Sensory Deficits in a Mouse Model of Christianson Syndrome

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April 2019

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science.

Abstract

Christianson Syndrome (CS) is a recently characterized X-linked neurodevelopmental disorder caused by loss-of-function mutations in the gene *slc9a6,* encoding the endosomal Na^+/H^+ Exchanger 6 (NHE6). The disorder is associated with developmental delay, intellectual disability, loss of motor coordination, mutism, ataxia, epilepsy, as well as autistic features. In addition to these symptoms, CS patients exhibit elevated pain thresholds to noxious stimuli as well as discomfort at normally innocuous stimuli. The underlying causes of these sensory deficits are yet to be determined. Hence, this study aims at understanding how loss-of-function of NHE6 affects transmission and processing of pain.

To this end, we examined the expression of NHE6 in peripheral and central neurons implicated in sensation and interpretation of pain. Additionally, we characterized the nociceptive behaviour of NHE6 knockout (KO) mice using a battery of behaviour tests. Our immunohistochemical experiments demonstrate that NHE6 is highly expressed in nociceptive, small-diameter dorsal root ganglia (DRG) neurons. Moreover, mice lacking NHE6 display decreased responses to noxious mechanical, thermal and chemical stimuli but are more responsive to noxious cold than wild-type littermates. Interestingly, immunohistochemical characterization of DRG tissue from aged NHE6 null mice indicates a decrease in some neuronal subsets suggesting cell death. Finally, using light brush-induced Fos activation in the dorsal horn, we found that the spinal processing of innocuous stimuli is not different between wildtype and NHE6 knockout mice.

According to our results, mice deficient in NHE6 can model sensory deficits seen in CS patients. However, it is unclear whether this dysfunction is a result of defects in nociceptors, dorsal horn circuits or supraspinal pain processing centers. Therefore, the findings of this study form a base upon which the sensory pathologies of CS can be further elucidated.

Résumé

Le syndrome de Christianson (CS) est une maladie neuro-développementale récemment caractérisée et associée à une pathologie du chromosome X. Cette maladie est causée par des mutations de type « perte de fonction » du gène *slc9a6*, encodant le transporteur sodium/proton 6 (NHE6) exprimé dans les endosomes. Ce syndrome est associé à plusieurs désordres, tels que les délais de développement, la déficience intellectuelle, la perte de coordination motrice, le mutisme, l'ataxie, l'épilepsie, ainsi que des traits du spectre de l'autisme. En plus de ces symptômes, les enfants atteints du syndrome ont un seuil de tolérance à la douleur plus élevé, tout en présentant un inconfort face à des stimuli de faible intensité tels que le toucher. En ce moment, les mécanismes contribuant à ces anomalies sensorielles demeurent inconnues. Donc, le projet de recherche vise à déterminer comment l'absence de NHE6 affecte la transmission et le traitement de la douleur.

En premier lieu, nous avons examiné l'expression de NHE6 dans les circuits neuronaux impliqués dans la sensation et la perception de la douleur. Puis, nous avons caractérisé le phénotype sensoriel d'un modèle murin de CS, une souris knockout (KO) pour le transporteur NHE6. Nos résultats indiquent que NHE6 est fortement exprimé dans les nocicepteurs. Par ailleurs, les souris NHE6 KO sont moins sensibles aux stimuli douloureux mécaniques, thermiques, et chimiques, mais sont plus sensibles aux stimuli douloureux froids. En inspectant les nocicepteurs des souris NHE6 KO âgées, nous avons trouvé une diminution dans quelques sous-types de neurones sensoriels suggérant une mort cellulaire. Finalement, le niveau d'expression de la protéine Fos induite par des stimuli de faible intensité n'est pas différent dans les deux génotypes, indiquant que le traitement des stimuli tactiles est similaire entre les souris WT et NHE6 KO.

Nos résultats démontrent qu'en l'absence du transporteur NHE6, les souris peuvent reproduire les symptômes des enfants atteints du CS. Cependant, il reste à déterminer si ces anomalies sensorielles sont causées par une pathologie des nocicepteurs, soit des circuits neuronaux de la corne dorsale ou soit des centres d'interprétation de la douleur du cerveau. Ainsi, les résultats de cette étude nous permettront de développer davantage notre compréhension de la maladie de CS.

Acknowledgements

I would first like to thank Prof. Reza Sharif-Naeini for his supervision. It was an honour to contribute to a field as exciting as this, with your guidance. I appreciated your patience and support during my time at the lab and learned greatly with your encouragement. I wish the lab all the best new exciting projects and the continued success of current projects.

Next, I would like to thank Dr. Hugues Petitjean for your advice and guidance on technical, theoretical and statistical concepts for this project. I am thankful to Dr. Marine Christine and Albena for kindly teaching me protocols and providing helpful organizational tips during my time at the lab. I would also like to thank Prof. John Orlowski and Dr. Alina Ilie for providing the NHE6 antibody and engaging theoretical discussions on this project.

Finally, I would like to thank the facilities that were essential to the completing this project. Thank you to the Advanced Bioimaging Facility for lending their imaging equipment, Prof. Alfredo Ribeiro Da Silva's lab for lending their cryostat and the staff of the Comparative Medicine and Animal Resources Centre of McGill for their assistance and expertise in managing mice colonies.

Abbreviations

%	Percent
ACC	Anterior Cingulate Cortex
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AS	Angelman Syndrome
ASD	Autism Spectrum Disorder
BLA	Basolateral Amygdala
BMP	Bone Morphogenetic Protein
CaV	Voltage-gated Ca^{2+} channel
CGRP	Calcitonin Gene Related Peptide
CHC22	Clathrin Heavy Chain 22
CIP(A)	Congenital Insensitivity to Pain (with Anhidrosis)
CMARC McGill	Comparative Medicine and Animal Resources Centre of McGill
CNS	Central Nervous System
CS	Christianson Syndrome
CV	Conduction Velocity
DH	Dorsal Horn
dI1-6	Dorsal horn interneurons early born 1-6
$dIL_{A/B}$	Dorsal horn interneurons late born A/B
DMSO	Dimethyl Sulfoxide
DRG	Dorsal Root Ganglion
EE	Early Endosome
EGF	Epidermal Growth Factor
FXS	Fragile X Syndrome
g	grams
GABA	Gamma-aminoautyric acid
GERD	Gastroesophageal reflux disease
GINIP	Gαi-interacting protein
HST	Horse Serum with Triton X-100
HTMR	High Threshold Mechanoreceptor
IB4	Isolectin B4
IC	Insular Cortex
ID	Intellectual Disability
K2P	Two pore domain potassium channel
KO	Knockout
Kv	Voltage-gated K^+ channel
LAMP	Lysosomal-associated membrane protein
LE	Late Endosome
LOF	Loss of function
LTMR	Low Threshold Mechanoreceptor
LTP	Long Term Potentiation
Lys	Lysosome
Nav	Voltage-gated Na^+ Channel
NeuN	Neuronal nuclei specific antigen
NF200	Neurofilament 200

NGF	Nerve Growth Factor
Ngn1/2	Neurogenin1/2
NGST	Normal Goat Serum with Triton X-100
NHE6	Na^+/H^+ Exchanger 6
Nrp1	Neuropilin1
PAG	Periacqueductal Gray
PBN	Parabrachial Nucleus
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PNS	Peripheral Nervous System
PV	Parvalbumin
RE	Recycling Endosome
RVM	Rostral Ventromedial Medulla
s	seconds
S1	Primary Somatosensory Cortex
Sema3A	Semaphorin3A
Syn13	Syntaxin13
TGN	Trans-Golgi Network
TH	Tyrosine Hydroxylase
TrkA/B/C	Tyrosine kinase receptor A/B/C
TRP	Transient Receptor Potential
ug	micrograms
ul	microlitres
V-ATPase	Vacuolar type H^+-ATPase
VPL	Ventral Posterolateral Nucleus
WT	Wildtype
XLID	X-Linked Intellectual Disability
α	Alpha
β	Beta
δ	Delta

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1. Introduction

1.1. Christianson Syndrome

Christianson syndrome (CS) is a recently described rare X-linked neurodevelopmental disorder, affecting males and resulting in broad nervous system dysfunction.[1] Loss-of-function (LOF) mutations in the *slc9a6* gene encoding the endosomal Na^+/H^+ exchanger 6 (NHE6) protein cause CS.[2] The condition is characterized by symptoms such as post-natal microcephaly, intellectual disability (ID), absence of verbal language despite normal hearing, truncal ataxia, epilepsy, strabismus and developmental delays.[1,3,4] Furthermore, patients exhibit autistic features, hyperkineticism, a generally happy demeanour and interestingly, an apparent elevated threshold to pain but abnormal responses to normally innocuous sensory experiences.[4-7]

1.1.1. Clinical Overview

The disorder CS was first described by Christianson *et al.*, in a large South African family of patients.[1] Since then, at least 60 cases have been clinically reported in literature on CS.[1-4,8-21] The prevalence of CS is estimated to be 1:16,000 to 1:100,000 given that the *slc9a6* gene was in the top six most recurrently mutated genes in a screen of X chromosome exons in pedigrees of X-linked Intellectual Disability conditions (XLIDs).[8,22] The incidence of CS does not seem limited to a specific ethnic group as patients have been identified all over the world: South Africa, Egypt, Sweden, the United Kingdom, Norway, Japan, India and the United States.[1,8,12,14,16,17] A variety of mutations were discovered in CS patients to date, the majority being protein truncating.[2,10,12,13] Splicing mutations removing transmembrane domains, as well as single number and copy number variant mutations have also been identified.[4,9,11] Many insertion, deletion, and missense mutations have also been found in CS patients with varying phenotypes.[4,14] The *slc9a6* gene is located on the X-chromosome and as such, CS is inherited from carrier mothers heterozygous for the mutation.[1,2]

While most cases are of inherited mutations, approximately 40% of clinically reported cases of CS have been due to *de novo* mutations.[8] Patients present very similar phenotypes with some variation in severity. It is currently unknown whether the severity of CS is dependent on the type of mutation.[8]

Patients of CS are usually born at term after uneventful pregnancies with normal growth parameters except for head circumference which can range from slightly low to average.[1,3,9-12] However, developmental delays become apparent early in infancy. Gross motor acquisition is delayed with head control occurring at 4-5 months and sitting up at 8-9 months.[3,4,11] Assisted or unassisted walking is delayed until 1-3 years of age and with unsteady gait due to truncal ataxia, the loss of coordination of muscles in the trunk involved in gait stability.[2-4,9-13] Patients also acquire little or no fine motor skills.[3,17] Verbal language does not develop in CS patients except for a few words at most although patients respond normally to auditory cues indicating normal hearing.[1,3,4,17] When administered non-verbal intelligence tests, CS patients score within the ID range when tested in infancy, adolescence or adulthood.[1,4,17] Regardless of the patients' actual age, CS patients have shown the age equivalent of 5 months to 1 year when tested in realms such as receptive and expressive language, daily living skills, as well as social functions like interpersonal relationships, play and leisure time, coping skills.[2,4] Almost all patients have strabismus, hyperkinetic behaviour and a visibly happy demeanour with unprovoked laughter.[1-4,9,11] Many patients have problems with feeding and digestion due to swallowing difficulties, regurgitations and Gastro-Esophageal Reflux Disease (GERD).[2-4,9] Additionally, many patients also exhibit sleeping problems.[4]

A major comorbidity of CS is epilepsy. All patients are typically diagnosed with epilepsy with the seizure onset ranging from 4 months to 3 years of age.[1-4,9,13,16] Patients can have varying seizure types but most typically have tonic-clonic, generalized or focal-onset seizures.[1,3,4,12,14]

Patients can have seizures around ten times a week or several times a day and are usually treated with medication such as phenobarbital to lower seizure frequency down to 0-2 seizures a year.[3,11] More than half of CS patients examined undergo regression of symptoms later in life following seizure clusters or severe illness.[3,4,10] As they age, gross and fine motor skills, walking, motor coordination as well as body stature and weight deteriorate greatly.[3,4,16] Intellectual and social functions such as minor verbal vocabulary, eye contact, facial and body language expression also decline or are lost completely.[3] The onset of regression has occurred anywhere from 15 months to 16 years in patients examined to date.[3]

Neuroanatomical signs of CS have been observed in patients through neuroimaging and in post mortem examinations. Post-natal microcephaly and enlarged ventricles of the brain are seen in almost all patients.[1,3,12,17] Patients also show mild to severe atrophy of the cerebellum and brainstem that worsens with age.[1,2,14,17] Some patients have diffuse neuronal loss and decreased size in the cerebral cortex and hippocampus.[1,12,17] Interestingly, gliosis and tau pathology have been found in the cerebral cortex, hippocampus and brainstem of patients post mortem.[17]

A definitive diagnosis for CS is given after the presence of a *slc9a6* mutation has been found by genetic sequencing.[8] However, many CS patients receive an initial diagnosis of Angelman Syndrome (AS) due to the phenotypic similarities in both disorders.[4,8] Symptoms of CS such as ID, limited speech, ataxia, unsteady gait, happy disposition with unprovoked laughter, seizures, microcephaly and sleep problems are all typical of AS.[23] Unlike CS, AS is caused by the absence of the maternal *ube3a* gene encoding the ubiquitin ligase, E3A.[23] However, due to the phenotypic similarities mentioned, CS was referred to as an “Angelman-like syndrome” despite having a distinct genetic etiology.[1,8] Other patients were often initially diagnosed as having an autism spectrum disorder (ASD) due to the autistic features of CS displayed by many patients.[1,12]

Patients show symptoms such as absence of social play or interest in sharing as well as lack of appropriate facial expressions, eye contact and other body language expression or other emotional response.[4] Patients also exhibit behaviours such as using caregivers' hands as tools, stereotyped repetitive movements and an occupation with unusual sensory interests which are all typical of ASD.[4] As such, many patients often test as severely autistic when administered standard ASD diagnostic tests.[17]

Unlike male patients of CS, female carriers of the *slc9a6* mutation mildly display some symptoms of CS but with varied penetrance.[1] Carriers of an *slc9a6* LOF mutation can be unaffected or present mild impairments in learning, speech and fine motor control.[1,10,12] That said, most female carriers are usually functional, can attend school and have mostly intact motor function and speech.[3] Thus far, there have been no reported cases of a female patient homozygous for a *slc9a6* mutation.[8]

At present, there is no cure for CS. Patients of the condition require parental or caregiver guidance well throughout life.[8] Existing treatments for CS patients include medications for epilepsy and sedatives to aid proper sleep.[3,5,8]

1.1.2. Somatosensory Deficits

Although not previously clinically examined, recent reports by parents of CS patients suggest that the patients may have abnormal sensory function.[4,6] Normally injurious events will elicit little or no response from patients.[4] Patients are reported to show alarmingly little reaction to incidents such as severe cuts, bone fractures, burns and other noxious experiences both from mechanical and thermal stimuli.[5,6] On the other hand, some patients are reported to have an unusually strong aversion to certain innocuous stimuli, such as clothing tags, textured toys as well as certain shoes and sandals.[5,7] Moreover, patients also seem to be hypersensitive to cold, as

suggested by some patients' exaggerated shivering in mild cool temperatures and extreme displeasure when touching cold water.[5] Not all patients experience these symptoms to the same degree, but pain tolerance and hypersensitivity to innocuous stimuli will paradoxically present in the same patient.[5,7] Furthermore, CS patients also have behavioural peculiarities relevant to sensory stimuli. Caregivers report that while averse to certain textures and tactile sensations, patients can have an unusual affinity to touching specific toys and materials.[4,5] How the loss of NHE6 can result in the development of these symptoms is unclear.

