Abnormal electrophysiological properties in sensory neurons from a swine model of cystic fibrosis

A Thesis Submitted to the College of Graduate and Postdoctoral Studies

In Partial Fulfillment of the Requirements for the Degree of Master of Science

In the Department of Anatomy, Physiology and Pharmacology (APP) University of Saskatchewan, Saskatoon, SK

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ABSTRACT

Cystic fibrosis (CF) results from dysfunction of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein. CF has traditionally been considered a pediatric disease, but the median predicted survival age in Canada is 55 years. As a result, patients are more likely to experience chronic complications of CF. One such complication is abnormal function of the peripheral nervous system, i.e., peripheral neuropathy. Recent findings from a swine model of CF (CFTR-/-), shows these animals are born with peripheral neuropathy. However, whether a lack of CFTR expression directly affects neuronal function remains to be explored. We hypothesize that the lack of functional CFTR in sensory neurons leads to electrophysiological abnormalities that contribute to the pathology of CF. We propose that malfunction in dorsal root ganglion (DRG) neurons innervating the GI tract contributes to gut-related complications. Using patch clamp electrophysiology, we found that DRG neurons from CFTR-/- (CF) swine had reduced generation of action potentials, compared to wild-type (WT) swine. Additionally, we observed a reduced percentage of neurons with T-type calcium currents in the CF swine, with respect to WT at postnatal day 7. Furthermore, our study showed that DRG neurons from CF swine, which displayed T-type calcium currents, had abnormal activation (G/Gmax) and inactivation (τ) kinetics. Moreover, DRG neurons from CF swine showed a reduction in capsaicin-evoked currents with respect to WT, at both postnatal day 0 and 7. Taken together our data indicate that lack of CFTR expression in sensory neurons is linked to a depression of sensory function in DRG neurons in the first week postnatal. Our data strongly suggest that in

addition to the effects of CF in epithelial cells, impairments in sensory neurons may contribute to the pathology of CF.

ACKNOWLEDGEMENTS

I would like to sincerely express my gratitude to my supervisors, Dr. Verónica Campanucci and Dr. Juan Ianowski. You have been truly inspiring and admirable mentors. It has been a privilege working with you and you have both given me a unique and invaluable experience, which very much contributed in shaping my academic career in research. Thank you for always making time out of your extremely busy days when we require help and advice.

To Zeinab and Maricris who taught me most of the things I know in this lab. It would have been a considerably more difficult journey without your help and guidance. To everyone in the lab (Joe, Wafa, Muniba, and Nicolas), who has made every day in the lab enjoyable and exciting. It was always a pleasure learning new things about everyone's life, cultures and languages.

To my committee members (Dr. John Howland and Dr. Valerie Verge), thank you for keeping me on track and giving me constructive criticism and advices to better my research and academic career.

To the College of Medicine, for providing me with the opportunity to study and practice my research abilities in a scholarly and efficient manner.

Lastly, to my friends and family, most especially my parents, for continually giving me their unconditional support, love and encouragement every single day.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
AP	Action Potential
BDNF	Brain-derived neurotrophic factor
Cav	Voltage-gated calcium
CF	Cystic fibrosis
Cm	Membrane capacitance
CAP	Capsaicin
CNS	Central Nervous System
CTL	Control
DRG	Dorsal Root Ganglion/Ganglia
ECa	Reversal Potential for calcium
ECF	Extracellular Fluid
EGTA	Ethylene Glycol-bis(2-aminoethylether)-N,N,N’,N’-Tetraacetic Acid
Gmax	Maximal Conductance
Gca	Calcium conductance
I_{Ca}	Calcium Current
Lido	Lidocaine
LC	Locus coeruleus
MAPK	Mitogen-Activated Protein Kinase
MgATP	Adenosine 5'-Triphosphate Magnesium Salt
MSD1	Membrane-spanning domains 1
MSD2	Membrane-spanning domains 2
Nav	Voltage-gated Sodium
NRM	Nucleus raphe magnus
NRGC	Nucleus reticularis gigantocellularis

NSAIDs	Nonsteroidal anti-inflammatory drugs
NFκB	Nuclear Factor kappa Beta
P0 and P7	Postnatal day 0 and 7
mPAG	Midbrain periaqueductal gray
P-loop	Pore loop
PKC	Protein Kinase C
PI3K	Phosphoinositide 3-kinases
R	Regulatory
RVM	Rostral ventral medulla
S1-S6	Segments 1-6
SEM	Standard Error of Mean
TRPV1	Transient Receptor Potential for Vanilloid 1
TRPC	Transient receptor potential canonical
TRPM	Transient receptor potential melastatin
TRPML	Transient receptor potential mucolipins
TRPA	Transient receptor potential ankyrin
TRPP	Transient receptor potential polycystins
TTX	Tetrodotoxin
TTX-R	Tetrodotoxin-sensitive
TTX-S	Tetrodotoxin-resistant
trkB	Tropomyosin receptor kinase B
V50	Membrane Potential for Half Maximal Activation
WT	Wild Type

CHAPTER 1

INTRODUCTION

Cystic fibrosis (CF) results from mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein. In Canada, CF is the most prevalent lethal genetic disorder. The disease was first described by an American pathologist, Dr. Dorothy Andersen in 1938, through her autopsy findings from children that were dying of malnutrition (Andersen, 1938).

Lack of functional CFTR in CF patients was originally identified as leading to reduced life expectancies due to gastrointestinal complications. However, prognosis has drastically improved over the past 50 years (Elborn et al., 2016). Thus, although CF was traditionally considered a pediatric disease, with significant advances in CF research and management, most patients now survive well into adulthood. According to The Canadian Cystic Fibrosis 2018 Registry Report the median predicted survival age of CF patients is 52.1 years and it is expected to increase further in the coming years (Boyle et al., 2013; Eldredge et al., 2014).

Manifestations of the disease range from single-organ to a multisystem disease (Bell et al., 2020). With higher survival age, patients are more likely to experience chronic complications that contribute to the pathology of CF. This negatively affects CF patient's quality of life and places a large burden of care on their families as well as the healthcare system. One such

complication is abnormal function of the peripheral nervous system, i.e. peripheral neuropathy. Over the last few years an increasing body of evidence suggests that patients and animal models of CF suffer from peripheral nerve pathology (Reznikov et al., 2013; Chakrabarty et al., 2013; McNarry and Mackintosh, 2016). Unfortunately, the mechanisms involved and how they may contribute to CF disease is not fully understood. The role of CFTR in nervous tissue has only been investigated to a limited extent (Reznikov et al., 2013).

1.1. The CFTR protein and CF

CFTR is an ATP-gated anion channel permeable to chloride and bicarbonate (Rees et al., 2009). CFTR is a member of the ATP-binding cassette (ABC) superfamily, which is characterized as having two membrane-spanning domains (MSD1 and MSD2) and two nucleotide-binding domains (NBD1 and NBD2) (Rees et al., 2009). However, CFTR is the only member of the family known to be an ion channel. Gating of CFTR requires not only binding but also hydrolysis of Adenosine triphosphate (ATP), which makes it the only ligand-gated ion channel that consumes its ligand (ATP) during the gating cycle (Bear et al., 1992).

The regulatory (R) region consists of approximately 200 residues and is referred to as a region rather than a domain to reflect its lack of a stable, folded globular structure (Baker et al., 2007). It has multiple effects on CFTR channel activity. Modulation of CFTR function is mediated by phosphorylation by PKA. Moreover, the PKA phosphorylation sites are found primarily in the

R region, as well as within the NBD1 sequence (Lewis et al., 2003). Therefore, channel gating is controlled by both PKA phosphorylation of the R region and ATP binding and hydrolysis at the NBDs (Baker et al., 2007). These multiple phosphorylation sites generally act additively to control CFTR channel opening. For example, the majority of the phosphorylation sites at the R region stimulate channel activity, but the Ser737 and Ser768 residues are inhibitory sites and substitutions at these residues result in increased channel conductance (Wilkinson et al., 1997; Baker et al., 2007). The R region is intrinsically disordered and must be phosphorylated at multiple sites for full CFTR channel activity. There are several sites in the R region that mediate interactions with NBD1. The R region has direct phosphorylation-dependent interactions with the NBD. Nucleotide binding and hydrolysis at the NBDs of CFTR are required for channel gating. NBD1 is one of the two domains containing sites for the predominant CF-causing mutation, delta-F508, which is an in-frame phenylalanine 508 deletion that produces a mutant version of CFTR that remains largely trapped in the endoplasmic reticulum. Channel gating occurs through ATP binding in an NBD1-NBD2 nucleotide sandwich that forms upon displacement of NBD1 regulatory segments (Fig. 1.1) (Lewis et al., 2003).

In addition to delta-F508, about 2000 disease causing mutations have been described to date (Lukacs and Verkman, 2012). These mutations are classified in six different classes based on the type of mutations and the severity of the phenotype (Veit et al., 2016). Class I mutations result in no protein production. Class II mutations result in the retention of a misfolded protein at the endoplasmic reticulum, which causes subsequent degradation in the proteasome. Class III mutations result in impaired channel gating. Class IV mutants result in decreased ion flow

through the channel. Class V mutations cause substantial reduction in mRNA and/or protein production. Lastly, Class VI mutations cause substantial plasma membrane instability (Elborn, 2016). However, the end result for all mutations is a reduced CFTR activity in several organ systems, including the lungs and gastrointestinal (GI) that are the source of most of the morbidity and mortality in CF patients (Turcios, 2019). In the last few years other phenotypes have become more apparent, as the CF patient population reaches older ages, such as abnormalities in the nervous system.

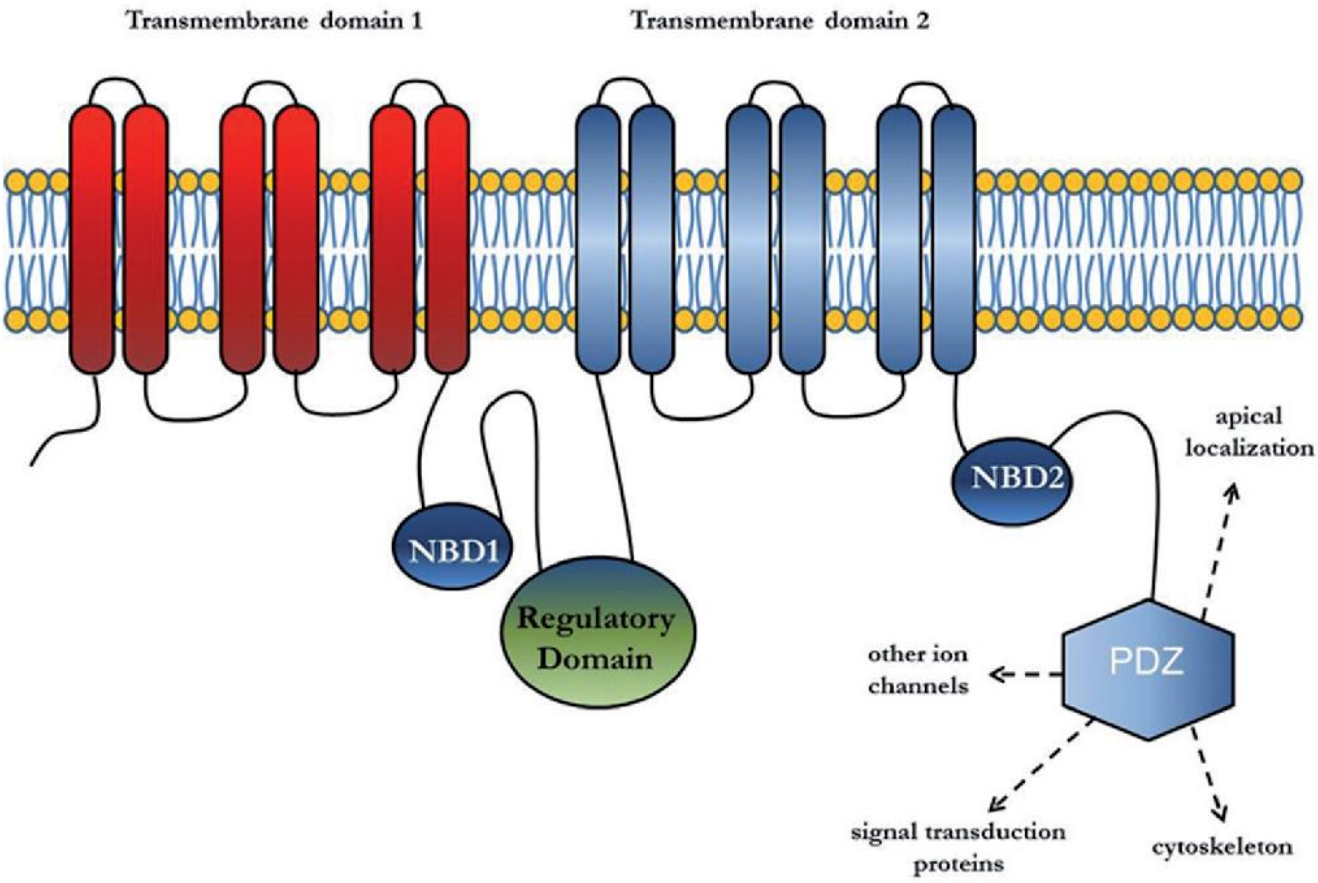

Figure 1.1. CFTR structure is composed of different domains. The nucleotide binding domains known as NBD1 and NBD2 that bind to ATP. The regulatory domain is involved in the opening of

the channel by the protein kinase A (PKA)-dependent phosphorylation. The PDZ domain allows CFTR to interact with the cytoskeleton, different ion channels, and intracellular signalling molecules (reproduced with permission from Anon,2017).

