THE ROLE OF THE CYSTIC FIBROSIS TRANSMEMBRANE REGULATOR (CFTR) IN CHLORIDE HOMEOSTASIS AND EXCITABILITY IN SWINE SENSORY NEURONS

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ABSTRACT

Cystic Fibrosis (CF) is a common fatal disorder associated with mutations of recessive inheritance in the gene that encodes for the cystic fibrosis transmembrane regulator (CFTR) protein. In the past, the life expectancy of patients with CF was 6 months due to respiratory failure and gastrointestinal (GI) complications. However, with current advances in the treatment and management of the disease, the life expectancy of CF has improved drastically. As a consequence, now adult CF patients are exposed to previously unknown chronic complications of CF such a peripheral neuropathy. Initially, peripheral neuropathy was considered a consequence of malnutrition and vitamin E deficiency, however, later studies showed that CFTR is widely expressed in the nervous system and may be an important regulator of chloride homeostasis in neurons. Unfortunately, the role of CFTR in neurons and its relevance in CF neuropathology has not been studied in detail.

Therefore, this project aimed to investigate the cellular consequences of the lack of CFTR function in dorsal root ganglion (DRG) neurons. First, we quantified chloride levels in DRG primary cultures from WT and CFTR-/- swine using a chloride sensitive dye (i.e., MQAE) and perforated patch-clamp electrophysiology. We found that CFTR-/- DRG neurons have a lower intracellular concentration of chloride compared to WT neurons. Second, we used a CFTR inhibitor (i.e., CFTR (inh)-172) and current-clamp electrophysiology to determine if short-term inhibition of CFTR had abnormal effects in DRG neuronal excitability and GABA mediated inhibition of action potentials. We observed that CFTR inhibition in DRG neurons had no effect on DRG neuronal excitability. Moreover, our findings indicate that GABA did not modulate action potential firing in DRG neurons from swine. Taken together, our data suggest that CFTR has an active role in the regulation of chloride transport in DRG neurons. However, if these changes have a significant effect on neuronal excitability remain to be studied in more detail.

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LIST OF ABBREVIATIONS

$[Cl^-]_i$	Intracellular Concentration of Chloride
ABC	Adenosine 5`-Triphosphate-Binding Cassette
ANOVA	Analysis of Variance
ARA-C	Cytosine Arabinoside
ATP	Adenosine 5`-Triphosphate
BDNF	Brain-Derived Neurotrophic Factor
BNaCs	Mammalian Brain Sodium Channels
CCC	Cation Chloride Cotransporter
CCI	Chronic Constriction Injury
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Regulator
CFTR-/-	Cystic Fibrosis Transmembrane Regulator Knock Out
CGRP	Calcitonin-Gene Related Peptide
Cl-	Chloride
C_m	Capacitance
DHN	Dorsal Horn Neurons
DIOS	Distal Intestinal Obstruction Syndrome
DMEM	Dulbecco`s Modified Eagle Medium
DRG	Dorsal Root Ganglia
ECF	Extracellular Fluid
E_{Cl}	Equilibrium Potential of Chloride
EGTA	Ethylene Glycol-bis(2-aminoethyl ether)-N,N,N',N'- Tetraacetic acid
F	Faraday Constant
GABA	ϒ-aminobutyric acid
GAD65	Glutamate Decarboxylase 65
GAD67	Glutamate Decarboxylase 67
GAT	ϒ-aminobutyric acid Transporter
GHK	Goldman-Hodgkin-Katz
GI	Gastrointestinal

H^+	Hydrogen ion
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
ICF	Intracellular Fluid
iFABP	Fatty Acid -Binding Protein
IV	Current-Voltage
K^+	Potassium ion
KCC2	Potassium Chloride Cotransporter-2
K_{SV}	Quenching Rate Coefficient or Stern-Volmer Constant
MgATP	Adenosine 5'-Triphosphate Magnesium Salt
MQAE	N-Ethoxycarbonylmethyl-6-Methoxyquinolinium Bromide
NBD	Nucleotide Binding Domain
NKCC1	Sodium-Potassium Chloride Cotransporter-1
OH^-	Hydroxide ion
OSR1	Oxidative Stress Responsive Kinase 1
P0	Postnatal Day 0
P7	Postnatal Day 7
PAG	Periaqueductal Gray
PBS	Phosphate-Buffered Saline
pCFTR	Porcine Cystic Fibrosis Transmembrane Regulator
PKA	Protein Kinase A
PKC	Protein Kinase C
PNI	Peripheral Nerve Injury
R	Universal Gas Constant
R_a	Access Resistance
R_m	Membrane Resistance
RVM	Rostral Ventral Medulla
SEM	Standard Error of the Mean
SPAK	SPS1-Related Proline/Alanine rich Kinase
T	Temperature
VGAT	Vesicular ϒ-aminobutyric acid Transporter
V_m	Membrane Potential

WNK	With no Lysine
WT	Wildtype
Z	Valence
τ	Fluorescence Intensity at a specific concentration of chloride
τ_0	Maximum Fluorescence Intensity

CHAPTER 1.

INTRODUCTION

1.1.Cystic Fibrosis (CF)

Cystic Fibrosis (CF) is a multisystem disorder with genetic etiology predominantly associated with the progressive failure of the respiratory system (Donaldson & Boucher, 2006). The occurrence of CF varies widely according to ethnicity and geographic location, being more common in populations with northern European ancestry (Lubamba et al., 2012). There are more than 80,000 people worldwide and approximately 4,000 Canadians living with CF (Cystic Fibrosis Foundation, 2018; Lubamba et al., 2012). Moreover, in Canada, the clinical complications of CF account for a cumulative of 26,000 days expended in hospital, which affects the quality of life of CF patients and their caregivers, and can result in high medical care expenditures (Cystic Fibrosis Foundation, 2018).

In 1938, Dorothy Andersen first described CF of the pancreas (Andersen, 1938). At that moment, the life expectancy of patients with CF was 6 months due to respiratory failure, meconium ileus, and malnutrition (Davis, 2006). However, with current advances in controlling lung infection, improving mucus clearance, and correcting CFTR function, most patients survive well into adulthood (Elborn, 2016). Indeed, the estimated median projected age of survival in Canada for CF patients is approximately 52.1 years (Cystic Fibrosis Canada, 2018).

CF is caused by mutations of recessive inheritance that result in complete or partial dysfunction of the cystic fibrosis transmembrane regulator (CFTR) (Elborn, 2016). So far in humans, more than 2,000 variants of the CFTR channel have been described, and 360 mutations have been associated with the occurrence of CF, which prevents practical classification of the disease according to mutation type (Cutting et al., 2013; Elborn, 2016). Therefore, CF mutations are classified into five categories depending on their cellular phenotype. Mutations that affect transcription, processing, and gating of the channel (Class I, II, and III, respectively) usually result in a more severe form of the disease. In contrast, mutations that alter the conduction properties or reduce the number of functional channels at the membrane (Class IV and V, respectively) are milder and tend to conserve limited CFTR activity (Cutting et al., 2019).

1.1.1. CFTR

The CFTR is an integral membrane glycoprotein that belongs to the ATP-Binding Cassette (ABC) superfamily and is encoded by the CFTR gene (Riordan et al., 1989). The CFTR gene is highly conserved across different species highlighting its physiological significance. CFTR is responsible for chloride (Cl^-) and bicarbonate transport across the membrane (Hwang & Kirk, 2013). Additionally, there is growing evidence indicating that CFTR regulates the activity of a variety of channels (e.g., sodium-potassium chloride cotransporter 1 (NKCC1), epithelial sodium channel (eNaC), and anoctamin-1 channel (ANO1) through direct or indirect interaction expanding the role of CFTR in preserving osmotic and ionic homeostasis (Berdiev et al., 2009; Kunzelmann, 2003; Lérias et al., 2018; Lubamba et al., 2012; Shumaker & Soleimani, 1999).

CFTR consists of two transmembrane domains, each containing six transmembrane alpha-helices and an intracellular nucleotide-binding domain (NBD). The two domains are linked by a regulatory domain that contains a variety of phosphorylation sites for protein kinase A (PKA), and protein kinase C (PKC) *(Figure 1.1)*. Phosphorylation of the regulatory domain in conjunction with adenosine triphosphate (ATP) hydrolysis at the NBDs appears to favour an open conformation and increased affinity to Cl^- (Cutting et al., 2019; Hwang & Kirk, 2013; Lubamba et al., 2012).

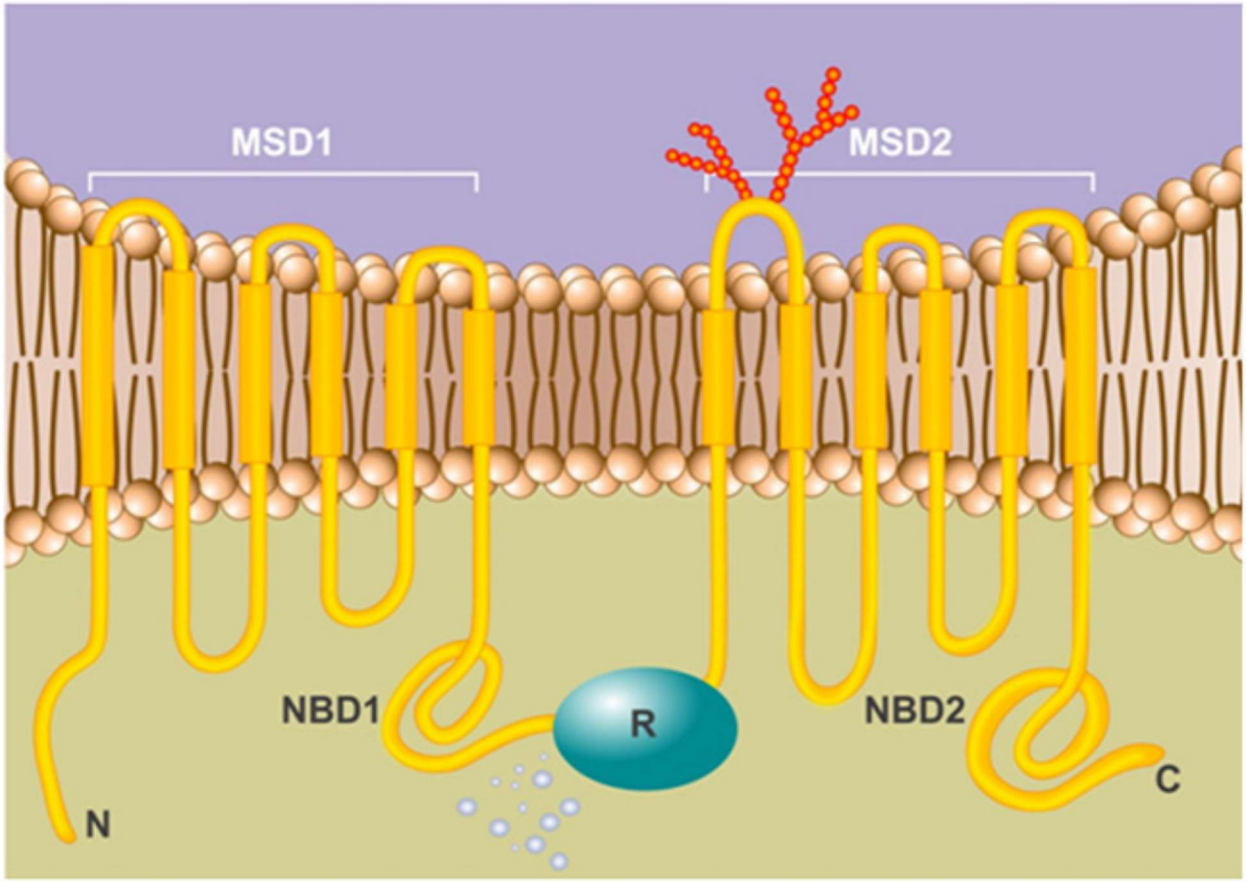

Figure 1.1. The CFTR channel. The CFTR channel is a membrane glycoprotein composed of two transmembrane domains. Each domain is attached to a nucleotide-binding domain (NBD) that binds to ATP and regulates channel opening. In addition, a regulatory domain (R) links both transmembrane domains and serves as a phosphorylation site for protein kinase A (PKA) and C (PKC) (Reproduced with permission from Lubamba, 2012).