1.2. The Sodium Proton Exchanger 6

The NHE6 protein, encoded by the *slc9a6* gene, is one of 9 mammalian NHE isoforms.[24] Like its isoforms NHE1-9, NHE6 has 12 transmembrane helices in the N-Terminus that forms the ion transport domain and a cytosolic C-terminus that is proposed to bind regulatory factors.[24] While NHE1-5 are associated with the plasma membrane, NHE6-9 are found on organelles inside the cell.[25] Organellar NHEs are broadly expressed by various tissues. The NHE6 exchanger itself is found in lower amounts in lung, liver, kidney, pancreatic tissue but expressed highly in brain, skeletal muscle and the heart.[26] Inside the cell, NHE6 localizes to the membranes of early and recycling endosomes.[27] The rest of the organellar NHEs are found in different intracellular compartments, with NHE7 and NHE8 found on the trans-Golgi network (TGN) and NHE9 in recycling and late endosomes.[25,28] Like the other characterized organellar NHEs, NHE6 transports either Na^+ or K^+ into the endosomal lumen and in exchange transports H^+ out of the endosome, therefore acting as a proton leak.[24,25] Thus, it works in opposition to Vacuolar type H^+ - ATPase (V-ATPase) which acidifies the intraluminal pH of endosomes and other compartments of the endosomal pathway.[29] In this manner, NHE6 functions in cohort with other organellar NHEs as

well as Cl^- and H^+ antiporters to regulate the intraluminal pH of compartments in the endosomal pathway.[28]

1.2.1. Endosomal Pathway

The endosomal pathway is an organellar network which regulates trafficking, recycling and degradation of proteins.[30] The pathway is composed of the Early Endosome (EE), Recycling Endosome (RE), Late Endosome (LE) and Lysosome (Lys).[31] Extracellular material and plasma membrane proteins are endocytosed in vesicles that are then sent to the EE. In a simplistic model, cargo from the EE is then sorted to REs to be recycled back to the plasma membrane locally or translocated to the plasma membrane at another area of the cell. Alternatively, the EE can also route cargo to LEs which then fuse with Lys's for the material to be degraded.[31] Compartments of the endosomal pathway have separate identities and functions maintained in part by differences in their associated regulatory proteins and intraluminal pH.[32]

The EE acts as a sorting station and is the first to receive incoming endocytosed cargo. The EE can receive cargo through clathrin-dependent as well as clathrin-independent pathways that are caveolar or mediated by proteins such as ARF6 and GEEc.[32,33] The EE forms from the fusion of primary endocytic vesicles and has many vacuolar and tubular domains.[32] These domains initiate the sorting of endocytosed materials to be recycled or degraded. As such, while the EE is typically enriched with Rab5, EEA1 and APPL1, regulatory proteins specific to the EE, there are also domains containing Rab4 and Rab11 that are usually found in local and long distance REs.[33,34] Additionally, the EE also communicates with the TGN to receive regulatory proteins as well as acid hydrolases that start the degradative identity in some domains of the EE.[33] However, these acid hydrolases are not yet active due to the only slightly acidic intraluminal pH of EEs. The intraluminal pH of EEs tends to be within the range of 6.8 to 6.0, which is thought to promote

certain sorting mechanisms and dissociation of ligands from bound receptors but is not acidic enough yet to allow degradative reactions.[31,33]

There are some similarities between the associated proteins and pH of EEs and REs. The intraluminal pH of REs does not differ greatly from that of EEs sharing the range of 6.8 to 6.0.[32] REs are highly associated with regulatory proteins such as Rab4, Rab11 as well as endosomal SNARE fusion proteins Syntaxin13 (Syn13) and vti1a.[33-35] These proteins are found in EEs as well, although in lower levels and only in certain domains possibly due to the cycling of cargo between the RE and EE compartments.[33] The LEs, as part of the degradative compartment of the endosomal pathway, are distinct in their pH and protein composition. These organelles are derived from domains of EEs. To fully mature as LEs, the vesicles go through steps such as a shift in fusion partners from the EE-associated Rab5 to the LE-associated Rab7.[32,35] Additionally, there is a drop in acidic pH to the range of 6.0 – 4.8 in LEs and the further acquisition of lysosomal components like Lysosome Associated Membrane Proteins (LAMPs) and acid hydrolases.[32,33] At the Lys, endosome-associated proteins are shed, LAMP proteins become more enriched and the acidic intraluminal pH of 4.5 aids the activity of degradative enzymes.[32] The purpose of the intraluminal pH gradient across the endosomal pathway is not completely understood. Thus far, it is known that the acidic pH aids processes such as the sorting of cargo, ligand release for recycling receptor proteins as well as hydrolytic reactions. The intraluminal pH gradient is also considered to give the cargo a “sense” of its position along the pathway, from the less acidic sorting and recycling compartments to the more acidic degrading compartments.[35]

The current understanding of the structure and function of the endosomal pathway comes mainly from studies in non-polarized cells. The endosomal system, in polarized cells with specialized functions such as neurons is more complex and unclear. The main components of the

endosomal system are present in neurons, although differing in intracellular localization and protein composition.[31] For example, recycling endosomes are clustered in non-polarized cells but are seen spread throughout the somatodendritic and axonal domains of neurons.[31] Furthermore, there seems to be variation in the distribution of proteins such as EEA1 and Rab5 between EEs in the somatodendritic space and axons of the neuron.[34] There are also neuron-specific endosome-associated proteins such as NEEP21.[31,34] This variation is expected due to the many specialized mechanisms observed in neurons. Protein recycling and degradation pathways in neurons must accommodate processes such as the turnover and degradation of domain-specific channels and membrane receptors, retrograde neurotrophic signaling, axonal pathfinding in development, synaptic plasticity as well as vesicle loading and recycling.[29,31,34]

There is mounting evidence demonstrating the significance of endosomes in neuron specific processes. Neurotrophin signaling is mediated in part by endosomes. During development, neurotrophins expressed at the distal axon get endocytosed upon binding ligands.[34] The endocytosed ligand-receptor complex can be dissociated soon after endocytosis with the receptor locally recycled back to the plasma membrane.[34] Alternatively, the ligand-receptor complex is sorted to a long-distance recycling endosome which travels retrogradely to the soma as a signalling endosome complex.[36] At the soma, the complex can initiate signaling cascades to activate transcriptional regulation in the nucleus or regulate processes such as the clustering of post synaptic density components at the dendrites.[34,36] In the case of the Tyrosine receptor kinase A (TrkA) and Nerve Growth Factor (NGF) signaling, downstream effects of this signaling complex at the plasma membrane differ from the effects of the signaling complex when it is endocytosed. Specifically, signaling at the surface-mediated survival whereas signaling while internalized mediates differentiation.[34,37] As such, neurotrophic signaling is aided by endosomal function.

Likewise, the endosomal system has been implicated in synaptic regulation in neurons. Members of the Rab family of GTPases associated with endosomes and endosomal SNARE proteins have been found on endosome-like structures in the presynaptic compartment.[33] There is evidence suggesting endosomes mediate dendritic spine development and maturation. Long Term Potentiation (LTP)-dependent spine development and maturation in the hippocampus can be blocked by interfering with the activity endocytic recycling proteins such as Syn13 and Rab11.[38] Blocking these proteins also resulted in rapid spine loss.[38] Endosomal fusion proteins Syn13, Syn6 and vti1a were also found localized to synaptic vesicles.[39] Inhibiting these proteins decreased the readily releasable population of synaptic vesicles and therefore reduced synaptic vesicle release.[33,39] Therefore, endosomal activity can regulate synaptic development and function. Given recent evidence implicating endosomal NHEs in neurodevelopmental and neurodegenerative disorders such as CS, data is emerging to clarify the function of NHE6 in the endosomal systems, notably in neurons.

1.2.2. NHE6 Regulation of Endosomes

There is much to be understood of the role of NHE6 in the endosomal pathway, especially in neurons, but studies conducted in heterologous expression experiments and polarized cells including neurons provide some insight into its function. According to findings in HeLa cells, NHE6 is involved in clathrin-mediated endocytosis of transferrin.[40] The exchanger mediates the early phase of internalization and is not associated with proteins endocytosed by clathrin-independent mechanisms such as Epidermal Growth Factor (EGF).[25,40] In the polarized hepatoma cell line, HepG2 that expresses NHE6 endogenously, the exchanger is implicated in maintaining the apical plasma membrane lipid composition to keep basolateral-apical cell polarity.[24,28] Another

polarized cell type, hair cells found in the inner ear, express the endosomal NHE6 and NHE9 exchangers which control pH in hair bundles of the cell separate from the cell soma.[28,41]

Recent studies in neuronal tissue have demonstrated roles for organellar NHEs, including NHE6, in neuronal development and activity. As mentioned above, endosomal function is important for dendritic spine development. In the hippocampus, NHE6 has been observed in GluA1-containing endosomes in dendritic spines after LTP, suggesting a role for NHE6 in the recruitment of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors.[42] Furthermore, NHE6 was also shown to be associated with TrkB in the hippocampus to promote neuronal development and dendritic spine maturation.[43] Another NHE isoform, NHE5 which cycles between plasma membrane and recycling endosomes, is shown to regulate pH in the synaptic compartment which assists in the regulation of dendritic spine density and maturation.[28,44] Organellar NHEs have also been implicated in synaptic vesicle loading and release. In synaptic vesicle loading, uptake of monoamines gamma-aminobutyric acid (GABA) and glutamate is coupled to the electrochemical gradient across the vesicle membrane.[29,45] Interestingly, NHE6 was found associated with vesicular GABA transporter (VGAT)- and vesicular glutamate transporter 1 (VGLUT1)-enriched synaptic vesicles through mass spectrometry.[46] The data mentioned above implicate NHE6 and possibly other organellar NHEs in synaptic vesicle loading. Therefore, there is growing evidence suggesting the contribution of NHE6 to proper neuronal development and activity as in other cell types. Thus, it follows that NHE6 LOF could have such deleterious effects as observed in CS. The mechanisms resulting in this pathology at the intracellular and physiological level are now being examined by the field.

1.2.3. Loss of NHE6

As NHE6 transports Na^+ and K^+ into the endosome and H^+ out, the exchanger therefore acts as a proton leak channel to de-acidify the endosome.[24,25] Therefore, the loss of NHE6 must lead to overacidification of the endosomal pH. Indeed, knockdown of NHE6 in HeLa cells leads to overacidification of transferrin positive vesicles.[40,47] The same result is seen in studies examining endosomal pH in the absence of NHE6 in the AP-1 cell line and primary mouse hippocampal neuron cultures.[43,48,49] The greater cellular and physiological consequences of this endosomal overacidification have not been fully elucidated.

At the cellular level, NHE6 LOF results in a decrease of the early endocytosis of cargo.[40,48,49] Overexpression of a CS-associated mutant form of NHE6 is also found to induce apoptosis in AP-1 cells, the neuroblastoma line SH-SY5Y, and primary hippocampal neuron cultures.[48,49] Decreases in neuronal branching and dendritic spine genesis and maturation were also demonstrated due to NHE6 LOF in mouse hippocampal neurons linked to decreased TrkB signaling.[43,48,49] Furthermore, neurons of the amygdala, hippocampus and piriform cortex in NHE6 null mice show robust GM2 ganglioside accumulation which is a sign of lysosomal storage disease.[50,51] Additional cellular pathologies seen in mice lacking NHE6 are, abnormal aggresomes and accumulation of unesterified cholesterol in the amygdala as well as marked Purkinje cell death in the cerebellum.[50,51] There are also discernible changes in the brain growth and degeneration of nervous system structures in NHE6 null mice. Post-natal brain growth in the NHE6 null mice is delayed in the cerebrum and cerebellum resulting in significantly smaller brain sizes, decreased area of the hippocampus and striatum and finally decreased thickness of the cortex and spinal cord.[52] Cortical degeneration progresses significantly faster in mice lacking NHE6 compared to WT mice.[52]

At the behavioural level, mice lacking NHE6 have behavioural phenotypes which model some symptoms seen in CS patients. Deficits in spatial memory, hyperkineticism and loss of motor coordination with regression in old age are observed in NHE6 null mice.[50,51] While the pathologies seen in CS and the mechanisms responsible are beginning to be revealed by current literature, there is very little known about the somatosensory abnormalities now associated with CS.

1.3. Physiology of Pain

The somatosensory system is essential to our organism as it facilitates the exploration of our environment and protection from harm through the perception of touch and pain.[53] The perception of external stimuli requires specific structures in the nervous system. These structures mediate the detection, integration, modulation and affect that can be associated with touch and pain.[54]

Somatosensation begins at sensory neurons located in the periphery. These primary afferents are the first responders of the somatosensory pathway.[54] The somas of these sensory neurons reside in clusters next to the spinal cord forming the dorsal root ganglia (DRG).[54] These neurons have a pseudo-unipolar morphology whereby an axon extends from the cell body and projects one axonal branch to peripheral targets and another axonal branch to post-synaptic targets in the central nervous system (CNS).[55] Peripheral terminals of primary afferents innervate visceral organs to mediate visceral somatosensation or innervate the skin to mediate cutaneous sensation.[54,55] Primary afferents exist as modality- and intensity-specific subsets which send central terminals to different targets in the spinal cord and other supraspinal structures.[56,57]

The next destination of sensory input is the dorsal horn (DH) of the spinal cord. While the first synapse for primary afferents from the head and neck can be found in the brainstem, primary afferents from the rest of the body form their first synapses in the DH.[58] The DH is the first site of

sensory integration and has two functionally separate domains: the deep DH and the superficial DH.[59] The deep DH receives collaterals from innocuous touch sensitive afferents which then project through the dorsal column to contact dorsal column nuclei of the brain stem.[57,59] From the dorsal column nuclei, the low intensity touch information is sent to the thalamus via the medial lemniscal pathway, where the signal is relayed to the primary somatosensory cortex (S1).[54]

Nociceptive inputs follow a different pathway. The superficial DH is innervated by nociceptive and heat sensitive afferents which synapse pain projection neurons.[54,59] These pain projection neurons relay nociceptive signals to structures in the brainstem and brain through the anterolateral tract.[54] From the anterolateral tract, the signal is transmitted to the ventral posterolateral nucleus (VPL) of the thalamus.[57,60] From the VPL, nociceptive signals are sent to the S1 to mediate the localization of the stimulus.[60] The anterolateral tract also contacts the parabrachial nucleus (PBN) in the brain stem which sends nociceptive signals to the anterior cingulate cortex (ACC) via the amygdala.[61,62] The ACC mediates the affective response to the noxious stimulus therefore aiding in the interpretation of the stimulus as an unpleasant painful experience.[61]

In addition to the detection and interpretation of pain, supraspinal structures can also influence incoming nociceptive information. Nociceptive projection neurons from the DH contact the periaqueductal gray (PAG) and rostral ventral medulla (RVM), brainstem structures involved in downstream modulation of nociceptive input and response.[63] The PAG is controlled by the hypothalamus, amygdala and cerebral cortex and subsequently influences RVM activity.[63,64] Descending signals from the RVM then regulate dorsal horn activity and therefore shape ascending nociceptive input.[64] There are other minor pathways that exist to project somatosensory information to the brain and even more pathways within the brain and brainstem that contribute to

pain perception, forming the pain "neuromatrix"; however, the previously mentioned structures and pathways are its major components.[54,65,66]

1.3.1. Primary Afferents

Peripheral sensory neurons can detect a wide array of stimuli due to the diverse functional, morphological and biomolecular profiles found in DRG neurons. The primary afferents are broadly classified based on conduction velocity (CV) and myelination into the following classes in order of descending CVs: Aα-, Aβ-, Aδ- and C-fibres.[56] These classes roughly correspond to functional subsets, such that the large diameter, thickly myelinated and fast conducting Aα-fibres and Aβ-fibres are proprioceptive afferents and low threshold mechanoreceptors (LTMRs) respectively.[57] The small diameter thinly myelinated Aδ- and unmyelinated C-fibres are most often high-threshold nociceptors.[56] That said, there are Aδ-LTMRs and C-LTMRs functionally separate from the nociceptor populations.[57] The expression of biomolecular markers further segregate subset populations within this broad classification scheme.

The nociceptive C-and Aδ-fibre populations contain subsets responding to noxious mechanical, heat and other stimuli. Within the unmyelinated C-fibres, a polymodal subset responds to noxious heat and noxious mechanical stimuli.[56] The C-fibre population can also be separated by their expression or lack of neuropeptides. Peptidergic C-fibres express the neuropeptides substance P and Calcitonin Gene Related Peptide (CGRP) as well as the neurotrophin receptor TrkA.[56] Non-peptidergic C-fibres can be identified by their binding of the Isolectin B4 (IB4) and express the C-Ret receptor.[54,56] The thinly myelinated Aδ-nociceptors can be divided into the Type I high threshold mechanoreceptors (Type I – HTMRs) which respond to noxious mechanical and chemical stimuli as well as the Type II Aδ-nociceptors which mainly respond to noxious heat.[54]

Apart from size and conduction velocity, primary afferents types differ in the end organs they form at peripheral terminals. LTMR populations form specialized end organs in both hairy and glabrous skin.[67] The A-β LTMRs innervate formations in the skin such as the Ruffini's end organs, Pacinian corpuscles, Meissner's corpuscles as well as associate with Merkel cells to detect pressure, stretch, vibration and texture discrimination.[67] The A-δ LTMRs form lanceolate endings at hair follicles along with C-LTMRs which also innervate hairy skin.[57,67] Cutaneous nociceptors form free nerve endings which terminate in the skin epidermis.[57] However, recently nociceptive HTMRs were identified forming circumferential endings around guard hair follicles.[68] At the peripheral terminals, low intensity encoding afferents as well as nociceptors express transducer proteins.[56,67] These are activated by external stimuli and transduce the stimulus into a signal to be transmitted by the primary afferent.

