of mammalian blood-nerve barrier tight junction formation

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Peripheral nerves coordinate signal transduction from the periphery to the central nervous system for processing and transmission back as required for normal mammalian function. Peripheral nerves and nerve roots are structurally divided into three compartments: the outermost epineurium, inner perineurium that surrounds nerve fascicles and the innermost endoneurium (Mizisin and Weerasuriya, 2011). As with all organs, peripheral nerve vascularization occurs during development and is maintained in health. Adaptations are expected dependent on tissue-specific functions and physiologic state. The peripheral nerve internal microenvironment is tightly controlled to facilitate coordinated and regulated axonal transmission. Peripheral nerves and nerve roots are perfused by extrinsic blood vessels called the vasa nervorum. These macrovessels penetrate the outer epineurium to give rise to epineurial arteries and arterioles and receive blood from epineurial venules and veins. Macrovessels subsequently transverse the inner perineurium, formed by multiple concentric layers of specialized epithelioid myofibroblasts, to perfuse the innermost endoneurium, in which reside myelinated and unmyelinated axons in a loose array of collagen fibers. Non-fenestrated tight junction-forming capillary-like microvessels exist within the endoneurium which are in direct contact with circulating blood. Thus, these microvessels are considered to form the blood-nerve barrier (BNB) (Ubogu, 2020). Tight junction-forming perineurial myofibroblasts provide a critical interface between the endoneurial and epineurial interstitial fluid compartments that further regulate the endoneurial microenvironment; however, these cells are not in direct contact with circulating blood. Peripheral neuropathies affect over 100 million people worldwide, and a common consequence of peripheral nerve disease is chronic pain, which may affect as many as 1% of individuals during their lifetimes. Understanding peripheral nerve molecular and biophysical microvascular adaptations and BNB function during development and in normal physiological states, as well as in diseases, including peripheral nerve injury, provides an avenue to understand how peripheral nerve regeneration and homeostatic restoration may occur. These processes could be harnessed for therapeutic development in peripheral neuropathies and chronic neuropathic pain to support neural regeneration and restore normal biological function.

Human blood-nerve barrier: In mammals, the BNB is considered the second most restrictive mammalian microvascular barrier after the blood-brain barrier (Allt and Lawrenson, 2000). Studies on the molecular and biophysical properties of the human BNB in vivo or in situ are limited; however, we have made significant advances over the past 12 years, catalyzed by the isolation and purification of primary human endoneurial endothelial cells (pHEndECs) (Yosef et al., 2010). Our recent work elucidated the normal adult human BNB transcriptome based on conserved transcripts expressed by early- and late-passage pHEndECs and laser-capture microdissected endoneurial microvessels from four histologically

normal adult sural nerve biopsies (free database access via the National Center for Biotechnology Information Gene Expression Omnibus [GEO] #: GSE107574). This transcript and network/pathway identification database has provided an essential framework to elucidate the structural and functional characteristics of the human BNB and evaluate biologically relevant mechanisms in normal physiological and pathophysiological conditions (Palladino et al., 2017), supported further by access to normal and diseased fixed and cryopreserved human peripheral nerve biopsies (Ubogu, 2020). In situ validation is essential, as transcript expression does not necessarily imply functional protein expression, and we have recently discovered that expression of putative intercellular junctional complex proteins does not necessarily imply expression at electron-dense intercellular contacts expected of restrictive tight junction proteins.

