

Investigating rare genetic variants in common migraine

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Abstract

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Migraine is a highly prevalent headache disorder imposing a significant burden of disability on human health worldwide. The headache is believed to arise from activation of trigeminal pain pathways, with CNS regions also playing an integral role in attack initiation and progression. Recent genetic associations have been made, but there is a need to convert these into relevant experimental models to study underlying disease mechanisms. Herein, I detail functional analysis of two deleterious variants in the genes *KCNK18* and *SLC12A3*, that segregate with migraine with aura in one large pedigree. Gene function has been studied in a range of cell models, from heterologous expression systems and primary neuronal cultures, to Induced Pluripotent Stem (iPS) cell-derived nociceptors. In this context, the protein products of *KCNK18* and *SLC12A3* have been shown to modulate parameters of neuronal excitability, including baseline membrane properties and firing patterns. Migraine attacks are not wholly attributable to perturbations in peripheral pathways. I have shown that these genes are also expressed within the CNS in a small number of discreet regions, suggesting a possible role in central processing. Utilizing recently defined genetic variants and physiological cell-based models, will provide a platform for mechanistic insights into migraine pathogenesis and allow for the development of drug screening assays for new migraine therapies.

Declaration

The work presented in this thesis is wholly attributable to the author (Gregory A Weir) unless stated otherwise. The research was carried out during 2010-2014 at the MRC Functional Genomics Unit, Department of Physiology, Anatomy and Genetics and the Weatherall Institute of Molecular Medicine, University of Oxford, UK. No part of this thesis has been submitted for any other degree at this, or any other university or institute of learning.

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Abbreviations

3V	Third cerebroventricle
4-AP	4-aminopyridine
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BOLD	Blood oxygenation level dependent
CA3	Field CA3 of hippocampus
CC	Corpus callosum
CGRP	Calcitonin gene-related peptide
CIPS	Calcineurin Inhibitor-Induced Pain Syndrome
CQ	5-Chloroquinolin-8-ol
CSD	Cortical spreading depression
DRG	Dorsal root ganglion
ECoG	Electrocorticography
ESC	Embryonic stem cell
EtOH	Ethanol
FBS	Fetal bovine serum
FHM	Familial Hemiplegic Migraine
GABA	γ -Aminobutyric acid
GDNF	Glial-derived neurotrophic factor
GWAS	Genome-wide association study
HCTZ	Hydrochlorothiazide
IBA	Isobutylalkenyl amide

iPSC	Induced pluripotent stem cell
K _{2P}	Two-pore domain K ⁺ channel
KSR	Knockout serum replacement
MA	Migraine with aura
MMA	Middle meningeal artery
MO	Migraine without aura
NC	Neural crest
NCC	Na ⁺ -Cl ⁻ co-transporter
NGF	Nerve growth factor
NO	Nitric Oxide
NTG	Nitroglycerin
Opt	Optic tract
P75 ^{NTR}	p75 Neurotrophin receptor
PACAP-38	Pituitary adenylate cyclase activating peptide
PAG	Periaqueductal grey
Pax3	Paired box 3
PMA	Phorbol 12-myristate-13-acetate
PNS	Peripheral nervous system
PVN	Paraventricular nucleus
RMP	Resting membrane potential
RUNX1	Runt-related transcription factor 1
RVM	Rostral ventromedial medulla
SON	Supraoptic nucleus;
SSN	Superior salivatory nucleus
TEA	Tetraethylammonium
TG	Trigeminal ganglion
TGVS	Trigeminovascular system

TNC	Trigeminal Nucleus Caudalis
TPA	Tetrapentylammonium
TRESK	TWIK-related spinal cord K ⁺ channel
TrkA	Neurotrophic tyrosine receptor kinase-A
TRPV1	Transient receptor potential cation channel subfamily V member 1
VIP	Vasoactive intestinal polypeptide

1. Introduction

Often trivialised as “just a headache,” migraine has had a long-standing impact on the health of individuals and society as a whole (Lipton, Stewart et al. 1995). Migraine causes significant discomfort and suffering in a large proportion of the population, yet current approaches to treating this disorder are inadequate (Ferrari, Goadsby et al. 2002) and the pharmaceutical industry have dis-invested in migraine drug discovery programs. This is somewhat surprising considering its high prevalence and life-long impact, which constitutes an attractive disease profile for industry. The lack of knowledge, as to the pathobiology of migraine susceptibility and the mechanism of attack initiation, may be a contributing factor. Despite the documentation of migraine for thousands of years, fundamental questions still remain and debate continues: Which brain region(s) trigger the cascade of a migraine attack? Is there common pathobiology amongst migraine subtypes? How is the pain of the headache phase generated and transmitted? Where and how do current therapeutics act? Answering these questions will be of upmost importance in the development of novel and effective therapeutic strategies.