1.3.1.1. Sensory Transduction

Heat-gated channels were among the first to be discovered as transducers. There are distinct channels sensitive to warmth and noxious heat. The transient receptor potential cation channel subfamily V 1 (TRPV1) is expressed by the majority of thermo-nociceptors, in both C-fibre and A-δ fibres.[69] The TRPV1 channel is thermally gated but also can be activated by binding capsaicin.[70] Other heat-gated channels are also involved, such as the TRPV2 channel which is found in A-δ fibres and activates at a higher heat threshold than TRPV1.[71] The TRP channels TRPM3 and TRPA1 have also been implicated in noxious heat sensing, working with TRPV1.[72]

At the opposing end of the thermal spectrum, cold sensations are also detected by TRP channel family members. The TRPM8 channel detects cool temperatures and is menthol sensitive.[73,74] The channel is expressed by a subset of C-fibres as is the TRPA1 channel.[75] While TRPM8 is sensitive to cool temperatures, TRPA1 detects noxious cold and is activated by menthol

and icilin.[76] Therefore, these two TRP channels cover a range of cool to noxious cold temperatures together.[75] The TRP channels also mediate response to noxious chemicals. Namely, TRPA1 is activated by the pungent compounds, isothiocyanates and thiosulfinates in addition to TRPV1 and its ligand capsaicin.[71,77,78]

In contrast to the thermosensitive and chemosensitive channels identified, not many mechanosensitive channels have been identified in mammals. The Piezo1 and Piezo2 channels are mechanoreceptors mediating proprioception discovered in mammals.[79,80] These channels have also been implicated in light tactile sensitivity and respiratory pathology as seen by patients lacking the Piezo channels.[81,82] Another set of mechanically-gated channels known in mammals are two pore potassium channels TREK1,2 and TRAAK.[83-85]

Signal transducers convert an external stimulus into an electric signal that can be transmitted by the primary afferent axon. Channels expressed along the axon length and at central terminals of primary afferents aid in the propagation and transmission of this signal.[86] Voltage-gated sodium, potassium and calcium channels contribute to proper sensory transmission.

1.3.1.2. Signal Transmission

Sodium channels mediate fast membrane depolarization and therefore are important for the propagation of action potentials throughout an axon.[87] Voltage-gated sodium channels (Nav) expressed by peripheral sensory neurons are no different. Primary afferents express Nav1.1, 1.6, 1.7, 1.8 and 1.9 where Nav1.7-9 are expressed in nociceptors.[86] The Nav1.7, 1.8, and 1.9 channels have slightly different contributions to action potential generation and propagation. Both Nav1.7 and Nav1.9 set the gain, enhancing small depolarizations which will eventually lead to action potential upstroke, but these two channels do not contribute to the actual upstroke.[86] Instead, Nav 1.8 produces the inward current contributing to the action potential upstroke.[86] The Nav1.7 and

1.9 channels are important for setting neuronal excitability such that their loss of function can lead to severe cases of pain insensitivity.[88]

Repolarization is achieved by potassium channels. Primary afferents express the voltage-gated Kv channels, two pore potassium channels (K2P) as well as calcium or sodium activated K channels.[86] Multiple types of Kv channels are expressed in afferent fibers such as Kv1, 2, 3, 4, 7 and 9.[86] These Kv channels regulate spike duration and determine neuronal firing frequency. Their function negatively regulates membrane excitability.[86] The K2P channels maintain hyperpolarized resting membrane potential. Primary afferents express the TRESK, TRAAK, TASK, TREK, THIK channels, some of which are also mechanically or thermally-gated such as the TREK and TRAAK channels.[84,86] These channels also negatively regulate primary afferent excitability and sensitivity to mechanical or thermal stimuli. The calcium and sodium activated K channels K_{Ca} and K_{Na} contribute to action potential repolarization and therefore, in part, regulate neuronal firing patterns.[86]

Lastly, peripheral sensory neuron activity is also aided by voltage-gated calcium channels (CaV). At the periphery, Cav3.2 T-type channels mediate afferent fibre excitability and are thought to facilitate opening of colocalized Nav channels.[89] At the central terminals, CaV2.2 and 3.2 T-Type as well as N-type channels can contribute to synaptic transmission at the dorsal horn by mediating neurotransmitter release.[86,89] Therefore, CaV channels contribute to both the initial generation of an action potential as well as mediate the synaptic transmission of the electric signal at the central terminals.

1.3.2. The Dorsal Horn

As discussed earlier in this chapter, the DH is the first site to receive and integrate peripheral somatosensory information.[59] The functional and morphological organization is much

more complex than described above. The DH is arranged in Rexed laminae that are distinguishable due to bands of molecularly and morphologically diverse interneurons.[59,90] The functional separation between the superficial laminae (LI-II_{Outer}) and the deeper laminae (LII_{Inner} -IV) is partly maintained by the direction of incoming nociceptive inputs to the superficial laminae and the LTMR inputs to the deeper laminae.[57,91] However, circuits formed by the distinct populations of excitatory and inhibitory interneurons actively integrate sensory inputs to separate these two DH domains.[59] The gate control theory was the first to assert the existence of DH circuits through which light touch can dampen noxious signals.[92,93] The theory proposed a circuit in which non-nociceptive Aβ-fibres and nociceptive C-fibres contact inhibitory interneurons and nociceptive projection neurons of the DH.[92,93] The theory also predicted that Aβ-fibres excite inhibitory interneurons to ultimately inhibit the projection neurons from conveying incoming nociceptive inputs from C-fibres.[92,93] Remarkably, the prediction that Aβ-fibres, C-fibres, and inhibitory interneurons work together in the DH to modulate the output of spinal projection neurons was later supported by discoveries made in the field.[92,94] In fact, there are connections between the LTMR-innervated deep DH and the nociceptive superficial DH, such that light touch inputs can activate nociceptive projection circuits through this pathway.[95] However, this crosstalk is gated by tonic inhibition mediated by GABAergic and glycinergic inhibitory neurons in the deep DH which are also contacted by light touch inputs.[57,96,97] Disinhibition of these connections by inhibiting GABAergic and glycinergic neuron activity reveals circuitry present for touch-evoked pain.[97,98] Abraira *et al.* described 11 populations of interneurons, 7 of which are excitatory and 4 inhibitory populations, with each population displaying distinct morphologies and firing properties.[99] Moreover, these interneurons receive input from peripheral sensory neurons, from descending cortico-spinal projections as well as other DH interneurons.[99] This integration of LTMR inputs is

essential for proper tactile sensitivity and texture discrimination.[99] Processing of nociceptive input also occurs by excitatory and inhibitory interneurons before projection to supraspinal targets.[55,100]

1.3.3. Development

Neurons of the somatosensory system have many diverse functions and accordingly diverse morphologies and biomolecular identities.[101] Many developmental events occur during the embryonic and early post-natal stages of an organism's growth to establish a proper somatosensory function.

1.3.3.1. Primary Afferent Differentiation and Innervation

Primary afferents are derived from early neural crest cells (NCCs). The early NCCs are induced by the influence of bone morphogenic proteins (BMPs) and the Wnt signaling factors.[101] The transcription factors, Islet1 and Brn3a, supress DH and other neuronal fates to ultimately induce peripheral sensory neuron fate in these NCCs.[102] The sensory neuron precursors begin migrating to form DRGs starting from E8.5 and finishing at E11 in the rodent.[101] The DRGs are formed by three waves of proliferation, the first two being NCCs and the last being boundary cap cells. The first two waves are mediated by neurogenin2 and 1 (ngn2/1) which form the TrkC/B lineage and some TrkA lineage DRG neurons respectively.[102] The third and last wave of boundary cap cells forms the remaining TrkA lineage sensory neurons.[102] The three Trk populations are specified by E13-14 in the rodent with the help of Runx1 and Runx3 factors.[103] The Runx3 factor expression separates TrkC and B expressing subsets of DRG neurons while Runx1 expression isolates the TrkA expressing subset.[101] In this way the expression of the Trk family of trophic factor receptors is the earliest marker of sensory neuron subtypes such that: TrkC is expressed mainly by large diameter proprioceptive neurons, TrkB is expressed by LTMRs and TrkA expressing neurons are nociceptors and thermoreceptors.[103] Further separation within the

nociceptors occurs post-natally with the expression of the Ret trophic factor receptor inhibiting TrkA expression in half the original TrkA lineage neurons.[102] This separates the non-peptidergic TrkA negative, Ret expressing nociceptors and the peptidergic TrkA expressing, Ret negative nociceptors.[102] The expression of the Ret and Trk family of trophic factor receptors is essential for neuronal survival and full phenotypic maturation of the sensory neuron subsets.[103] For example, TrkA is necessary for the expression of nociceptor specific ion channels and neuropeptides.[103,104]

Apart from neuronal specification, DRG neurons also extend axons to innervate peripheral targets and central targets before birth.[105] The sensory neurons innervate characteristic skin dermatomes in the periphery and form the typical somatotopic pattern in the DH.[105] This innervation is in part aided by growth factors and the expression of receptors for chemoattractants and chemorepellents.[101] Chemokine receptors expressed on DRG neuron axons include neuropillin (nrp1) and plexinA4/3 which can both interact with the chemorepellent semaphorin3A (Sema3A).[105,106] The Sema3A chemorepellent causes the collapse of axon growth cones upon binding nrp1 therefore inhibiting axon outgrowth where it is expressed.[107] During the rodent equivalent of E11-14, Sema3A is expressed in the DH when A-LTMRs form projections through the dorsal column.[105] The expression of Sema3A persists in the ventral horn but decreases in the DH starting at E14-15 when A-LTMRs begin to send collateral branches to innervate the DH.[105,108] Interestingly, A-fibre axons expressing TrkC and B tend to express less nrp1 and therefore extend deeper into the DH.[108] Whereas, C-fibre axons expressing TrkA express more nrp1 and therefore, stay near the superficial DH where there is little Sema3A.[108] Innervation of the DH begins during the embryonic stage but proper maturation of DH circuits is not accomplished until the early post-natal period in rodents.[109]

1.3.3.2. Dorsal Horn Differentiation and Maturation

The DH has a variety of neuronal subsets arranged in specific laminae due to the action of several transcription factors during development. The spinal cord itself forms from the vertebrate neural tube. Discrete domains form in the neural tube containing combinations of transcription factors that define different cell types.[110] The domains include dorsal interneuron 1-6 (dI1-6) as well as dorsal interneuron late born A-B ($dIL_{A/B}$) as well as the dorsal progenitor and ventral domains.[110] These domains express transcription factors such as lbx1, tlx1/3, pax2 and many more.[55] The DH neurons are born between E10-12.5 for early born and E11-13 for late born and migrate from these discrete domains to form laminae by E15.[110,111] By this time, the primary afferents begin to enter the DH and form connections with DH interneurons.[112] The low threshold A-fibre collaterals enter the DH at E15-17 and C-fibres enter later at E18-20.[105] The central terminals of the sensory neurons enter during the embryonic stage, but the formation and maturation of proper DH circuitry happens during the first post-natal weeks.[105,109]

In rodents, synaptic connections between primary afferents and DH neurons are immature at birth as demonstrated by exaggerated and uncoordinated responses to low threshold mechanical stimuli.[105,109] During the late embryonic stage, while C-fibres tend to specifically innervate the superficial LI-II layers of the DH, A-fibres extend from the deeper LIII-IV to the superficial LI-II laminae as well.[105,109] In fact, A-fibre innervation of LI-II is more functional than C-fibre innervation during the early post-natal stage. Stimulation of A-fibres during early post-natal causes robust activation of superficial DH neurons.[113,114] Proper C-fibre evoked activity in the superficial DH occurs after the second post-natal week.[115] Although C-fibres can be activated by electrical stimulation or chemical irritants in the first post-natal week, this stimulation does not yet activate superficial dorsal horn neurons.[116,117] At this stage, nociceptive activity is mediated only by Aδ-

fibre input.[109] Nociceptive and low threshold responses mature after the third post-natal week when A-LTMRs retract from the superficial laminae and C-fibre contacts onto superficial DH neurons increase.[109] In addition, glycinergic inhibition, required for the gating of LTMR input from nociceptive projection circuits, is matured after the third post-natal week.[96,109] It has been demonstrated that the maturation of glycinergic synapses in the DH and the retraction of A-fibre input from the superficial laminae is dependent on C-fibre activity during the second post-natal week.[109,118] Ablating C-fibres by excess capsaicin administration after birth prevents A-fibre withdrawal, the maturation of glycinergic inhibition and also has consequences for GABA-mediated inhibition and descending inhibitory signals.[119-121] Additionally, selective block of C-fibre activity over the second post-natal week delays functional glycinergic inhibition that gates touch-evoked nociceptive circuit activation.[109,118] By the fourth post-natal week, glycinergic inhibition matures in the DH allowing for the functional separation between LTMR and nociceptive input processing as seen in the adult.[55]

1.4. Known Developmental Pain Pathologies

In healthy conditions, the perception of pain is only activated in the presence of intense noxious stimuli.[54] The protective quality of pain comes from its ability to activate a withdrawal reflex and elicit an unpleasant emotional response upon detecting harm.[53] The absence of either of these actions of the pain mechanism can lead to serious injury whereas, an excess or impairment of either of these actions can be debilitating.[53] According the reports from parents of CS patients, patients have unusually elevated pain threshold while also presenting an aversion to tactile stimuli.[4,7] The observed sensory abnormalities have not yet been clinically examined specifically in CS patients. However, developmental or congenital conditions displaying similarly aberrant pain or tactile sensitivity have previously been investigated.

1.4.1. Pain Hyposensitivity

Congenital Insensitivity to Pain (CIP) is a condition present from birth that is characterized by an insensitivity to pain but typically intact sensitivity to other modalities.[122] Patients of CIP often suffer from self-inflicted lesions, painless fractures and neglected injuries due to the absence of pain as an alerting mechanism.[123] The condition has been associated with voltage-gated sodium channelopathies and loss of the neurotrophic factor receptor TrkA with its ligand NGF.[122]

The sodium channels Nav1.7 and 1.9 are both expressed in nociceptors and regulate nociceptor excitability by setting the gain.[86] Mutations in genes encoding Nav1.7 and Nav1.9 have been discovered in patients of CIP. Patients lacking Nav1.7 have no sensitivity to pain and are anosmic, or unable to perceive odor, but can perceive light touch, warmth, cold and pressure.[124,125] Patients have normal intelligence and show no sensory neuropathy in nerve biopsy and have normal brain scan.[124] The condition has been modeled using mice lacking Nav1.7, recapitulating insensitivity to noxious mechanical, thermal and chemical stimuli but innocuous mechanical sensitivity is intact.[126,127] While it is considered that a loss of Nav1.7 causes CIP due to reduced nociceptor excitability, it has also been suggested that the loss of Nav1.7 increases expression of met-enkephalin, an endogenous opioid.[126] This latter finding indicates that Nav1.7 LOF-associated CIP may be due to overactive opioid-mediated analgesia.[126] Apart from Nav1.7 LOF, mutations in Nav1.9 are also found in patients with CIP.[128,129] However, Nav1.9-associated CIP can present severe itching, intolerance of temperature changes and absent gut peristalsis.[128] Contrarily to the case of CIP-associated Nav1.7 mutations, both loss of function and gain of function mutations of Nav1.9 can lead to CIP.[128,129] Electrophysiological characterization of the CIP-associated Nav1.9 mutants demonstrates the contribution of Nav1.9 to the resting membrane potential (RMP).[130] Both

loss and gain of function Nav1.9 mutants disturb the RMP, impairing action potential generation.[129,130]

The CIP condition can also be caused by loss of function of TrkA or it ligand NGF, where the condition is referred to as CIPA because of the additional symptom of anhidrosis, the absence of perspiration.[131] Therefore, CIPA has a slightly different clinical presentation than found with the Nav channelopathies. Patients lacking TrkA exhibit insensitivity to visceral and superficial pain with anhidrosis, or the inability to perspire, as well as intellectual disability.[131] These patients have intact touch and position senses.[123] The loss of NGF also causes complete insensitivity to pain with anhidrosis and intellectual disability.[131,132] Nerve biopsies of patients with a loss of TrkA or NGF shows a decrease in C and A-δ fibres.[132] Studies conducted in model mice show that the loss of TrkA or NGF leads to the survival failure of NGF dependent neurons such as C and A-δ nociceptors during late embryonic development, sympathetic postganglionic cells mediating autonomic mechanisms such as sweating, as well as basal forebrain and striatal cholinergic neurons.[131,133,134] Therefore, neuronal death in these three structures must contribute to pain insensitivity, anhidrosis and intellectual disability respectively.