1.1.2. Peripheral Neuropathy in CF

With a projected survival rate of approximately 52.1 years (Cystic Fibrosis Foundation, 2018), CF patients are exposed to previously unknown chronic complications of the CF pathology. Peripheral neuropathy is one of the most relevant extrapulmonary pathologies and significantly impacts the quality of life of adult CF patients (Cavalier & Gambetti, 1981; O'Riordan et al., 1995).

Peripheral neuropathy involves the aberrant function of sensory, motor, and autonomic nerves (Yagihashi et al., 2011). From the cellular perspective, peripheral neuropathy has been studied mostly in ion channels, receptors, and signaling pathways associated with cations (Belmonte & Viana, 2008; Campbell & Meyer, 2006; Lai et al., 2004; Naziroğlu et al., 2012; Tomlinson & Gardiner, 2008; Wilke et al., 2020). However, evidence shows that alterations in Cl^- homeostasis can also play a critical role in the regulation of neuronal excitability and the development of peripheral neuropathy (Funk et al., 2008; Lorenzo et al., 2020; Morales-Aza et al., 2004; Sung et al ., 2000; Wilke et al., 2020). This is a relevant subject of research in the context of peripheral neuropathy in CF; On one hand, CFTR is itself permeable to Cl^- ions, and on the other, it regulates surface expression of Cl^- transporters that are responsible for setting the intracellular levels of Cl^- in peripheral neurons *(*see *section 1.2.1).* Thus, CFTR function may be directly linked to both Cl- homeostasis and changes in neuronal excitability.

A variety of clinical, epidemiological, and animal studies support the idea that a lack of functional CFTR expression may be relevant to the pathogenesis of peripheral neuropathy. First, CFTR is expressed in dorsal root, sympathetic, and paracervical ganglia (Niu et al., 2009; Su et al., 2010). Second, reports from CF patients show reduced conduction velocity in both motor and sensory nerves (Chakrabarty et al., 2013; O'Riordan et al., 1995). Third, CF patients express clinical and subclinical signs of autonomic dysfunction including abnormal adrenergic and cholinergic responses in the pupil, sweat glands, and the cardiovascular system. These abnormalities are also present in asymptomatic individuals with partial CFTR function, which suggests that they are indeed the result of autonomic neuropathy rather than a secondary complication of CF (Davis & Kaliner, 1983; Niu et al., 2009; Rubin et al., 1963; Sibinga & Barbero, 1961; Sullivan et al., 1986). Fourth, autopsies from CF patients show significant axonal dystrophy of the central branch of large primary and secondary afferent neurons in the nucleus

gracilis and cuneatus of the brainstem dorsal columns. Interestingly, the degree of axonal dystrophy was correlated with the length of survival of the patient (Cavalier & Gambetti, 1981; Sung, 1964). Finally, CFTR-/- swine have symptoms of peripheral neuropathy at birth, which correlates with loss of Schwann cell function and reduced conduction velocities of trigeminal and sciatic nerves (Reznikov et al., 2013).

Because the nervous system controls and tunes all systemic organ functions, the occurrence of peripheral neuropathy in CF patients underscores its possible contribution to the multisystem complications linked to the CF pathology (Reznikov, 2017). In sensory neuropathy, sensory neurons trigger local inflammatory cascades that could contribute to inflammation in the CF pathology (Balestrini et al., 2021; Barnes, 2003; Pisi et al., 2009). Primary sensory neurons are known to play vital roles in the gastrointestinal (GI) tract. They provide extrinsic modulation of GI motility, locally as well as through relay stations in the spinal cord (Smith-Edwards et al., 2019). Thus, the occurrence of sensory neuropathy in CF may contribute to the development of GI dysmotility and GI-related complications characteristic of CF. Evidence supporting these ideas come from diseases also associated with damage of sensory fibers, such as diabetes mellitus, in which patients develop GI complications and share some symptoms with CF patients (Azpiroz & Malagelada, 2016). Therefore, this project aims to investigate the cellular mechanisms that may lead to the development of sensory abnormalities in CF.

1.2.Primary afferent neurons

Primary afferent neurons are responsible for transducing and transmitting a vast range of sensory information from the periphery or the viscera to the CNS (Lai et al., 2004). In vertebrates, primary afferent neurons are located in dorsal root ganglia (DRG), trigeminal ganglia, or the nodose/jugular complex (Nascimento et al., 2018).

DRG neurons are pseudo-unipolar (i.e., they have one process protruding from the cell body which bifurcates into the peripheral and central branches). The DRG peripheral branch travels in parallel with the ventral roots arising from motor neurons in the spinal cord to form 31 pairs of spinal nerves that innervate skin, muscles, and internal organs (Haberberger et al., 2019; Zhang et

al., 2015). Conversely, the DRG central branch enters the spinal cord through the intervertebral foramen to synapse with secondary sensory neurons in the CNS (Marani, 2014).

DRG neurons are considered a very heterogeneous population (Lai et al., 2004), and they generate a variety of sensory fibers. These primary afferent fibers are usually classified according to their diameter and conduction velocity. Thus, there are four groups of afferent fibers: large diameter myelinated fibers (Aα), medium diameter myelinated fibers (Aβ), small diameter myelinated fibers (Aδ), and non-myelinated fibers (C). Aα and Aβ fibers are responsible for conveying proprioceptive and mechanosensory information, while Aδ and C fibers are responsible for relaying nociceptive information (Duce & Keen, 1977; Harper & Lawson, 1985a, 1985b; Lai et al., 2004). In terms of their anatomical organization, the first site of synapse and the ascending pathway, both in the CNS, also varies according to the type of DRG neuron. Aδ and C fibers typically synapse with dorsal horn neurons (DHN) at laminae II (substantia gelatinosa). The ascending processes of the DHNs travel contralaterally along the spinothalamic tract towards the thalamus (i.e., spinothalamic pathway). On the other hand, the central process of Aα and Aβ fibers will ascend ipsilaterally through the dorsal column and synapse with secondary neurons at the nucleus gracilis or the nucleus cuneatus internus of the brainstem. From there, secondary neurons will travel contralaterally to convey information to the thalamus (i.e., dorsal column-medial lemniscal pathway). All sensory information will be relayed to the thalamus and then projected to the somatosensory cortex (Bear, 2016; Løseth et al., 2021; Marani, 2014) (*Figure 1.3A*).

Traditionally, secondary sensory neurons were considered the first filtering point for sensory information, before reaching the higher sensory regions of the brain. However, the fact that DRG neurons express the receptors for the major regulatory neurotransmitters (i.e., ϒ-aminobutyric acid (GABA), glutamate) suggests that they may act as an earlier filtering point for sensory information (Du et al., 2017). Moreover, the inhibitory effect of Cl^- fluxes through $GABA_A$ receptors seem to play an essential role in the filtering of sensory information at the level of the DRG *(*see *section 1.3)* (Du et al., 2017).

1.2.1. Cl^- homeostasis in DRG neurons

In the nervous system, intracellular Cl^- levels ($[Cl^-]_i$) are mainly regulated by the action of two cation Cl^- cotransporters (CCC): the potassium Cl^- cotransporter-2 (KCC2) and NKCC1. KCC2 is responsible for the efflux of Cl^- while NKCC1 mediates its influx (Wilke et al., 2020).

Changes in the expression levels of these transporters during development are responsible for the "developmental shift" of Cl^- homeostasis in the CNS, resulting in a drastic decrease of the $[Cl^-]_i$ after birth (Mao et al., 2012). At first, the high functional expression of NKCC1 causes the intracellular accumulation of Cl^-, which upon activation of $GABA_A$ receptors result in depolarizing Cl^- currents. However, in the adult brain active extrusion of Cl^- through KCC2 outweighs the activity of NKCC1 resulting in net outward hyperpolarizing Cl^- currents and acute inhibition (Dzhala et al., 2005).

In contrast to the maturation process that occurs in CNS neurons, DRG neurons undergo a rather subtle developmental transition in $[Cl^-]_i$ in which there is no reversion in the polarity of Cl^- currents. In the peripheral nervous system, the low or lack of expression of KCC2 prevents the shift in Cl^- concentration as described in mature central neurons (Funk et al., 2008; Gilbert et al., 2007; Mao et al., 2012). These differences between the regulation of Cl^- homeostasis in the central and peripheral nervous systems is of significant relevance since an increase in the membrane conductance for Cl^-, such as when $GABA_A$ receptors get activated, will have different effects in DRG neurons compared to central neurons. However, it is worth mentioning that the depolarizing effect of GABA in the PNS is still inhibitory since it contributes to sodium channel inactivation and shunting electrical propagation of signals (i.e., decrease in membrane resistance and sodium driving force)(Du et al., 2017; Zeilhofer et al., 2012) (*Figure 1.2*).

Nevertheless, Cl^- homeostasis in DRG neurons is subject to regulation and is a relevant topic in the investigation of sensory pathology due to the significance of the electrochemical gradient of Cl^- in the inhibitory effect of GABA.