In this thesis I will first seek to address the generalities of migraine and our current knowledge of the pathobiology - from the genetic to the systems level. With the advent of new screening techniques, genetic associations have slowly emerged for migraine (Silberstein and Dodick 2013); Chapters 3 and 4 will provide functional evidence in support of a role for two such genetic associations, namely the genes *KCNK18* and *SLC12A3*. Migraine research relies heavily upon human clinical/imaging studies, but also upon molecular and systems research in animal models. Often these two fields fail to reach consensus (Olesen and Jansen-Olesen 2012). Chapter 5 and 6

describe the development of a humanized cellular model of migraine, utilizing the rare variants previously discussed and Induced Pluripotent Stem Cell technology.

1.1 Characteristics of migraine

1.1.1 The burden of migraine

Migraine is a highly prevalent headache disorder, imposing a significant burden of disability on human health worldwide. In Europe headache disorders have a one-year prevalence of 51%. Migraine itself represents roughly one quarter of these cases, with 43 million Europeans listed as migraine sufferers (Andlin-Sobocki, Jonsson et al. 2005). The prevalence is unequal across gender, with a cumulative lifetime incidence of 43% for women compared to 18% for men (Stewart, Wood et al. 2008). There are also differences across socio-economic groups, with individuals on low income 60% more likely to suffer attacks than those on higher income (Stewart, Lipton et al. 1992). Such figures may only represent a fraction of total migraine cases as the majority go undiagnosed or unreported (Lipton, Stewart et al. 1998). Due in part to its debilitating nature and also its sheer prevalence, migraine should be acknowledged in terms of its burden on society, both at a social and economic level.

A classical migraine attack comprises several phases, including a severe, throbbing and usually unilateral headache. The headache phase will typically last between 4-72 hours and can be associated with secondary symptoms such as photophobia and nausea (Headache Classification Committee of the International Headache 2013). While clearly there exists a spectrum of symptoms and severity, with some patients experiencing only infrequent and mild migraine attacks, the majority of migraineurs report more than one attack per month, and 13% report more than one per week (Steiner, Scher et al. 2003). Migraine is known to be a severe stressor in life with impact on both family relationships (Lipton, Bigal et al. 2003) and career prospects

(Trust 2004). Additionally, depression is three times more common in the migraine population (WHO 2004).

The prevalence of migraine varies with age and generally maximally impacts upon those in the most productive years of their lives (Stewart, Lipton et al. 1992), with median age of onset at around 25 years of age (Stewart, Wood et al. 2008). On average in the UK, migraineurs lose 5.7 working days per year due to migraine attacks, resulting in 25 million lost work days nationally per year (Steiner, Scher et al. 2003). Treatment costs and the effect on the workforce, makes migraine a significant economic burden- costing Europe €27 billion annually and coming 3rd in the rank of most costly neurological disorders (Andlin-Sobocki, Jonsson et al. 2005). Despite the economic burden it imposes, migraine is heavily underfunded- being the least publicly funded of all neurological disorders relative to its economic impact (Olesen, Lekander et al. 2007).

1.1.2 Clinical aspects of migraine

Migraine is diagnosed based on attack descriptions given by the patient and the exclusion of possible secondary causes of headache. Criteria for diagnosis have been set out by the Headache Classification Committee of the International Headache Society (Table 1.1). Migraine can be grouped into two major subtypes: migraine with aura (MA) and migraine without aura (MO). The aura is a reversible focal neurological symptom that classically manifests as disturbances of the visual system, but can take other forms including sensory or motor disturbance. Symptoms typically develop gradually for 5-20 minutes before or during the headache phase and can last up to one hour (Headache Classification Committee of the International Headache 2013). Roughly one third of migraine patients are defined as MA sufferers but it is not uncommon for patients to experience both forms of migraine (Russell, Rasmussen et al. 1995).