Lastly, a recently described CIP condition is caused by the loss of function of the *cltcl1* gene encoding the minor clathrin heavy chain 22 (CHC22).[135] Patients with a LOF mutation in this gene are insensitive to pain and soft touch, have developmental delays but have intact motor function and thermal perception.[122,135] The CHC22 protein is involved in endosomal sorting and is expressed in the human cortex, hippocampus, striatum and sensory neuron precursors prenatally.[135,136] In culture, sensory neuron precursors require the downregulation of CHC22 to initiate differentiation and neurite outgrowth.[135] Therefore, according to *in vitro* studies, CHC22

expression is vital for properly timed differentiation as well as the growth of sensory neurons and its LOF may cause CIP by severely disrupting sensory neuron development.[122]

A lack of sensitivity to pain, as in the described CIP conditions, has serious consequences for health and well-being.[53] Conversely, other equally debilitating developmental conditions exist that confer sensory impairments such as hyper-responsiveness to usually innocuous sensations.

1.4.2. Abnormal Sensory Processing in ASD

Patients of ASD exhibit aspects of sensory impairments in a variable manner.[137] They can exhibit hypersensitivity or an unusual aversion to certain low intensity stimuli.[138-140] They can also exhibit increased pain thresholds or decreased response to painful experiences.[141-143] Mouse models of ASDs have allowed the examination of physiological mechanisms underlying these sensory abnormalities. For example, patients of Fragile X syndrome (FXS) seem to experience tactile hypersensitivity.[144] Mice modelling FXS show exaggerated somatosensory barrel cortex responses to tactile stimuli and no adaptation to repetitive tactile stimuli.[145,146] Moreover, when testing a mouse model of AS caused by loss of maternal e3 ubiquitin ligase, global mutant mice recapitulated mechanical and thermal hyperalgesia seen in AS patients but not mice with a deletion in peripheral sensory neurons.[147] Although both these studies showed sensory abnormalities caused by dysfunction of higher order structures, there have also recently been studies implicating peripheral mechanisms for ASD-associated sensory deficits.

In a mouse model of Rett syndrome, caused by a loss of the epigenetic regulating mecp2 protein, mice lacking mecp2 only in peripheral neurons reproduced the tactile hypersensitivity and behavioural phenotypes of human patients.[148] The study further discovered that the tactile hypersensitivity was due to a mecp2 LOF induced decrease in GABAr expression by LTMRs which disrupted inhibition of LTMR input in the dorsal horn.[148] Similarly, another study examined

decreased pain sensitivity seen in ASD-associated with loss of the synaptic scaffolding protein, SHANK3.[143,149] Mice lacking SHANK3 had attenuated heat hyperalgesia and decreased response to chemical and neuropathic pain models due to decreased TRPV1 surface expression in the absence of SHANK3.[150] As such, while sensory deficits in ASD patients have been attributed to cognitive impairments in the past, there is increasing evidence implying peripheral mechanisms.

1.5. Aim

In addition to many neurodevelopmental defects, patients of CS exhibit both a decreased sensitivity to noxious stimuli and an aversion to normally innocuous tactile experiences.[4,7] At the cellular level, loss of NHE6 was demonstrated to attenuate neurotrophic signaling and subsequent neuronal development as well as the surface expression of membrane bound proteins.[43,48,49] Nociceptor function depends on neurotrophic signaling for proper development as well as the surface expression of various ion channels and other effector proteins for detection and transmission of the nociceptive signal.[56,103] Nociceptor function also contributes to the maturation of dorsal horn circuitry.[109] Developmental conditions such as CIPs and ASDs present similar sensory phenotypes as observed in these patients. These conditions demonstrate that disrupted development of nociceptors or improper function and expression of ion channels by nociceptors can cause pain insensitivity.[122,123] These conditions also show that aberrant dorsal horn circuitry can result in improper processing of innocuous stimuli.[148]

1.5.1. Hypothesis

We extrapolate from previous studies of developmental sensory disorders that pain hyposensitivity and tactile hypersensitivity can be caused by nociceptor dysfunction and the subsequent irregular development of dorsal horn circuitry. Therefore, we posit that the loss of NHE6 must impair synaptic transmission by nociceptors which would then impair proper

development of the dorsal horn circuits. This would ultimately result in the hyposensitivity to noxious stimuli and the tactile hypersensitivity observed in CS patients.

1.5.2. Project overview

This study was comprised of three aims. Firstly, we determined the expression profile of the NHE6 protein in selected structures involved in the detection, integration, modulation and perception of noxious and non-noxious stimuli. Next, we characterized the sensory phenotype of an NHE6 knockout mouse line as a model of CS using tests for mechanical, thermal and cold sensitivity. Finally, we examined three mechanisms that when disrupted could lead to the observed sensory deficits: signal transduction tested by observing neurogenic inflammation, nociceptor growth and survival investigated by sensory neuron counts between mutant and wildtype mice and lastly, dorsal horn processing of peripheral inputs tested by brush induced Fos induction.

2. Methods

2.1. Animals

All animal use protocols were approved by the Comparative Medicine and Animal Resources Centre (CMARC) of McGill University. Mice were housed in 12-hour light and dark cycles with free access to standard rodent chow and water.

Immunohistochemistry experiments examining the expression of NHE6 in wild type (WT) tissue were performed on C57BL/6 mice. The NHE6 knockout (KO) mice were purchased from Jackson Laboratories (B6.129P2-Slc9a6tm1Dgen/J, Stock No. 005843). The Slc9a6 gene was inactivated in these mice by inserting a LacZ-Neo cassette into exon 6 of the gene. Mice were genotyped by PCR using the forward primers 5’-GGG TGG GAT TAG ATA AAT GCC TGC TCT-3’ and 5’-AAC AGC TGT GGA GGG ATA TGT GCT-3’ for mutant and WT respectively and the reverse primers 5’-AGC TGG CTT TGC GCA TGG AGC ATT C-3’. The PCR product

shows a band at 432 bp for mutants and 224 bp for WT with heterozygotes showing both bands. Breedings were made with a WT male C57BL/6 mouse and two $NHE6^{-/+}$ females to obtain $NHE6^{+/Y}$ (referred to here as WT) and $NHE6^{-/Y}$ (referred to here as KO) mice littermates for behaviour and immunohistochemistry experiments. Mice were backcrossed to C57BL/6 line prior to experimentation.

2.2. Immunohistochemistry and Imaging

WT and NHE6 KO mice were administered a lethal dose of ketamine for anaesthesia then transcardially perfused with a 4% paraformaldehyde (PFA) made in phosphate buffered saline (PBS). The brain, spinal cord and lumbar DRG were dissected and post-fixed in 4% PFA for 3 hours and cryoprotected overnight in 30% sucrose made in PBS. The brain, spinal cord and DRG samples were then cryosectioned into 40um, 25um and 14um sections respectively. Brain and spinal cord sections were kept floating in PBS at 4°C while DRG sections were mounted directly on Fisherbrand™ Superfrost™ Plus microscope slides (Thermo Fisher Scientific, Rockford, IL) and kept at -20°C until processed for immunofluorescence staining. Brain and spinal cord sections were floating while stained. DRG sections were stained while mounted on microscope slides.

Tissue sections were blocked in 10% normal goat serum with 0.33% triton x-100 (NGST) in PBS for 1 hour at room temperature. Then, they were incubated with primary antibodies diluted in 2.5% NGST for 48 hours at 4°C. The primary antibody reaction was stopped by 3x10 minute rinses in 1% NGST at room temperature. The tissue sections were then incubated with the secondary antibodies diluted at 1:500 in 1% NGST for 1 hour at room temperature. Following the secondary antibody incubation, the tissue was rinsed for 3x10 minutes with PBS and 1x10 min with distilled water. Brain and spinal cord sections were then mounted on microscope slides. Microscope slides with the stained tissue were dried and cover-slipped using Aqua-Poly/Mount

mounting medium (Polysciences Inc., Warrington, USA). Immunohistochemistry experiments on DRG tissue involving co-staining for tyrosine hydroxylase (TH) were performed sequentially with a sheep anti-TH antibody (1:400; Millipore). Tissue was first blocked in 2% horse serum with 0.33% triton x-100 (HST) in PBS for 1 hour at room temperature. Next, the tissue was incubated overnight at room temperature with the sheep anti-TH primary antibody diluted in 2% HST followed by 3x10 min rinses in 2% HST and a 1-hour incubation in a donkey anti-goat alexa fluor conjugated secondary antibody. After 3x10 minute rinses in PBS, the tissue was stained with other primaries as previously mentioned. The following primary antibodies were used: rabbit anti-NHE6 (1:250, generated and validated by Orlowski *et al.*)[42], mouse anti-NF200 (1:500, Sigma-Aldrich), rat anti-GINIP (1:1000, generated and validated by Dr. Aziz Moqrich)[151], mouse anti-CGRP (1:500, Sigma-Aldrich), biotinylated-IB4 (1:1000, Sigma-Aldrich), biotinylated mouse anti-Neun (1:500, Millipore). Alexa Fluor conjugated antibodies raised in goat against rabbit, mouse, or rat were used as secondary antibodies. Streptavidin conjugated to Alexa Fluor 647 was used to detect biotinylated primary antibodies. Stains were then imaged using the inverted fluorescent Zeiss LSM 710 Confocal Microscope. Images were analyzed using Image J software.

2.3. Behaviour Experiments

Behaviour tests were performed on KO and their WT littermates as the control group. Mice were tested three times at 8 weeks of age and again at 24 weeks of age unless otherwise indicated. Tester was blind to genotype of mice during the tests. Mice were acclimatized to each behavioural apparatus for 3 sessions of 30 minutes prior to testing. Mice were allowed to settle in the behavioural apparatus for 30 minutes before each testing session.

2.3.1. Thermal Sensitivity

Sensitivity to heat was assessed using the Hargreaves' test (Hargreaves Apparatus, Stoelting, USA). In this test, mice are placed in individual plexiglass restrictors on an elevated glass platform. A noxious thermal stimulus in the form of radiant heat is shone through the glass on the plantar surface of the hind paw. The latency to withdrawal of the hind paw away from the heat source is measured automatically. To avoid tissue damage, the cut-off latency was set at 20 seconds. Three to five trials were conducted with at least 5 minutes between each trial.

2.3.2. Mechanical Sensitivity

The von Frey test was used to observe mechanical sensitivity. Mice were placed in plexiglass restrictors on an elevated wire mesh. Calibrated von Frey filaments were used to apply varying weights to the plantar hind paw. Each filament was applied 5 times against the hind paw within a period of 30 seconds. The number of nociceptive responses such as withdrawal, paw fluttering or guarding and licking or biting of the paw were counted per 5 applications of each filament. The filament which elicits 3 nociceptive withdrawal responses out of 5 applications is taken as the mechanical withdrawal threshold. Starting at a filament exerting 20 mg of pressure, the mice were tested with filaments delivering increasing weights until they exhibited 5 withdrawal responses with 5 minutes of rest between each filament. To prevent injury, the highest filament weight tested was 2g.

2.3.3. Cold Sensitivity

Sensitivity to cold was assessed using the Cold Hot Plate Test apparatus (Bioseb, France). During the habituation and acclimatization sessions, the cold plate was set to 25℃. Mice testing protocol was adapted from a study by Miyake *et al.*[152] Mice were placed individually in a plexiglass restrictor on the cold plate set at specific temperatures ranging from room temperature

to noxious cold for set durations depending on the temperature. The following are the temperatures and durations used during testing: 25℃, 15℃ for 120s; 10℃, 5℃ for 90s; -0.5℃ for 60s. Mice behaviour in response to the set temperature was recorded for the previously mentioned durations. The behaviour recordings were divided into 10 second bins which were each given a score according to the following scheme: 0 – no response, 1 – flinching, 2 – jumping. The scores were averaged across the bins for each temperature to give one overall score per temperature for each mouse.

2.3.4. Neurogenic Inflammation Model

Intraplantar injection of capsaicin was used as a model of neurogenic inflammation. NHE6 KO and WT mice at 24 weeks were first acclimatized to an elevated glass platform, in individual plexiglass restrictors. The dorso-ventral width of both hind paws was measured prior to injection for each mouse. Mice were then unilaterally given an intraplantar injection of 5 μg of capsaicin emulsified in the following to make a 1μg/μl solution: 2.5% ethanol, 1.25% Tween-80, 2.5% dimethyl sulfoxide (DMSO) and sterile physiological saline. Immediately after the injection, mice were returned to the glass platform and their spontaneous behaviour was recorded for 5 minutes. The duration of licking behaviour during this time was observed as a measure of their spontaneous nociceptive response to capsaicin. The mice were allowed to rest for a total of 30 minutes after the injection, at which time the width of the injected hind paw was measured again to assess paw edema as an indication of neurogenic inflammation.

2.3.5. Fos Induction

Fos induction by light brush stimulation of the hind paw was used to assess the dorsal horn integration of light touch. NHE6 KO and WT mice at 24 weeks were kept under isofluorane gas anaesthesia. One hind paw of each mouse was brushed with a flat paint brush on the plantar aspect

from the heel to toe in one smooth motion at a frequency of 1 stroke s^{-1} for 10 minutes. The mouse was then kept under isoflurane anaesthesia for 90 minutes without any further stimulation. After 90 minutes, mice were euthanized and transcardially perfused with saline and 4% PFA. The lumbar spinal cords of these mice were dissected, post-fixed and processed for immunohistochemistry as described above. Using the mentioned immunohistochemistry protocols, dorsal horn sections were immunolabeled for the Fos protein (rabbit anti c-Fos mAb, Cat#2250, Cell Signaling Technology), IB4 for a laminar marker as well as NeuN as a marker for neuronal cell bodies. Images were captured and analyzed as mentioned above.

3. Results

3.1. NHE6 Expression in the Sensory Pathway

3.1.1. CNS and PNS Structures Express NHE6

We hypothesized that if the absence of NHE6 leads to improper processing of pain and touch signals, that the exchanger may be expressed in structures of the somatosensory pathway. Using a previously validated NHE6 antibody, we examined the expression of the exchanger in the S1 and ACC which are involved in the localization and emotional valence of the painful stimulus respectively.[54,60,61] In addition, we stained the PAG, involved in the modulation of the pain response, using NeuN as a general neuronal marker.[63,153] The anti-NeuN antibody used, has been used and cited as a reliable probe for NeuN in mouse CNS tissue.[154,155] Our data indicates that the exchanger is expressed in a small percentage of the neurons in these regions (Fig. 1 B: 20.1 ± 3.74% of NeuN expressing neurons co-expressed NHE6 in the ACC, 20.8 ± 1.21% in S1 and 10.7 ± 2.58 % in the PAG; n=3 WT mice).

We next examined the expression of NHE6 in the DH of the spinal cord, which constitutes the first relay station where peripheral sensory inputs are processed.[59] Although no neuronal cell

bodies were positive for NHE6, we found a strong signal in the superficial regions of the dorsal horn (Fig. 1, A). The signal presents a crest-like profile resembling the innervation of the dorsal horn by primary afferents. This suggests that the signal might originate from sensory neurons in the DRG. Indeed, when we stained the DRG, we found more than half of the neurons in the sensory ganglia were immuno-positive for NHE6 (Fig. 1 B: 54.82 ± 9.55% neurons, n = 3 WT mice). We next sought out to determine if the NHE6 expressing DRG neurons belonged to a particular subset of sensory afferents.