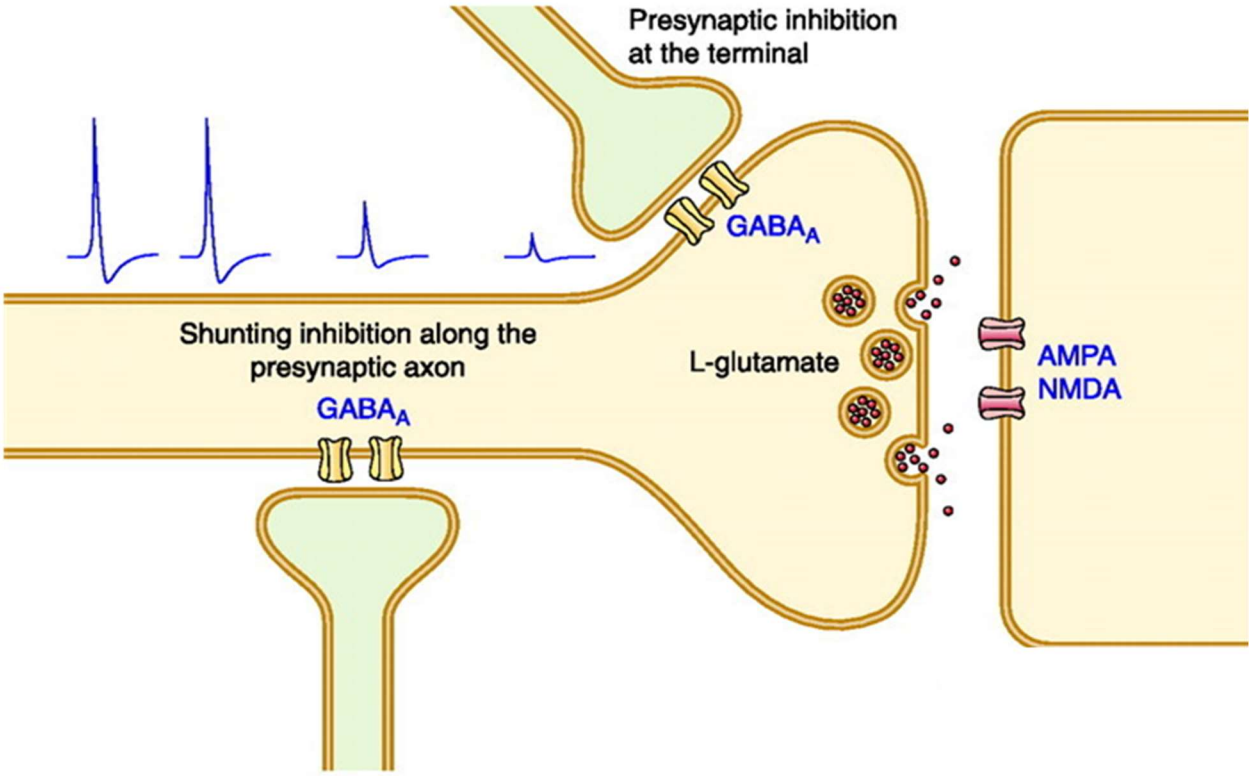

Figure 1.2. GABA-mediated inhibition of action potential propagation at the level of the DRG. Activation of $GABA_A$ receptors in DRG neurons promotes the depolarization of the membrane through the efflux of Cl^- resulting in a voltage-dependent inactivation of sodium channels and a decrease in membrane resistance. This will decrease action potential propagation along the axon, and the release of excitatory neurotransmitters at the central terminal of the DRG neuron. (Modified from Zeilhofer et al., 2012; and reproduced with permission).

1.3. GABA

GABA is considered the main inhibitory neurotransmitter in the nervous system. The synthesis of GABA is catalyzed by the enzyme glutamate decarboxylase 65 and 67 (GAD65 and GAD67). There are two major GABA receptors expressed in DRG neurons: $GABA_A$ and $GABA_B$ receptors. The $GABA_A$ receptor is a ligand-gated ion channel that mediates the flux of Cl^- across the membrane (Wu & Sun, 2015). The structure of the $GABA_A$ receptor in humans is characterized by a heteropentameric conformation composed of different permutations of eight different subunits (α(1-6), β (1-3), ϒ(1-3), ρ(1–3), δ, ε, Θ, and π) with binding sites for GABA, benzodiazepines, barbiturates, and neurosteroids (Sigel & Steinmann, 2012; Treiman, 2001). The most common isoform in the peripheral nervous system consists of two α1, two β2, and one ϒ2 subunit (Sigel & Steinmann, 2012). Conversely, the $GABA_B$ receptor is a metabotropic G protein-coupled receptor that mediates slow inhibitory effects through attenuation of calcium currents and augmentation of potassium currents at the central branch of DRG neurons (Towers et al., 2000).

Generally speaking, GABA-mediated inhibition of DRG neurons occurs at two different levels: the central branch and the soma (Du et al., 2017; Løseth et al., 2021). The inhibitory mechanisms at the central branch involve neurons in the rostral ventral medulla (RVM) and periaqueductal gray (PAG), which give rise to descending pathways that are essential in the modulation of pain. Previously it was thought that these pathways mediated antinociceptive effects solely through the release of GABA and enkephalins onto DH neurons of the spinal cord. However, evidence indicates that these neurotransmitters are also released onto the central branch of DRG neurons. Moreover, inhibition of these descending pathways results in behavioural hypersensitivity to heat, suggesting that GABA released from the brainstem is required to suppress pain under physiological conditions (Løseth et al., 2021; Zhang et al., 2015) (*Figure 1.3B*). In addition to the descending pathways from the brainstem, there are also local GABAergic interneurons in the spinal cord that establish axo-axonic synapses with the DRG neurons. GABA from neurons in the brainstem and the spinal cord induces presynaptic inhibition at the DRG and reduces the release of excitatory neurotransmitters such as substance P and glutamate (Campbell & Meyer, 2006; Moore et al., 2002).

It was previously accepted that the $GABA_A$ receptors highly expressed in the soma of DRG neurons were a by-product of receptor trafficking to the DRG central branch, and that they did not

have any physiological relevance on neuronal excitability, particularly since there was no GABAergic input from the CNS onto the DRG (Du et al., 2017; Fukuoka et al., 1998; Ma et al., 1993). However, recent evidence shows that there is a local GABAergic system at the DRG that is maintained by subpopulations of neurons that release GABA upon stimulation. (Du et al., 2017). First, DRG neurons express GAD65 and GAD67 meaning that they have the molecular machinery for the synthesis of GABA. Second, DRG neurons express the vesicular GABA transporter (VGAT) required to pack GABA into vesicles and various isoforms of the GABA transporter (GAT) required for the extracellular removal of GABA, a function also supported by satellite cells withing the DRG (Du et al., 2017; Schon & Kelly, 1974). Third, sniffing patch-clamp experiments demonstrate that there is somatic release of GABA at the DRG. Finally, *in vitro*, and *in vivo* experiments indicate that somatic activation of $GABA_A$ receptors impedes the propagation of action potentials and reduces pain transduction (Du et al., 2017).

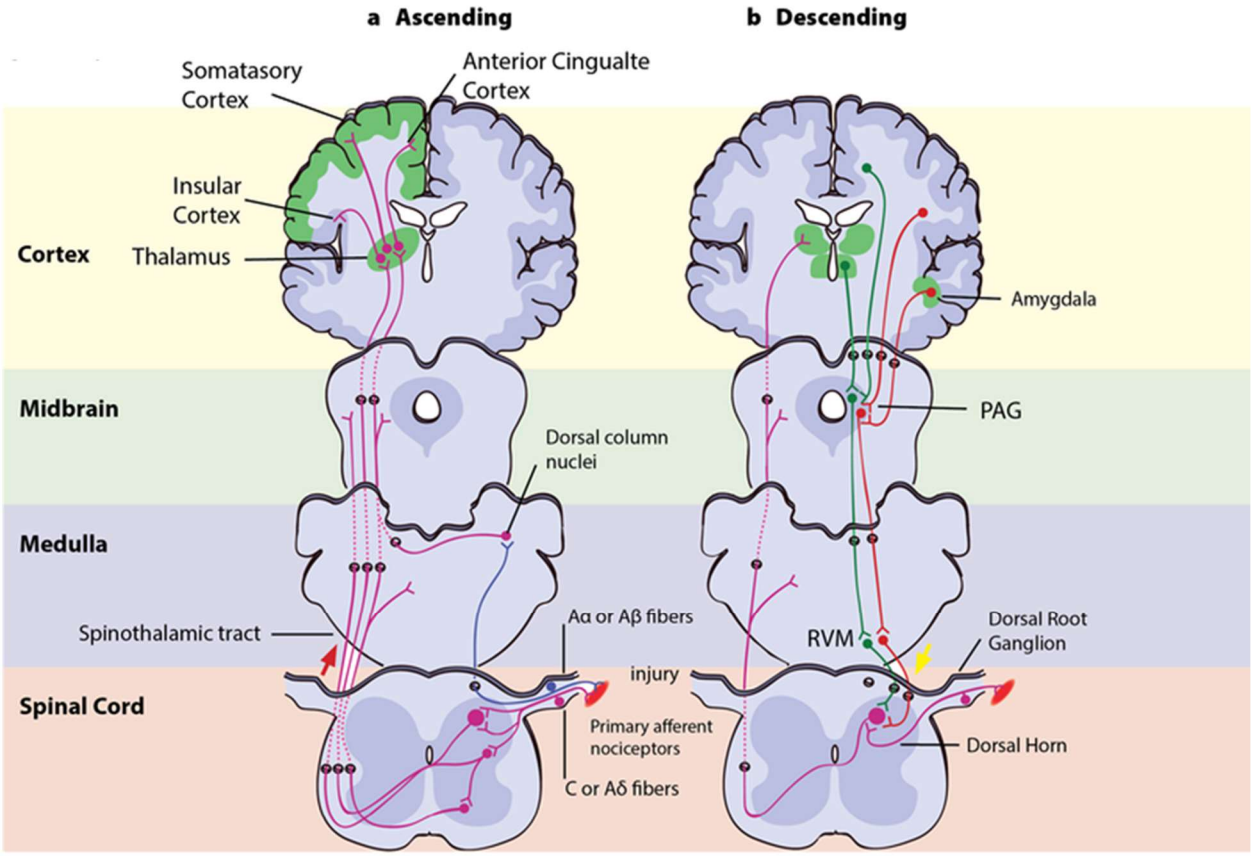

Figure 1.3. Ascending and descending pathways in the sensory nervous system. (A) Ascending sensory pathways. In the spinothalamic pathway (Purple line), primary sensory neurons will synapse immediately with secondary sensory neurons (aka DHNs) in the spinal cord. The axon of secondary sensory neurons will decussate and ascend through the spinothalamic tract towards the thalamus. On the other hand, in the dorsal column-medial lemniscal pathway (Blue), the central branch of primary sensory neurons will ascend towards the medulla and synapse with secondary sensory neurons at the dorsal column nuclei, which consist of the nucleus gracilis and the nucleus cuneatus. From there secondary neurons will decussate and synapse with neurons in the thalamus. Neurons from the thalamus then project towards the somatosensory cortex. **(B)** Descending sensory modulatory pathways. Neurons from the periaqueductal gray (PAG) receive input from higher sensory regions of the brain. PAG neurons project towards the rostral ventromedial medulla (RVM) in the brainstem, which contain neurons that mediate the release of GABA and endogenous opioids in the dorsal horn. (Modified with permission from Løseth et al., 2021).

1.3.1. The GABAergic system and sensory neuropathy

GABA is an essential modulator of neuronal excitability at different levels of the sensory nervous system. Therefore, abnormal GABAergic function has been implicated in the development of sensory neuropathy.

For instance, in the CNS, peripheral nerve injury (PNI) of the sciatic nerve causes increased excitability of DHNs and neuropathic pain. The latter is associated with a decrease in the number of GABAergic synapses at the level of the DHNs and a switch in Cl^- homeostasis mediated by brain-derived neurotrophic factor (BDNF) that causes depolarizing GABA responses instead of the typical hyperpolarization (Coull et al., 2003; Lorenzo et al., 2020).

Similarly, sensory abnormalities have been linked with dysregulation of the GABAergic system and abnormal concentration of Cl^- at the level of the DRG neurons. For example, PNI and chronic constriction injury (CCI) are correlated with decreased expression of $GABA_A$ receptors in DRG neurons (Fukuoka et al., 1998; Obata et al., 2003). Additionally, Funk and colleagues showed that exposure to inflammatory mediators results in accumulation of $[Cl^-]_i$ in DRG neurons suggesting a possible Cl^- dependent mechanism for the generation of inflammatory hyperalgesia and increased neuronal excitability (Funk et al., 2008). This seems to be a direct consequence of increased expression of NKCC1 and reduced expression of KCC2 (Funk et al., 2008; Morales-Aza et al., 2004). Moreover, electrophysiological experiments indicate that DRG neurons from NKCC1 mutant mice have abnormal GABA-evoked currents that result from depletion of $[Cl^-]_i$. Furthermore, behavioural tests in these mice suggest that they have a higher pain threshold compared to control mice (Sung et al., 2000). All of the above demonstrates that GABA-mediated signaling is an essential modulatory mechanism at the level of DRG, and abnormal expression of Cl^- transporters may contribute to the development of sensory abnormalities.