Human blood-nerve barrier tight junctional complex: The human BNB transcriptome consists of 133 intercellular junctional complex molecules (22 tight junction or junction-associated, 45 adherens junction or junction-associated and 52 cell junction-associated or adaptor molecules), with in situ endoneurial endothelial microvessel protein expression of α1 catenin (CTNNA1), cadherin-5 (CHD5), cadherin-6 (CDH6), claudin-4 (CLDN4), claudin-5 (CLDN5), crumbs cell polarity complex component lin-7 homolog A, gap junction protein A1, multiple PDZ domain crumbs cell polarity complex component, protocadherin-1, vezatin, ZO-1 and zyxin demonstrated by indirect fluorescent immunohistochemistry (Palladino et al., 2017; Ouyang et al., 2019). Our in situ studies performed using histologically normal adult peripheral nerves showed that many of these junctional complex proteins are not restricted to tight-junction forming microvessels or located at intercellular contacts. In order to accurately determine how the BNB forms during development, adapts physiologically and is pathologically altered, it is imperative to know its precise molecular composition. By high resolution confocal microscopy, we recently ascertained that CLDN4 is most likely associated with BNB tight junctions and CDH5 associated with adherens junctions, with CTNNA1 serving as an adapter molecule linking the F-actin cytoskeleton with tight junctions (Ubogu, 2020), as shown in **Figure 1A**.

Molecular regulation of the mammalian blood-nerve barrier: pHEndECs express specific mitogen receptors for molecules such as glial-derived neurotrophic factor (GDNF; GFRα1), vascular endothelial growth factor (VEGF; VEGFR2), basic fibroblast growth factor (bFGF; FGFR1), transforming growth factor-β1 (TGF-β1; TGFRI/II) and glucocorticoids in vitro and in situ (Yosef and Ubogu, 2012; Reddy et al., 2013; Ubogu, 2013; Palladino et al., 2017) implying that autocrine or paracrine mitogen secretion by endothelial cells, Schwann cells, pericytes, mast cells or endoneurial fibroblasts could regulate BNB composition and function. VEGF has been shown to support pHEndEC proliferation and sterile micropipette wound healing, as well as microvascular angiogenesis, in

picomolar and nanomolar concentrations in vitro (Reddy et al., 2013), as observed with endothelial cells from other tissues. This implies that VEGF could be essential for peripheral nerve vascularization during development and adaptive revascularization following nerve injury. We sought to determine which mitogen(s) and signaling pathway(s) could be essential for BNB development and restoration after injury during peripheral nerve regeneration, recognizing the overall importance of vascularization in normal tissue function and importance of regulating the endoneurial microenvironment to restore physiological peripheral nerve axonal signal transmission.

Using a published in vitro human BNB barrier model (Yosef et al., 2010), we tested the effect of GDNF, bFGF, TGF-β1 and hydrocortisone (molecules previously implicated in enhancing restrictive microvascular tight junction properties at the blood-brain barrier) in restoring restrictive physiological properties (transendothelial electrical resistance, solute permeability) in confluent cultures following serum withdrawal (which induces cellular detachment and loss of intercellular contacts). We ascertained that GDNF (1 ng/mL, 0.03 nM) sufficiently induced maximal BNB recovery within 48 hours dependent on RET tyrosine-kinase signaling pathways. This restoration was slightly enhanced by cyclic adenosine monophosphate-protein kinase A signaling in combination with maximal concentrations of multiple redundant mitogens (Yosef and Ubogu, 2012). GDNF typically signals via its receptor GFRα1 complexed with RET-Tyrosine kinase, and we subsequently determined that Ras-mitogen activated protein kinase (MAPK) signaling was employed for this biological effect at the human BNB in vitro (Dong and Ubogu, 2018).

Our published initial data using RNA-interference support the hypothesis that the observed GDNF-mediated restoration of human in vitro BNB function following injury is dependent on CREB1 transcription factor-driven synthesis and SEC31A transport of essential junctional complex molecules cortactin (CTTN), CTNNA1 and CLDN4, as well as GDNF-mediated SRC kinase activation required to phosphorylate CTTN, downstream of RET-Tyrosine kinase-MAPK signaling (Ubogu, 2020). We also observed that GDNF induced F-actin cytoskeletal filament reorganization, resulting in more continuous intercellular membrane contacts between pHEndECs, supporting the notion that restrictive junctional complex formation and cytoskeletal dynamics are intimately linked (Yosef and Ubogu, 2012; Ubogu, 2020). In support of our in vitro human BNB work, we have also demonstrated that GDNF restores murine sciatic nerve BNB macromolecular impermeability within 14 days following non-transecting crush nerve injury using a Tamoxifen-inducible conditional GDNF knockout transgenic mouse strain (Dong et al., 2018). Further preliminary studies have demonstrated dynamic GDNF-mediated in situ changes in murine BNB CTNNA1-phosphorylated CTTN-CLDN4 expression and an important role of SRC kinase expression during sciatic nerve recovery from compression nerve injury (Ubogu, 2020).