1.2 The sensory nervous system

There is evidence that neurons express CFTR and that the nervous system is affected by CF (Reznikov, 2017). The implications of these findings are not well understood. However, since the sensory nervous system conveys sensory information from the external or internal milieu, it is logical to hypothesize that proprioception and pain perception may be altered in CF patients.

In the case of noxious (painful) stimuli, the sensory nervous system is responsible for connecting the receptive sites in the periphery (skin, mucosa, viscera, muscles and joints) to pain-processing regions in the central nervous system (CNS) (Krames, 2014). Thus, injury of peripheral tissues results in activation of the primary sensory neurons, such as those located in dorsal root ganglia (DRG). DRG neurons are pseudo-unipolar cells that extend processes to both, the periphery and the CNS. In the CNS, these peripheral processes synapse with second-order neurons in the superficial laminae of the dorsal horn of the spinal cord (Sapunar et al., 2012; Basbaum et al., 2009). Projection neurons within the spinal cord transmit information to the thalamus, which is then conveyed to the somatosensory cortex to provide the brain with information regarding the location and intensity of the painful stimulus. Moreover, the emotional component of pain perception includes other groups of projection neurons that send information

to the brain stem and amygdala, which then conveys this to the cingulate and insular regions of the brain.

The axons of the projection neurons within the dorsal horn cross the midline and travel contralaterally up to the nuclei in the brainstem and thalamus. This forms the ascending pathways that underlies pain transmission. There are also descending pathways that positively and negatively influence pain transmission at the level of the spinal cord. Neurons of the rostral ventral medulla (RVM) and midbrain periaqueductal gray (PAG) are part of the descending feedback system, which regulates the output from the spinal cord (Basbaum et al., 2009) (Fig. 1.2). Endogenous mechanisms diminish pain through net inhibition, which inhibits incoming pain information at the spinal cord level. The circuit also consists of the locus coeruleus (LC), the nucleus raphe magnus (NRM) and the nucleus reticularis gigantocellularis (NRGC), all contributing to the descending pain suppression pathway (Burchiel, 2002). The descending modulatory circuit is an opioid-sensitive circuit and is therefore well studied in terms of development of chronic pain. It is also a pathway that many pain-relieving drugs, such opiates, cannabinoids, nonsteroidal anti-inflammatory drugs (NSAIDs), and serotonin/norepinephrine reuptake blockers act on to produce pain-relief (Ossipov et al., 2010).

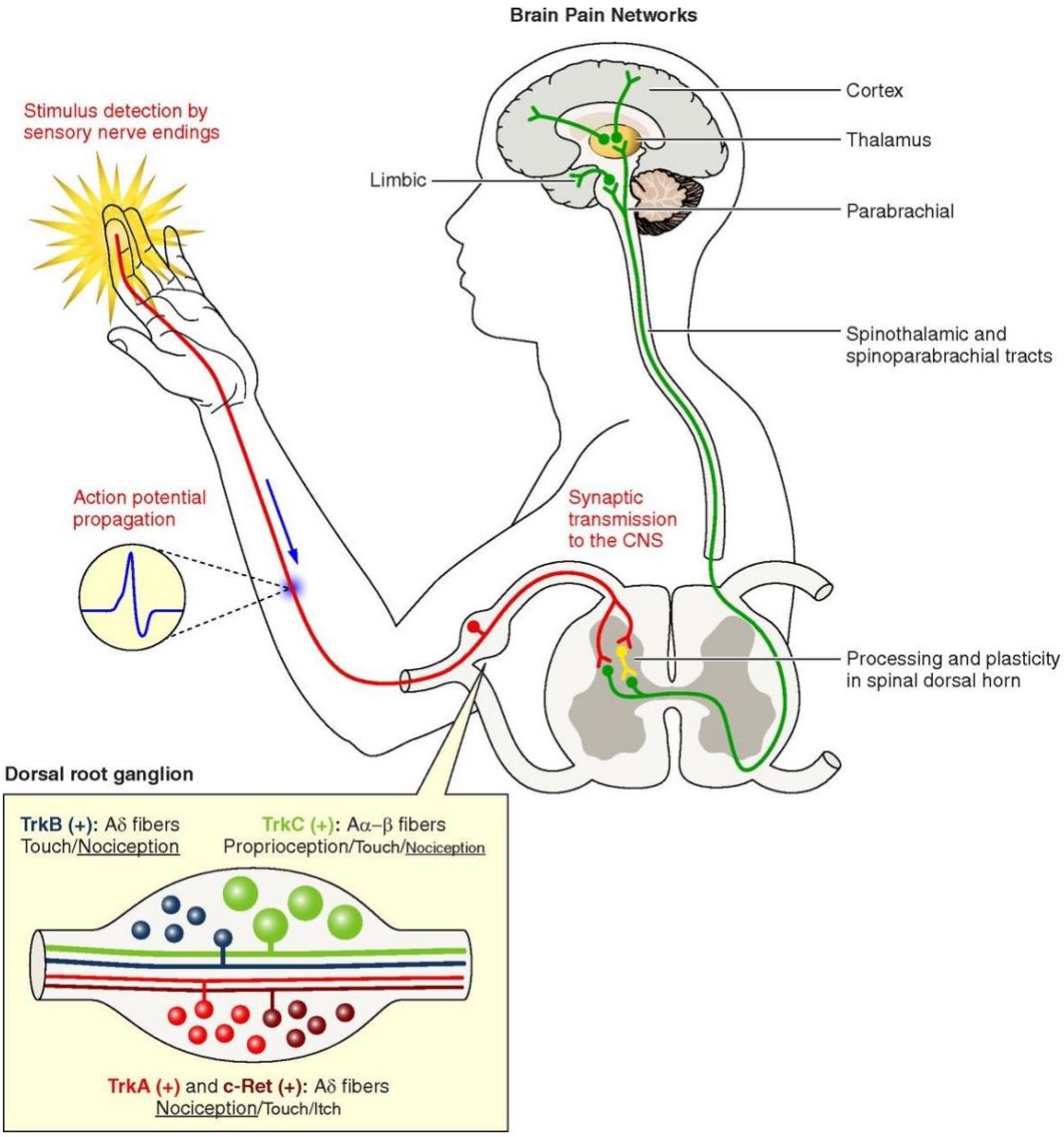

Figure 1.2. Pain pathway. DRG neurons are the pain sensing neurons in the peripheral nervous system. Afferent sensory fibers from these neurons innervate distal regions such as the viscera where they detect noxious stimuli. This leads to generation of action potential that travel along these fibers to the DRG neuron soma and then relayed to the neurons in the spinal dorsal horn. DRG neurons are diverse and categorized based on the expression of neurotrophin receptors. trkA- and c-Ret-positive small-diameter afferent sensory fibers correspond to unmyelinated C-fibers. They are mainly involved in nociception. trkB- and trkC-positive myelinated larger diameter afferent sensory fibers correspond to A-δ and A-α/β fibers, respectively. The convey touch and proprioception signals. Both categories contain nociceptive neurons (reproduced with permission from Bourinet et al., 2014).

DRG neurons are morphologically and neurochemically varied. They are classified into three main groups known as group A, B, and C fibers. Group A fibers are myelinated and can be further divided into Aα, Aβ, and Aδ. These fibers terminate in laminae I, III, IV, and V of the dorsal horn of the spinal cord. Preganglionic nerve fibers of the autonomic nervous system and visceral sensory afferent fibers are part of the thinly myelinated Group B fibers. Group C nerve fibers are unmyelinated and are nociceptive in function. These fibers terminate in laminae I and II of the spinal cord. C-fiber nociceptors are polymodal because they are activated by thermal, mechanical, and chemical stimuli. C-fiber neurons and small/medium, thinly myelinated Aδ fibers of the DRG mediate the sensation of pain (Lai et al., 2004).

1.3 GI abnormalities in CF

GI symptoms are a hallmark of CF (Dray et al., 2004; Robertson et al., 2006; van der Doef et al., 2011) and patients suffer from GI tract complications that can be life threatening. The majority of the GI complications in CF have been associated with a lack of CFTR in epithelial cells of the GI tract and abnormal ion transport (Zhang et al., 2010; Chen et al., 2010; Wilschanski and Durie, 1998). However, peripheral neuropathy may be a contributing factor to these GI complications because the sensory, autonomic, and Enteric Nervous System (ENS) innervate the GI tract and regulate its function. Obstruction, infection, as well as inflammation causes dysfunction of the GI tract, similar to what is observed in the lungs of CF patients (De Lisle et al., 2013; Dray et al., 2004; Constantine et al., 2004; Chaudry et al., 2006; Bodet-Milin et al., 2006;

Wilschanski and Durie, 1998; Tabori et al., 2017). Therefore, mutations of the CFTR gene in sensory, autonomic, and enteric neurons can contribute to the dysfunctions of the GI system (Xue et al., 2016). The possible contribution of peripheral neuropathy to CF gastrointestinal complications has not been previously studied.

Sensory innervation of the GI tract is critical for normal intestinal function, including the traffic of intestinal contents. The GI tract receives extrinsic (autonomic and sensory) and intrinsic innervation. Intrinsic neurons within the gut wall, which are part of the Peripheral Nervous System (PNS) and can also be affected by neuropathy, from the ENS. The ENS is composed of the myenteric and submucosal plexuses. In order to move contents along the GI tract in the oral-anal direction, activation of the ENS is required (Useaka et al., 2016). The ENS is also responsible for simple reflexes during passive distension of the gut wall. In addition, extrinsic neurons monitor the physical and chemical environment of the gut. Information is then sent to the CNS, which triggers motor adjustments and control of secretions.

Sensory innervation of the GI tract is comprised of extrinsic neurons located in spinal (DRG) and nodose/jugular ganglia (Fig. 1.3). Vagal sensory neurons, with cells bodies in the nodose/jugular ganglia, mainly innervate the proximal small intestine and partially the colon (Wang and Powley, 2000). In addition, the spinal thoracolumbar DRGs innervate the small intestine and the lumbosacral DRGs innervate the large intestine (Tan et al., 2008).

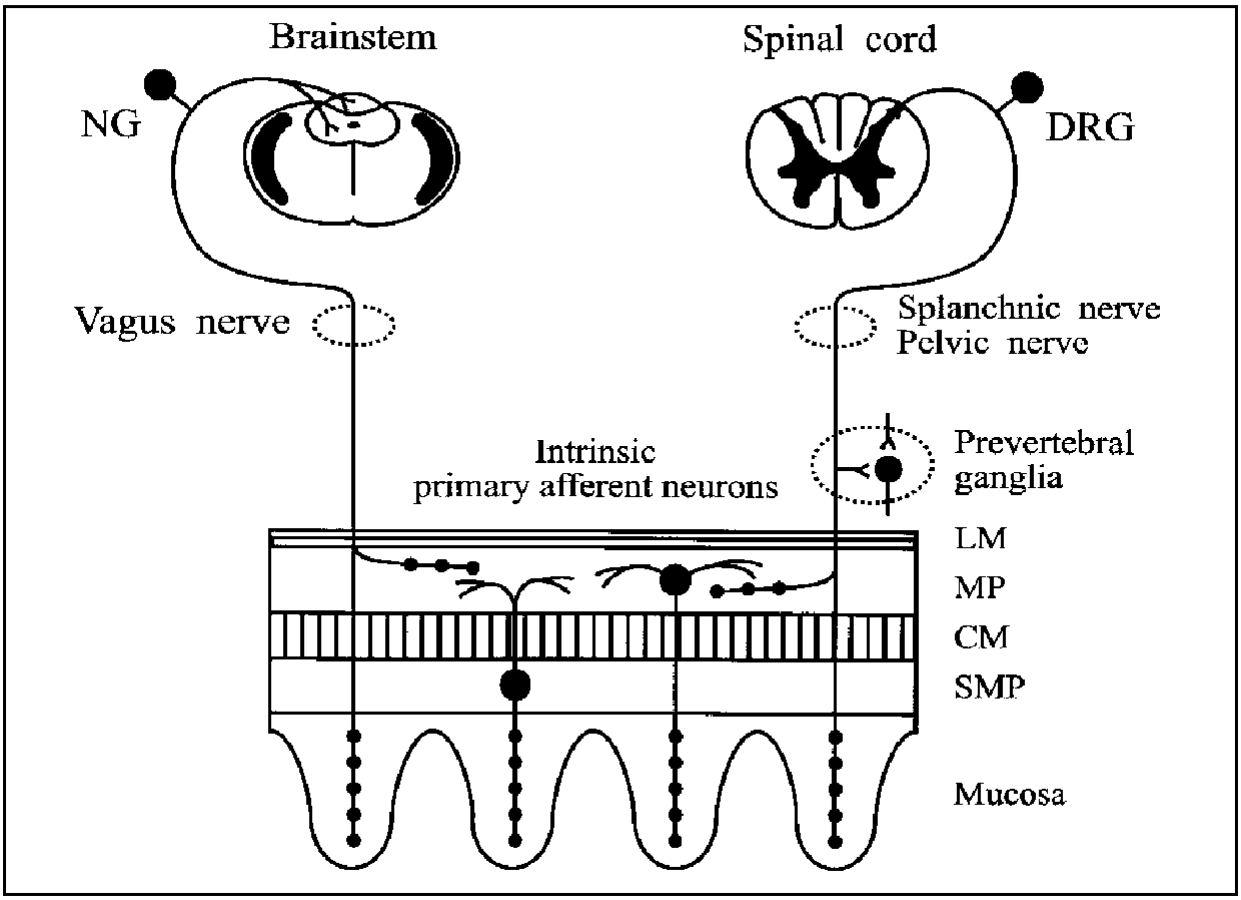

Figure 1.3. Intrinsic and extrinsic sensory neurons innervate the GI tract. There are two populations of intrinsic primary afferent neurons. They originate in the submucosal plexus (SMP) and myenteric plexus (MP). The two populations of extrinsic neurons are vagal afferents originating from the nodose ganglia (NG) and the spinal afferents originating from the DRG (reproduced with permission from Holzer et al., 2001).