To the best of our knowledge, there are no studies to date that have investigated the relevance of CFTR in Cl^- homeostasis in the DRG neurons. However, multiple studies in other cell types showed that under physiological conditions, CFTR regulates the expression and activity of a variety of other transporters that are important in Cl^- homeostasis in DRG neurons (Ostroumov et al., 2007). For instance, CFTR directly upregulates the expression of NKCC1 (Adam et al., 2005; Shumaker & Soleimani, 1999). In terms of the nervous system, Ostroumov and colleagues reported that the expression of NKCC1 and CFTR function were positively correlated in motor neurons of

the spinal cord (Ostroumov et al., 2011). Additionally, acute inhibition of CFTR in the same neurons results in an immediate decrease in the $[Cl^-]_i$ and a negative shift in the E_{Cl}. This, in conjunction with the fact that CFTR directly upregulates the expression of NKCC1 in other tissues, suggests that CFTR is also required to maintain normal expression of NKCC1 in the nervous system (Ostroumov et al., 2011; Shumaker & Soleimani, 1999). Moreover, CFTR inhibits amiloride-sensitive sodium channels, which are highly expressed in DRG neurons and are referred to as the mammalian brain sodium channels (BNaCs) (Berdiev et al., 2009; García-Añoveros et al., 2001; König et al., 2001). In the absence of CFTR, increased activity of BNaC would result in intracellular sodium loading and a subsequent reduction in the electrochemical gradient of sodium. This will indirectly reduce secondary active transport of Cl^- across the membrane through the NKCC1 transporter (Adam et al., 2005; A. Ostroumov et al., 2011). Taken together, all of the above suggests that CFTR acts as an important regulator of NKCC1. Moreover, since NKCC1 is the main Cl^- cotransporter in the periphery, CFTR dysfunction may result in significant alterations of Cl^- homeostasis in DRG neurons.

Therefore, in the current thesis, we will explore the effect of GABA and Cl^- homeostasis in the context of sensory abnormalities in an animal model of CF which lacks expression of CFTR.

1.4. Rationale, hypothesis, and experimental objectives

Previously, CF was considered a pediatric disease, but with improvements in early specialized care and management, the median age of survival has increased over time (Elborn, 2016; Lubamba et al., 2012). Thus, adult patients are more likely to experience chronic complications of CF. Peripheral neuropathy has been identified as a chronic complication of CF, and it has been associated with higher chronological age. Furthermore, considering the broad regulatory function of the nervous system, peripheral neuropathy may also contribute to the development of other CF complications such as GI tract disorders (Cavalier & Gambetti, 1981; O'Riordan et al., 1995; Park, 1981; Reznikov, 2017).

So far, the mechanisms underlying peripheral neuropathy in CF have not been explored. Nevertheless, the fact that CFTR is expressed in the PNS (Niu et al., 2009; Su et al., 2010) and peripheral neuropathy is present in CF patients and animal models of the disease (Cavalier &

Gambetti, 1981; Chakrabarty et al., 2013; O'Riordan et al., 1995; Reznikov et al., 2013; J. H. Sung, 1964) suggests that CFTR might be more important in the PNS than previously considered.

Furthermore, other reports showed that sensory neuropathy is associated with dysregulation of Cl^- homeostasis in DRG neurons (Funk et al., 2008) and that CFTR actively modulates functional expression of NKCC1 and Cl^- homeostasis in spinal motor neurons (Adam et al., 2005; Ostroumov et al., 2011; Shumaker & Soleimani, 1999). Changes in Cl^- homeostasis have also been linked to the dysregulation of the GABAergic system and abnormal excitability of DRG neurons (Sung et al., 2000). Therefore, we hypothesized that lack of CFTR expression leads to the dysregulation of Cl^- homeostasis in sensory neurons, which in turn causes abnormal excitability.

To test this hypothesis, we will concentrate on the following experimental objectives:

1.4.i Determination of intracellular chloride concentrations and E_{Cl} in DRG neurons from wild type and CFTR -/- swine.

1.4.ii. Effect of pharmacological inhibition of CFTR on DRG neuron excitability.

1.4.iii. Effect of pharmacological inhibition of CFTR on GABA mediated modulation of DRG neuron excitability.

CHAPTER 2

MATERIALS AND METHODS

2.1. Animals

All experiments were approved by the University of Saskatchewan´s Animal and Research Ethics Board and adhere to the Canadian Council of Animal Care guidelines for humane animal use.

We used neonatal swine, wild type, or CFTR knock-out (CFTR-/-), at postnatal day 0 (P0) or day 7 (P7). Wild-type swine were obtained from the Prairie Swine Center (University of Saskatchewan) and served as controls. Gut-corrected CFTR-/- swine were purchased from Exemplar Genetics (Iowa, USA). Gut correction involves partial expression of CFTR in the intestine through transfection of the porcine CFTR (pCFTR) gene so that is under the control of the intestinal fatty acid-binding protein (iFABP) promoter. Gut correction is required since 100% of CFTR -/- piglets develop meconium ileus requiring surgery for survival. Gut corrected swine express approximately 20% of the normal level of CFTR in gut epithelia (not in the nervous system or muscle tissues), which is sufficient to alleviate meconium (Stoltz et al., 2013).

2.2. Primary lumbar DRG cultures

The methods to generate lumbar DRG primary cultures are a modified version from a previous protocol used in neonatal mice models (Lam et al., 2018). Lumbar (L1-L5) DRGs from neonatal swine (P0 or P7) were used for culture. The extraction of the vertebral column was performed through two parallel incisions at the level of the iliac crest and the inferior vertebrae of the thoracic region. The vertebral column was immediately submerged in cold phosphate-buffered saline (PBS) solution. Lumbar DRGs were dissected in a sterile environment and kept submerged in Dulbecco`s Modified Eagle Medium (DMEM) containing sodium bicarbonate. DRGs were digested with 0.1% collagenase (180-200 U/mL; Worthington, Freehold, NJ) in DMEM containing HEPES (N-2-hydroxyethylpiperazine-N'-2-rthanesulfonic Acid) (pH: 7.4) at 37ºC for 1 hour in a water bath. Next, the ganglia were washed and digested in 0.1% trypsin (180-200 U/mL; Worthington, Freehold, NJ). After digestion, DRGs were mechanically dissociated with a fire-polished pipette

until the solution had a cloudy appearance, and enzymes were inactivated using the same volume of serum-containing media. Cells were precipitated by centrifugation and resuspended in growth media containing DMEM-sodium bicarbonate and supplemented with nutrients, horse serum, antibiotics (penicillin-streptomycin), and nerve growth factor (10 ng/mL). Then, neurons were plated on laminin-coated glass-bottom Petri dishes (35 mm) made in-house. The cultures were maintained at 37ºC in a 95% air/ 5% CO_2 environment and fed every five days with fresh growth media. To eliminate non-neuronal cells, cultures were treated with cytosine arabinoside (ARA-C; 10μM) from days 2 to 4. Finally, cultures were treated with either vehicle (Dimethylsulfoxide (DMSO)) or the CFTR inhibitor 172 (CFTRinh-172), a selective inhibitor that prevents chloride flux through CFTR by stabilizing the closed conformation of the channel (Verkman et al., 2013).

2.3. Quantification of Cl^- levels in live cells

2.3.1. N-Ethoxycarbonylmethyl-6-methoxyquinolinium bromide (MQAE)

The intracellular concentration of Cl^- was examined using the Cl^--sensitive dye N-ethoxycarbonylmethyl-6-methoxyquinolinium bromide (MQAE). Cultures were incubated at 37ºC for two hours with 5 mM MQAE dissolved in growth media. Next, cultures were washed three times with an extracellular solution and imaged in an inverted microscope (Axio observer, Carl Zeiss, Oberkochen, Germany) under continuous perfusion. The excitation wavelength used was 470 nm (Colibri 2.0 LED illumination system (Zeiss)) and collected at 510-550 nm wavelength emissions with an AxioCam camera (Zeiss) controlled by Zen software (Zeiss). The intracellular fluorescence intensity (excluding the nucleus) was subtracted from the background fluorescence. The mean MQAE fluorescence intensity, which is inversely proportional to the intracellular levels of Cl^-, was quantified in WT and CFTR-/- cultures (Kaneko et al., 2002).

2.3.2. Determination of $[Cl^-]_i$ and equilibrium potential of Cl^- (E_{Cl})

Calibration of the intracellular MQAE fluorescence intensity was done using a double ionophore technique (Myung et al., 2015) and the Stern-Volmer Equation (Eq.2.1 and 2.2) (Kaneko et al., 2002; Krapf et al., 1988; Myung et al., 2015).

$$\frac{\tau_0}{\tau} = 1 + K_{sv}[Cl^-]_i \text{.....(2.1)}$$

$$[Cl^-]_i = \frac{\frac{\tau_0}{\tau} - 1}{K_{sv}} \text{.....(2.2)}$$

Where τ_0, is the fluorescence in the absence of Cl^-; τ, is the fluorescence intensity in the presence of Cl^-; K_{sv}, is the Stern-Volmer quenching constant for Cl^-.

First, cells were incubated with MQAE for two hours as described above. To determine the mean fluorescence intensity at a zero-quencher concentration (τ_0) the cells were subsequently incubated for 30 minutes in a 0 mM Cl^- solution containing the ionophores nigericin (which enables K^+/H^+ exchange; 100 mM) and tributyltin (which enables Cl^-/OH^- exchange; 100 mM). Second, cells were incubated in standard solutions to determine the fluorescence intensity (τ) at different concentrations (50 mM and 100 mM). Third, using the Stern-Volmer relationship and linear fitting, the quenching rate coefficient (K_{SV}) was estimated to be 17.6 M^{-1}. Fourth, the $[Cl^-]_i$ in each group was determined using their respective ratio of fluorescent intensity (τ_0/τ) and a rearranged version of the Stern-Volmer equation (Eq.2.2). Finally, the equilibrium potential of Cl^- (E_{Cl}) in each group was calculated using the Nernst equation (Eq. 2.3).

$$E_{Cl} = \frac{RT}{Z_{Cl}F} \ln\left(\frac{[Cl^-]_{out}}{[Cl^-]_{in}}\right) \text{.....(2.3)}$$

Where R, is the universal gas constant (8.314 JK^{-1} mol^{-1}); T, is the temperature (298.15 K (25ºC); Z, is the valence of Cl^- (-1); F, is the Faraday constant (96,485.340 C mol^{-1}); $[Cl^-]$, is the intracellular (in) and extracellular (out) concentration of Cl^-.

2.4.Patch Clamp Electrophysiology

Recordings were generated under the gramicidin-perforated patch version of the whole-cell patch-clamp configuration to keep the intracellular Cl^- concentration undisturbed during experiments. Gramicidin generates membrane pores impermeable to anions, preserving the normal $[Cl^-]_i$ (Kaneko et al., 2002). A stock solution of gramicidin (50 mg/mL of DMSO) was prepared every 2 days and stored at -20ºC. The final concentration of gramicidin in the intracellular solution was 100 µg/mL. To improve solubility, the gramicidin solution was warmed for 7 minutes in a water bath and sonicated for 10 minutes. The solution was replaced every 2 hours. To facilitate the

giga-seal formation, the tip of the electrode was dipped in intracellular fluid (ICF) for 2 seconds and then backfilled with gramicidin containing ICF (Ishibashi et al., 2012).