Migraine can be described by several phases that may or may not occur for a given attack. Premonitory symptoms, including fatigue, neck stiffness, difficulty

concentrating, nausea and heightened sensitivity to light, present up to 48hrs preceding the headache phase. When present, aura will typically manifest within 30 minutes preceding headache pain (Queiroz, Rapoport et al. 1997). Migraine headaches are often rated as moderate to severe in intensity, commonly unilateral and throbbing in their nature. The period of headache pain is variable, but can last up to several days and is associated with a number of secondary symptoms including nausea, photophobia, vomiting and phonophobia. The characteristics of the postdrome phase of an attack differ markedly between patients and typically last several days. Patients can report euphoria following an attack, or a sense of depression coordinated with cognitive difficulties, mood changes and other symptoms (Headache Classification Committee of the International Headache 2013).

Diagnosis	Criteria
Migraine without aura (MO)	<u>At least 5 attacks fulfilling criteria A-D</u> A) Headache lasting 4-72 hours B) Headache has at least two of the following characteristics: <i>Unilateral localization</i> <i>Pulsating quality</i> <i>Moderate or severe pain severity</i> <i>Aggravation by, or causing avoidance of routine physical activity</i> C) During headache at least one of the following <i>Nausea and/or vomiting</i> <i>Photophobia and phonophobia</i> D) Not attributable to another disorder
Migraine with aura (MA)	<u>At least 2 attacks fulfilling criteria A-C</u> A) One or more of the following fully reversible aura symptoms <i>visual</i> <i>sensory</i> <i>speech and/or language</i> <i>motor</i>

	e) brainstem f) retinal B) At least two of the following: a. <i>At least one aura symptom spreads gradually over ≥ 5 minutes, and/or two or more symptoms occur in succession</i> b. <i>Each individual aura symptom lasts 5-60 minutes</i> c. <i>At least one aura symptom is unilateral</i> d. <i>The aura is accompanied, or followed within 60 minutes, by headache.</i> C) Not attributable to another disorder and transient ischaemic attack has been excluded.
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Table 2.1 Migraine diagnosis criteria. Adapted from (Headache Classification Committee of the International Headache 2013).

1.2 Pathophysiology of migraine

1.2.1 The Aura

As a premonitory symptom to the headache, aura has classically been viewed as a tractable way of studying the earliest phases of a migraine attack. Most MA sufferers experience a visual aura (Russell and Olesen 1996) that can consist of a propagating scintillating scotoma or zigzag shapes that move gradually across the visual field. In some cases the visual perception of aura has spatial and temporal characteristics remarkably similar to Cortical Spreading Depression (CSD) (Lashley 1941, Wilkinson 2004). This observation lead to the assumption that CSD is the neurophysiological correlate underlying the aura. CSD was first described by Leao in 1944 in a series of experiments that demonstrated how electrical stimulation of the brain surface in rabbits could induce a measurable wave of depolarization, followed by hyper-polarization that travelled across the cortex at a rate of 3-5mm/min (Leao 1944, Leao 1944, Leao 1945, Leao 1947). The wave of CSD is propagated through neuronal and glial cell activity resulting in a disruption of normal ionic balance (Hansen and Zeuthen 1981). In

addition to the neuronal/glial waveform, there is profound vasodilation followed by vasoconstriction of associated cerebral blood vessels (Brennan, Beltran-Parrazal et al. 2007).

Animal models of CSD are well established and provide strong evidence associating CSD with migraine pathogenesis (van den Maagdenberg, Pietrobon et al. 2004, Ayata, Jin et al. 2006, Eikermann-Haerter, Dilekoz et al. 2009). CSD-like events in humans have been shown. Electrocorticography (ECoG) recordings direct from the cortex of patients undergoing surgery for brain injury, demonstrate repetitive CSD events around the site of injury (Dreier, Major et al. 2009, Woitzik, Hecht et al. 2013). However, there is a paucity of direct evidence to confirm that these CSD events occur during a migraine attack. Standard EEG recordings with scalp electrodes are unlikely to detect the spreading depression and due to the invasiveness of the technique, ECoG has not been performed on migraine patients. Functional imaging has provided indirect evidence for CSD events during aura. Blood oxygenation level dependent (BOLD) signal changes with characteristics consistent with CSD have been observed in three subjects during a visual aura (Hadjikhani, Sanchez Del Rio et al. 2001). Further, a DC shift suggestive of a CSD waveform has been observed using Magnetoencephalography during spontaneous and visually stimulated migraine aura (Bowyer, Aurora et al. 2001). Lack of a direct observation of CSD during a migraine attack, in addition to the structural differences between human and rodent cortex, leave open the possibility that the migraine aura may be due to an event differing from classical experimental CSD.