It is well established that the main role of DRG neurons innervating the viscera is the transmission of noxious stimuli and visceral sensations (Lai et al., 2017; Holzer et al., 2001). However, a more complex role for these neurons was recently proposed. DRG neurons, which innervate the gut, can also modulate gut motility as a consequence of their activation by noxious stimuli (Smith-Edwards et al., 2019).

1.4 Sensory neuropathy in CF

CFTR expression has been detected in both the CNS and PNS, but its role has not been studied extensively. CFTR is expressed in the brain and spinal cord of the central nervous system (Guo et al., 2009a; Guo et al., 2009b; Johannesson et al., 1997; Mulberg et al., 1995; Mulberg et al., 1998), and in DRGs, sympathetic ganglia, paracervical ganglia, and enteric nervous system (Xue et al., 2016; Kanno and Nishizaki, 2011; Su et al., 2010; Niu et al., 2009). Although the role of CFTR in the nervous system remains under investigation, there is evidence suggesting it is important in normal peripheral neuronal function.

CF patients suffer from peripheral nerve dysfunction or neuropathy (Chakrabarty et al., 2013), a condition also reported in swine models of CF at birth (Reznikov et al., 2013). The latter strongly suggest CFTR is required for normal nervous system physiology. Studies investigating peripheral nerve dysfunction in pediatric CF patients found that neuropathy in CF patients was not related to sex, age, type of CF symptoms, body mass index, or duration of disease (El-Salem

et al., 2010). Moreover, in CF patients the autonomic and enteric nervous system function is abnormal (Xue et al., 2016). Therefore, nerve conduction studies in CF pediatric patients showed evidence of peripheral neuropathy, and these abnormalities may contribute to GI complications.

1.5 Mechanism of neuropathy in CF

A possible mechanism by which CFTR may affect nervous system function is by modulating neuronal excitability, which is tightly affected by changes in chloride homeostasis. The intracellular concentration of chloride in the adult CNS is kept low by the function of the chloride extruder K(+)-Cl(-) cotransporter (KCC2). In sensory afferent neurons the Na(+)-K(+)-2Cl(-) cotransporter (NKCC1) accumulates chloride intracellularly, which keeps the chloride concentration higher inside these neurons. Thus, activation of chloride ion channels, such as GABA-A receptors, leads to inward chloride currents and membrane hyperpolarization in the CNS, but in DRG neurons causes depolarizations through chloride efflux (Mao et al., 2012). This current dogma has been challenged by a report revealing that in motor neurons of the spinal cord CFTR operates together with NKCC1 to accumulate chloride intracellularly. Blocking CFTR shifts the chloride equilibrium potential negatively, indicating that CFTR actions promote depolarizing transmission in response to inhibitory neurotransmission (GABA/Gly) (Ostroumov et al., 2011). Thus, CFTR may play an important role in chloride homeostasis and the loss of CFTR may, thus, contribute to development of neuropathy in CF patients by altering intracellular chloride concentration. There is currently limited information on the role of CFTR in intracellular

ion homeostasis and the interaction with voltage-gated sodium and calcium channels, which are responsible for the generation of action potentials and burst firing activity in neurons.

1.6 Voltage-gated sodium channels

Voltage-gated sodium channels (Nav) are found throughout the nervous system. These channels are essential for the upstroke of action potentials in electrically excitable cells (Hille, 2001). The structure of these channels is comprised of a pore-forming α-subunit associated with either one or two β-subunits (De Lera Ruiz and Kraus, 2015). The α-subunit forms the ion-selective and voltage-sensitive domain of the channel (Lauria et al., 2014). There are four homologous domains known as domains I-IV in the α-subunit. Each of the domains are made up of six α-helices known as segments 1 to 6 (Wang et al., 2011). The pore of the channel is composed of segments (S) 5 and 6 and a pore-loop (P-loop) that connects the two segments (De Lera Ruiz and Kraus, 2015). The voltage-sensing domain is formed by S4. The latter regulates the channel gating during depolarization (De Lera Ruiz and Kraus, 2015). The β-subunit modulates the channel gating and surface expression in the plasma membrane (Lopez-Santiago et al., 2006; Benarroch, 2007).

Membrane depolarization causes the S4 domain to spiral outward towards the extracellular space (Catterall, 1992; Waxman et al., 1999). This conformational change in structure opens the pore of the channel (Wang et al., 2011). Channel opening results in an influx of sodium ions, which further depolarizes the membrane (Catterall, 1992). Another important

structural feature of the Nav channels is the inactivation gate. It is formed by the intracellular loop between domains III and IV. When the cell depolarizes, the inactivation gate plugs the pore of the channel in response to membrane depolarization (De Lera Ruiz and Kraus, 2015). Consequently, Nav channels are said to exist in three different functional states: closed, activated and inactivated (Bagal et al., 2015). These channels are responsible for the generation of action potentials, and thus, the transmission of sensory signals to the CNS.

A variety of sodium channels are expressed in DRG neurons, and they are divided in two main populations. All Nav channels, except Nav 1.5, 1.8 and 1.9, can be blocked by nanomolar concentrations of Tetrodotoxin (TTX) and thereby are termed TTX-sensitive channels. Thus, Nav 1.5, Nav 1.8, and Nav 1.9 are termed TTX-resistant (Wang et al., 2011). Small DRG neurons, which give rise to nociceptive C and Aβ fibers, display sodium current components that are both TTX sensitive and resistant. These Nav channels are essential in nociceptive transmission through their role in the conduction of action potentials (Bennett et al., 2019). It has been demonstrated that DRG voltage-gated sodium channels participate in the induction of neuropathic pain and an increased expression of these channels have been observed. The mechanism underlying changes in expression levels in neuropathic pain are still unclear. Channelopathies involving Nav channels have been shown to induce either painful neuropathies, an insensitivity to pain, or alterations in smooth muscle function. Understanding how Nav channels contribute to the function of both neuronal and non-neuronal tissues of the GI tract will ultimately help researchers better understand its role in normal gut function and in chronic visceral pain that involves altered intestinal motility (Erickson et al., 2018).

DRG neurons innervating the gut express Nav channels. In fact, activation of NaV 1.6 using the β-scorpion toxin Cn2 leads to profound changes in neuronal responses to mechanical stimuli *in vivo* and is therefore required for the transduction of mechanical information in sensory afferents innervating the viscera (Israel et al., 2019; Schiavon et al., 2006). Moreover, Salvatierra et al. demonstrated that the Nav 1.1 subunit regulates the excitability of sensory nerve fibers responsible for the transmission of mechanical pain stimuli to the spinal dorsal horn in individuals with visceral hypersensitivity and chronic abdominal pain (Salvatierra et al., 2018).

1.7 Voltage-gated calcium channels

Voltage-gated calcium channels (Cav) shape action potentials and influence neuronal excitability. The structure of voltage-gated calcium channel subunits consists of α1, β, α2δ, and, γ subunits (Catterall, 2000; Hofmann et al., 1999; Kang et al., 2001). These heteromeric proteins are composed of an α1 pore forming subunit and the modulatory subunits a2δ, a2β, or a2γ. Regulation of current density and kinetics of activation and inactivation is through the a2δ subunit (Luo et al., 2001). The α1 subunit of calcium channels is similar in structure to the α-subunit of Nav channels. This subunit is essential for voltage sensing, ion selectivity, and calcium permeation of the channel. Moreover, the α1 subunit is used to classify the ten different classes of voltage- gated calcium channels: L (α1S, α1C, α1D, and α1F), P/Q (α1A), N (α1B), R (α1E), and T (α1G, α1H, and α1I) types (Miller, 2001; Perez- Reyes, 2003; Tsien et al., 1991). The following section will be focused specifically on the transient opening (T-type) group of calcium channels.

DRG neurons are presynaptically located in relationship to the spinal dorsal horn. They are involved in conveying sensory information to the thalamus, and subsequently to cortical structures of the pain matrix. The low voltage-activated T-type calcium channel is responsible for burst firing and is expressed in the DRG neurons and central endings of these cells in the dorsal horn of the spinal cord. These channels activate during small depolarization of the cell membrane, causing a transient surge of calcium entry into excitable cells during the early phase of an action potential. The activation threshold for T-type channels is more negative than that of Nav channels, which make these channels instrumental in controlling cell excitability (Iftinca and Zamponi, 2009; White et al., 1989; Marger et al., 2011). Moreover, the T-type calcium channels drive sensory transduction by increasing intracellular calcium concentration in response to depolarization (Miller, 2001), and are able to modulate the function of peripheral and central pain pathways by influencing fast synaptic transmission and neuronal excitability. T-type calcium channels are involved in both the development and maintenance of physiological (nociceptive) and pathological (neuropathic) pain.

There are three different types of T-type calcium channels known as Cav 3.1, 3.2, and 3.3. Activation of T-type calcium channels depolarizes the membrane potential, which allows the cell to reach the threshold for action potential firing during sub-threshold membrane depolarizations (Nelson et al., 2005; 2007b; Jagodic et al., 2007). Cav 3.2 is expressed in small to medium-size DRG neurons and is involved in the generation of high-frequency burst firing (Nelson et al., 2005; 2007b; Jagodic et al., 2007). This channel has been reported to contribute to mechanisms

underlying neuropathic pain in DRG neurons (Todorovic et al., 2011), and thought to be involved in the pathophysiology of primary sensory neurons and neuronal transmission of painful stimuli as the membrane firing can potentially contribute to symptoms of neuropathic pain, such as hyperalgesia and allodynia (Jacus et al., 2012; Todorovic et al., 2011; Bourinet et al., 2005; Jagodic et al., 2008; Latham et al., 2009; Joksimovic et al., 2018). Notably, T-type calcium channels are expressed by C fibers and are upregulated under pathophysiological states. The transcription factors early growth response 1 (Egr1) and the repressor element 1-silencing transcription factor (REST) bi-directionally regulate CaV 3.2 promoter activity and mRNA expression, which have critical implications for the regulation of neuronal calcium homeostasis (van Loo et al., 2012). Cav 3.2 is expressed in colonic DRG neurons and they contribute to the exaggerated pain perception in irritable bowel syndrome (Marger et al., 2011). Increased Cav 3.2 channel density in DRG neurons innervating the colon exacerbates visceral hypersensitivity and inhibitors of these channels prevent the development of visceral hypersensitivity (Marger et al., 2011; Picard et al., 2019).

Neurotrophins, such as brain-derived neurotrophic factor (BDNF), are essential for neuronal development, survival, and differentiation (Lewin and Barde, 1996). BDNF promotes axonal growth of sensory neurons and is involved in the modulation of the pain circuitry. Some of the actions of BDNF are mediated by modulating ion channel function. For example, the T-type calcium currents can be enhanced by BDNF binding to tropomyosin receptor kinase B (trkB). BDNF was shown to alter the steady-state inactivation and induced a significant depolarizing shift in the inactivation potentials of T-type channels (Wang et al., 2019), resulting in increased

neuronal excitability. Phosphoinositide 3-kinases (PI3K) and p38 mitogen-activated protein kinases (MAPK) are involved in the BDNF-induced stimulation of PKA and inhibition of PKA prevents the increase in T-type calcium current that is mediated by BDNF. Therefore, BDNF is able to reversibly and dose-dependently enhance T-type calcium currents through the stimulation of trkB coupled to PI3K-p38-PKA signaling. This was shown to affect trigeminal ganglion neuronal excitability and consequently pain perception (Wang et al., 2019).

1.8 Transient receptor potential vanilloid member 1 (TRPV1)

TRP ion channels play a critical role in sensory physiology as these channels enable individual cells to sense changes in their local environment. TRP channels are ligand-gated channels found on the cell surface of sensory neurons. They respond to a variety of intracellular and extracellular stimuli, and the function of some members of this family is to respond to noxious, irritating, and inflammatory stimulants. TRP members are categorized by their subunits, these are grouped into Canonical (TRPCs), Vanilloid (TRPVs), Melastatin (TRPMs), Mucolipins (TRPMLs), Polycystins (TRPPs), and Ankyrin repeat (TRPA) (Wu et al., 2010). TRPV1 is part of the TRPV subfamily of the large TRP ion channel super family.

The structure of the TRPV1 channels is composed of six transmembrane (TM) domains along with a short pore-forming hydrophobic region between the fifth and sixth TM domains (Caterina et al., 1997). The channel has several regions and amino acids that are associated with

the many functions that this channel has including multimerization, capsaicin action, proton action, heat activation, desensitization, permeability, phosphorylation and modulation by lipids (Fig. 1.4). The long amino terminus of this channel contains three ankyrin-repeat domains and a carboxyl terminus containing a TRP domain close to the sixth TM, which is similar to other members of the TRP channels. The ankyrin repeats are capable of binding to many cytosolic proteins such as the calcium-binding messenger protein calmodulin (Rosenbaum et al., 2004).

Capsaicin, the active ingredient found in chili peppers, and exposure to noxious heat, greater than approximately 43°C, and low pH all activate TRPV1, which causes peripheral terminal depolarization (Szallasi et al., 2007). If the signal is strong, calcium entry through this channel causes activation of Nav channels and the subsequent firing of action potentials. TRPV1 expression is found in small diameter, unmyelinated C-fibers and thinly myelinated Aδ fibers (Cavanaugh et al., 2008; Helliwell et al., 1998). Since TRPV1 plays an important role in nociceptive neurons, detecting painful thermal and chemical stimuli, there have been several studies investigating its potential use as a therapeutic target in analgesia (Brown et al., 2015). TRPV1 sensitisation involves protein kinase C-mediated phosphorylation of intracellular sites located within the loop connecting TM3 and TM4 and the C-terminal tail (Bhave et al. 2003, Cesare & McNaughton 1996, Numazaki et al. 2002, Premkumar & Ahern 2000, Vellani et al. 2001; Julius, 2013).