To assess the formation of gramicidin pores, we monitored access resistance (Ra) and the generation of voltage-gated currents. Patch perforation usually occurred between 15 and 30 minutes. At the end of each experiment, further confirmation of perforated patch configuration was achieved by applying negative pressure and obtaining the whole-cell configuration (Ishibashi et al., 2012).

Patch pipettes were made from borosilicate glass (World Precision Instruments, Sarasota, FL, USA) using a vertical puller (PC10; Narishige Scientific Instruments, Sarasota, Fl, USA) with a resistance of 5-10 MΩ when filled with ICF solution. Micropipettes were filled with the following intracellular solution (in mM): 130 KCl, 5 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES (N-2-hydroxyethylpiperazineN'-2-ethanesulfonic acid), 10 EGTA (ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'- tetraacetic acid) and 2 Mg-ATP (adenosine 5'-triphosphate magnesium salt), and pH was adjusted to 7.2 with KOH (all from Sigma–Aldrich). Cultured neurons were perfused continuously at 2 mL/min with extracellular fluid (ECF) consisting of (in mM): 140 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 0.44 KH_2PO_4, 2.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 5.0 glucose (all from Sigma-Aldrich); pH was adjusted to 7.4 with NaOH. . A fast-step perfusion system was used to deliver either control or GABA (500 μM; Sigma-Aldrich)-containing extracellular solution at 2 ml/min perfusion rate. Membrane currents were recorded at room temperature using an AxoPatch 200B amplifier (Molecular, Devices, Palo Alto, CA, USA) equipped with 1 GΩ cooled head stage feedback resistor and Digidata 1400A analog to digital converter (Molecular Devices) and stored on a personal computer. Voltage-clamp and current-clamp protocols and analysis were performed using pClamp 10 (Molecular devices) and Origin 2020 software (Origin Lab Corp., Northampton, MA, USA)(Chandna et al., 2015; Lam et al., 2018).

2.4.1. Voltage-clamp electrophysiology: Determination of E_{Cl} and $[Cl^-]_i$ from GABA-evoked currents

Cultured DRG neurons from both genotypes were used in the perforated patch-clamp experiments (Ishibashi et al., 2012). Voltage steps (-100 to +100 mV, at 20 mV intervals) were

used to evoke GABA currents. The E_{Cl} was calculated for each cell by linear regression, and then averaged per age group for each genotype. The $[Cl^-]_i$ in each group was calculated using a modified version of the Nernst equation (Eq. 2.4):

$$[Cl^-]_i = \frac{[X]_{out}}{e^{(E_{Cl}/(-\frac{RT}{Z_{Cl}F}))}} \quad \text{.....(2.4)}$$

Where R, is the universal gas constant (8.314 JK^{-1} mol^{-1}); T, is the temperature (298.15 K (25ºC); Z, is the valence of Cl^- (-1); F, is the Faraday constant (96,485.340 C mol^{-1}); $[Cl^-]$, is the intracellular (in) and extracellular (out) concentration of Cl^-.

2.4.2. Current-clamp electrophysiology: effect of GABA on DRG neuron excitability

Primary DRG cultures from WT swine maintained in media containing either vehicle or CFTR(inh)-172 (10 μM; Tocris Bioscience; >48 hours) were used in perforated patch-clamp experiments (Ishibashi et al., 2012). To determine DRG neuronal excitability, action potentials were generated by the injection of depolarizing current steps (100 pA intervals for 500 ms). To quantify the inhibitory effect of GABA, action potentials were generated by the injection of a depolarizing current ramp from 0 to 0.8 nA (1-second duration) in the presence of control and GABA (500 μM; Sigma-Aldrich)-containing extracellular solution.

2.5.Statistical Analysis

All data are reported as mean ± standard error of the mean (SEM). The equilibrium potential of Cl^- was calculated from parameters obtained by linear regression. Two-way ANOVA followed by a Tukey´s *post hoc* test was used to analyze the Cl^- imaging, voltage clamp, and current-clamp data. A student´s *t*-test or Mann-Whitney test was employed to analyze the action potential frequency within groups for the current-clamp data. In all cases, data with *p* values less than 0.05 were considered statistically significant. All statistical analyses were performed using Prism 8 (GraphPad Software Inc., La Jolla, Ca, USA).

CHAPTER 3

RESULTS

3.1. Determination of intracellular chloride concentrations and E_{Cl} in DRG neurons from WT and CFTR -/- swine

3.1.1. MQAE live-imaging

We observed a significant difference in the mean MQAE fluorescence intensity in neurons from CFTR-/- piglets relative to those from WT. DRG neurons from CFTR-/- swine had higher mean MQAE fluorescence (P0, 82.3±1.24 AU; P7, 83.3±1.3 AU) than neurons from WT swine (P0, 63.3±1.1 AU; P7, 67.0±1.2 AU). Since the MQAE fluorescence is inversely proportional to the concentration of Cl^-, our data indicate that lack of CFTR expression resulted in lower intracellular Cl^- levels in DRG neurons *(Figure 3.1A-B)*.

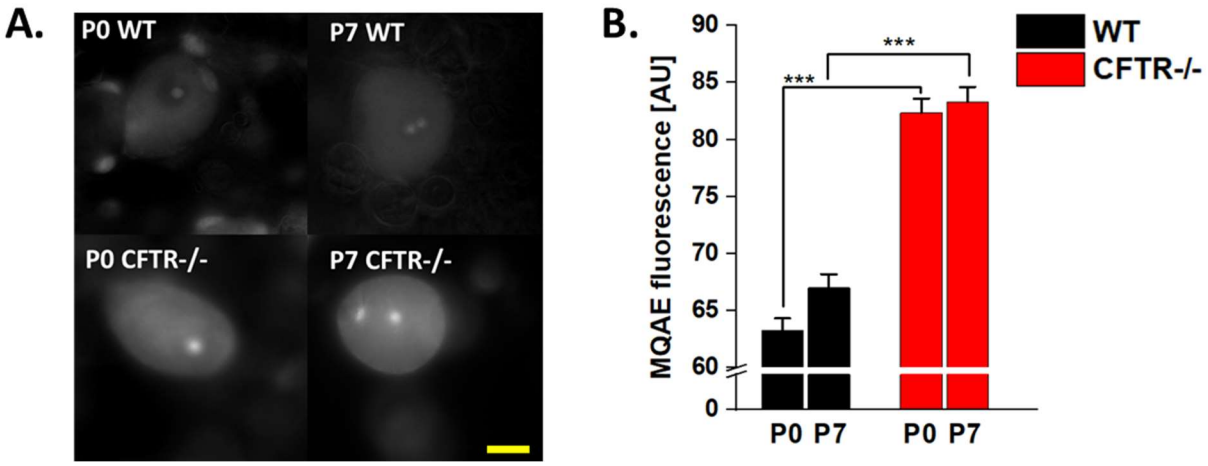

Figure 3.1. DRG neurons from CFTR-/- swine displayed higher MQAE fluorescence intensity. **(A)** Representative fluorescent images of cultured DRG neurons incubated with MQAE from the following groups: WT P0 (n=1052), WT P7 (n=792) P0 CFTR-/- (n=1195), and P7 CFTR-/- (n=859). **(B)** The bar graph summarizes the mean MQAE fluorescence intensity for all groups. Data are expressed as the mean ± SEM arbitrary fluorescent units [AU]. Statistical analysis was performed by two-way ANOVA followed by a Tukey´s *post hoc* test; ***p<0.001. ns p-values: P0 WT vs P7 WT p=0.1551, P0 CFTR-/- vs P7 CFTR-/- p=0.9424. Scale bar in A represents 30 µm.

Next, we calculated the $[Cl^-]_i$ for each group using the MQAE fluorescent data. To calibrate the mean MQAE fluorescence intensity we used a double ionophore technique (Myung et al., 2015) in combination with the Stern-Volmer equation**,** which allowed us to determine $[Cl^-]_i$ from the kinetic interactions of fluorescent molecules (See *Materials and Methods; Figure 3.2A*) (Kaneko et al., 2002; Myung et al., 2015)

The calculated $[Cl^-]_i$ in neurons from P0 (28.4±1.8 mM) and P7 (20.4±1.6 mM) CFTR-/- swine was significantly less than those in WT swine (P0, 53.0±2.3 mM; P7, 44.3±2.3 mM) *(Figure 3.2B).* Additionally, we calculated the E_{Cl} in each group using the Nernst equation. We found a significant negative shift in the calculated E_{Cl} of neurons from CFTR-/- swine (P0, -43.4 ±1.6 mV; P7, -51.7 ±1.9 mV) with respect to those from WT swine (P0, -27.3±1.6 mV; P7, -31.9±1.3 mV) *(Figure 3.2C).* Thus, our results provide supporting evidence that CFTR is required for the physiological accumulation of Cl^- in DRG neurons.

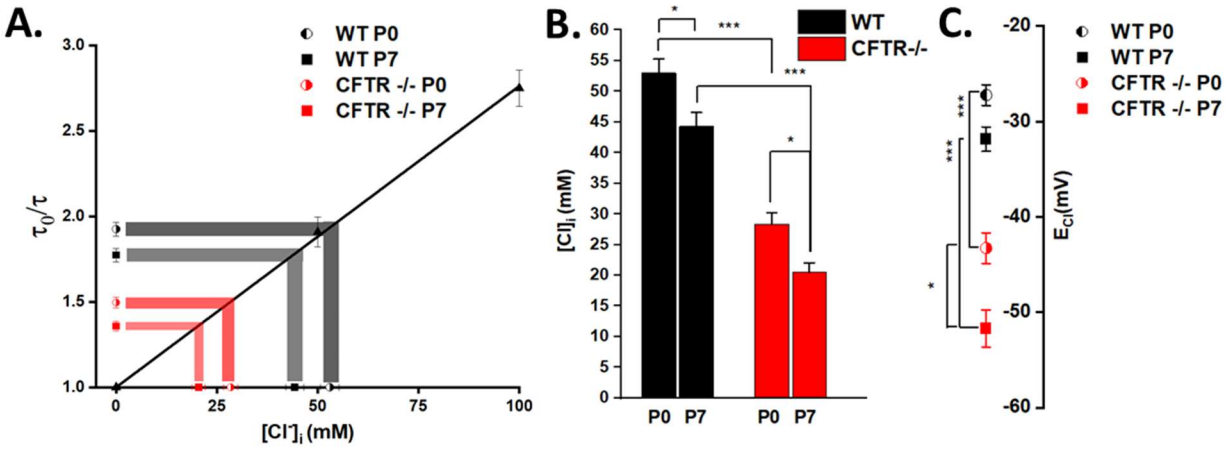

Figure 3.2. DRG neurons from CFTR-/- swine had less $[Cl^-]_i$ and a negatively shifted E_{Cl}. (A) Determination of $[Cl^-]_i$ using the Stern-Volmer relationship. The $[Cl^-]_i$ was extrapolated for the following groups: 0 mM (n=325), 50 mM (n=292) and 100 mM(n=227) using a conventional linear fit. Collected results yielded a K_{sv} of 17.6 M^{-1} **(B)** Mean intracellular Cl^- concentration in WTP0, WTP7, CFP0, and CFP7 cells. **(C)** The calculated mean E_{Cl} for each group (see *Materials and Methods*). Data are expressed as the mean ± SEM. Statistical analysis performed by two-way ANOVA followed a Tukey´s *post hoc* test; *p<0.05, **p<0.01, ***p<0.001. (C) ns p-values: P0 WT vs P7 WT p=0.2068.