It is now clear that CSD can activate pain pathways. Spreading activation followed by deactivation releases multiple factors including: nitric oxide (NO) (Obrenovitch, Urenjak et al. 2002), calcitonin gene-related peptide (CGRP) (Colonna, Meng et al. 1994) and ATP (Schock, Munyao et al. 2007), all of which can activate or sensitize pain signaling pathways. Induction of CSD in anaesthetized and awake animals causes activation of the nociceptive trigeminovascular system (Ingvar, Laursen et

al. 1997, Fioravanti, Kasasbeh et al. 2011) and behavioral studies suggest that in awake rodents, CSD can induce pain-associated facial expressions (Karatas, Erdener et al. 2013).

The relevance of CSD to migraine is still to be fully elucidated. Whilst it appears likely that CSD-like events do occur during migraine aura, the role of CSD in migraine without aura is fiercely debated. It thus remains an open question as to whether in human migraine, CSD is a causative step in the cascade that results in headache pain, or is simply an epiphenomenon.

1.2.2 The Vascular theory for the origin of the headache

Perhaps the most contested aspect of migraine pathology today, is the origin and signaling pathways of the headache pain during an attack. Harold Wolff initially popularized the vascular theory of migraine in the mid-twentieth century. Experiments on awake patients undergoing neurosurgery showed that electrical stimulation of dural and cerebral arteries induced nausea and headache pain (Wolff 1948). Eighty percent of migraineurs experience pain of a pulsatile and throbbing nature consistent with a role for arteries (Ray BS 1940). Furthermore, headache can be induced by balloon dilation of cerebral arteries (Nichols, Mawad et al. 1990) and is a commonly seen symptom of cerebral arteritis (Headache Classification Subcommittee of the International Headache 2004). Finally, one of the earliest therapeutics for migraine, ergotamine, and several subsequent efficacious therapies including triptans (Jansen, Edvinsson et al. 1992, Petersen, Lassen et al. 2005) are potent vasoconstrictors (Maier 1926).

1.2.3 Vascular changes are not causative of headache pain during migraine

There exists accumulating evidence that changes in blood flow may not be the driver of migraine pain. Blood flow studies have noted a temporal discrepancy between hypoperfusion and headache (Olesen, Friberg et al. 1990). Pituitary adenylate cyclase

activating peptide (PACAP-38) and vasoactive intestinal polypeptide (VIP) both cause marked cranial vasodilation but only PACAP-38 can initiate migraine (Rahmann, Wienecke et al. 2008, Schytz, Birk et al. 2009). In addition, many current efficacious migraine therapies have no documented effect on the vasculature (Goadsby 2012). Whilst Nitroglycerin (NTG) is a potent vasodilator, no changes in extracranial or intracranial vessels were observed during NTG -induced migraines (Schoonman, van der Grond et al. 2008). An interesting finding by Ahn (Ahn 2010) and followed up recently by Mo (Mo, Maizels et al. 2013), suggests that the throbbing nature of headache does not necessarily correlate temporally with heart rate. Perhaps most conclusively, it has now been shown using magnetic resonance angiography that there is no extracranial and only minor intracranial vasodilation during spontaneous migraine attacks (Amin, Asghar et al. 2013).