The neurotrophin BDNF can also affect the expression of TRPV1 channels. Ciobanu et al. (2009) demonstrated that exposure of DRG neurons to BDNF *in vitro* increased the TRPV1

expression, leading to increased response to capsaicin. Growth factors such as NGF can also functionally potentiate TRPV1 in DRG neurons and regulate the sensitization of the colonic primary afferent fibers, especially through overexpression of ion channels, inducing abdominal discomfort in irritable bowel syndrome (IBS) patients (Chen et al., 2015). Biopsy studies have reported increased TRPV1 expression in peripheral nerve endings taken from these patients (Scanzi et al., 2016).

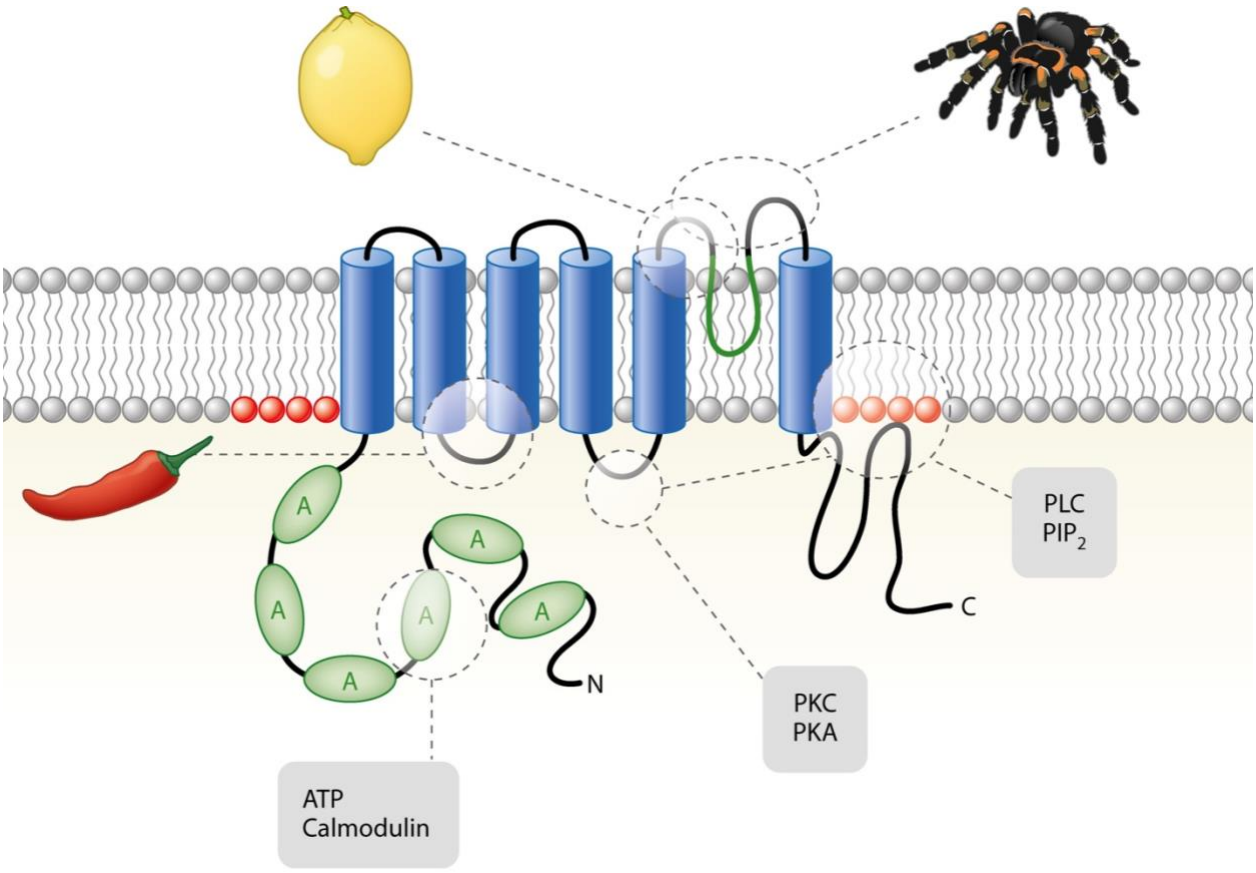

Figure 1.4. TRPV1 structure. The various sites of TRPV1 domains that are sensitive to various stimuli or contribute to physiological modulation of the channel downstream of metabotropic receptors are shown above. Capsaicin (chili pepper), extracellular protons (lemon), and peptide toxins from tarantula (spider) are several stimuli that activate this channel as well as cellular proteins and cytoplasmic second messengers that modulate the channel (reproduced with permission from Julius, 2013).

1.8 Rationale and hypothesis

Reports from CF patients and animal models of CF indicate that peripheral nerve dysfunction, or neuropathy, is part of the myriad of complications that patients may experience in the CF pathology. Neuropathy can affect all components of the peripheral nervous system, including sensory nerves. The latter may serve as an important contributor to the most reported extra pulmonary complication, GI abnormalities of CF. Therefore, in the current thesis we hypothesize that lack of CFTR expression causes electrophysiological abnormalities in sensory neurons from the DRGs that innervate the GI tract, which could contribute to the GI pathology in CF. We will be specifically concentrating on the function of voltage-gated and ligand gated ion channels in sensory neurons obtained from WT and CFTR-/- swine, as electrophysiological parameters to determine sensory function in an animal model of CF.

CHAPTER 2

MATERIALS AND METHODS

2.1 Primary DRG cultures

We used gut-corrected CFTR−/− swine purchased from Exemplar Genetics (Iowa, USA) and wild-type swine from Prairie Swine Center (University of Saskatchewan) that served as controls. A sow implanted with cloned embryos with the gut-corrected CFTR-/- genotype (CFTR-/-;TgFABP > pCFTR pigs) (Stoltz et al., 2013) was used to generate DRG primary cultures . Bilateral thoracic (L1-L5) DRG neurons were harvested and cultured from dorsal root ganglia (DRG) from wild-type (WT) and CFTR knock-out (CFTR-/-) neonatal (P0 and P7) piglets as previously described (Lam et al. 2018). Briefly, ganglia were removed under sterile conditions and enzymatically dissociated at 37°C in DMEM/F12 containing collagenase type 2 (1mg/ml; Worthington, Freehold, NJ, USA) for the first stage and DMEM/F12 containing trypsin (1mg/ml; Worthington, Freehold, NJ, USA) for the second stage. The resulting cell suspension was washed twice in serum-containing DMEM/F12 medium to inactivate the trypsin and plated on laminin-coated glass-bottom Petri dishes (35 mm) made in-house. The neurons were grown in DMEM/F12 medium supplemented with vitamins, cofactors, penicillin–streptomycin, 5% rat serum and NGF (10 ng/ml; Alomone Labs, Jerusalem, Israel). Cultures were maintained at 37°C in a humidified atmosphere of 95 % air 5 % CO2 and fed every 5 days with growth media. To eliminate non-

neuronal cells, cultures were treated with cytosine arabinoside (10 µM; Sigma, St. Louis, MO, USA) from days 2 to 4. Established DRG cultures were incubated for 24h at 37oC; (5% CO2; humidified) in growth media.

2.2 Immunocytochemistry

Cultured DRG neurons were fixed with 4% paraformaldehyde for 1 hour at room temperature (Lam et al 2018). After washing with PBS (3 times for 3 min), the cells were incubated overnight at 4°C with a rabbit anti-neurofilament (NF200; 1:1000, abcam) and Anti-CGRP (1:1000, abcam) antibodies diluted in PBS containing 1% BSA and 0.5% Triton X-100. The next day, the samples were washed in PBS (3 x 3 min) and then incubated in the dark for 1 hour at room temperature with a goat anti-rabbit secondary antibody conjugated to FITC (1:500; Invitrogen - Thermofisher, Carlsbad, CA, USA). Samples were covered with an anti-photobleaching reagent (Vectashield; Vector Laboratories, Burlingame, CA, USA) and viewed with an epifluorescence microscope (AxioObserved; Zeiss). Fluorescence intensity was quantified as the difference in intensity of the region of interest (neuronal cell body, excluding the nucleus) to the background.

2.3 Patch clamp electrophysiology

Patch pipettes were made from borosilicate glass (WPI, Sarasota, FL, USA) glass using a vertical puller (PC 10; Narishige Scientific Instrument Lab., Tokyo, Japan). Micropipettes had a resistance

of 8–11 MΩ when filled with intracellular recording solution, and formed gigaseals of 1–8 GΩ. Recording electrodes were filled with the following solution (in mM, all from Sigma): 60 KAc, 70 KF, 5 NaCl, 1 MgCl2, 1 CaCl2, 2 MgATP, 10 EGTA, and 10 HEPES, and pH was adjusted to 7.2 with KOH. The external solution contained (in mM): 140 NaCl, 5.4 KCl, 0.33 NaH2PO4, 0.44 KH2PO4, 1 MgCl2, 2.8 CaCl2, 10 HEPES, and 5 glucose, and pH was adjusted to 7.4 with NaOH. Whole-cell currents or membrane potentials were recorded at room temperature with the aid of an Axopatch 200B or Multiclamp amplifier (Molecular Devices, Palo Alto, CA, USA) equipped with a 1 GΩ headstage feedback resistor, and sampled at 1kHz for CAP experiments respectively, with a Digidata 1440A. Voltage clamp protocols, data acquisition and analysis were performed using pCLAMP 10 software (Molecular Devices) and Origin 7 (OriginLab Coorporation, Northampton, MA, USA). Once the whole-cell configuration was achieved, cells were allowed to stabilize for 5 minutes before recording. Control and extracellular solutions containing CAP, TTX, TEA, or Lidocaine were applied using a pressurized perfusion fast-step system (SF-77B, Warner Instruments, Hamden, CT, USA) that provided a constant flow of 2–3 ml/min.

Ionic currents were generated in voltage-clamp mode. To isolate the components of the inward whole-cell current we used the voltage-gated Na channels blockers tetrodotoxin (TTX; 1µM) and lidocaine (100 µM), and the potassium voltage-gated channel blocker tetraethylammonium (TEA, 20 mM). To generate inward Nav currents the membrane potential was held at -60 mV followed by a hyperpolarizing prepulse (-120 mV) to remove Nav inactivation and subsequent step voltages (-100mV to +20mV). In order to generate inward Cav currents, the membrane potential was held at -60mV followed by a hyperpolarizing prepulse (-90mV, 3s) and subsequent step

voltages (-70mV to +29mV, 250ms). The decay τ time constant for T-type calcium currents was calculated as the current amplitude decays from its maximum (100%) to 37% of the response.

Conductance values were obtained by the following formula:

$$G_{Ca} = \frac{I_{Ca}}{V_m - E_{Ca}}$$

Where G_{Ca} is the conductance for calcium ions, I_{Ca} is the peak current at a specific test pulse, V_m is the voltage step, and E_{Ca} is the reversal potential for calcium. G_{Ca} was normalized to maximum conductance (Gmax). Steady-state activation curves were acquired by fitting normalized conductance (G_{Ca}/Gmax) values in the following Boltzmann equation:

$$\mathrm{y} = \frac{(\mathrm{x} - \mathrm{ECa}) * \mathrm{Gmax}}{Gmax\ 1 + e\ (x - V_{50})/dx}$$

Where, E_{Ca} is the reversal potential for calcium, Gmax is the maximal conductance, and V_{50} is the membrane potential at half-maximal activation.

Capsaicin-evoked currents were recorded at a holding potential of -60mV by exposing the cells to capsaicin (CAP; 1uM; 1 s). Action potentials were generated in current clamp mode by the injection of depolarizing current steps (100 pA intervals for 500 ms).

2.4 Statistical analysis

All data are expressed as mean ± SEM. A two-way ANOVA followed by multiple comparisons test was used to analyze voltage-clamp and current-clamp data. In all cases, p values less than 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad InStat 3.0 or Prism 8 GraphPad (GraphPad Software Inc., La Jolla, CA, USA).

CHAPTER 3

RESULTS

To evaluate the effect of lack of CFTR expression in sensory neurons we generated primary cultures from lumbar DRG neurons from WT and CFTR-/- swine at postnatal day 0 (P0) and postnatal day 7 (P7). Cultured DRG neurons were maintained in culture conditions for up to four weeks. Figure 3.1 shows representative examples of cultured DRG neurons labeled with the peptidergic marker calcitonin gene-related peptide (CGRP) and the neuronal marker neurofilament. These neurons were used in patch-clamp electrophysiology experiments.

A comparison of passive membrane properties recording during whole-cell patch clamp electrophysiology for cultured DRG neurons from WT and CF swine, are summarized in Table 3.1. A statistical comparison of membrane potential (V_m), and cell capacitance (C_m) at P0 and P7 stages showed no significant changes between the groups.