3.1.2. Gramicidin-perforated patch-clamp electrophysiology

First, we determined the mean passive membrane properties for all groups (WTP0, WTP7, CFTR-/- P0, and CFTR-/- P7) as reported in *Table 3.1.* We did not find any significant differences in the membrane capacitance (C_m) and membrane potential (V_m) between groups.

	C_m(pF)	V_m(mV)	n
WT P0	23.11±4.22	-61.96±1.94	17
CFTR -/- P0	22.60±2.70	-64.98±1.75	12
WT P7	29.47±4.67	-63.48±1.85	19
CFTR -/- P7	26.25±4.65	-63.44±2.36	9

Table 3.1. Passive membrane properties of WT and CFTR -/- DRG neurons from P0 and P7 swine. Data are expressed as the mean ± SEM. Statistical analysis was performed by two-way ANOVA followed by a Tukey´s *post hoc* test. Cm, membrane capacitance (pF); Vm, membrane potential (mV); n, number of cells tested. ns p-values (Cm): P0 WT vs P7 WT p=0.6679, P0 CFTR-/- vs P7 CFTR-/- p=0.9598, P0 WT vs P0 CFTR-/- p=0.9998, P7 WT vs P7 CFTR-/- p=0.9642. ns p-values (Vm): P0 WT vs P7 WT p=0.9298, P0 CFTR-/- vs P7 CFTR-/- p=0.9665, P0 WT vs P0 CFTR-/- p=0.7109, P7 WT vs P7 CFTR-/- p=0.9999.

Next, to provide further evidence supporting our hypothesis that CFTR is required to maintain physiological Cl^- levels in DRG neurons, we determined the E_{Cl} in DRG neurons from WT and CFTR -/- swine using gramicidin-perforated patch-clamp electrophysiology and GABA evoked currents *(Figure 3.3A)*.

Consistent with the available literature, our results suggest that the mean E_{Cl} in DRG neurons is highly heterogenous, ranging from -60 to -20 mV, which corresponds to $[Cl^-]_i$ from 10 to 80 mM (Gilbert et al., 2007; Wilke et al., 2020). Using GABA-evoked currents at different test potentials (-100 to +20 mV, for 3000 ms) we plotted the current-voltage (IV) relationship and calculated the E_{Cl} per cell, which was then used to estimate the mean per group. *(Figure 3.3B-D).* We found no significant differences in the mean E_{Cl} of DRG neurons from P0 (-19.7±1.3 mV) and P7 (-20.6±2.1 mV) CFTR-/- swine compared to neurons from P0 (-25.0 ±0.9 mV) and P7 (-27.1±0.8 mV) WT swine. Additionally, and contrasting with our live-imaging $[Cl^-]_i$ calculations, when we used a rearranged form of the Nernst equation *(Figure 3.3E)* to calculate the $[Cl^-]_i$ from GABA-evoked currents, we found no differences between postnatal ages or genotypes.

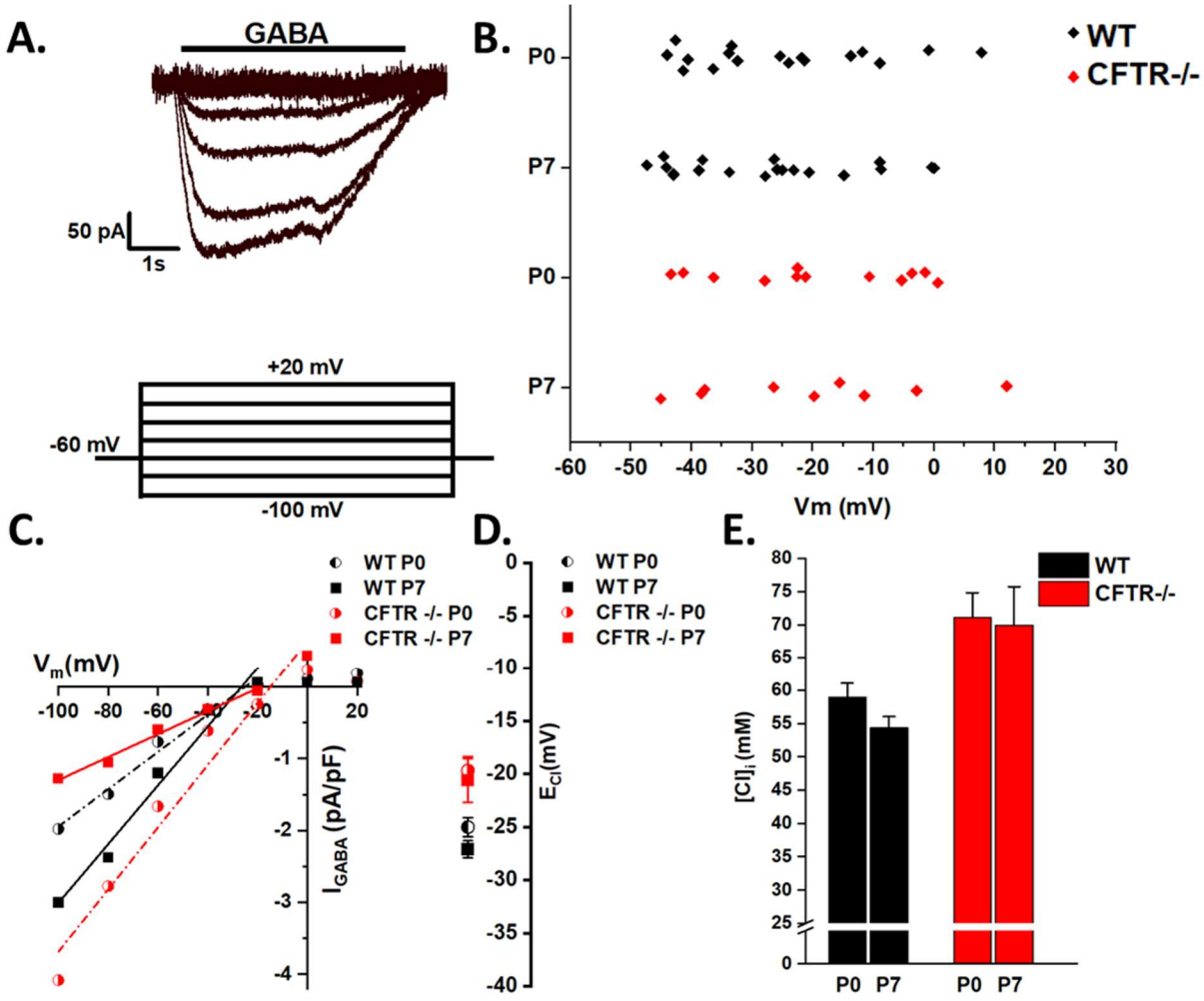

Figure 3.3. Gramicidin-perforated patch-clamp recordings indicate that DRG neurons from CFTR-/- swine have similar E_{Cl} compared to WT swine. (A) Sample current trace from perforated patch configuration experiments, in which currents were evoked by the application of GABA at different step voltages (-100 to +20 mV, at 20 mV intervals). **(B)** Individual E_{Cl}, **(C)** representative Current vs Voltage (I-V) relationship **(D)** average E_{Cl} for WT and CFTR-/- DRG neurons from P0 (WT, n=17; CFTR-/-, n=12) and P7 (WT, n=19; CFTR-/-, n=9) swine. Data are expressed as the mean ± SEM. Statistical analysis was performed by two-way ANOVA followed by a Tukey´s *post hoc* test. Inset in A represents the stimulation protocol. (D) ns p-values: P0 WT vs P7 WT p=0.8666, P0 CFTR-/- vs P7 CFTR-/- p=0.9817, P0 WT vs P0 CFTR-/- p=0.3856, P7 WT vs P7 CFTR-/- p=0.7713. (E) ns p-values: P0 WT vs P7 WT p=0.6029, P0 CFTR-/- vs P7 CFTR-/- p=0.9513, P0 WT vs P0 CFTR-/- p=0.0722, P7 WT vs P7 CFTR-/- p=0.0554.

3.2.Pharmacological inhibition of CFTR had no effect on DRG neuron excitability

First, we determined the mean passive membrane properties of all groups (Vehicle P0, Vehicle P7, CFTR(inh)-172 P0, and CFTR(inh)-172 P7) studied as reported in *Table 3.2.* We did not find significant differences in the C_m and V_m between groups.

	C_m(pF)	V_m(mV)	n
WT P0	29.92±3.20	-58.82±5.50	27
CFTR(inh)-172 P0	34.52±4.29	-61.16±1.53	14
WT P7	30.48±4.63	-69.93±1.98	14
CFTR(inh)-172 P7	26.98±3.75	-64.79±2.86	14

Table 3.2. Passive membrane properties of WT DRG neurons from P0 and P7 swine exposed to vehicle and CFTR(inh)-172 . Data are expressed as the mean ± SEM. Statistical analysis performed by two-way ANOVA followed by a Tukey´s *post hoc* test.; Cm, membrane capacitance (pF); Vm, membrane potential (mV); n, number of cells tested. ns p-values (Cm): P0 WT vs P7 WT p=0.9996, P0 CFTR-/- vs P7 CFTR-/- p=0.6068, P0 WT vs P0 CFTR-/- p=0.8233, P7 WT vs P7 CFTR-/- p=0.9396. ns p-values (Vm): P0 WT vs P7 WT p=0.3015, P0 CFTR-/- vs P7 CFTR-/- p=0.9588, P0 WT vs P0 CFTR-/- p=0.9823, P7 WT vs P7 CFTR-/- p=0.8927.

Next, we concentrated on evaluating the role of CFTR on neuronal excitability. Unpublished results from our laboratory revealed that DRG neurons from CFTR-/- swine had a significantly reduced generation of action potentials (Givzad, 2020). We further explored this effect by using the CFTR selective inhibitor, CFTR(inh)-172, and subsequently evaluating neuronal excitability in cultured DRG neurons from WT swine. To quantify excitability, we employed current-clamp electrophysiology and exposed neurons to depolarizing current steps for 0.5 s, from 0 to 1500 pA (at 100 pA intervals), preceded by a hyperpolarizing step to remove partial inactivation of voltage-gated sodium channels (-300 pA for 0.1s) *(Figure 3.4A).* Moreover, to avoid disturbances of $[Cl^-]_i$, these experiments were done in the perforated patch-clamp configuration.