1.2.4 Neurovascular inflammation

The vascular theory as it was first proposed, has largely been replaced by a hypothesis based on neurovasculature dysfunction. Here, the headache pain involves the trigeminovascular system (TGVS) (Figure 1.1) that consists of intracranial meningeal blood vessels and the pain fibers that innervate them. The dura is extensively innervated by unmyelinated (C-fibers) and thinly myelinated (A δ -fibers) nociceptors originating in the trigeminal ganglion (TG) and C1-3 dorsal root ganglion (DRG) (Andres, von Düring et al. 1987). Stimulation of the dura is a well accepted migraine model (Bergerot, Holland et al. 2006, Akerman, Holland et al. 2013) and results in increased activation of both first order trigeminal neurons (Levy, Zhang et al. 2008) and second order trigeminal neurons residing in the Trigeminal Nucleus Caudalis (TNC) (Holland, Akerman et al. 2012).

There is strong evidence from animal models indicating that CSD events directly lead to TGVS activation, supporting the theory that CSD, and hence its correlate the aura,

precipitates headache pain. The wave of activity associated with CSD induces the release of a range of signaling factors including glutamate, ATP and H⁺ and K⁺ ions from neurons and glia, and CGRP and NO from perivascular nerve terminals (Levy 2012). Early work detailed the ability of CSD to induce long lasting blood flow changes in the Middle Meningeal Artery (MMA), which corresponded with ipsilateral activation of second order trigeminal neurons. Activation could be abolished by sumatriptan or chronic sectioning of the trigeminal nerve (Bolay, Reuter et al. 2002). Direct electrophysiological recordings have now shown that CSD can activate meningeal nociceptors (Zhang, Levy et al. 2010), however questions remain as to whether factors released by CSD can induce delayed and prolonged activation of trigeminal afferents consistent with the temporal characteristics of aura and prolonged headache pain. A recent study has provided evidence that through the opening of neuronal Panx1 mega channels CSD can induce a parenchymal inflammatory cascade resulting in MMA vasodilation and TGVS activation (Karatas, Erdener et al. 2013). In contrast to transient release of neurotransmitters and ions, inflammatory pathways may act to provide a delayed and prolonged stimulus for TGVS activation. In support for the involvement of peripheral pathways, mediators that cross the blood-brain barrier (BBB) slowly, or not at all, such as histamine and CGRP, can induce migraine attacks (Lassen, Thomsen et al. 1995, Lassen, Haderslev et al. 2002).

1.2.5 The central theory of migraine

In contrast to the viewpoint that peripheral nociceptors drive the headache phase, an alternative theory postulates that migraine pathogenesis resides mostly within the CNS (Akerman, Holland et al. 2011). Eighty-two percent of migraineurs experience a prodromal phase (Schoonman, Evers et al. 2006) whereby premonitory symptoms are experienced hours or days before the headache pain. These can include circadian rhythm disturbances, heightened sensitivity to light, fatigue and changes in thirst, mood,

appetite and urination (Giffin, Ruggiero et al. 2003). Such symptoms strongly argue for a dysfunction intrinsic to the CNS during the earliest phase of a migraine attack. Classically, efficacy of anti-migraine drugs has been attributed to action in the periphery (Humphrey, Feniuk et al. 1990). However it has been shown in animal models that when applied locally, triptans can act on central targets (Storer and Goadsby 1997). Systemic infusion of sumatriptan can reduce the activity of TNC neurons as measured by c-Fos expression (Shepherd, Williamson et al. 1995) and single unit recordings (Kaube, Hoskin et al. 1993). It must be noted that this effect was only observed after blood-brain barrier (BBB) disruption, which has yet to be robustly demonstrated to occur during migraine (Edvinsson and Tfelt-Hansen 2008). Indeed cilostazol, a robust migraine provocation, strengthens the BBB (Horai, Nakagawa et al. 2013). Recent experiments have highlighted the possibility that CSD events can activate TNC neurons without a contribution from peripheral afferents. Injection of lignocaine into the TG can block spontaneous and dural/facial evoked responses in second order trigeminal neurons. Lignocaine injection during or before CSD triggering had no effect on discharge rates in the TNC (Lambert, Truong et al. 2011). From these results it was concluded that activation of the TGVS following CSD can occur without activation of peripheral inputs, and instead signals are sent directly between the cortex and brainstem. The study findings have however been questioned due to technical aspects of the study design (Burstein, Strassman et al. 2012). Furthermore it is particularly unclear how a large-scale wave of activity such as CSD, would selectively activate only the central pain pathways associated with headache. Finally, a CNS-restricted mechanism has so far not been able to explain the delayed nature of TNC activity following CSD; increased discharge is consistently seen only after 10-20 minutes following CSD (Zhang, Levy et al. 2010, Lambert, Truong et al. 2011).