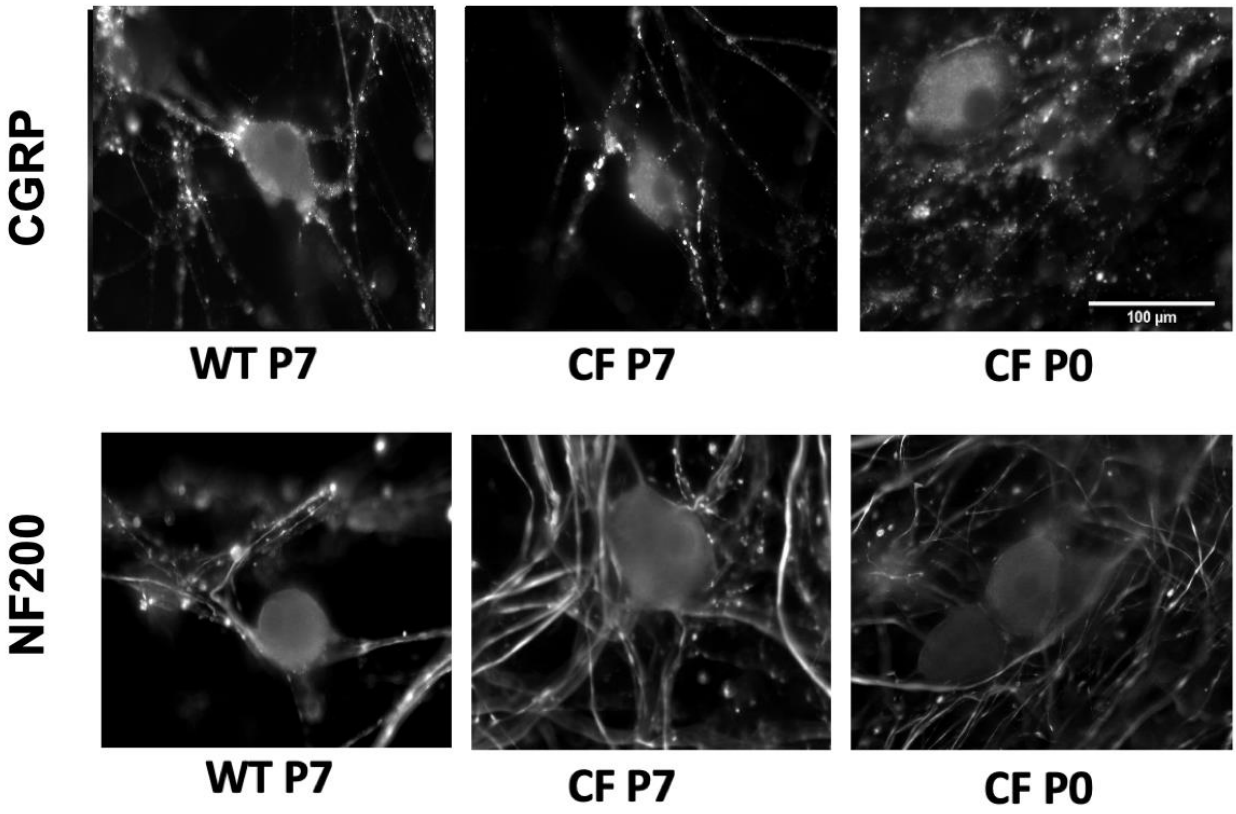

Figure 3.1. Cultured swine DRG neurons maintained for 21 days in culture conditions. Representative images for CGRP and neurofilament fluorescence in DRG neurons of WT P0, CF P0, WT P7, and CF P7 (40x magnification).

	V_m (mV)	C_m (pF)	n
WT P0	**-59.84 ± 0.02**	**58.56 ± 18.32**	**5**
CF P0	**-59.89 ± 0.05**	**55.58 ± 3.46**	**13**
WT P7	**-59.48 ± 0.33**	**58.99 ± 5.91**	**9**
CF P7	**-59.86 ± 0.06**	**52.65 ± 5.028**	**8**

Table 3.1. Passive membrane properties for cultured DRG neurons from WT and CF swine. Table summarizes the membrane potential (V_m), and cell capacitance (C_m) obtained during whole-cell recording of cultured DRG neurons from WT and CF swine at P0 and P7 stages. Statistical analysis performed by two-way ANOVA followed by a Tukey's post hoc test. There were no significant differences found between the genotypes and age groups.

3.1 DRG neurons from CFTR-/- animals had reduced excitability compared to those from wild type, at P7

As a measure of neuronal excitability, current-clamp electrophysiology was utilized to study action potential (AP) firing frequency in cultured DRG neurons. APs were generated by the injection of depolarizing current at 100 pA increments for 500 ms. APs were compared between WT and CFTR-/- groups at different postnatal stages (P0 and P7), and at the 600 pA injection, which provides the maximal AP frequency in WT (Fig. 3.2C). First of all, we observed a significant increase in AP frequency at P7, with respect to P0, in neurons from WT swine. This pattern was not observed in the CFTR-/- genotype. Second, we observed no change in AP frequency between the genotypes at P0 (Fig. 3.2 A-C), with frequencies of 0.84 ± 0.10 and 1.88 ± 0.44 in the WT and CFTR-/- group, respectively. Third, at P7 however, there was a significant reduction in the AP firing frequency in CF group compared to WT. The AP frequency at 600 pA for these groups were 4.44 ± 0.86 and 1.68 ± 0.10 ($p<0.01$).

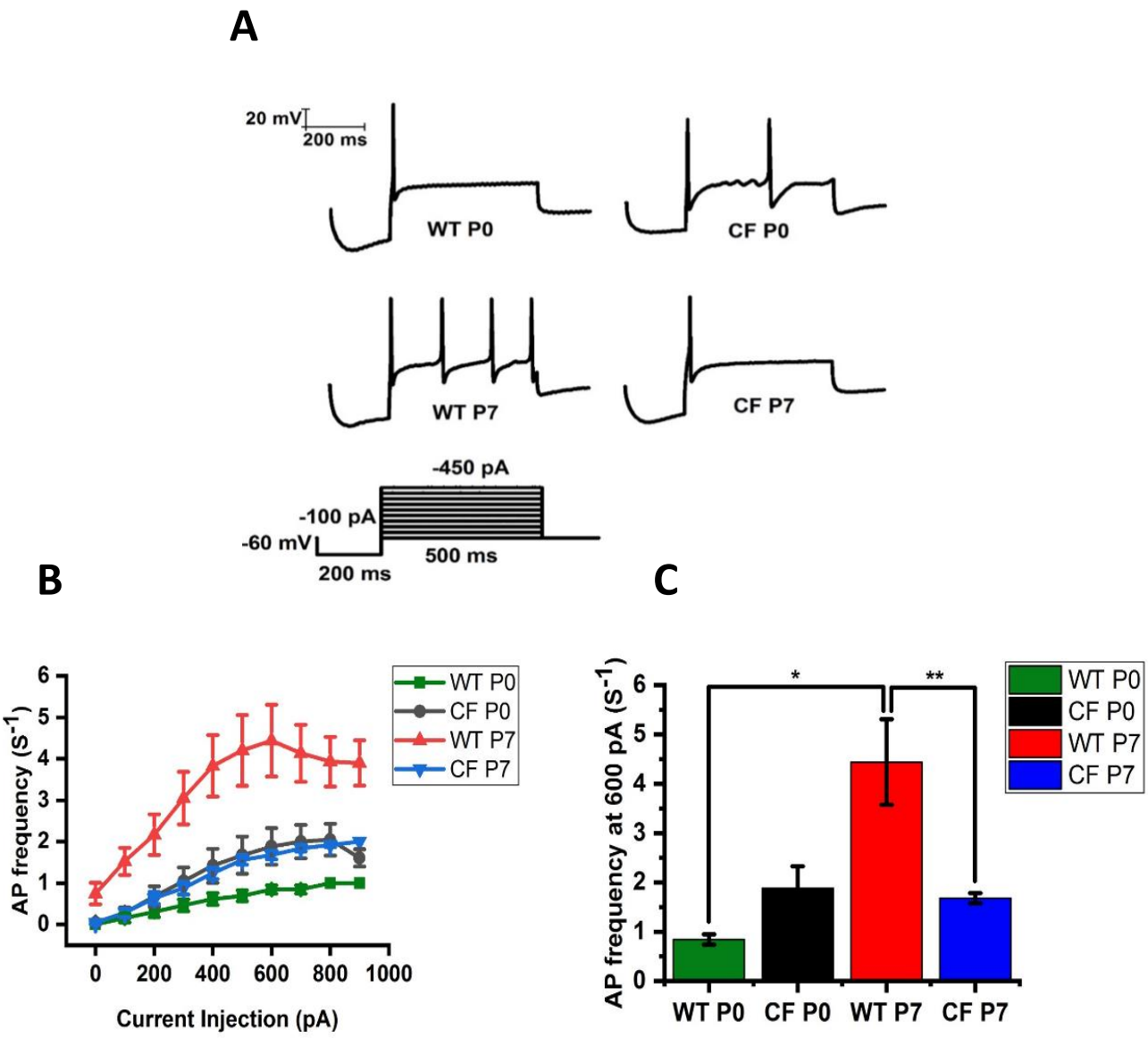

Figure 3.2. DRG neurons from CFTR-/- animals at P7 had reduced excitability than wild type P7 neurons. A. Representative traces of action potentials (AP) generated in cultured DRG neurons from wild WT P0, CF P0, WT P7, and CF P7 at 600 pA injection step**.** Stimulation protocol: 200 ms hyperpolarisation at -70 mV. Step current injections 0 to 900 pA at 500ms per step. **B.** Line graph shows a reduction in AP frequency in DRG neurons from CFTR-/- animals at P7. **C.** Comparison of the AP frequency at 600 pA indicates a significant reduction between WT P7 and CF P7 animals. Statistical analysis performed by two-way ANOVA followed by a Tukey's post hoc test; *, $p < 0.05$; **, $p < 0.01$. There was a significant main effect between ages ($F_{(1, 161)} = 4.589$, $P = 0.0337$). There was a significant interaction between genotype and age ($F_{(1, 161)} = 5.758$, $P = 0.00176$). WT P0 (n=13), CF P0 (n=43), WT P7 (n=59), CF P7 (n=50). All data are represented as mean ± SEM. Inset in A represents the stimulation protocol used to generate action potentials.

3.2 DRG neurons from CFTR-/- animals had normal voltage-gated sodium current.

In order to better understand the mechanisms underlying the decrease in AP firing, we first concentrated on Nav currents in neurons from WT and CFTR-/- animals at P0 and P7. Voltage-clamp electrophysiology was used to generate whole-cell currents (Fig. 3.3A, B). We isolated whole-cell inward currents with the help of the voltage-gated potassium channel blocker, TEA. TTX and lidocaine were used to identify the TTX-sensitive and TTX-resistant component of the whole-cell inward current. In DRG neurons, from both genotypes, and at the two postnatal time points studied, the inward current was mainly carried by TTX-sensitive channels. A comparison of the Nav currents at the maximal peak current density (-10mV) showed no significant differences ($p < 0.05$) between genotypes and age groups (Fig. 3.4). The maximum peak current densities (pA/pF) for each group were - 50.71 ± 15.5 for WT P0, -39.60 ± 5.12 for CF P0, -39.62 ± 6.97 for WT P7, and -31.74 ± 3.50 for CF P7.

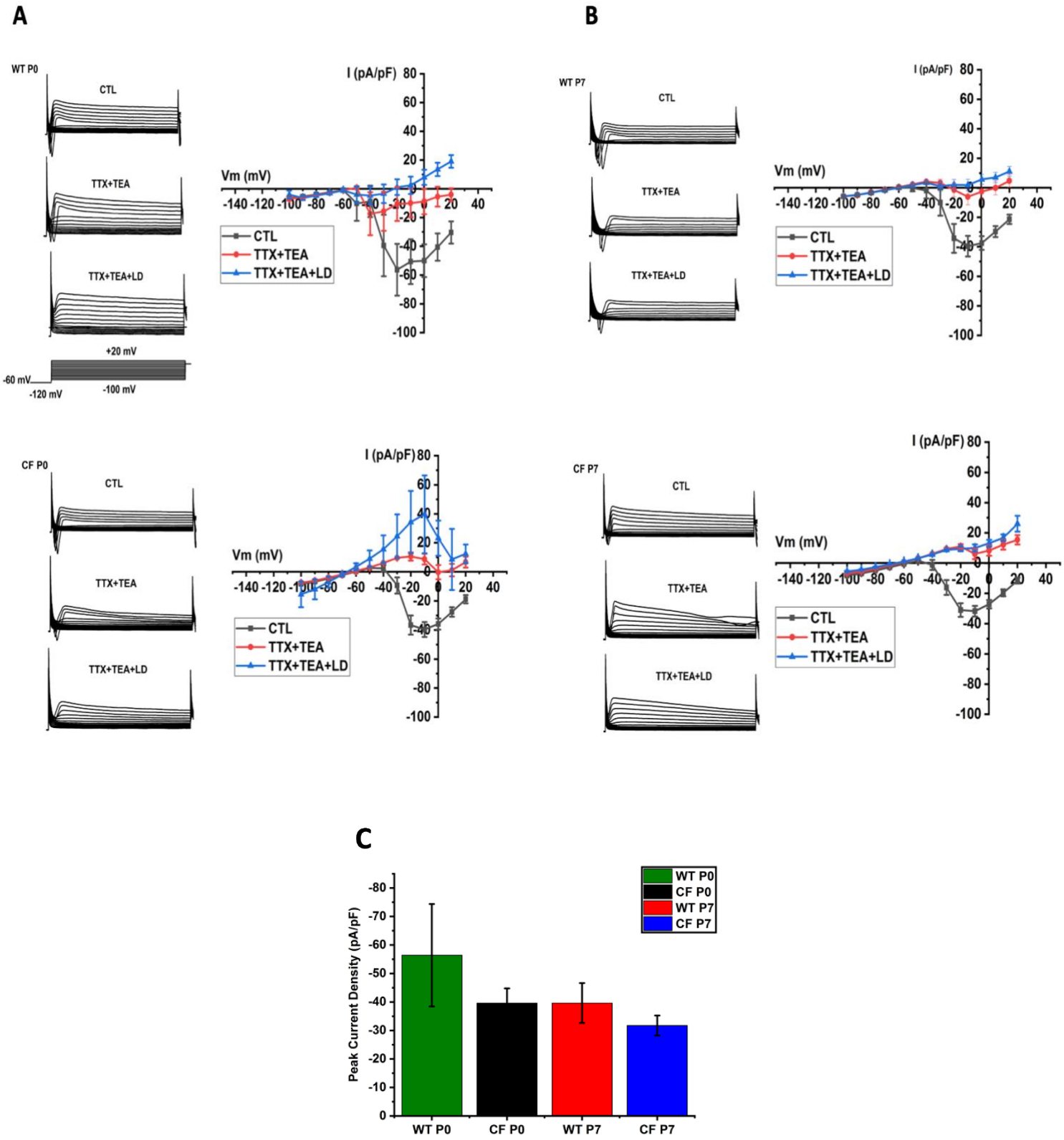
A
WT P0
CTL
TTX+TEA
TTX+TEA+LD
+20 mV
-60 mV
-120 mV
-100 mV
I (pA/pF)
Vm (mV)
CTL
TTX+TEA
TTX+TEA+LD
CF P0
CTL
TTX+TEA
TTX+TEA+LD
I (pA/pF)
Vm (mV)
CTL
TTX+TEA
TTX+TEA+LD
B
WT P7
CTL
TTX+TEA
TTX+TEA+LD
I (pA/pF)
Vm (mV)
CTL
TTX+TEA
TTX+TEA+LD
CF P7
CTL
TTX+TEA
TTX+TEA+LD
I (pA/pF)
Vm (mV)
CTL
TTX+TEA
TTX+TEA+LD
C
Peak Current Density (pA/pF)
WT P0
CF P0
WT P7
CF P7
WT P0
CF P0
WT P7
CF P7