3.1.2. NHE6 is expressed by small diameter unmyelinated nociceptors

Peripheral sensory neuron subtypes can be differentiated based on their expression of well-known biomarkers and their cell soma size. To identify the population of NHE6 expressing sensory neurons, we compared the expression pattern of NHE6 to that of subset specific biomarkers in the DRG (Fig. 2, n = 3 WT mice). Of the NHE6 positive neurons, only 5.2 ± 1.04% seem to express parvalbumin (PV), a marker of proprioceptive neurons. A marker of myelinated neurons, neurofilament-200 (NF200), was found in 29.11 ± 4.43% of NHE6 expressing neurons. Interestingly, unmyelinated neurons accounted for much of the NHE6 expressing population. Tyrosine Hydroxylase (TH), which labels C-low threshold mechanoreceptors (C-LTMRs), was expressed by 13.15 ± 0.52% of NHE6 positive neurons. A marker of peptidergic nociceptive C-fibres, Calcitonin Gene Related Peptide (CGRP) was expressed by 18.74 ± 1.32% of NHE6 neurons. Non-peptidergic nociceptive fibres which bind Isolectin B4 (IB4) accounted for 33.94 ± 6.54% of NHE6 neurons. Finally, 44.70 ± 3.56% of NHE6 positive neurons express a recently characterized Gαi¬-Interacting Protein (GINIP) which labels non-peptidergic nociceptors and C-LTMRs.[151]

We continued our characterization by determining the cell size distribution of the NHE6 expressing population of sensory neurons (Fig. 2, C). When compared to the cell size distributions of a large diameter subset such as the parvalbumin expressing proprioceptors and a small diameter subset such as the non-peptidergic IB4 binding nociceptors, the NHE6 positive neurons tend to be of a smaller size. Taken together, our data indicates that NHE6 is expressed mostly in unmyelinated small diameter nociceptor neurons.

3.2. Sensory Profile of CS Model Mice

To gain a better understanding of the function of NHE6 in the transmission and processing of pain and touch, we performed behavioural tests on a mouse model of CS, in which the *slc9a6* gene is inactivated by the insertion of a *LacZ-Neo* cassette, effectively knocking out NHE6.

3.2.1. Thermal sensitivity

We used the Hargreaves' test to examine the thermal sensitivity of the KO mice and their WT littermates. At 8 weeks of age, KO mice had similar paw withdrawal latencies to their WT littermates, suggesting similar sensitivities to noxious thermal stimuli (Fig 3. A: 9.09 ± 0.81s for WT compared to 10.73 ± 0.91s for KO, n = 10 WT and 5 KO at 8 weeks, Independent samples T-Test). However, when tested at 24 weeks the NHE6 null mice had significantly increased paw withdrawal latencies compared to WT littermates (Fig 3. A: 8.92 ± 1.14s for WT and 13.82 ± 2.01s for KO, n = 8 WT and 5 KO; $p < 0.05$, Independent samples T-Test). This suggests that KO mice are less responsive to noxious thermal stimuli at 24 weeks.

3.2.2. Mechanical Sensitivity

We also tested the mechanical sensitivity in these mice using the von Frey test. At 8 weeks of age, KO mice and their wildtype littermates had similar mechanical thresholds (Fig. 3, B: 1.12 ± 0.16g for WT and 1.23 ± 0.14g for KO, n = 9 WT and 8 KO, Independent samples T-Test). At

24 weeks, the KO mice have higher mechanical thresholds than the WT mice (Fig. 3 B: 0.74 ± 0.11g for WT and 1.55 ± 0.16g for KO, n = 10 WT and 10 KO, Independent samples T-Test, $p < 0.001$). When tested at suprathreshold filaments, the wildtype mice steadily increase the number of nociceptive responses with the increase in intensity of the mechanical stimulus (Fig 3 C). However, the responses of KO mice plateaued shortly after they reached the mechanical threshold. Notably, KO mice had significantly less nociceptive responses to stimulation with 2g compared to WT littermates at 8 weeks (Fig. 3C: Independent samples T-Test, $p < 0.01$). Moreover, the difference between the number of nociceptive responses of the KO and the WT mice at supra-threshold filaments is more pronounced at 24 weeks of age (Fig. 3 D: Independent samples T-Test, $*p < 0.05$, $**p < 0.01$). Taken together, this means that KO mice have normal sensitivity to static mechanical stimuli below the mechanical threshold but are less responsive to noxious mechanical stimuli than their WT littermates. Moreover, this effect is more visible in the older KO mice.

3.2.3. Cold sensitivity

To further investigate the sensory profile of KO mice as a model of CS, we observed their sensitivity to cold. Using the cold plate assay, KO and WT mice were subjected to temperatures of 25.0℃, 15℃, 10℃, 5℃ and -0.5℃ to observe their behavioural response to mild and noxious cold temperatures. As expected, mice did not exhibit any nociceptive behaviour at 25℃, regardless of genotype or age. At 8 weeks, the response to both mild and noxious cold was similar in both KO and WT mice (Fig. 3 E: n = 9 WT and 9 KO at 8 weeks, 2-way ANOVA). At 24 weeks, whereas WT mice displayed very similar responses to all cold temperatures tested, KO mice showed exaggerated nociceptive behaviours starting at 15℃. We observed significantly more nociceptive flinching and jumping from the 24-week-old KO at temperatures from 15℃ to -0.5℃ than the wildtypes (Fig. 3 E: n = 16 WT and 9 KO at 24 weeks, 2-way ANOVA, $**p < 0.01$, ***p

< 0.001). This suggests, that KO mice are hypersensitive to mild and noxious cold temperatures which can be observed at 24 weeks of age.

3.3. Peripheral Contribution to Sensory Abnormalities of CS Model

Given that the KO mice exhibit sensory abnormalities much like the symptoms reported for CS patients, mice lacking NHE6 seem to model relevant characteristics of CS. We had hypothesized that sensory defects in this disorder could be due in part to the dysfunction of peripheral sensory neurons. Therefore, using the NHE6 null mice as a tool to study this phenomenon, we assessed possible models for the contribution of peripheral sensory neurons to the dysregulated pain and touch transmission observed in CS.

3.3.1. Neurogenic Inflammation

In addition to sensing and propagating information from external stimuli, sensory neurons also mediate protective and healing mechanisms in the periphery, one of which is neurogenic inflammation. Upon activation by noxious stimuli, peptidergic nociceptors release neuropeptides that act on vascular tissue and immune cells to induce inflammation of the injured area.[156,157] The application of capsaicin, a potent agonist of TRPV1, is often used as a model of neurogenic inflammation.[78] Here, we unilaterally injected capsaicin into the plantar hind paw of KO and WT mice at 24 weeks, to test the ability of peptidergic neurons in these mice to induce a nociceptive response and subsequently, inflammation.

Immediately after the intraplantar injection of capsaicin, WT mice displayed classic spontaneous pain behaviours, such as licking of the injected hind paw. Contrarily, KO mice exhibited spontaneous pain behaviours for less than half the duration of their wildtype littermates (Fig. 4 A: 18.84 ± 2.52s for WT and 8.38 ± 1.69s for KO in the 1st minute bin, n = 9 WTs and 7 KOs, Independent samples T-Test, $p < 0.01$). The dorso-ventral width of the mice hind paws was

measured before and after the capsaicin injection. The injected hind paws of both the WT and KO mice showed an increase in width after the capsaicin treatment (significant by paired T-test comparing pre and post injection widths, analysis not shown). However, when the paw widths were compared between the two groups post injection, we observed much less swelling in the KO mice' hind paws than in their wildtype littermates (Fig. 4 B: 137.93 ± 6.01% swelling for WT and 119.21 ± 4.49% swelling for KO, Independent samples T-Test, $p < 0.05$). Taken together, the decreased nociceptive response and the decreased paw edema suggests that the capsaicin induced inflammation is not as robust in the KO mice as it is in healthy WT mice.

3.3.2. Sensory neuron loss

Studies examining cells in culture as well as brain structures such as the hippocampus and cerebellum, have demonstrated that NHE6 is necessary for healthy cell development and survival due to its association with membrane bound signaling proteins.[43,49,50] Therefore, it is possible that the loss of NHE6 may interfere with peripheral sensory neuron development and health through similar mechanisms as previously demonstrated in CNS structures. Peripheral sensory neurons are stimulus modality and intensity specific and exist in well characterized subset populations. To assess an effect of the loss of NHE6 on sensory neuron health and survival, we examined the subset populations by staining DRG tissue from 8 and 24-week-old WT and KO mice for biomarkers of nociceptor neurons.

We focused on the NF200, IB4 and CGRP populations as these are populations that contain nociceptors and stained highly for NHE6. The antibodies used in this study have been previously validated and cited to probe for NF200,[158,159] IB4[160-162] and CGRP[163-165] in mouse DRG tissue. When staining for NF200, we found significantly less NF200 expressing neurons in the KO DRGs compared to wildtype tissue at 8 weeks (Fig. 4 C: 34.63 ± 1.66% in WT and 22.56 ± 1.60% in KO

at 8 weeks, n = 3 WT and 3 KO; $p<0.05$, 2-way ANOVA). Although there seems to be less NF200 positive neurons in the KO DRGs compared to the WTs at 24 weeks, this difference is not statistically significant (Fig. 4 C:25.12 ± 0.79% in WT and 17.32 ± 2.88% in KO at 24 weeks, n = 2 WT and 5 KO; $p > 0.05$, 2-way ANOVA). We found no significant difference between the IB4 binding neuron counts for WT and KO at either 8 or 24 weeks. When counting CGRP expressing neurons, we found no significant difference between WT and KO and 8 weeks (15.09 ± 2.10% in WT and 23.38 ± 3.76% in KO at 8 weeks, n = 3 WT and 3 KO; $p > 0.05$). However, we counted significantly less CGRP immunoreactive neurons in the KO at 24 weeks (21.74 ± 2.30% in WT and 12.45 ± 2.25% in KO at 24 weeks, n = 3 WT and 6 KO; $p < 0.05$). Taken together, these results provide some evidence for changes in nociceptor populations that may be attributable to cell death.

3.3.3. Sensory Integration

According to parent accounts, patients of CS exhibit an unusual aversion to innocuous mechanical stimuli.[5,7] This apparent mechanical hypersensitivity can be caused by improper integration of mechanical inputs by the DH.[55] Although LTMRs primarily innervate the deeper laminae of the DH, there is a relay circuit that connects the deep DH to the superficial DH laminae, such that LTMR input could activate nociceptive projection circuits.[59] In a healthy adult spinal cord, this crosstalk is prevented by tonically active inhibitory circuits in the DH.[55] However, these inhibitory circuits require C-fibre activity to be functionally mature.[109] As our data suggest C-fiber dysfunction in the NHE6 KO mice, it is possible that inhibition in the DH may not have matured properly in these mice. If this is the case, light mechanical stimuli would activate superficial nociceptive DH neurons.[118] To test this possibility, we used a Fos induction assay to observe neurons activated by light touch in the deep and superficial DH in NHE6 KO and WT mice.

We gave light brush stimulation unilaterally to the plantar hind paw of anaesthetized mice before euthanizing 90 minutes post stimulation. The lumbar spinal cord segment was then fixed and stained for the Fos protein using an antibody commonly used to detect Fos in mouse nervous tissue.[166-168] The number of Fos-immunoreactive neurons were counted in the ipsilateral dorsal horn using the contralateral dorsal horn as a negative control. The neurons counted were separated according to their location: the nociceptive superficial laminae and the deeper laminae encoding receiving touch inputs. In both the WT and KO mice dorsal horns, we counted approximately 1-2 neurons per section of dorsal horn in either the ipsilateral or contralateral sides. There was no difference in the distribution of neurons counted between the superficial or deep laminae regardless of genotype of the mice tested (Fig. 4 D, E; n = 5 WT and 5 KO at 24 weeks, 20 sections per mouse, Independent samples T-Test). These results may suggest that there is no difference in the interpretation of touch inputs by either genotype, due to similar Fos expression in the dorsal horns of both groups. That said, Fos expression requires strong and consistent neuronal activation. Though light brush stimulation can strongly induce Fos expression in the dorsal horn during the early post-natal period, gentle brush does not induce robust Fos expression in adult dorsal horns.[114] Therefore, the results most likely indicate that this assay did not have the resolution to detect any differences in dorsal horn neuron activity after a stimulation as gentle as light brush application.

4. Discussion

Recent anecdotal reports by parents of CS patients indicate that the condition may come with sensory deficits. The patients often display little or no response to noxious thermal or mechanical events but are extremely averse to light mechanical sensations as well as cold stimuli.[4-7] The aim of this study was to investigate how the loss of function of NHE6 leads to this sensory phenotype and whether structures in the somatosensory system are directly involved. To start, we

found NHE6 expression in cortical structures such as the S1 and ACC as well as the PAG of the brainstem. Notably, NHE6 expression was highest in small diameter unmyelinated nociceptive neurons of the DRG. Next, we examined the sensory phenotype of NHE6 KO mice. The NHE6 null mice displayed decreased responses to noxious thermal and mechanical stimuli while being over-responsive to cold stimuli, modelling traits found in CS patients. To test the involvement of the nociceptors in these sensory abnormalities, we observed neurogenic inflammation as a measure of nociceptor activity, nociceptor subpopulations as an indication of growth and survival of sensory neurons and finally, brush induced Fos induction as a measure of mechanical gating in the DH. We found decreased neurogenic inflammation and decreases in nociceptor subpopulations in the NHE6 KO mice suggesting sensory neuron dysfunction.

4.1. NHE6 KO as mouse model of CS

The NHE6 KO mouse strain has been previously used to model and study mechanisms involved in CS, specifically in the context of broad neurodegeneration, motor functioning and memory. According to certain behavioural assays, NHE6 null mice display mild but similar behavioural patterns to CS patients. In open field tests, mice lacking NHE6 show hyperkineticism as seen with patients of CS.[2-4,11,50,51] In addition to hyperkineticism, CS patients also display truncal ataxia, unsteady gait and occasionally loss of motor function.[1,3,4,9,12] Similarly, NHE6 KO mice do not show gross ataxia but deficits of motor coordination and unsteady gait are apparent when observed on tests of motor coordination such as balance beam and rotarod assays.[50,51] Mice lacking NHE6 also show deficits in spatial memory, although more learning and memory tests should be performed to conclude if these mice model the intellectual disability status of CS patients.[8,51] Furthermore, these behavioural phenotypes found in NHE6 null mice show regression as also seen in CS patients.[2,4,10,12,16,50,51] Therefore, NHE6 KO mice have modelled at least some

motor functioning related symptoms of CS and may also model memory deficits of the patients as well.

Notably, patients of CS show many signs of aberrant neurodevelopmental growth and accelerated neurodegeneration as seen in post-mortem and neuroimaging analyses. Post-natal microcephaly is observed in every patient as well as generalized atrophy of the cerebrum, hippocampus and brainstem with dilated ventricles in most cases.[1,3,11,12,17] The cerebellum is also often severely atrophied.[1,11,17] Tau pathology in the cerebral cortex and hippocampus has been observed in post-mortem analyses of some CS patients.[17] All these neuropathologies have been observed in the NHE6 KO mice. General brain growth of NHE6 KO is slower than normal from early postnatal to adolescence, with degeneration of brain size occurring at a faster rate than normal.[52] There are also significant decreases in cortical thickness, hippocampal area and significant purkinje cell loss in the cerebellum which increases with age.[50,52] Moreover, the amygdala, hippocampus and sparsely in the cerebral cortex contain neurons with GM2 ganglioside accumulation, hyperphosphorylated tau and aggresomes.[50-52] Therefore, NHE6 null mice display similar neuropathologies to those seen in CS patients in addition to the behaviour phenotypes previously mentioned.

As such, mice lacking NHE6 were a potentially useful tool to investigate the recently reported sensory abnormalities of CS patients. Patients of CS were reported as being unusually under responsive to incidents involving injury by noxious mechanical and thermal stimuli, thereby showing elevated pain thresholds.[4,6] Patients can sustain deep cuts, fractures and severe burn wounds without a typical reaction.[5,7] Conversely, the same patients showing high thresholds to pain are also extremely averse to light tactile stimuli and rough textures as well as exhibiting a hypersensitivity to cold.[5] In our study, we observed a decreased response to noxious thermal and

mechanical stimuli from NHE6 KO mice compared to wildtype littermates. While tactile and texture sensitivity was not tested, the NHE6 KO mice displayed an increased response to cold as found in CS patients. A recent study also investigating sensory deficits in CS using NHE6 null mice as a model, has similar results wherein NHE6 KO mice have a higher threshold for noxious thermal and mechanical stimuli.[169] Considering these results, our data further confirms the NHE6 KO mouse as a valuable tool for modeling and studying sensory or other phenotypes of CS patients.