Schwann cells are unique glial cells restricted to the peripheral nervous system. Schwann cells are known to secrete GDNF in vitro and in vivo (Ibanez and Andressoo, 2017), with higher levels expressed during peripheral nerve injury to support axonal regeneration. GDNF has been shown to influence microvascular endothelial and epithelial tight junction barrier function in mammalian blood-brain, blood-retina, blood-testis and intestinal barriers in vitro or in vivo (Yosef and Ubogu, 2012; Ubogu, 2020), implying a generalized critical importance in restrictive junctional complex biology. Our work implies that Schwann cells, through GDNF, may directly regulate mammalian
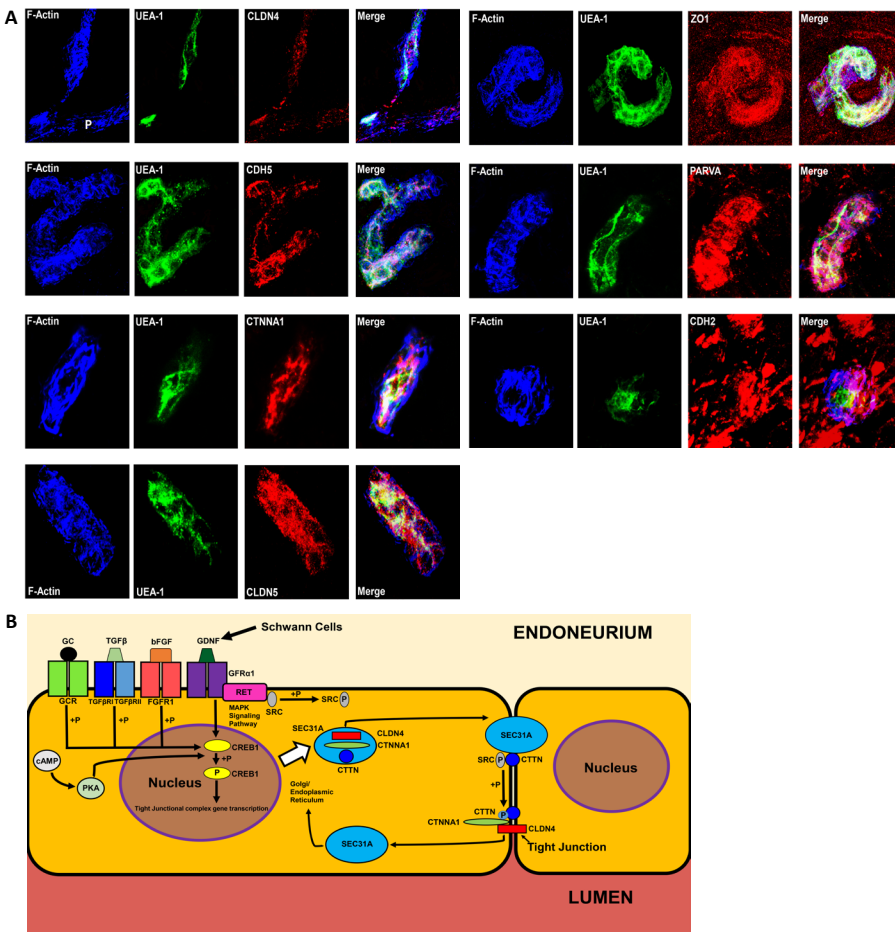
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Figure 1 │ Human blood-nerve barrier *in situ* and *in vitro*.
(A) Human blood-nerve barrier (BNB) junctional complex. Digital high resolution indirect immunohistochemistry confocal photomicrographs of normal adult endoneurial microvessels stained with Rhodamine Phalloidin to detect microvessel F-actin cytoskeleton (pseudocolor blue), and fluoresceinated Ulex Europaeus Agglutinin-1 (UEA-1) to detect microvessel cell membranes were generated to determine cellular localization of hypothesized junctional complex molecules (red) claudin 4 (CLDN4), cadherin 5 (CDH5), α-1 catenin (CTNNA1), claudin 5 (CLDN5), zona occludens-1 (ZO-1), α-parvin (PARVA) and cadherin 2 (CDH2). The punctate CLDN4 expression and membrane co-localization suggest that this molecule is an essential component of BNB tight junctions. This molecule is also expressed in the multilayered perineurium (P), implying that it may be an essential component of its restrictive barrier. The linear, continuous CDH5 expression and membrane co-localization in endoneurial microvessels are consistent with an intercellular adherens junction protein. The plaque-like linear CTNNA1 expression and strong membrane and cytoskeletal co-localization suggest a role as an adapter molecule that links the endoneurial microvessel membrane to the cytoskeleton. CLDN5, ZO-1, PARVA and CDH2 demonstrate diffuse intracellular expression with additional strong continuous membrane and cytoskeletal co-localization, implying that they are not structural components of BNB tight junctions. Low endoneurial background expression levels of ZO-1 and PARVA suggest possible roles in maintaining structural integrity in normal adult peripheral nerves, while high focal CDH2 expression suggest expression in surrounding axons, as previously published with cadherin 6. The figure is sourced from the authors' laboratory. (B) Human BNB junction complex formation: The GDNF-CREB1-SRC-SEC31A-CTTN/ CTNNA1/ CLDN4 hypothesis. This figure illustrates a hypothesized pathway by which paracrine GDNF secretion by Schwann cells mediates restrictive human BNB formation dependent on GFRα1-RET-MAPK signaling, including specialized junctional complex protein transport for further study. Potential redundancy is demonstrated via bFGF, TGF-β1 and glucocorticoids. +P indicates a phosphorylation and P indicates an activated phosphorylated protein. bFGF: Basic fibroblast growth factor; BNB: blood-nerve barrier; CLDN4: claudin-4; CTTN: cortactin; GC: glucocorticoids; GDNF: glial-derived neurotrophic factor; TGF-β1: transforming growth factor-β1.