Figure 3.3. DRG neurons from CFTR-/- animals had similar sodium currents compared to WT animals A. Representative traces of voltage-gated currents and I-V plots shows the mean (±SEM) inward current densities generated by different voltage-steps in cultured DRG neurons from WT P0 and CF P0. **B.** Representative traces of voltage-gated currents and I-V plots shows the mean (±SEM) inward current densities generated by different voltage-steps in cultured DRG neurons from WT P7 and CF P7. WT P0 (n=12), CF P0 (n=30), WT P7 (n=20), CF P7 (n=26). **C.** Bar graph shows peak current density ±SEM. Statistical analysis performed by two-way ANOVA followed by a Tukey's post hoc test. There were no significant differences. WT P0 (n=12), CF P0 (n=30), WT P7 (n=20), CF P7 (n=26). Inset in A represents the stimulation protocol used to generate inward sodium currents.

3.3 A reduced proportion of DRG neurons from CFTR-/- swine had transient calcium currents compared to WT.

Since our analysis of the Nav currents showed no significant differences between the genotypes that could have potentially explained the reduced AP firing observed in the CF group, we next concentrated on Cav channels. We studied Cav currents in WT and CFTR-/- animals at P0 and P7 (Fig. 3.5). To generate low-voltage activated T-type currents we used the voltage-step protocol previously described by Khomula et al. (2013). To isolate calcium currents from the rest of the voltage-gated currents we used TTX, lidocaine and TEA. A comparison of the calcium currents at the maximum peak current density showed no significant difference between the groups ($p < 0.05$). The maximum peak current densities (pA/pF) for each group are -38.82 ± 15.94 for WT P0, -24.83 ± 2.73 for CF P0, -24.83 ± 2.73 for WT P7, and -28.67 ± 4.88 for CF P7 (Fig. 3.5D). We identified three relevant changes in the T-type currents between these groups. First, we observed a reduction in the percentage of neurons expressing transient calcium currents in the CF

genotype. Although this change in percentages was not evident at P0 between genotypes, there was a reduction trend observed at P7 (Fig. 3.5E). While 41% (40.90 ± 10.72) of neurons from WT animals showed calcium currents that were consistent with T-type kinetics, only 17% (16.66 ± 5.43) of cells from CF animals displayed similar currents. In contrast, this change was not observed at P0, when 21% (20.83 ± 7.77) and 27% (27.08 ± 6.48) of neurons from WT and CF swine, respectively, had voltage-gated currents consistent with the expression of T-type calcium channels.

Second, to study the activation kinetics of Cav currents in DRG neurons from CF swine, we generated activation curves shown in Figure 3.5F. A significant difference in the membrane potential for half-maximal activation (V_{50}) was observed between WT P0 (-45.05 ± 5.27mV) and CF P0 (-7.45 ± 8.36mV) groups (Fig. 3.5G). There were no significant differences between WT P0 (-45.05 ± 5.27mV), WT P7 (-26.44 ± 4.82), and CF P7 (-18.37 ± 2.66) ($p < 0.05$) (Fig. 3.5G).

Third, to study the inactivation kinetics of Cav currents in DRG neurons from CF swine we quantified the maximal Cav current decay τ (Fig. 3.5I), which showed a significant change at P0 between genotypes. The values of the decay τ were 4.57 ± 1.67 and 3.55 ± 0.42 for WT P0 and WT P7, respectively; and 1.23 ± 0.12 and 1.85 ± 0.34 for CF P0 and CF P7, respectively.

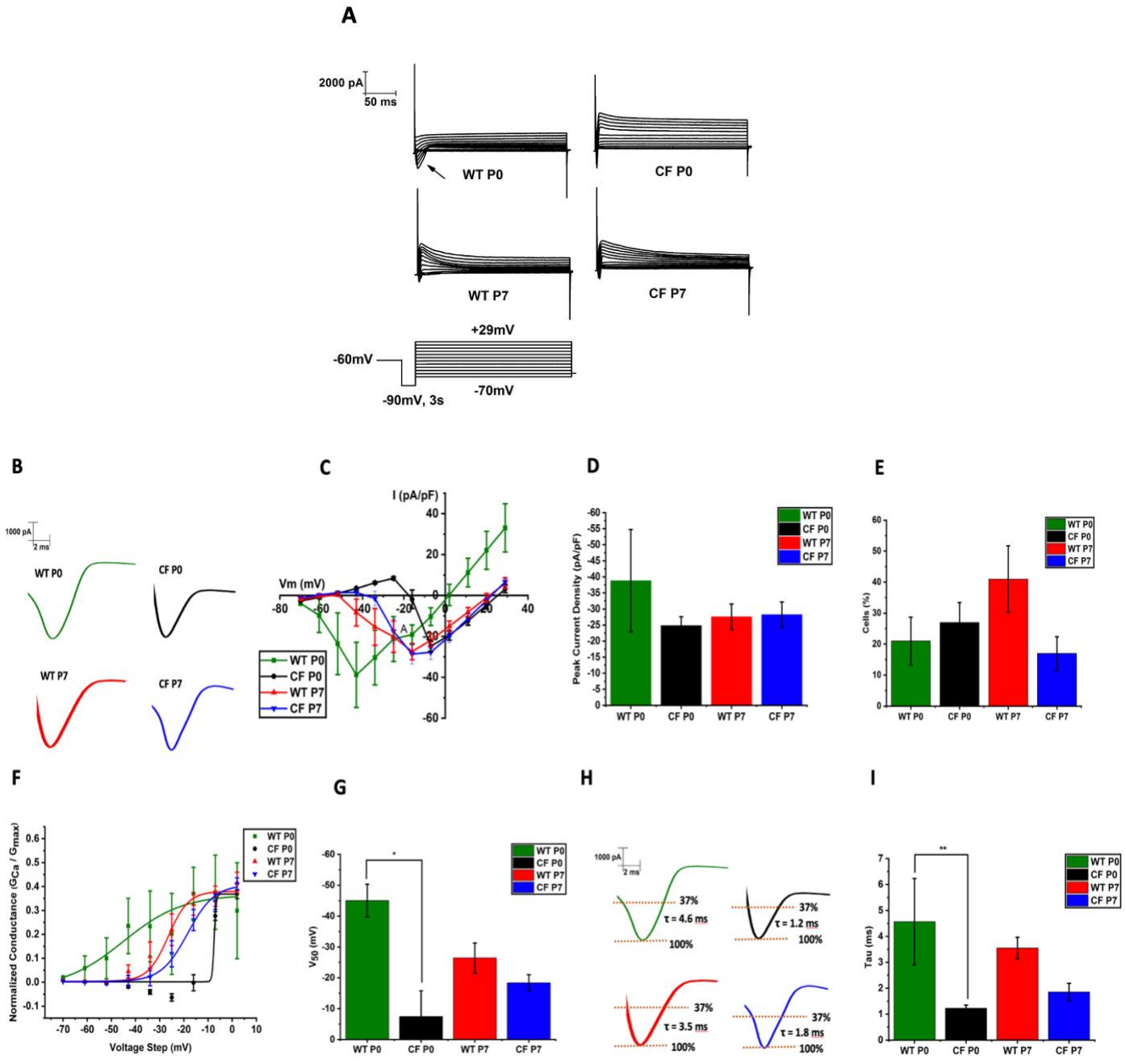
A
2000 pA
50 ms
WT P0
CF P0
WT P7
CF P7
+29mV
-60mV
-90mV, 3s
-70mV
B
1000 pA
2 ms
WT P0
CF P0
WT P7
CF P7
C
I (pA/pF)
Vm (mV)
WT P0
CF P0
WT P7
CF P7
D
Peak Current Density (pA/pF)
WT P0
CF P0
WT P7
CF P7
E
Cells (%)
WT P0
CF P0
WT P7
CF P7
F
Normalized Conductance (GCa / Gmax)
Voltage Step (mV)
WT P0
CF P0
WT P7
CF P7
G
V50 (mV)
*
WT P0
CF P0
WT P7
CF P7
H
1000 pA
2 ms
37%
τ = 4.6 ms
100%
37%
τ = 1.2 ms
100%
37%
τ = 3.5 ms
100%
37%
τ = 1.8 ms
100%
I
Tau (ms)
**
WT P0
CF P0
WT P7
CF P7

Figure 3.5. DRG neurons from CFTR-/- animals had similar calcium currents to WT animals. A. Representative traces of voltage-gated calcium currents generated in cultured DRG neurons from WT P0, CF P0, WT P7and CF P7. **B.** Representative traces showing peak current density (Imax). **C.** The I-V plots show the mean ±SEM inward current densities generated at different voltage steps for WT P0, CF P0, and WT P7 and CF P7. All data are represented as mean ±SEM. **D.** Bar graph shows peak current density ±SEM. Statistical analysis performed by two-way ANOVA followed by a Tukey's post hoc test. There was no significant interaction effect. **E.** Bar graph showing the percentage of neurons that displayed currents consistent with T-type kinetics. Statistical analysis performed by two-way ANOVA followed by a Tukey's post hoc test. There was a significant interaction effect ($p = 0.0497$) **F.** Activation curves for voltage-gated inward currents in DRG neurons. Current-voltage curves from Fig. 3.5C were converted to conductance-voltage relationships and fitted into a Boltzmann equation described in Materials and Methods (WT P0 $r^2 = 0.96$; CF P0 $r^2 = 0.88$; WT P7 $r^2 = 0.93$; CF P7 $r^2 = 0.98$). Representative traces showing τ for each group. **G.** The membrane potential for half-maximal activation (V_{50}). Statistical analysis performed by two-way ANOVA followed by Sidak's multiple comparison test; *, $p < 0.05$. There was a significant main effect between genotypes ($F_{(1, 31)} = 9.317$, $P = 0.0046$). There was no significant interaction effect. **H and I.** Example of T-type calcium current traces and time constant (τ), measured as the time required for the maximum current to decay to 37%. Statistical analysis performed by two-way ANOVA followed by a Tukey's post hoc test; **, $p < 0.01$. There was a significant main effect between genotypes ($F_{(1, 31)} = 20.01$, $P < 0.0001$). There was no significant interaction effect.

3.4 DRG neurons from CFTR-/- animals had reduced capsaicin-evoked currents

In order to evaluate the responses of DRG neurons to sensory stimuli, we tested the function of the TRPV1 receptor using capsaicin (1μM; 1 s). Capsaicin currents were compared between WT and CFTR-/- groups at different postnatal stages, P0 and P7. DRG neurons lacking CFTR expression had reduced capsaicin currents in both P0 and P7 groups (Fig. 3.6A). This reduction in current density was statistically significant at P7 ($p < 0.001$). The peak current density for WT P7 and CFTR-/- P7 were -30.24 ± 3.35 and -17.53 ± 1.86, respectively. There were no significant differences ($p < 0.05$) between WT P0 and CF P0 (Fig. 3.6B). The peak current density (pA/pF) for these groups were -11.40 ± 1.51 and -4.63 ± 1.07, respectively. In addition, and contrasting with our observations in AP generation and Cav currents, there was a significant increase in the peak amplitude of CAP-evoked current from P0 to P7 in both genotypes (Fig. 3.6B).

The comparison of the maximal capsaicin-evoked current charge (Q_{max}) showed, that DRG neurons from CFTR-/- swine had reduced Q_{max} (pC/pF) in both P0 and P7 groups (Fig 3.6C). The reduction was statistically significant in WT P7 and CFTR-/- P7 ($p < 0.05$). The Q_{max} (pC/pF) for WT P7 and CFTR-/- P7 were 8.23 ± 1.19 and 4.78 ± 0.75, respectively. There were no statistically significant differences ($p < 0.05$) between WT P0 and CF P0. Q_{max} (pC/pF) for these groups were 3.07 ± 0.34 and 1.41 ± 0.35, respectively.