4.2. NHE6 LOF and Sensory Deficits

The function of NHE6 in the somatosensory system had not yet been studied within or without the context of CS and its comorbid sensory deficits. However, comparing the reports of sensory abnormalities observed of CS patients to other known developmental conditions of pain insensitivity and abnormal sensitivity, informed our hypothesis. Some well-known cases of CIP are caused by disrupted nociceptor activity or improper nociceptor growth and differentiation. Channelopathies involving loss of function of Nav1.7 or Nav1.9 prevent signal transmission by nociceptors resulting in an absence of pain sensation.[126,129,130] Other similar cases of CIP are caused by loss of function of TrkA or its ligand NGF which are both essential for nociceptor differentiation, growth and survival.[131,132,156] Another recently described case of CIP is caused by the loss of function of CHC22 which is also involved in nociceptor differentiation events.[135] The NHE6 exchanger has been implicated in trafficking of certain ion channels as well as trophic factor receptors in the brain, specifically the hippocampus.[42,43] Given that NHE6 is expressed in nociceptors, the loss of the exchanger could either interfere with neuronal activity or proper growth and differentiation of nociceptors which could then confer pain insensitivity as seen in the mentioned CIP cases. This would explain CS patients' under responsiveness to injurious

experiences. However, the paradoxical presentation of mechanical and cold hypersensitivity along with pain hyposensitivity according to parent reports was not accounted for with nociceptor dysfunction alone. Mechanical hypersensitivity, specifically allodynia, is prevented by tonically active inhibitory circuits in the spinal cord dorsal horn that gate light tactile stimuli from activating superficial nociceptive circuits.[98,100,154,170] Interestingly, these inhibitory circuits finish developing during the early post-natal stage and require c-fibre activity to fully mature.[105,109,118] Early post-natal ablation of c-fibres or silencing c-fibre activity during this period results in aberrant inhibition and activation of superficial dorsal horn neurons in response to light mechanical stimulation.[105,109,120,121] Therefore, we posited that if the loss of NHE6 disrupts nociceptor function this could ultimately prevent the maturation of dorsal horn inhibitory circuits resulting in mechanical hypersensitivity. Moreover, it has been demonstrated that c-fibre nociceptors are involved in regulation of cold sensation such that c-fibre activity suppresses dorsal horn neurons encoding cold sensitivity.[171,172] Hence, the loss of NHE6 disrupting nociceptors may also reduce the dampening of cold responsive circuits in the dorsal horn, causing the hypersensitivity to cold seen in CS patients.

At the core of our hypothesis is the consequence of the loss of NHE6 on nociceptor function. Our behaviour results indicate that NHE6 null mice are less responsive to noxious mechanical and thermal stimuli. While this suggests that nociceptors may not be as active to noxious stimulation as in healthy mice, the tests used do not rule out the contribution supraspinal circuit dysfunction in addition to or in place of defective nociceptors. One mechanism mediated mainly by nociceptors that does not strongly involve CNS pain structures is neurogenic inflammation. Once sufficiently activated by an external noxious stimulus, peptidergic nociceptors release neuropeptides in the periphery, such as CGRP and substance P, where they act on

surrounding vascular tissue and immune cells to induce inflammation in the site of the assault.[156,157] This mechanism requires neuronal activation of the nociceptor which sends both an input towards the spinal cord dorsal horn but also an antidromic signal back to the peripheral terminals of the nociceptor to induce neuropeptide release.[157] Observing the following inflammation would give an indirect measure of the nociceptors' ability to transduce and locally respond to a given noxious stimulus. To this end, we induced neurogenic inflammation in NHE6 null mice and their littermates by injecting capsaicin, an agonist of the TRPV1 channel expressed on peptidergic c-fibre nociceptors and measured the resulting edema as well as spontaneous pain behaviour. Mice lacking NHE6 displayed less spontaneous pain behaviour in response to the capsaicin injection which matched results of our other pain behaviour assays. More importantly, NHE6 KO mice also had decreased edema after the capsaicin injection, indicating less inflammation. In our assay, capsaicin induced neurogenic inflammation can be inhibited in part by improper signal transduction by c-fibre nociceptors which can result from decreased surface expression of the capsaicin receptor, TRPV1.[150] It is possible that membrane surface expression and recycling of TRPV1 may be affected by the loss of NHE6 in c-fibres of KO mice.

It has been demonstrated that capsaicin bound TRPV1 is endocytosed in a clathrin dependent manner and NHE6 was found to regulate clathrin-mediated endocytosis.[40,173] While there is no known association between TRPV1 and NHE6, and the involvement of NHE6 in clathrin-mediated endocytosis of certain cargo does not necessitate an association between TRPV1 and NHE6 in nociceptors, TRPV1 surface expression can be examined in NHE6 KO nociceptors to explain this decrease in apparent inflammation. The decrease in neurogenic inflammation in NHE6 KO mice can also be attributed to an inability of the c-fibre nociceptors to mount an action potential to induce neuropeptide release. Assuming that NHE6 can be associated with other cargo

endocytosed in a clathrin-mediated manner, NHE6 could be involved in the surface expression and recycling of any number of ion channels contributing to neuronal excitability.[29,174-176] On the other hand, neurogenic inflammation also involves the action of immune cells such as dendritic cells, mast cells and T-lymphocytes.[156,157] According to mRNA expression analyses, NHE6 is highly expressed in mast cells of the mouse as well as dendritic cells and T-lymphocytes in humans.[177,178] Considering this, the functional consequence of the loss of NHE6 in these immune cells could contribute to the decreased inflammation observed in NHE6 KO mice as well. Immune function has not been examined in the NHE6 KO mice or clinically in CS patients and as such, there is not enough literature to rule out or confirm a negative affect of NHE6 loss on immunity. That said, in addition to decreased edema and therefore inflammation in response to capsaicin, NHE6 KO mice also had a lesser behavioural nocifensive response in accordance with their sensory phenotype found in our other pain behaviour assays. Taken together, the results suggest that the under responsiveness of NHE6 KO mice to noxious stimuli may be due in part to a defect in nociceptor function. It is unclear whether the deficit in nociceptor function is in the transduction of the noxious stimulus, the activation and firing of the nociceptor or simply due to a loss of nociceptors.

In addition to affecting aspects of nociceptor activity, the loss of NHE6 could have consequences on nociceptor health and survival. In previous studies investigating neuropathology in mice lacking NHE6, neurons in the cerebral cortex, hippocampus and amygdala had neuronal loss as well as metabolic and structural abnormalities, such as GM2 ganglioside accumulation, tau pathology and aggresomes.[50-52] Studies done *in vitro* have also observed decreased branching and increased apoptosis of hippocampal neurons lacking NHE6.[43,48,49] It has been suggested that this defect in growth and survival of neurons in the absence of NHE6 is due to decreased surface

expression of trophic factor receptors resulting from aberrant endosomal protein trafficking and recycling.[43] Trophic factors and receptors play a major role in the differentiation, growth and survival of sensory neurons.[101,102] Therefore, we examined DRG tissue from NHE6 KO mice and their littermates to detect changes in nociceptor subpopulations that may be due to improper nociceptor growth or survival. The CGRP and NF200 expressing as well as the IB4 binding populations were chosen as many neurons in these populations expressed NHE6 in WT DRG tissue. We found decreases in the number of NF200- and of CGRP-immunoreactive neurons in the NHE6 KO compared to WT mice. The decrease in CGRP-immunoreactive neurons was statistically significant while comparing NHE6 KO and WT at 24 weeks but not at 8 weeks; whereas, the decrease in NF200-immunoreactive neurons was significant at 8 weeks but not at 24 weeks. It should be noted that the sample sizes were not consistent across age and genotype, ranging from 2 – 6 mice per group. The smaller sample sizes of n = 2 – 3 mice may have hindered statistical significance. Perhaps, statistical significance could be generated if the sample size were increased to 5 – 6 mice per group. That said, the observed decrease could be due to neuronal loss or decreased expression of NF200 and CGRP. The CGRP neuropeptide is a well-known biomarker for peptidergic C-fibre nociceptors that are typically heat sensitive but can be polymodal, heat and mechanosensing.[56,68,179] The NF200 expressing population also accounts for medium diameter Aδ-fibre nociceptors, both mechanosensitive and thermosensitive, as well as large diameter Aβ-LTMRs.[56,180,181] We found a large proportion of both CGRP and NF200 expressing neuron subsets express NHE6 in WT tissue. While the CGRP expressing subset is mostly nociceptors, the NF200 expressing subset has both nociceptors and LTMRs. The NHE6 positive sensory neurons were small to medium diameter, suggesting that within the NF200 population, the NHE6 expressing neurons may be the Aδ-fibre population which are mostly nociceptors. Interestingly, the

expression profile of NHE6 among the subsets of nociceptors is similar to the expression of TrkA in adult mice.[102,179] Given the association of NHE6 with the TrkB trophic factor receptor in the hippocampus, it is possible that NHE6 may also be associated with TrkA in these nociceptors.[43] The TrkA receptor and its ligand NGF are essential for the early specification, growth and survival of nociceptors.[101-103] While there is a small but significant decrease in the NF200 and CGRP neuron populations in the NHE6 KO mice, this is not an excessive sensory neuron loss as typically seen in cases of a congenital loss of TrkA-NGF signaling.[131,134,182] Hence, TrkA-NGF signaling in nociceptors is likely intact during development. In the adult, TrkA is required for the survival of nociceptors but also the expression of neuropeptides and ion channels necessary for maintaining nociceptor identity.[34,103,104] Accordingly, assuming the loss of NHE6 did influence TrkA expression in adult NHE6 KO mice, the decrease in immunoreactive neurons observed could be due to decreased expression of the marker as opposed to neuron loss. For example, a decrease in the neuropeptide CGRP expression can also explain inhibited neurogenic inflammation observed in NHE6 KO mice in the previously described assay. It would be important to examine TrkA surface expression in DRG neurons of NHE6 KO mice to confirm if a deficiency of TrkA-NGF signaling due to the loss of NHE6 could be interfering with protein expression and consequently nociceptor function. Conversely, if neuronal loss is responsible, it would be important to measure signs of neuronal health in DRG tissue of mice lacking NHE6. Signs of malfunctioning protein recycling-degradation mechanisms such as GM2 ganglioside accumulation, aggresomes and tau pathology were found in neurons of brain structures in NHE6 KO mice.[50-52] A recent study also examining sensory deficits associated with CS did not observe GM2 ganglioside accumulation in neurons of the DRG in NHE6 null mice.[169] Other indicators of protein recycling-degradation mechanisms have not yet been found in DRG neurons.

We cannot conclude a specific form of defect occurring in the nociceptors of NHE6 KO mice. Nevertheless, the previously described results do imply that improper function of nociceptors may contribute to the under-responsiveness to noxious mechanical and thermal stimuli observed in the mice lacking NHE6. According to our hypothesis, the mechanical hypersensitivity reported for CS patients could be caused by C-fibre inactivity affecting the maturation of inhibitory circuits in the spinal cord DH. The DH can be divided into the deep DH which receives collateral input from LMTRs and the superficial DH which receives input from nociceptors and has nociceptive projection neurons.[57,59] While relay circuits exist to provide cross-talk between these two regions, they are kept functionally separate by tonically active glycinergic inhibitory neurons.[57,96,170] In rodents, maturation of glycinergic inhibitory circuits in the DH occurs during the second post-natal week.[105] It has been demonstrated that this maturation event is dependent on C-fibre activity.[118] By the early post-natal stage, low threshold Aβ-fibres innervate the DH including the superficial nociceptive laminae, such that stimulation of these fibres activation of superficial DH neurons.[105,113,114] From the second post-natal week by the third post-natal week, Aβ-fibres retract from the LI-II laminae while C-fibres innervate these superficial laminae.[105,109] During this early post-natal phase, glycinergic synapses in LIIi and LIII contacting Aβ-fibre terminals and excitatory relay neurons fully mature, finalizing the gate between LTMR inputs and the superficial DH.[109] Interestingly, silencing C-fibre activity during this phase delays the maturation of these inhibitory connections such that Aβ-fibres continue to activate neurons in the superficial DH.[118] Moreover, ablating C-fibres prevents the withdrawal of Aβ-fibre terminals from the superficial DH.[105,119,120] Functionally, this results in light touch inputs being able to activate superficial nociceptive neurons therefore evoking a nocifensive response to an otherwise innocuous stimulus.[109,118] Our capsaicin assay indicated a defect in C-fibre function in NHE6 KO

mice. Therefore, we speculated whether irregular or absent C-fibre activity during the early post-natal stage could have affected the maturation of DH circuits, resulting in the aversion to touch and textures seen in CS patients.

To investigate if this is the case, we used a Fos induction assay to observe the location of DH neurons activated by a low intensity mechanical stimulus, such as gentle brushing, in NHE6 null and WT mice. The presence of Fos-positive neurons only in the deep DH would indicate that DH inhibitory circuits are intact. However, the presence of Fos-positive neurons in the superficial DH would indicate that the brush stimulus could be activating nociceptive neurons and therefore, being coded as a noxious stimulus. Neither NHE6 KO mice nor their WT littermates showed significant Fos expression in superficial DH or deep DH neurons in response to light brush stimulation. These results are inconclusive due to the resolution of the assay. Fos induction assays are well characterized and commonly used experiments to observe DH neuron activation in response to noxious stimuli such as capsaicin and formalin administration.[183] The previously mentioned noxious stimuli provide the robust and prolonged activation required for CNS neurons to express detectable levels of Fos, therefore making Fos a suitable marker for nociceptive activation of superficial DH neurons.[183,184] Conversely, light mechanical stimulation can strongly induce Fos expression in the superficial DH of neonates but is too weak to induce Fos in the DH of adult mice.[114,185] Therefore, it would be more fitting to conduct a light brush induced Fos expression assay in neonatal NHE6 KO mice and their littermates. In healthy WT neonates Aβ-fibres can activate superficial DH neurons from birth until the third post-natal week when the maturation of DH circuits finishes.[114,118] Examining Fos expression in NHE6 KO mice DH induced by brush stimulation before and after this developmental phase would indicate any defects in the

DH maturation of these mice. However, with the current results we are unable to conclude whether improper nociceptor activity affected the maturation of DH circuits in NHE6 KO mice.

There was one aspect of the sensory phenotype of the NHE6 KO mice that we did not investigate the peripheral cause, which was the apparent hypersensitivity to cold. The NHE6 KO mice displayed an increased response to cold temperatures than WT littermates. It has been recently demonstrated that C-fibre activity may also regulate DH neuron responses to cold stimuli. The ablation of CGRP expressing C-fibre nociceptors, results in stronger responses to cold stimuli by second order DH neurons.[171] The sensitivity of cold responsive TRPM8 expressing primary afferents is not affected, indicating that capsaicin responsive C-fibres monosynaptically inhibit cold responsive neurons in the DH.[171,172] Our behavioural data alone cannot explain the apparent hypersensitivity to cold exhibited by the NHE6 KO mice. The capsaicin assay discussed previously suggests that NHE6 KO mice may have impaired TRPV1-expressing C-fibre activity. Hence, it is possible that this defect in C-fibre activity could unmask responses of cold sensitive DH neurons in the NHE6 KO mice. That said, further investigation regarding the consequences of NHE6 LOF on cold responsive DH circuitry is required to support this speculation.

4.3. Assessing Insensitivity and Indifference in CS

This investigation indicates that NHE6 KO mice are less responsive to noxious mechanical and thermal stimuli while being hyper-responsive to cold stimuli. The findings imply but do not confirm that nociceptor dysfunction contributes to the sensory phenotype in mice lacking NHE6. Moreover, the findings do not rule out supraspinal mechanisms as a greater contributor to the observed sensory phenotype of NHE6 KO mice in addition or in absence to peripheral deficit. The question remains, whether the observed sensory abnormalities are a matter of sensitivity to stimuli or the valence associated with pain. Our investigation was mainly focused on nociceptors due to

the high expression of NHE6 in the mouse DRG and according to human gene expression databases, human DRG as well.[178] However, NHE6 is also expressed in structures of the brain and brainstem that have an active role in the perception of somatosensorily stimuli. Notably, previous studies have demonstrated significant neurodegeneration in these structures in CS patients as well as NHE6 KO mice.