Contrasting with the unpublished results from CFTR-/- swine, we observed no significant changes in the action potential firing frequency between P0 and P7 WT DRG neurons treated with vehicle and CFTR (inh)-172 (P0 Vehicle= 3.0±0.6 s^{-1}, P7 Vehicle= 3.4±1.2 s^{-1}, P0 CFTR(inh)-172=4.42±1.21 s^{-1}, P7 CFTR(inh)-172=2.5±0.6 s^{-1}) *(Figure 3.4-C).*

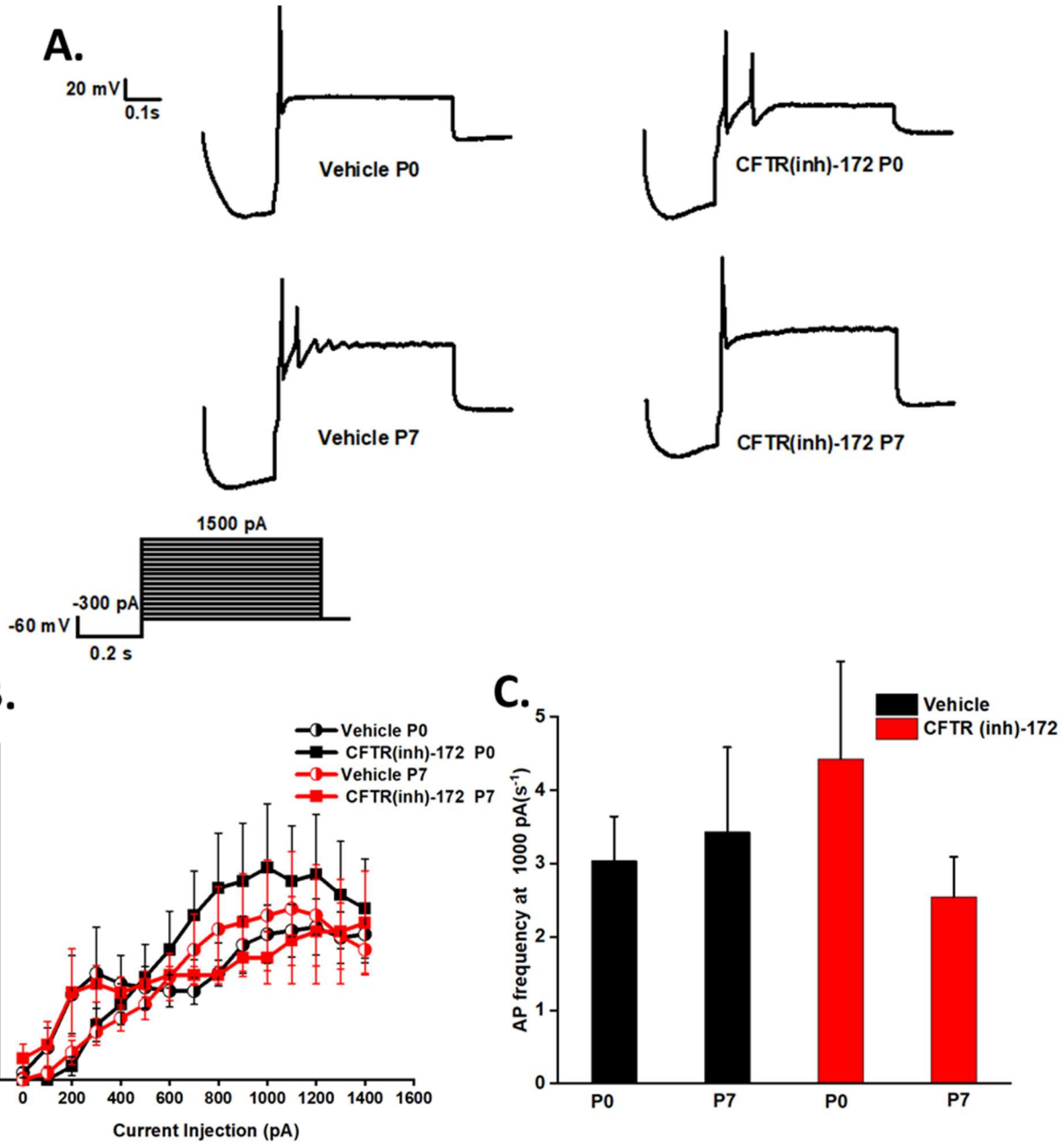

Figure 3.4. DRG neurons from WT swine treated with CFTR (inh)-172 had normal excitability. (A) Representative current-clamp traces of DRG neurons from P0 and P7 WT swine treated with CFTR(inh)-172 or vehicle. **(B)** Line graph summarizes action potential firing frequency in DRG neurons from P0 and P7 WT swine treated with CFTR(inh)-172 and vehicle. (**C)** The bar graph summarizes the action potential firing frequency at the 1000 pA injection step. We observed no significant differences in DRG neurons from P0 and P7 WT swine treated with CFTR(inh)-172 and vehicle. P0 Vehicle (n=27), P0 CFTR(inh)-172 (n=14), P7 Vehicle (n=14), and P7 CFTR (inh)-172 (n=11). Statistical analysis was performed by two-way ANOVA followed

by a Tukey´s *post hoc* test. ns p-values: P0 Vehicle vs P7 Vehicle p=0.9886, P0 CFTR (inh)-172 vs P7 CFTR (inh)-172 p=0.5938, P0 Vehicle vs P0 CFTR (inh)-172 p=0.6684, P7 Vehicle vs P7 CFTR (inh)-172 p=0.9348.

3.3.GABA does not modulate DRG neuron excitability in swine

It has been reported that GABA can decrease action potential frequency in DRG from mice (Du et al., 2017)**.** Therefore, considering that our MQAE fluorescence data suggest that lack of CFTR impairs Cl^- homeostasis in DRG neurons from swine, we next evaluated the effect of GABA on action potential frequency in DRG neurons from WT swine incubated with or without CFTR(inh)-172 *(Figure 3.5A).* As described above, we used the perforated patch configuration to avoid disturbances in $[Cl^-]_i$.

Similar to our previous findings (see *section 3.2*), we did not observe a change in action potential frequency between WT cells treated with vehicle or the CFTR (inh)-172 *(Figure 3.5B).* As expected, the application of GABA induced a mild depolarization on the DRG resting potential (Figure 3.5A, blue trace)**.** However, we did not observe a significant effect of GABA on action potential frequency between WT cells treated with vehicle and the CFTR (inh)-172 *(Figure 3.5C).* Therefore, our electrophysiology data suggest that inhibition of CFTR function, and GABA do not modulate DRG neuron excitability in the swine.

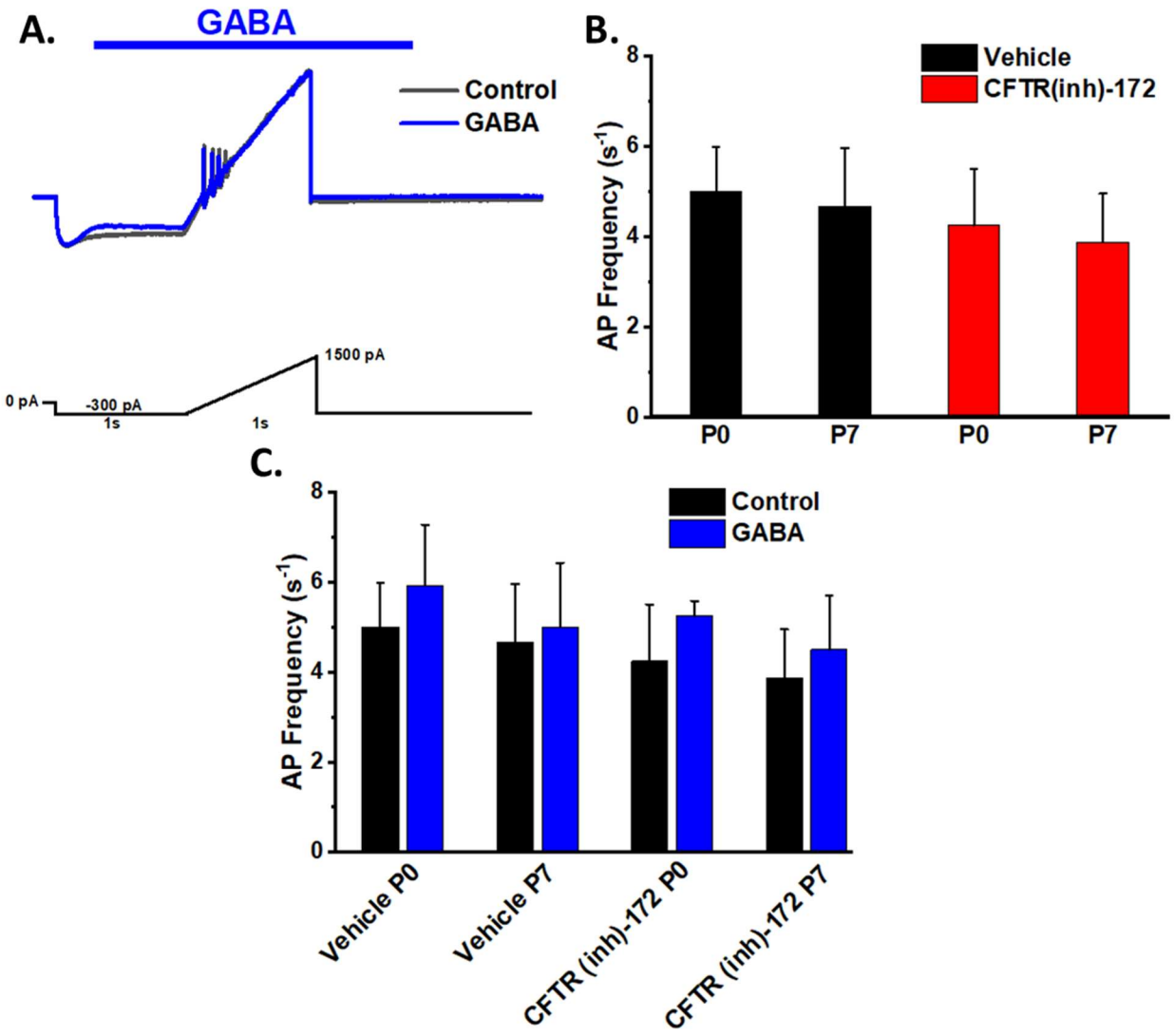

Figure 3.5. GABA does not affect the action potential firing frequency of DRG neurons. (A) Representative current-clamp recording indicates no change in action potential firing frequency following GABA application. **(B)** Action potential firing frequency is similar in P0 and P7 DRG neurons treated with CFTR (inh)-172 and vehicle **(C)** GABA has no significant effect on action potential firing frequency in P0 and P7 DRG neurons treated with CFTR (inh)-172 and vehicle. Data are expressed as the mean ± SEM. Statistical analysis was performed by a two-way ANOVA followed by a Tukey´s *post hoc* test and Mann-Whitney test. P0 Vehicle (n=14), P0 CFTR (inh)-172 (n=8), P7 Vehicle (n=9), and P7 CFTR (inh)-172 (n=8). Inset in A represents the stimulation protocol. (B) ns p-values : P0 Vehicle vs P7 Vehicle p=0.9963, P0 CFTR (inh)-172 vs P7 CFTR (inh)-172 p=0.9967, P0 Vehicle vs P0 CFTR (inh)-172 p=0.8940, P7 Vehicle vs P7 CFTR (inh)-

172 p=0.9686. (C) ns p-values: P0 Vehicle p=0.6870, P7 Vehicle p=0.8076, P0 CFTR (inh)-172 p= 0.3952, P7 CFTR(inh-172)= 0.8766.

CHAPTER 4

DISCUSSION

The association between peripheral neuropathy and CF was first described almost 60 years ago by J. H. Sung (Sung, 1964). Since then, epidemiological, clinical, and animal studies demonstrated that peripheral neuropathy is an important extrapulmonary complication of CF (Chakrabarty et al., 2013; Davis & Kaliner, 1983; Niu et al., 2009; O'Riordan et al., 1995; Reznikov et al., 2013; Rubin et al., 1963; Sibinga & Barbero, 1961; Sullivan et al., 1986). Originally, peripheral neuropathy was considered a secondary complication of malnutrition in CF (Cavalier & Gambetti, 1981). However, later studies found that CFTR is highly expressed in the PNS, suggesting that neuropathy could result from the functional absence of CFTR in peripheral neurons (Niu et al., 2009; Su et al., 2010).

To the best of our knowledge, the involvement of CFTR in the modulation of Cl^- homeostasis in neurons has only been studied in the CNS. It was reported that CFTR is an important regulator of Cl^- homeostasis and neuronal excitability in motor neurons in the spinal cord (Ostroumov et al., 2011). However, whether CFTR plays such a role in peripheral neurons, including sensory neurons, remained to be studied and it was the main focus of the current investigation.