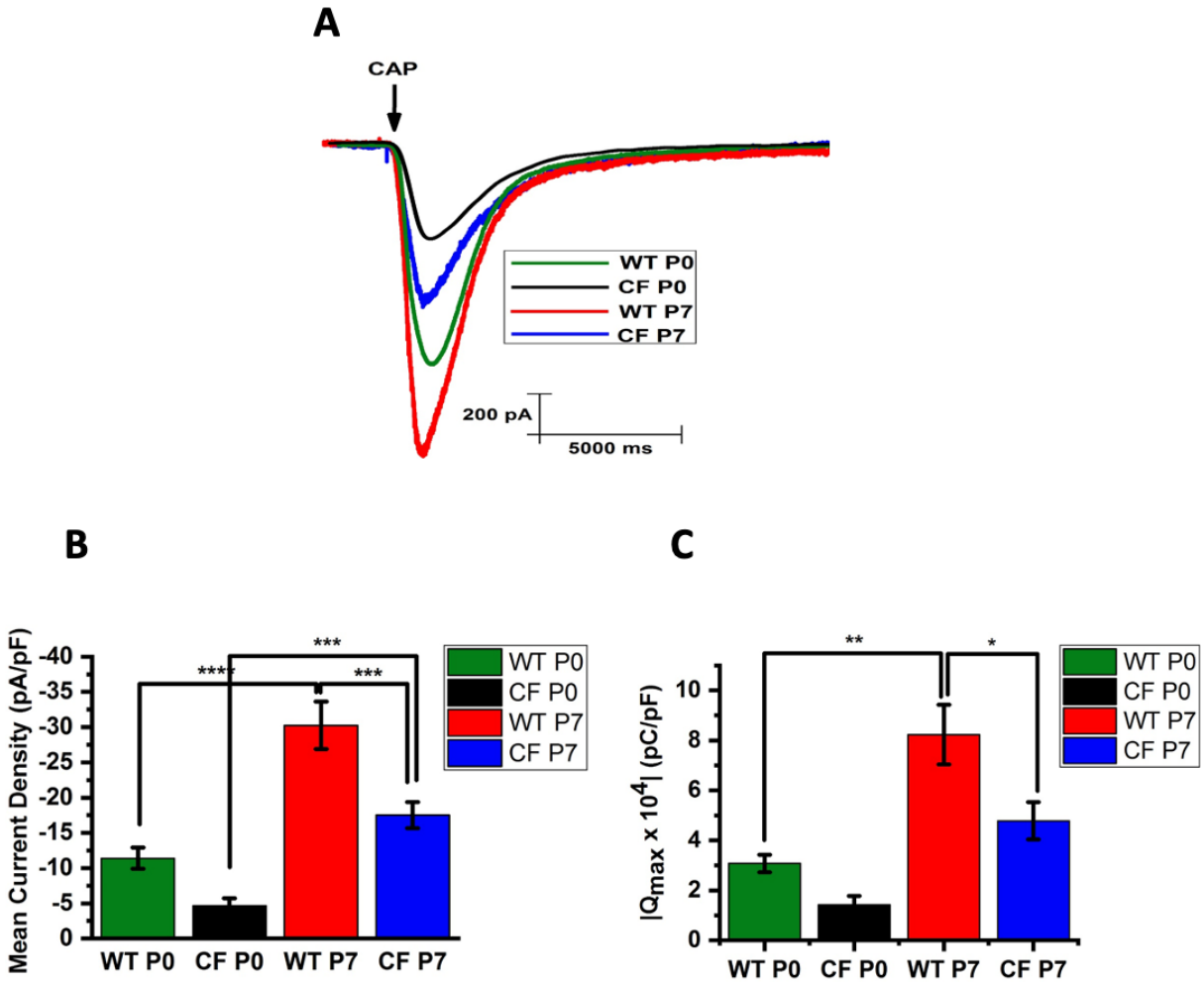

Figure 3.6. DRG neurons from CFTR-/- animals had reduced capsaicin-evoked currents. A. Representative capsaicin-evoked current traces from WT P0, CF P0, WT P7, and CF P7. **B.** Mean current density (pA/pF) values of capsaicin (1µM; 1s)-evoked currents (VH = -60mV) from cultured WT P0, CF P0, WT P7, and CF P7. Statistical analysis performed by two-way ANOVA followed by a Tukey's post hoc test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****,$P<0.0001$. There was a significant main effect between genotypes ($F_{(1, 74)} = 7.226$, $P = 0.0089$). There was no significant interaction effect. **C.** Bar graphs summarizing maximal current charge (Qmax) from cultured WT P0, CF P0, WT P7, and CF P7. WT P0 (n=12), CF P0 (n=14), WT P7 (n=16), CF P7 (n=36). All data are represented as mean ± SEM. Statistical analysis performed by two-way ANOVA followed by a Tukey's post hoc test; *, $p < 0.05$; **, $p < 0.01$. There was a significant main effect between genotypes ($F_{(1, 74)} = 15.12$, $P = 0.0002$). There was no significant interaction effect.

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 Findings

The experiments discussed in Chapter 3 were performed using whole-cell patch clamp electrophysiology in DRG neurons from WT and CFTR-/- swine, in order to generate voltage-gated currents, action potentials and to stimulate ligand-gated ion channels. Our findings revealed that sensory neurons from CF and WT swine had similar passive membrane parameters. In addition, cells from both genotypes survived under culture conditions for the duration of the *in vitro* experiment (up to 4 weeks).

The electrophysiological analysis between DRG neurons from WT and CFTR-/- swine (at P0 and P7) included in the current thesis, is the first attempt in the field to understand the mechanisms underlying peripheral neuropathy in CF. Our observations show that sensory neurons that innervate the viscera are less excitable in the CF swine, which could be related to an abnormal postnatal maturation process in these cells. In particular, the generation of action potentials, the percentage of cells expressing T-type calcium currents, as well as the responses to capsaicin, which were all similar between the genotypes at P0, but show significant differences at one week in postnatal life. The sensory neuron abnormalities that we report in the current thesis revealed

that lack of CFTR in DRG neurons can lead to sensory dysfunction through either direct or indirect mechanisms. These findings provide for the first time a possible link between neurogenic abnormalities and GI complications of CF.

4.2 Neuropathy in CF

Neurons in the peripheral nervous system, such as DRG neurons, express CFTR (Kanno and Nishizaki, 2011). However, until now, the role of CFTR in peripheral neuron physiology has not been explored. Immunocytochemical studies showed that CFTR was expressed in neurons alone, but not satellite cells, fibroblasts, or Schwann cells (Kanno and Nishizaki, 2011). Sensory neurons from the DRGs are responsible for detecting multiple stimulation modalities, including noxious stimuli. These neurons, which are known to contribute to pain pathologies, are sensitized by a combination of physiological and biochemical changes during chronic inflammatory conditions (Kim et al., 2016).

Peripheral neuropathy has been reported in people and animal models of CF (Chakrabarty et al., 2013; Reznikov et al., 2013). Until recently, abnormalities of the peripheral nervous system in CF patients were mainly attributed to secondary manifestations of the disease. However, further research in this field using newborn CFTR-/- swine show abnormal function of the peripheral nervous system at birth (Reznikov et al., 2013). In these animals, the loss of CFTR impacted Schwann cells, causing ultrastructural myelin sheath abnormalities. The Schwann cell pathology

developed in CFTR-/- swine shared similar peripheral nerve abnormalities with Charcot–Marie–Tooth disease, in which mutations in the myelin gene cause myelin infoldings, reduced axon density, slowed nerve conduction velocity, and subsequent neuropathy (Reznikov et al., 2013). These findings are consistent with previous studies showing that axonal and demyelinating neuropathy in CF patients (El-Salem et al., 2010; O'Riordan et al., 1995; Cynamon et al., 1988). Moreover, functional studies in pediatric CF patients showed that 56% of patients had reduced nerve conduction in sensory nerves (El-Salem et al., 2010).

CF can also impact the nervous system indirectly. GI complications of CF, are responsible for pancreatic insufficiency and impaired fat absorption in approximately 85% to 90% of people with CF. These individuals are also prone to malabsorption of the fat-soluble vitamins, including vitamin E (Dodge and Turck, 2006; Sinaasappel et al., 2002). Low vitamin E levels in CF patients put them at an increased risk of cerebellar ataxia, myopathy, pigmented retinopathy, loss of vision, and sensory motor neuropathy (Ueda et al., 2009; Suskind, 2009). The latter, which manifests as loss of reflexes and generalized weakness, is consistent with our own findings of depression of sensory function in DRG neurons of CFTR-/- swine.

4.3 The link between sensory neuron impairment and gut motility

The coordinated movement of ingested material through the digestive tract to absorb nutrients and expel waste is crucial for life (Rao, 2019). Intestinal dysmotility in CF can potentiate

infections, cause nutrient malabsorption, and manifest with debilitating symptoms, such as diarrhea and constipation. This in turn negatively affects CF patient health and quality of life.

The motor actions that are responsible for the traffic of intestinal contents are controlled and modulated by the combined action of intrinsic and extrinsic neurons of the GI tract, which express CFTR (Kanno and Nishizaki, 2011). The possibility that neuropathy in CF may contribute to the manifestation of abnormalities in gut motility has never been considered. Although all the cellular players essential for neurogenic peristalsis are within the gut, in practice, extrinsic innervation also has a role in modulating intestinal motility. Spinal afferent neurons, with cell bodies located in DRGs adjacent to the spinal cord, extend projections into the gut wall that form extensive networks around myenteric neurons; however, the functional significance of this innervation until now was unexplored.

DRG neurons innervating the gut are mainly associated with the transmission of noxious stimuli and visceral sensations (Lai et al., 2017). DRG neurons are highly diverse in terms of cell size, gene expression, and myelination levels. While small-diameter neurons are the pain-sensing neurons, medium-to-large diameter neurons preferentially detect low threshold non-painful mechanical stimulation (Basbaum and Woolf, 1999; Liu and Ma, 2011).

Recent studies have shown that DRG neurons have a more complex role. The DRG is an important site of visceral afferent convergence. Specifically, the DRG neurons that innervate the gut express TRPV1 and ingesting capsaicin stimulates gut motility. Spinal afferent neurons expressing TRPV1

are able to modulate the activity of enteric neurons and increase colonic contractions by activating parasympathetic pre-ganglionic neurons in the spinal cord (Smith-Edwards et al., 2019). This shows that DRG neurons can also function to tune intestinal traffic. For example, activation of DRG neurons positively regulates gut motility in a colonic in situ preparation that preserves the connections between the ENS, DRG, and spinal cord. This shows that a complete afferent-to-efferent extrinsic circuit is necessary for DRG neurons to modulate myenteric neurons and in turn affect gut motility. Therefore, the function of DRG neurons is not just inputs of visceral pain, as they are also able to influence gut motility during conditions that activate the pain circuitry. Activation of pain fibers innervating the gut can engage higher centers to integrate and tune the motility levels required for effective intestinal traffic. Based on these studies, we propose that the output resulting in adjustments to gut motility triggered by engaging DRG neurons can be potentially impaired in CF. Our electrophysiological results show the novel observation of a marked decrease in sensory function in the DRGs of CFTR-/- swine, strongly pointing at the possible involvement of impaired sensory responses and abnormal gut motility in the CF pathology.

4.3 Abnormal Cav currents and its relevance in DRG excitability

The low voltage-activated CaV3.2 channel that is responsible for generating T-type calcium currents in DRG neurons, controls neuronal excitability during sensory perception (Jacus et al., 2012; Garcia-Caballero et al., 2014). Our study revealed that in CF P7 swine there is a reduction

trend in the percentage of DRG neurons expressing T-type calcium currents, and that cells expressing these currents had abnormal activation and inactivation kinetics. Kinetic analysis of the T-type currents observed in DRG neurons from CFTR-/- swine showed a shift to more positive potentials in the activation voltage as well as faster decay. These values were significantly different from those of WT neurons at P0, and they showed a similar non-significant trend at P7. These findings indicate that Cav channels required a stronger depolarization to become activated, and once active, their effect was shorter in time resulting all together in less calcium influx and reduced AP firing. These observations are particularly relevant for synchronous DRG activity, DRG neurons express gap junctions and their electrical coupling is essential for sensory transduction. An increase in DRG coupling has been linked to the development of visceral pain (Huang et al., 2010). Kim et al. developed an imaging technique that simultaneously monitors the activities of thousands of DRG neurons in live mice indicating the cells were electrically coupled, in which adjacent neurons tend to activate together following tissue injury (Kim et al., 2016). This coupled activation occurs among various neurons and increases in injured animals. Blocking gap junctions attenuated neuronal coupling and mechanical hyperalgesia (Kim et al., 2016).

The reduction in the percentage of neurons expressing T-type calcium currents may contribute to tuning down the sensory system, further supporting the concept of decreased sensory function in CFTR-/- swine. At this point we are unable to draw any firm conclusions on how lack of CFTR may lead to a reduction in the number of DRG neurons expressing T-type calcium current, this requires further investigation. However, there is evidence from epithelial cells showing that CFTR has the ability to interact physically with other channels. By direct physical interaction, CFTR

inhibits the function of the Epithelial Sodium Channel (ENaC) (Gentzsch et al., 2010). ENaC function is critical in regulating sodium absorption by epithelia and helps to maintain total body salt and volume homeostasis (Benos and Stanton, 1999; Boucher, 2004; Schild, 2004). ENaC expression is not limited to non-neuronal tissues. In fact, ENaC and Cav3.2 are co-expressed in neurons from the lumbar DRGs, as well as in the thalamus, hypothalamus, hippocampus, and dorsal horn (Garcia-Caballero et al., 2019; Fricke et al., 2000; Drummond et al., 2000), and ENaC was found to regulate the expression levels of Cav3.2 channels. The Aβγ-ENaC form of the channels enhances Cav3.2 calcium channel trafficking to the plasma membrane. Cav3.2 channel expression also enhances β-ENaC trafficking to the cell surface. Thus, it is feasible that dysregulation of the CFTR-ENaC interaction in the DRG neurons of CF swine may alter expression and/or function of Cav3.2 channels (Garcia-Caballero et al., 2019). The latter is speculative at this point but highlights the need for further studies to understand the interactions that CFTR may have with other channels and intracellular pathways in the nervous system.