The cerebral cortex in CS patients was shown to have diffuse neuronal loss, decreased thickness and tau pathology.[14,17] The same was found in the cerebral cortices of mice lacking NHE6 along with abnormal protein aggresomes.[51,52] Cerebral regions such as the ACC and the insular cortex (IC) are both involved in coding the affective response to pain.[61] Patients with lesions to these areas can sense and localize a painful stimulus but do not find it unpleasant demonstrating the importance of valence to the pain experience.[61,186] Additionally, in the absence of NHE6, the basolateral nucleus of the amygdala (BLA) shows robust signs of neuropathology, such as GM2 ganglioside accumulation and tau pathology.[51,52] The amygdala contributes to the emotional affective dimension of pain as well as cognition related to pain and fear.[62] The BLA contains neurons that respond specifically to noxious stimuli and communicates with the ACC, IC and other regions of the amygdala to further shape pain related cognition.[187] Moreover, the cerebellum is greatly atrophied after loss of NHE6 function, with a marked loss of purkinje cells in the vermis.[1,2,11,17,50-52] The cerebellum receives nociceptive input and is consistently responsive to noxious stimuli in neuroimaging studies.[188,189] Interestingly, the cerebellum is also activated in cases of emotional pain and empathic behaviour suggesting its involvement in emotional cognition.[190,191] The previously mentioned structures have a demonstrated involvement in the affective quality of pain. Given that the loss of NHE6 causes neuropathologies in these structures, it is possible that the sensory phenotype that we observe both in CS patients and in the mouse

model may be due to aberrant coding of the emotional experience of pain. Adding on to the structures affected by NHE6 LOF, the brain stem is atrophied in CS patients and NHE6 null mice.[1,17,52] Within the brainstem, the PAG and RVM are involved in descending modulation of pain as well as endogenous mechanisms inducing opioid analgesia.[64,192] The PAG itself is controlled by the hypothalamus, amygdala and cerebral cortex and sends descending inputs from the RVM to control the ascending signal from the spinal cord.[63] This descending regulation shapes the withdrawal response according to different circumstances that can be hormonally and cognitively dictated.[64] The loss of NHE6 has an apparent effect on the health of the cerebral cortex, amygdala, brainstem and cerebellum in both CS patients as well as NHE6 null mice. Given that these structures are involved in forming the perception and response to a painful stimulus, it is possible that observed sensory abnormalities in CS may be attributed to dysfunctions in these regions in addition to any deficit in the periphery.

This investigation was inspired by the recent findings of apparent sensory abnormalities in CS patients. However, these findings come from anecdotal reports and questionnaires by caretakers of the CS patients.[5,7] The sensory phenotype of the patients has not yet been formally clinically examined. Given that the CS patients are of non-verbal status, have intellectual disability and may present autistic features, qualitative assessments can be complicated. While subjective pain reports are valuable for understanding the patients' experiences, in this case such qualitative assessments rely on interpreting the patients' expression of pain through known pain behaviours.[193] Facial and body language cues have been used previously as measures of pain behaviour in patients with intellectual disability and/or autistic features.[193] In some cases, individuals with ASD or ID will show lower than expected behavioural activity during painful medical procedures but more behavioural signs of distress after the procedure.[193,194] When monitoring both behavioural and

physiological cures for pain response, subjects had insignificant behavioural expressions of pain but elevated heart rates and plasma beta endorphin levels during a pain medical procedure.[194] Individuals with ASD or ID commonly have deficits in expressing and processing emotional faces.[195] Children with developmental delays or autistic features can display reduced or inappropriate facial and body language cues during normally positive social interactions.[196,197] This is also true of CS patients.[1,4,12,17] The autism-associated deficit in emotional expression of pain is more clearly observed in high-functioning ASD individuals who are not of non-verbal or ID status. According to self reports, some ASD individuals describe incidents where they experienced excruciating pain but were not aware that they should express it outwardly.[198] Moreover, high-functioning ASD subjects did not report pain on a "pain scale" during a normally painful medical procedure but were able to report extreme discomfort on a "comfort scale."[199] Taken together, the under responsiveness of CS patients to normally painful incidents may be due to decreased sensitivity to noxious stimulation, intact sensitivity but aberrant affective coding of the experience or intact sensitivity and affect but atypical expression of the pain response. Thus far, the current qualitative reports do not distinguish between these mechanisms.

Likewise, the tactile aversion exhibited by CS patients has also been associated with idiopathic and X-linked ASDs.[137] For example, individuals with XLID conditions such as Rett syndrome and Fragile X Syndrome in the autism spectrum commonly display abnormal tactile sensitivity.[144,200,201] As previously mentioned of CS patients, ASD individuals show strong negative reactions to normally innocuous sensations such as specific types of clothing or certain textures according to parent reports.[139,202] It is important to note that these reports do not distinguish between increased sensitivity to light mechanical stimuli and impaired adaptation to repetitive mechanical stimuli, both of which can elicit an aversive response.[137] Clinical studies and

studies using mouse models have demonstrated impaired habituation to tactile stimuli in ASDs. Neuroimaging studies show decreased neural habituation in the somatosensory cortex in response to light mechanical stimuli in FXS.[146,203] Similarly, electrodermal responses in FXS patients show lower rates of habituation to tactile stimuli along with lower mechanical thresholds.[144] In tests of sensory discrimination, the performance of adults with ASDs is not hindered after an initial adapting stimulus whereas it is hindered in typical adults.[204] Lack of adaptation to sensory information is a common in ASDs. Although CS patients often display autistic features, it is unclear whether their observed tactile aversion is due to a lack of adaptation with or without a decreased mechanical threshold. According to parent reports, CS patients who in infancy were very expressive of extreme aversion to certain shoes and clothing items seem to better tolerate wearing similar items in adulthood, though still showing a mild aversion.[5] Taken together, the sensory phenotype of CS patients may be due to a combination of peripheral and cognitive mechanisms.

4.4. Future Directions

Sensory deficits had not been previously addressed in the context of CS. Our findings along with another contemporary study examining pain hyposensitivity in CS, are the first to address and demonstrate sensory abnormalities in the mouse model of CS.[169] However, the specific mechanisms contributing to the sensory phenotype of NHE6 null mice and more importantly, that of CS patients, remain unclear. The study suggests but does not conclusively demonstrate impaired nociceptor function as a cause of the sensory deficits in the absence of NHE6. To further determine the consequences of the loss of NHE6 on nociceptor activity and subsequently the development and function of DH circuits dependent on nociceptors, electrophysiological characterization is required. We theorized that the loss of NHE6 may negatively affect ion channel membrane

trafficking and therefore, neuronal excitability. Measuring parameters such as neuron excitability as well as calcium, sodium and potassium ion current densities in DRG neurons lacking NHE6 can indicate changes in ion channel surface expression.[205] We also theorized that the surface expression of growth factor receptors can be compromised by a loss of NHE6 and disrupt key developmental events. These events include nociceptor differentiation, nociceptor biomarker expression as well as central and peripheral target innervation.[103] Therefore, electrophysiological recordings examining the response of post-synaptic DH neurons to nociceptive and low threshold afferent activation can reveal impairments in nociceptor innervation and synaptogenesis as a cause for disrupted signal transmission.[118] To rule out endogenous opioid-dependent analgesia mediated by the PAG and RVM, NHE6 KO and WT mice can be administered naloxone, an opioid receptor antagonist, and then tested in nociceptive behavioural assays.[64,126,206] Additionally, to rule out the involvement of defects elsewhere in the CNS, it is possible to design conditional KO mice where NHE6 is only deleted in the peripheral sensory neurons and repeat the same experiments done on global NHE6 KO mice.[148] This way, the contribution of NHE6 deficient nociceptors to the overall sensory phenotype can be isolated.

Due to the very recent discussion of sensory abnormalities of CS patients, clinical examination of the patients' sensory profiles is still at a preliminary stage. Thus far, only 60 cases have been identified spread all over the world in US, Norway, Sweden, South Africa, India and Japan which makes widespread and thorough clinical examination difficult.[1-3,8-18] Nevertheless, thorough clinical assessments of the patients' sensory phenotypes would further focus investigations addressing somatosensation in CS. The non-verbal status, ID and possible autistic features present in CS patients necessitates a nuanced approach to the evaluation of their sensory phenotypes.[5] As such, a combination of qualitative measurements examining facial and body

language expression of pain responses as well as measuring physiological signs like blood pressure and neuroimaging can give a detailed report of the patients' response.[193,207] Furthermore, there is considerable variation in the types of mutations found in CS patients and the presented symptoms.[8] Current efforts are targeted towards correlating the type of mutations with the resulting symptoms which would also further contextualize the variation reported in the sensory phenotype as well.[5,7,8]

5. Conclusion

The neurodevelopmental disorder CS poses a challenging but urgent scientific problem. Among other neurodegenerative symptoms, CS patients present an unusually elevated threshold for mechanical and thermal pain with a strong aversion to certain tactile and cold sensations.[2,4-7] This investigation sought out to determine the cellular mechanisms resulting in these recently described somatosensory abnormalities in CS patients. We hypothesized that the loss of NHE6 impairs nociceptor activity inhibiting nociceptive signal transmission and leading to a hyposensitivity to pain. We further proposed that NHE6 LOF induced nociceptor inactivity disrupts the development of inhibitory circuits in the DH and unmasks nociceptor-mediated cold suppression, causing tactile and cold hypersensitivity. To this end, we first identified the distribution of NHE6 in structures involved in the somatosensory pathway. Then we characterized the sensory phenotype of an NHE6 KO mouse strain to use as a model for CS. Lastly, we assessed the contribution of nociceptor dysfunction to the CS-associated sensory abnormalities. We found NHE6 expression in small diameter unmyelinated nociceptive DRG neurons. The NHE6 KO mice displayed decreased responses to noxious thermal and mechanical stimuli but exaggerated responses to cold. The KO mice exhibited decreased capsaicin induced neurogenic inflammation as well as a decrease in nociceptive subsets of DRG neurons implying nociceptor loss or inactivity. Tactile sensitivity and processing tested by brush induced Fos expression yielded no conclusive

results. Taken together the data suggests that the loss of NHE6 causes sensory deficits that can be attributed in part to nociceptor dysfunction. We are among the first to demonstrate sensory deficits in the mouse model of CS and the expression of NHE6 in the somatosensory pathway with another contemporary study showing similar results.[169] The findings of this investigation form a base on which assays employing electrophysiological characterization and conditional NHE6 KO strains can further elucidate the mechanisms implicated.

Pathologies of pain and touch can be severely debilitating. Conditions of excessive pain greatly disturb the quality of life whereas conditions of pain insensitivity result in undetected injuries, ultimately affecting life expectancy.[53] Other conditions of CIP as well as ASDs present sensory abnormalities comparable to those discussed here of CS.[122,131,199] There is currently a growing body of literature examining improper somatosensation in congenital and neurodevelopmental conditions. This investigation is a step towards clarifying the underlying causes of these deficits and ultimately to developing treatments which restore normal sensitivity in afflicted patients.

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7. Figure Legends and Figures

Figure 1. NHE6 is moderately expressed in pain centers in the CNS and highly expressed sensory neurons.

(A) Brain, spinal cord and DRG tissue from 8 week old wildtype mice stained for NeuN (green) and NHE6 (red). Representative images are shown (n=3 mice, scale bar: 50 μm). Arrows and arrow heads indicate cells negative and positive for NHE6 respectively. Tissue from NHE6 null mice DRGs shows no immunoreactivity for NHE6 antibody.

(B) Number of NHE6 positive neurons presented as a percentage of NeuN positive neurons in each structure.

Figure 2. NHE6 is expressed predominantly in small diameter unmyelinated neurons of the DRG.

(A) Double immunofluorescent stains for NHE6 (red) and well known markers (green) of sensory neuron subsets on DRG tissue from 8 week old mice (PV: parvalbumin, NF200: neurofilament 200, TH: tyrosine hydroxylase, GINIP: $G_{\alpha i}$-interacting protein, CGRP: calcitonin gene related peptide, IB4: isolectin-B4). Representative images are shown (n=3 mice, scale bar: 50 μm).

(B) Number of NHE6 neurons expressing marker indicated as percentage of total NHE6 positive neurons.

(C) Size distribution of NHE6 neurons alongside IB4 binding small neurons and PV expressing large neurons of the DRG. Frequency displayed as percentage of neurons counted in each bin, (n=3 mice).

Figure 3. NHE6 KO mice display hyposensitivity to noxious heat and mechanical stimuli and hypersensitivity to noxious cold stimuli.

(A) Hindpaw withdrawal latency measured in Hargreaves' test for heat sensitivity. Independent samples T-Test, * $p<0.05$ (n = 10 WT, 5 KO at 8 and 24 weeks).

(B) Mechanical withdrawal thresholds measured by von Frey filaments. Filament weight eliciting 3 hindpaw withdrawals out of 5 applications taken as threshold. Independent samples T-Test, *$p<0.05$, ***$p<0.001$ (n = 9 WT, 8 KO at 8 weeks; 11 WT, 10 KO at 24 weeks).

(C, D) Mechanical sensitivity shown as number of nociceptive responses per 5 applications of von frey filament at 8 weeks (C) and 24 weeks (D). Independent samples T-Test, *$p<0.05$, **$p<0.01$.

(E) Response to indicated temperatures on cold plate test scored according to the following scheme: 0 – no response, 1 – flinching, 2 – jumping. Independent samples T-Test, **$p<0.01$, ***$p<0.001$ (n = 9 WT, 9 KO at 8 weeks; 16 WT, 9 KO at 24 weeks).

Figure 4. Peripheral contribution to sensory abnormalities in NHE6 KO mice.

(A, B) Neurogenic inflammation tested by response to intraplantar capsaicin injection.

(A) Duration of licking behaviour observed for 5 minutes and quantified immediately after capsaicin injection in plantar paw. Independent samples T-Test, **$p<0.01$ (n = 9 WT, 7 KO at 24 weeks).

(B) Hindpaw width of injected paw used as a measure of edema to further examine capsaicin induced neurogenic inflammation. Hindpaw width displayed as percentage of baseline before and after capsaicin injection. Only injected paw shown. Independent samples T-Test, *$p<0.05$.

(C) DRG tissue from NHE6 null mice and WT at 8 and 24 weeks old stained for selected markers (NF200, IB4 and CGRP in cyan) and NeuN (magenta) to observe possible changes in sensory

neuron subtype populations. Left panel shows representative images, right panel shows number of marker expressing neurons as a percentage of total NeuN positive neurons (scale bar = 50 μm, n = 3 WT, 3 KO at 8 weeks; 2 WT, 5 KO at 24 weeks; tested with 2 way ANOVA).

(D, E) Brush induced fos expression examined to detect changes in dorsal horn integration of innocuous mechanical stimuli in mice at 24 weeks.

(D) Representative dorsal horn images stained for fos (green) and IB4 (red) as laminar marker (scale bar = 50 μm).

(E) Quantification of fos positive neurons in superficial (LI-II) and deeper laminae (LIII-IV). Number of fos positive neurons per 25 μm section on ipsilateral and contralateral dorsal horn (n = 5 WT, 5 KO at 24 weeks; at least 20 sections per mouse).