4.1. Lack of CFTR expression resulted in reduced $[Cl^-]_i$ in swine DRG neurons

In the current thesis, we used two independent methods, live Cl^- fluorescence imaging and electrophysiology, to investigate whether CFTR contributes to $[Cl^-]_i$ in DRG neurons of swine. Although the results obtained through these two methods were not consistent, we can interpret our findings and draw conclusions based on the experimental design and methodological considerations.

Our imaging experiments revealed that lack of CFTR is associated with lower $[Cl^-]_i$ in swine DRG neurons (from both ages tested). Similar results were reported by Ostroumov and collaborators in motor neurons from the spinal cord, in which acute inhibition of CFTR caused a decrease in $[Cl^-]_i$ and a negative shift in the E_{Cl} (Ostroumov et al., 2011). The change in Cl^- levels, and its corresponding E_{Cl}, that we report in DRG neurons, could be explained by the regulatory

interactions of CFTR with other proteins involved in Cl^- transport. CFTR increases the expression of NKCC1, which is responsible for Cl^- accumulation in DRG neurons (Ostroumov et al., 2007; Shumaker & Soleimani, 1999). CFTR also downregulates amiloride-sensitive sodium channels (i.e., BNaC), which indirectly stimulates the activity of NKCC1 (Adam et al., 2005; A. Ostroumov et al., 2011). Thus, lack of CFTR would result in decreased expression and reduced activity of NKCC1, leading to depletion of $[Cl^-]_i$, and a negative shift of the E_{Cl}, as observed in the MQAE fluorescence experiments. Consistent with this idea, DRG neurons from NKCC1 knock-out mice display reduced Cl^- levels and a negative shift in E_{Cl} as we observed in CFTR-/- swine. Interestingly, the absence of NKCC1 in mice was also associated with a higher pain threshold and the development of sensory dysfunction (Sung et al., 2000).

Contrasting with our MQAE findings, the perforated patch-clamp electrophysiology results showed that DRG neurons from WT and CFTR -/- swine had similar $[Cl^-]_i$. Although we can not provide a conclusive argument of these contrasting effects, we can, however, propose two possible explanations:

1. Lack of current reversibility in patch-clamp experiments: When the V_m of the cell is forced to values more positive than the E_{Cl} we are supposed to see outward GABA currents, which corresponds to Cl^- entering the cell. However, in our experiments, we observed an abnormal inward rectification of the IV relationship for GABA at membrane potentials more positive than the E_{Cl}. This deviation from the linearity in the IV relationship prevented the graphical determination of the E_{Cl} *(Figure 3.3C)*. Additionally, cells with a very negative E_{Cl} (e.g., -100, -80 mV) could not be analyzed due to the lack of current. The latter prevented us from quantifying a shift to more negative potentials in the E_{Cl} of neurons from CFTR-/- swine, which we were able to observe by MQAE fluorescence. Previous studies in DRG neurons from mice (Sung et al., 2000) and humans (Valeyev et al., 1996) show that GABA-mediated currents were linearly proportional to the driving force of Cl^- (V_m-E_{Cl}) at any voltage, which indicates no rectification in the IV relationship for GABA. However, according to the Goldman-Hodgkin-Katz (GHK) equation, in the presence of asymmetrical concentrations of Cl^-, the IV plot would follow a non-linear relationship since the flow of Cl^- would be favoured in one direction compared to the other (Clay, 2009; O'Toole & Jenkins, 2012). In our case, the fact that we conserved the physiological $[Cl^-]_i$ through perforated patch-clamp experiments could account for the unusual rectification observed

in the GABA IV relationship, and thus, for the lack of reversibility of the GABA-evoked currents at the $V_m > E_{Cl}$. In future experiments, we plan to generate the IV plot from each cell under both the gramicidin-perforated patch configuration, as well as under whole-cell after allowing sufficient time (approximately 15 min) for the Cl^- concentration to equalize across the membrane. This experiment will allow us to determine if the unusual GABA IV relationship reported in this thesis was due to GHK rectification.

2. Bias in the MQAE fluorescence experiments: The MQAE imaging experiments provided a robust quantification of Cl^- values, due to the combination of a large number and the fact that the technique prevents any possible cell damage, as may be induced by patching electrodes. However, there are limitations associated with this technique that may affect E_{Cl} measurements. Since MQAE fluorescence is inversely proportional to the Cl^- concentration, the dye would be completely quenched in cells with very high $[Cl^-]_i$, resulting in dark images. The latter introduces a bias of the mean $[Cl^-]_i$ towards lower concentrations and the E_{Cl} toward more negative values.

Taken together, the limitations inherent to the techniques used introduced bias in the determination of $[Cl^-]_i$ and E_{Cl} in the opposite end of the spectrum, which could account for some of the discrepancies observed in our results obtained by MQAE fluorescence and patch-clamp electrophysiology.

4.2. GABA did not modulate DRG neuron excitability in swine

To study whether changes in Cl^- concentration due to the absence of CFTR could result in changes in neuronal excitability and GABA mediated inhibition, we used current clamp electrophysiology in the perforated patch-clamp configuration. Unpublished observations from our laboratory (Givzad, 2020), showed that DRG neurons from CFTR-/- swine had impaired action potential generation. To further study this effect, we used WT DRG neurons from the same ages as in the previous study and incubated them in CFTR(inh)-172 conditions.

However, we observed no significant changes in the action potential firing frequency of WT DRG neurons treated with CFTR(inh)-172 when compared to vehicle. The discrepancies in these two sets of results from our own research team may be explained by the different developmental environments DRG neurons were exposed. In the knock-out swine, DRG neurons developed

without CFTR expression, while in the case of WT DRG neurons, CFTR function was only inhibited for 48 hr in culture conditions. Furthermore, the effects of inhibiting CFTR function may be different from lack of expression of the CFTR protein. The latter could be related to the intracellular pathways downstream and regulated by CFTR function, which may play different functions in the CFTR-/- swine.

We hypothesized that DRG neurons incubated with the CFTR(inh)-172 would have abnormal GABA mediated inhibition of action potential firing, based on previous reports in mice showing that acute inhibition of CFTR in central neurons had significant effects on the $[Cl^-]_i$ (Ostroumov et al., 2011), and that GABA mediated depolarization impeded action potential firing through the soma of DRG neurons (Du et al., 2017). However, our findings indicate that GABA did not modulate neuronal excitability in swine as it does in rodents. Recent research indicate that there are other Cl^- channels that closely interact with CFTR and seem to be relevant in regulating DRG neuron excitability, such as the calcium-activated cation channel, ANO1, also known as Transmembrane member 16A (TMEM16A) (Jin et al., 2013; Lérias et al., 2018). Therefore, for future experiments, we will study the relevance of this Cl^- channel in the excitability of DRG neurons.

4.3. Peripheral Neuropathy and Pathophysiology of CF

The extent to which peripheral neuropathy contributes to the pathophysiology of CF is unknown. However, studies of peripheral neuropathy in other conditions such as diabetes mellitus provide some insight into the overall pathological consequences that may arise from impaired function of the PNS (Bansal et al., 2006).

For instance, CF is associated with intestinal dysmotility, constipation, and serious intestinal blockages (Rafeeq & Murad, 2017). Primary manifestations of GI dysmotility in CF can occur as early as in the neonatal stage (i.e., meconium ileus) or at any age (i.e., distal intestinal obstruction syndrome (DIOS) (Ooi & Durie, 2016; Park, 1981). Although this has been widely attributed to the lack of functional CFTR in the gut epithelium, the possible contribution of a neurogenic component has not yet been studied (Ooi & Durie, 2016). Two important pieces of evidence indicate that peripheral neuropathy may be more important than previously considered in the

development of gut disorders in CF. First, elegant research from Smith-Edwards and collaborators found that afferent input from DRG neurons modulates smooth muscle contraction in the colon via the activation of a parasympathetic spinal circuit (Smith-Edwards et al., 2019). This suggests that sensory neuropathy may impair extrinsic neurogenic control of GI function and contribute to the development of GI tract abnormalities in CF. Second, there is substantial evidence that peripheral neuropathy in Diabetes mellitus alters intestinal motility, leading to a range of abdominal manifestations (e.g., constipation, intestinal distention, and abdominal pain) that share some similarities with those present in CF symptomatology (Azpiroz & Malagelada, 2016).

In CF, the most common and fatal symptom is local inflammation of the lungs. Primarily, inflammation of the lungs has been attributed to the mucus retention and chronic infection that results from improper airway clearance (Cutting et al., 2019; Elborn, 2016). However, evidence indicates that inflammation in CF may also have a neurogenic component (Borson et al., 1989). This idea arises from a unique form of retrograde signaling termed neurogenic inflammation, which involves the release of inflammatory substances such as substance P and calcitonin-gene-related peptide (CGRP) from afferent neurons (Willis, 2003). These receptors are widely distributed in the human airways and are current targets of investigation for the treatment of asthma (Balestrini et al., 2021; Barnes, 2003; Pisi et al., 2009). Moreover, there is evidence that abnormal intracellular levels of Cl^- can result in the generation of action potentials at the central branch of the DRG neurons, which propagate antidromically and potentially stimulate neurogenic inflammation in the periphery (Willis, 1999). Therefore, considering the close interaction between airway nerves and inflammation together with sensory neuropathy in CF, neurogenic inflammation could, at least partially, account for the development of airway pathology in CF.

4.4. Future Directions and Limitations

Our first limitation is that due to the cost and availability of CFTR -/- swine we could not perform all experiments in CFTR-/- DRG neurons. Therefore, we decided to culture WT DRG neurons and incubate them in the presence of CFTR(inh)-172. Although this could potentially mimic the short-term effects in the $[Cl^-]_i$, this method may not reliably account for the long-term changes that may result from functional absence of CFTR. In addition, a second limitation is that in our voltage-clamp experiments we observed a completely unexpected inward rectification of

GABA currents, which prevented the reliable measurement of the E_{Cl} at negative voltages. It would be interesting in the future to investigate the molecular mechanisms behind inward rectification of these channels in swine.

In addition, the current consensus is that CFTR regulates Cl^- homeostasis through direct and indirect interactions with other transporters. Although these mechanisms have been previously studied in the epithelium (Adam et al., 2005; König et al., 2001; Lérias et al., 2018; Shumaker & Soleimani, 1999), there are no studies that investigated the intracellular pathways downstream and upstream of CFTR in the nervous system. For instance, the WNK (With no lysine)–SPAK (SPS1-Related Proline/Alanine rich Kinase) / OSR1 (Oxidative Stress Responsive Kinase 1) complex, which is a Cl^- sensitive cascade that regulates the activity of NKCC1 and KCC2, could be a possible candidate since there is evidence that CFTR closely interacts with this pathway (Huang et al., 2019; Kim et al., 2020; Yang et al., 2007). Moreover, dysregulation of the WNK-SPAK/OSR1 pathway has been postulated as a key contributing factor in the development of neurological disorders that are associated with abnormal Cl^- homeostasis (Alessi et al., 2014; Watanabe & Fukuda, 2015). Therefore, taking into account the relevance of CFTR in the regulation of NKCC1 expression in other tissues and the possible interaction between CFTR and the WNK-SPAK/OSR1 pathway our next objective will be to study the expression levels of these proteins in DRG samples from CFTR -/- and WT swine through biochemical analysis (Western blotting).

CHAPTER 5

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