4.4 Postnatal maturation of DRG neurons

Neurotrophins play essential roles during embryogenesis, regulating growth, proliferation, and survival of neurons. The role of neurotrophins further extends after birth as they regulate physiology and differentiation of sensory neurons into different populations, including the expression of ion channels and cell surface receptors (Aloe, 2004; Yuen et al., 1996). The neurotrophin family is comprised of nerve growth factor (NGF), brain-derived neurotrophic

factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). Neurotrophins bind to neurotrophin receptor (NGFR or p75) and a family of tyrosine kinase receptors, tropomyosin-related kinase A (trkA), trkB, and trkC (Cohen et al., 2016). NGF preferentially binds trkA, while BDNF binds trkB, and NT-3 binds trkC (Cohen et al., 2016).

While trkA is widely expressed by sensory neurons during embryogenesis, the sensitivity to NGF declines postnatally. Findings from mice show that during embryogenesis, 80% of DRG neurons are trkA-positive (Farinas et al. 1998) at embryonic days 13 to 15 (White et al. 1996; Molliver and Snider 1997; Molliver et al. 1997; Benn et al. 2001), and this high percentage of trkA-positive cells can still be detected in both neonatal mice and rats three weeks after birth (Ernsberger, 2009). Immunohistochemical studies in rats and mice show a 60% reduction of trkA-positive DRG neurons at P7 (Bennett et al. 1996a; Molliver and Snider 1997; Benn et al. 2001). Studies conducted using adult rodents show that trkA-positive neurons reach approximately 40% by the third and second postnatal week in rodents (Luo et al. 2007). In contrast, the expression of trkB in DRG neurons during embryogenesis is much more restricted (Ernsberger, 2009). The BDNF sensitivity of DRG neurons increases after birth, and BDNF is required for the survival of a significant fraction of peptidergic and non-peptidergic DRG neurons during the postnatal period. BDNF null mice loose approximately 50% of DRG neurons during the first 2 weeks of life (Valdes-Sanchez et al., 2010). BDNF is predominantly produced within sensory ganglia, contrasting with the mostly peripheral (skin) production of NGF, and is more abundant than skin-derived NGF or BDNF (Valdes-Sanchez et al., 2010).

In addition to survival, NGF and BDNF signalling is essential for the differentiation of DRG neurons. NGF is required for peptidergic nociceptors that express the neuropeptides CGRP and substance P. Furthermore, TRP channel expression is also regulated by NGF signalling during development and the postnatal period (Ernsberger, 2009). BDNF signalling, for example, is required for myelination of some populations of DRG neurons (Xiao et al., 2009). Additionally, a strong relationship between NGF and pathological pain signalling has been established. The NGF–trkA complex modulates expression levels of a variety of cell surface receptors involved in nociception in DRG neurons. NGF has been linked to an increase in bradykinin receptors, acid-sensing ion channels (ASIC) 2/3, voltage-gated sodium channels, voltage-gated calcium channels, delayed rectifier potassium channels, putative mechanotransducers, as well as TRPV1 receptor-mediated currents (Cohen et al., 2016). The increase in TRPV1 currents can be due to a decrease in the TRPV1 activation threshold or an increase in receptor trafficking to the cell surface (McKelvey et al., 2013; Chuang et al., 2001; Stein et al., 2006; Cohen et al., 2016).

Our data revealed that action potential, CAP-evoked currents, as well as the percentage of cells expressing T-types currents normally increase in the WT genotype between P0 and P7. However, in the CFTR-/- genotype those values remained comparable to P0. The latter suggest that functional changes taking place during the postnatal period may be impaired in the CFTR-/- genotype. The generation of burst firing patterns in DRG neurons in the postnatal period, as well as the increase in T-type calcium currents, result from genetically programmed increases in channel density (Lovinger and White, 1989). Moreover, trophic input such as NGF from the spinal cord, periphery, or other cell types into the DRG can in combination contribute to the increase in

T-type calcium currents during postnatal development. A study by Lovinger and White (1989) in rats showed that the T-type calcium current correlated positively with the age of the animal, when compared between postnatal P1 and P12. It remains to be investigated whether neurotrophic factors are affected in the CFTR-/- genotype. In view of the strong involvement of neurotrophins signalling in ion channel expression and DRG neuron differentiation, an extensive study of neurotrophin receptors in the CFTR-/- genotype is required to better understand this point.

4.5 Role of TRPV1 in pain transduction

The capsaicin receptor, TRPV1 is a main component of signal transduction in nociception. Activation of the TRPV1 channel causes depolarization at the peripheral terminal, which in turn may cause AP firing. Sensory information is then conveyed from the peripheral terminal to the relay station in the spinal dorsal horn. This information is then sent to the brain to be integrated as sensory perception (Boron and Boulpaep, 2017). TRPV1 is selectively expressed in a subset of primary afferent nociceptors, and its expression is regulated by multiple factors. TRPV1 is expressed in both nociceptive C-fibers and A δ-fibers, which terminate in the spinal dorsal horn. Moreover, TRPV1 is localized to both pre- and post-synaptic neurons in lamina I and II, glial cells (Doly et al., 2004), as well as supraspinal localization that contributes to descending modulation of nociceptive stimuli (Maione et al., 2009). Our results show that capsaicin-evoked currents were significantly reduced in the CFTR-/- genotype at both P0 and P7 stages compared to WT. Based

on our electrophysiological data, we cannot conclude whether the reduction in CAP-evoked currents in the CFTR-/- genotype is the result of reduced TRPV1 expression or posttranslational modifications that lead to reduced surface expression or conductance of the channel. Further molecular and biochemical studies are required to address this point.

TRPV1 mRNA and receptor protein expression increases under pathophysiological conditions in DRG neurons. This is associated with hyperalgesia. The distribution and function of TRPV1 channel has made this channel one of the most researched in the context of different chronic pain conditions and numerous studies have shown altered TRPV1 expression in chronic pain. Several studies have documented upregulation of pro-nociceptive ion channels such as TRPV1. Interestingly, studies show that the changes in expression levels and currents of TRPV1 differ depending on the time after injury. For example, downregulation of TRPV1 is reported in DRG neurons two weeks following nerve transection or spinal nerve ligation (Hudson et al., 2001). In other similar studies, spinal nerve ligation at L5 caused decreased expression of TRPV1 in the damaged DRG (Fukuoka et al., 2002). Chronic constriction injury animal models also showed that one to three days after injury there were no changes in expression levels of TRPV1, but increased expression was observed seven to fourteen days after injury (Kanai et al., 2005). Moreover, Hong and Wiley found that membrane expression of TRPV1 protein in rats was increased in myelinated A-type fiber DRG cells, while it was decreased in small unmyelinated C-type nociceptive DRG cells in early painful diabetic neuropathy (Hong and Wiley, 2005). Not only do these studies provide compelling evidence for a pivotal role for TRPV1 channels in a variety of pain conditions but also, they show how pathological conditions can modulate its function, impacting sensory function.

4.6 CFTR-/- swine as a model of CF

In the current thesis we used the CF swine because they are the best animal models of the CF pathology. CF swine recapitulates the pulmonary and extrapulmonary aspects of the disease that manifests in humans. The latter is not seen in mouse models of the disease, which do not develop pulmonary complications and extrapulmonary defects develop more prominently than in humans. In particular, intestinal disease is the major defect in the CF mouse, which develops one week after birth (Rommens et al., 1989; De Lisle et al., 2012) and the spectrum of severity is highly variable (Olivier et al., 2015), interfering with the reproducibility of data.

Although, CF swine carrying the homozygous delta F508 CFTR mutation are commercially available, the only previous study demonstrating the development of peripheral neuropathy at birth in CF swine was carried in CFTR-/- piglets (Reznikov et al., 2013). Therefore, we have chosen this particular CF model for the current study. Our results, point at the relevance of extending our studies to other swine models of CF in order to better understand the onset of development of peripheral nervous system complications in CF.

An important consideration of swine models of CF is that they all suffer from meconium ileus at birth (Meyerholz et al., 2010; Rogers et al.,2008; Stoltz et al., 2010). Therefore, to circumvent this life-threatening complication, CFTR-/- gut-corrected animals have been commercially developed (Exemplar Genetics). Gut-corrected animals express intestinal-specific CFTR, under the intestinal

fatty acid-binding protein promoter, which has been developed to alleviate meconium ileus in newborn swine. Gut corrected swine express approximately 20% of the normal level of CFTR in gut epithelia. This is sufficient to alleviate meconium ileus, but the animals still develop other GI complications characteristic of the CF pathology, such as pancreatic insufficiency and constipation (Stoltz et al.,2013). As mentioned earlier, CFTR-/- swine present peripheral neuropathy at birth, suggesting that CFTR may have a critical role in normal nervous system physiology. The work of Reznikov et al. (2013) on CFTR-/- pigs shows peripheral nervous system complications including myelin sheath abnormalities, demyelination, and nerve conduction studies revealed the occurrence of sensory-motor neuropathy. Therefore, the research included in the current thesis aimed at providing knowledge on mechanisms linked to abnormal sensory neuron function in CFTR-/- that may underlie sensory neuropathy and may contribute to chronic CF complications such as GI abnormalities.

4.7 Conclusion and Future directions

The current study is the first to concentrate on the electrophysiological properties of DRG neurons from swine models of CF. The findings of this study suggest that the lack of CFTR expression profoundly impacts the normal functions of these cells, providing the basis for sensory impairment in CF. As a result, sensory dysfunction at the lumbar level potentially contributes to

the GI complications hallmark of CF. Dysregulation of TRPV1 channels is important in the development of abnormal pain signalling in DRG neurons. Our study shows that CFTR-/- DRG neurons have reduced capsaicin currents compared to WT. Further investigation is required to elucidate the physiological significance of these findings.

CFTR has been studied in various types of cells. The molecular mechanisms connecting abnormal CFTR function in DRG neurons to development of peripheral neuropathy in CF patients has not been addressed before. Studies looking at the effects of CFTR in nervous tissue show that this protein plays a variety of different roles in neurons. Oxidative stress is a significant complication of CF, both in adults and young children (Kettle et al., 2004; Brown and Kelly, 1994; van der Vliet et al., 1997). For example, lack of CFTR disrupts mitochondrial function and leads to mitochondrial oxidative stress (Zeng et al., 2014; Valdivieso and Santa-Coloma, 2013; Velsor et al., 2006; Shapiro, 1988). This in turn plays a key role in activating the apoptotic pathway that leads to increased neuronal apoptosis (Zhang et al., 2018). It is still unclear how CFTR depletion causes mitochondrial oxidative stress. Moreover, it is not known whether oxidative stress is an important feature early on the course of this disease. Therefore, it would be relevant to see how early on in the disease mitochondrial dysfunction occurs in neurons in CF and whether the changes in channel function in DRG neurons observed in our study are related to oxidative stress.

Another important aspect of CF that contributes to lung damage is inflammation, which has been extensively studied in adults. Inflammation in CF and its effect on the nervous system early on in the disease has not been studied. Studies have revealed that dysfunction of CFTR causes

exaggerated inflammatory responses in pulmonary tissue by increasing the gene expression of several proinflammatory molecules such as interleukin (IL)-1β, IL-8,IL-17 and toll-like receptor-2 (TLR2) (Osika et al., 1999; Aujla et al., 2008; Shuto et al., 2006). These reports suggest a role of CFTR as a suppressor for inflammation (Jacquot et al., 2008). Our study focused on the effects of CF early on at the postnatal stage. There is evidence showing that airway inflammation in CF begins early during the neonatal period. However, there are no definitive conclusions on whether inflammation that results in subsequent lung damage is a cause or a consequence of infection in young patients. Therefore, it is worth exploring the presence and role of inflammatory markers in nervous tissue and the subsequent development of neuropathy early on during the postnatal period. It would be interesting to see how early on in the disease inflammation is a contributing factor to peripheral neuropathy and gut dysmotility and whether this contributes to changes in Cav3.2 expression and capsaicin currents in DRG neurons.

Finally, pain sensitivity can be evaluated in larger animals such as swine using using mechanical nociception threshold (MNT) and thermal nociception threshold (TNT) tests (Tapper et al.,2013; Mohling et al., 2014). In the future, it would be relevant to this study to use MNT and TNT in behavioral tests in the swine model of CF in order to provide valuable information about the extent to which the pain circuitry is affected by lack of CFTR.

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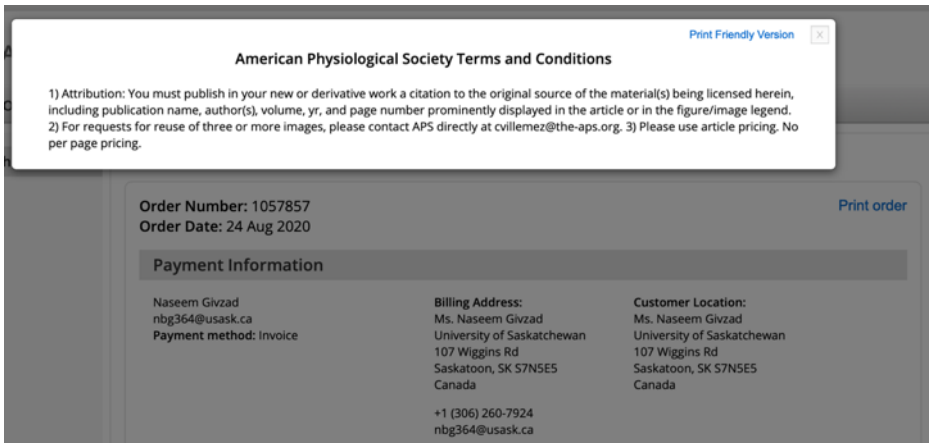

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