BNB formation during development and rapidly restore BNB function after injury, with potential redundancy mediated by bFGF, TGF-β1 and endogenous glucocorticoids (**Figure 1B**). We have demonstrated that GDNF is a sufficient essential molecular regulator of the human and mouse BNB *in vitro* and *in situ* following injury (Yosef and Ubogu, 2012; Dong and Ubogu, 2018; Dong et al., 2018). We advocate that restoring BNB function through GDNF-mediated mechanisms may be a necessary prerequisite to re-establish the tightly regulated microenvironment required to support functional axonal regeneration needed for normal physiologic signal transduction in peripheral nerve disorders. Failure to restore endoneurial homeostasis may result in chronic persistent neuropathic pain with associated morbidity and mortality.

Conclusions: The BNB is required for peripheral nerve homeostasis. Its molecular structure and determinants, as well as signaling pathways responsible for normal function are incompletely understood. We have made early significant advances, guided by nonbiased pHEndEC and endoneurial microvessel transcriptome and proteome bioinformatics. These are further supported by *in situ* observations of normal and well-characterized diseased adult peripheral nerve biopsies. In addition to comprehensively understanding human BNB function in health and how it adapts or fails to adapt in different disease states, a major goal of our dedicated efforts, considering the unique biology of the BNB, is to discover molecular targets for peripheral neuropathies and chronic neuropathic pain that enhance peripheral nerve axonal and microvascular regeneration and restore normal peripheral nerve and nerve root function in humans.

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