Figure 1

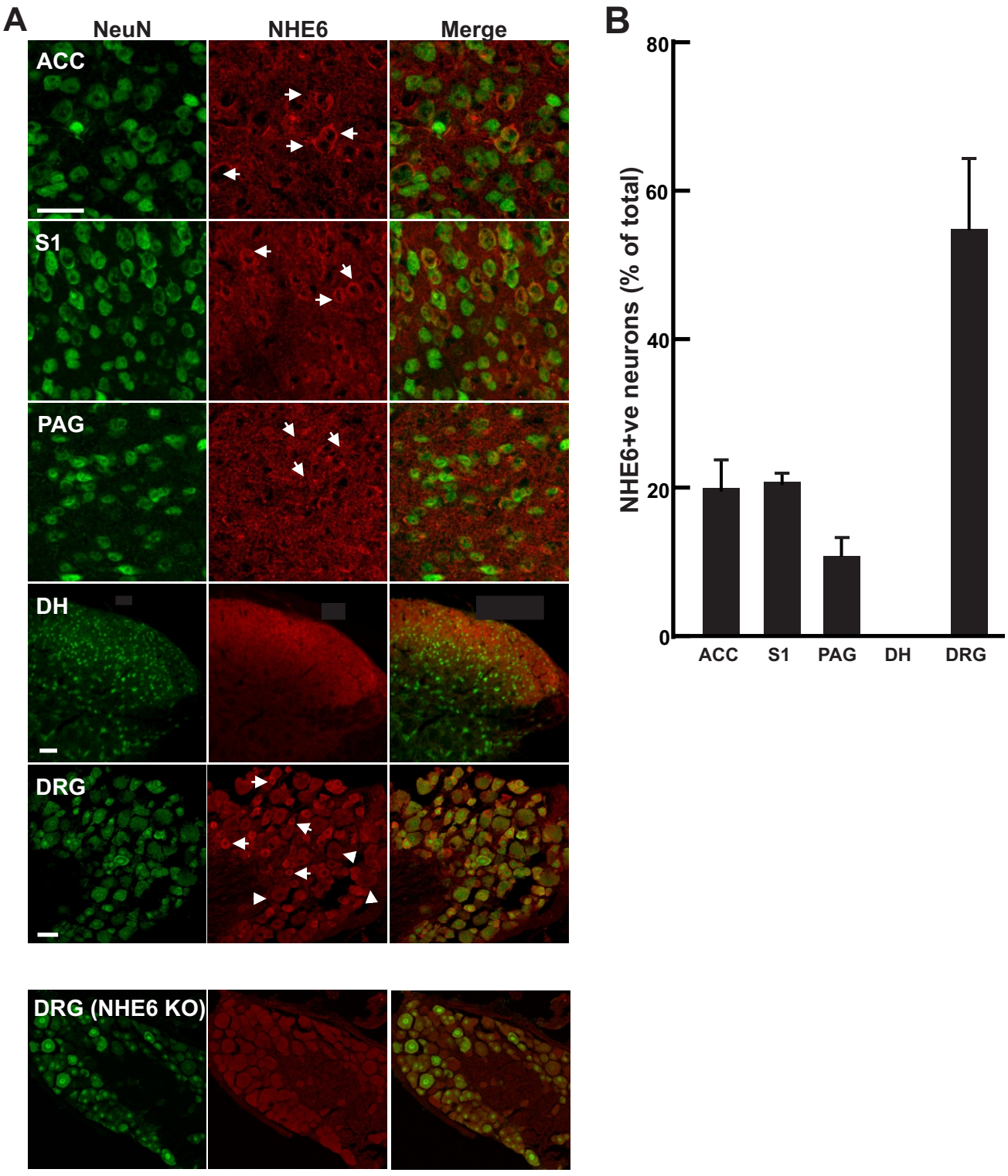

A
NeuN
NHE6
Merge
ACC
S1
PAG
DH
DRG
DRG (NHE6 KO)
B
NHE6+ve neurons (% of total)
80
60
40
20
0
ACC
S1
PAG
DH
DRG

Figure 2

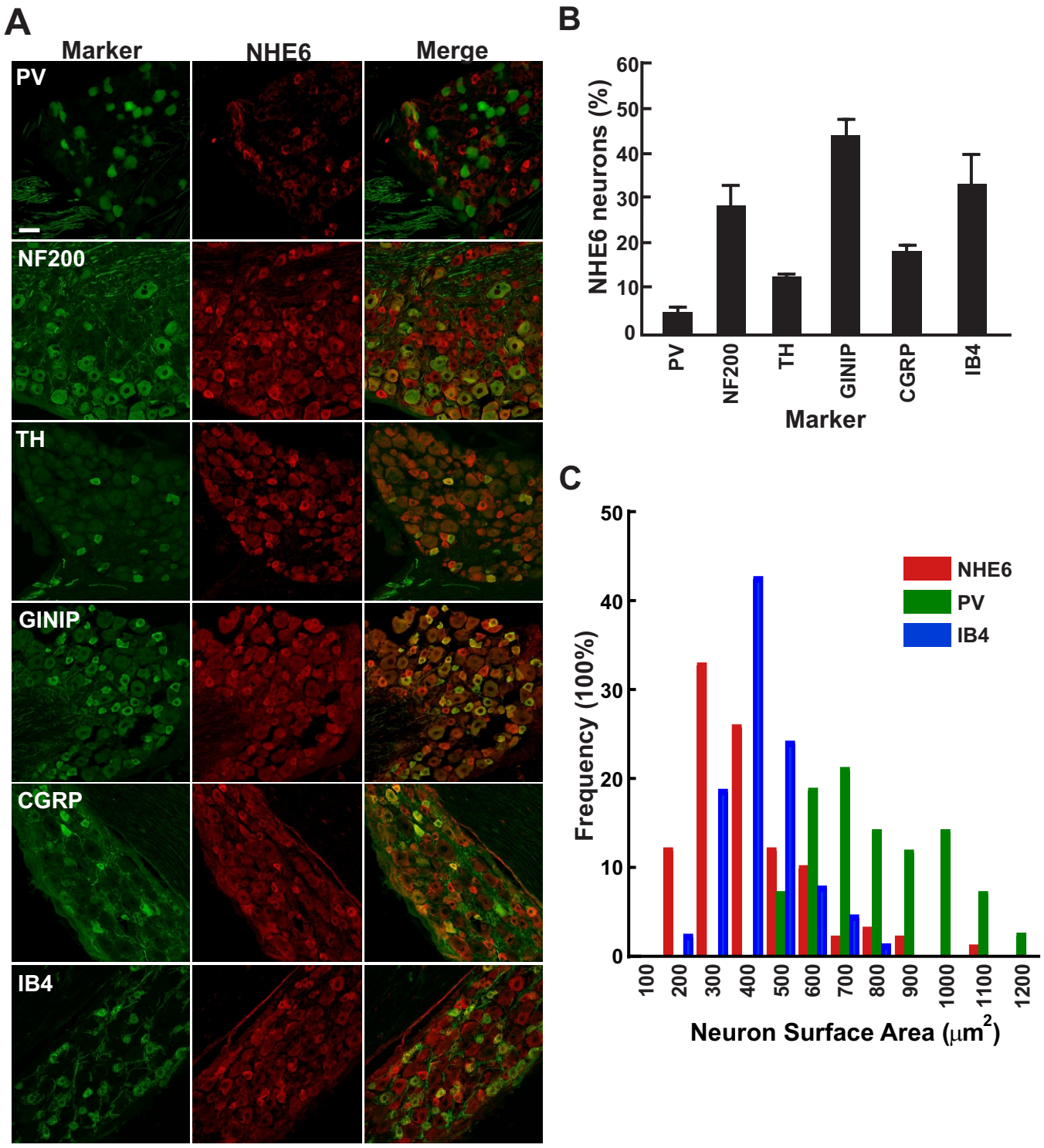

A
Marker
NHE6
Merge
PV
NF200
TH
GINIP
CGRP
IB4
B
NHE6 neurons (%)
0
10
20
30
40
50
60
PV
NF200
TH
GINIP
CGRP
IB4
Marker
C
Frequency (100%)
0
10
20
30
40
50
NHE6
PV
IB4
100
200
300
400
500
600
700
800
900
1000
1100
1200
Neuron Surface Area (μm2)

Figure 3

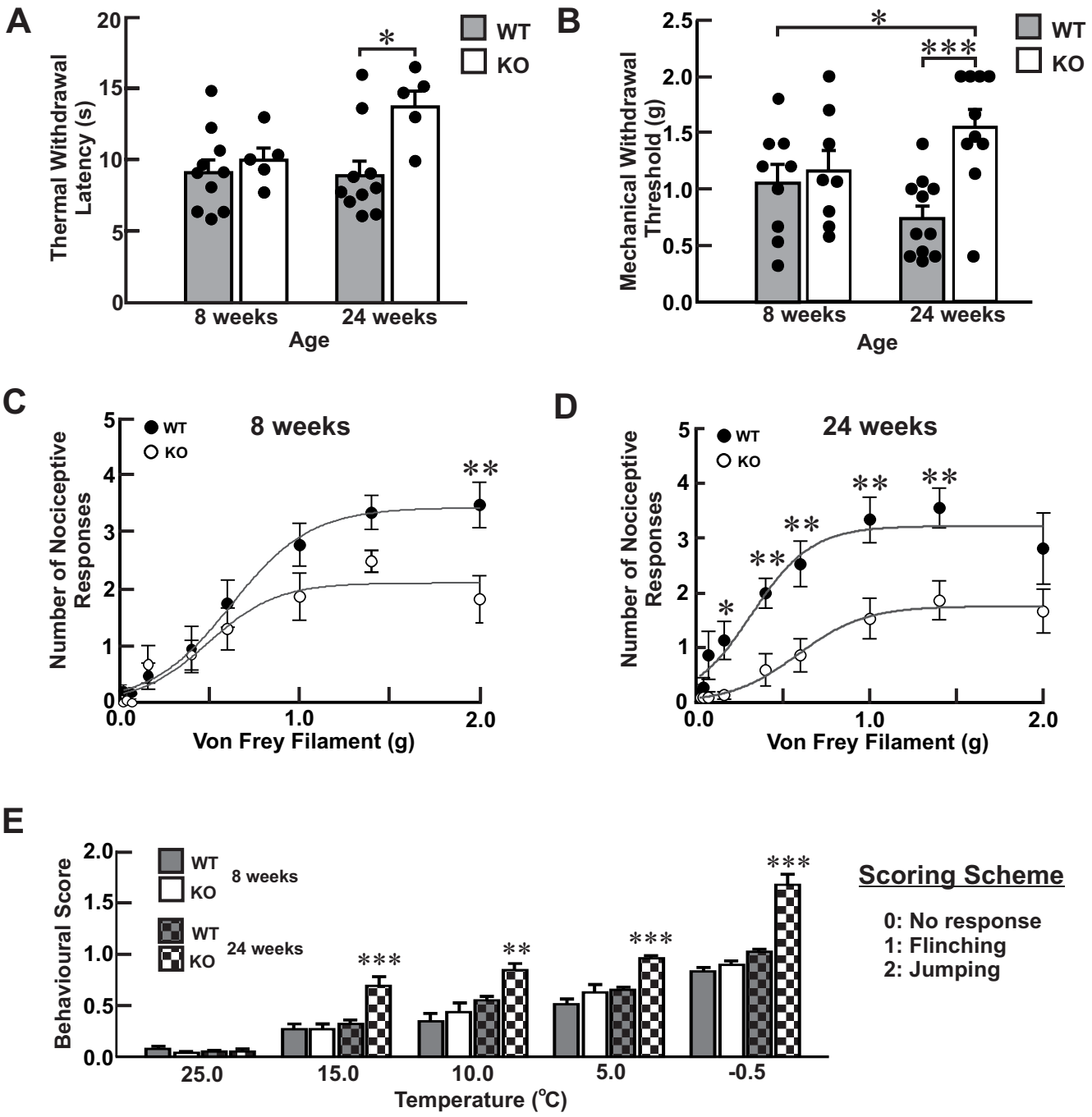
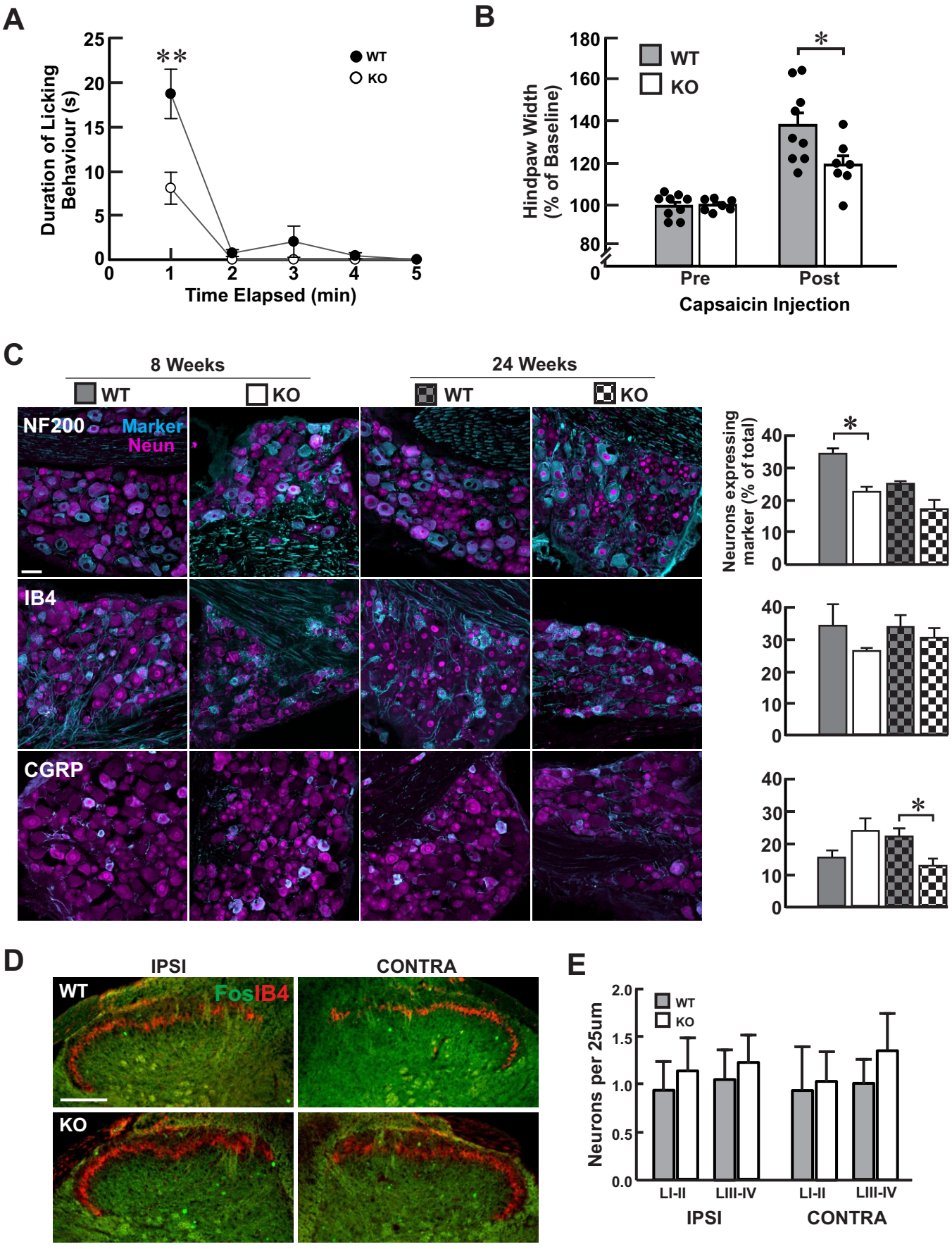

A
Thermal Withdrawal Latency (s)
20
15
10
5
0
*
WT
KO
8 weeks
24 weeks
Age
B
Mechanical Withdrawal Threshold (g)
2.5
2.0
1.5
1.0
0.5
0.0
*
***
WT
KO
8 weeks
24 weeks
Age
C
8 weeks
WT
KO
Number of Nociceptive Responses
**
0.0
1.0
2.0
Von Frey Filament (g)
D
24 weeks
WT
KO
Number of Nociceptive Responses
*
**
**
**
**
0.0
1.0
2.0
Von Frey Filament (g)
E
Behavioural Score
2.0
1.5
1.0
0.5
0.0
WT
KO
8 weeks
WT
KO
24 weeks
***
**
***
***
25.0
15.0
10.0
5.0
-0.5
Temperature (°C)
Scoring Scheme
0: No response
1: Flinching
2: Jumping

Figure 4


A
Duration of Licking Behaviour (s)
Time Elapsed (min)
WT
KO
**
B
Hindpaw Width (% of Baseline)
WT
KO
*
Pre
Post
Capsaicin Injection
C
8 Weeks
24 Weeks
WT
KO
WT
KO
NF200
Marker
Neun
IB4
CGRP
Neurons expressing marker (% of total)
*
*
D
IPSI
CONTRA
WT
FosIB4
KO
E
Neurons per 25um
WT
KO
LI-II
LIII-IV
LI-II
LIII-IV
IPSI
CONTRA