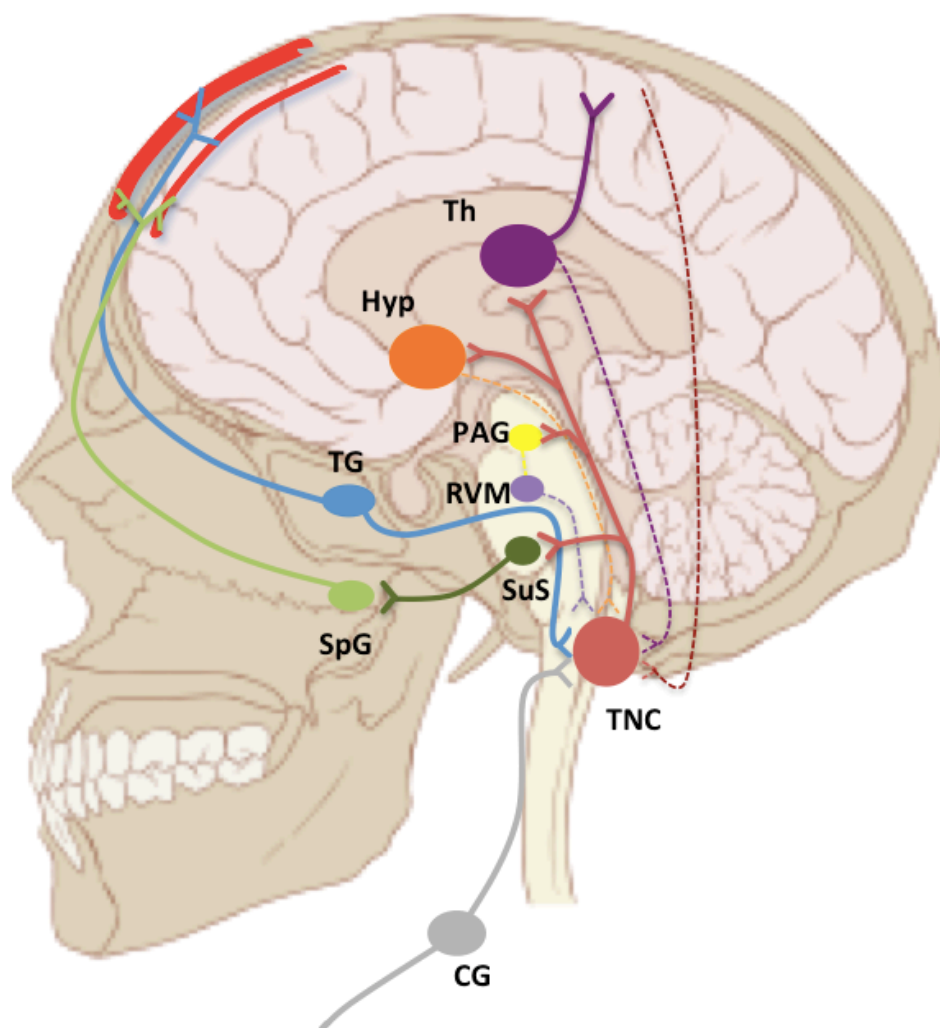


Figure 1.1 Neuronal pathways implicated in migraine. The cerebrovasculature is innervated by trigeminal afferents that project to second order neurons in the TNC. These neurons send signals to several nuclei within the thalamus, which in turn ascend to cortical regions including S1, anterior cingulate cortex and insular cortex, leading to the perception of pain. In addition to this, second-order trigeminal neurons project to the periaqueductal grey (PAG) and to hypothalamic nuclei. TNC neurons also synapse with neurons from the superior salivatory nucleus. From here, the sphenopalantine ganglion carries parasympathetic efferents to the cranial vasculature. This reflex likely contributes to the autonomic symptoms often associated with migraine. Descending modulation of TNC neurons originates from cortical, thalamic and hypothalamic regions as well as from the PAG, via the rostral ventromedial medulla (RVM). Solid lines represent afferent traffic and dashed lines represent descending modulation.

The periaqueductal grey (PAG) has consistently been shown to be activated during migraine (Afridi, Giffin et al. 2005, Denuelle, Fabre et al. 2007). Such activation remains beyond successful treatment with sumatriptan (Weiller, May et al. 1995). Electrical activation of the PAG inhibits dural evoked activation of TNC neurons (Knight and Goadsby 2001) and a small proportion of patients undergoing electrode implantation near the PAG report delayed migraine-like pain (Raskin, Hosobuchi et al. 1987). These findings spawned the idea that PAG dysfunction could be the mediator of migraine attacks. Anatomically, the PAG has extensive communication with the rostral ventromedial medulla (RVM) (Holstege and Kuypers 1982). Both areas have bi-directional connections with the TGVS and higher brain centers, such as the hypothalamus and thalamus (Droste and Roskamm 1990, Semenenko and Lumb 1992, Edelmayer, Vanderah et al. 2009). Specific dysfunction of the PAG acting as a driver for migraine attacks is an attractive way of explaining the many and varied premonitory and accessory symptoms of migraine. However it would not explain the specificity of the headache pain during migraine, since the PAG is activated during a wide range of pain syndromes (Hsieh, Stahle-Backdahl et al. 1996, Petrovic, Ingvar et al. 1999, Derbyshire, Jones et al. 2002). It is reasonable to assume from imaging and clinical observations that central regions have a clear modulatory role on TGVS traffic during headache, but until it is shown that dysfunction of these regions can activate previously quiescent trigeminal nociceptive pathways, it must be considered more probable that activation of primary trigeminal afferents is required for the headache pain.

1.3 Genetic background

1.3.1 Contribution of a genetic component

Migraine is often observed to segregate in families, suggestive of a genetic component. First-degree relatives of MO have a 1.9-fold increased risk (spouses have 1.4-fold increase), while with regard to the more genetically tractable MA, first-degree relatives have a 4-fold increase in risk (spouses have no increase) (Russell and Olesen 1995). Twin studies reveal that concordance is higher amongst monozygotic than dizygotic twins (Ulrich, Gervil et al. 1999) and that genetic and environmental factors likely play a roughly equal role in pathogenesis (Mulder, Van Baal et al. 2003). Complex segregation analysis suggests that Mendelian inheritance is unlikely, and that in most cases, disease is affected by a combination of multiple gene variants (Russell, Iselius et al. 1995).

1.3.2 Familial Hemiplegic Migraine (FHM)

FHM is a rare autosomal dominant condition caused by monogenic mutations. It is considered to be on the spectrum of migraine and patients share many clinical characteristics with those of typical MA. Distinctive of FHM, patients experience hemiparesis that occurs during the aura phase and also exhibit longer duration of aura and headache symptoms. FHM patients can also experience “typical” migraine attacks (Thomsen, Eriksen et al. 2002). While it seems genetic factors are not shared, FHM patients are at a higher risk than the general population for MA (Thomsen, Olesen et al. 2003), suggestive of a common pathophysiology. Due to its clinical similarity to typical migraine but more easily interpretable genetics, FHM has been used extensively as a model to define novel pathways and develop hypothesis for the pathogenesis of typical migraine.

To date, mutations in three genes have been identified as causing FHM (Figure 1.2): *CACNA1A* (FHM1) (Ophoff, Terwindt et al. 1996), encoding the $\alpha 1$ subunit of neuronal $\text{Ca}_v2.1$ Ca^{2+} channels; *ATP1A2* (FHM2) (De Fusco, Marconi et al. 2003), encoding the $\alpha 2$ subunit of Na^+/K^+ ATPase pumps; and *SCNA1* (FHM3) (Dichgans,

Freilinger et al. 2005), encoding the pore-forming $\alpha 1$ subunit of neuronal $\text{Na}_v1.1$ Na^+ channels. The described mutations do not explain all FHM cases and so it is likely that mutations in other genes are yet to be discovered. A mutation in *SLC4A4* (Suzuki, Van Paesschen et al. 2010), encoding the $\text{Na}^+\text{-HCO}_3^-$ co-transporter NBCe1, has been identified in sisters with FHM, however definitive evidence for causality is lacking.

The functional properties of FHM-associated proteins gives credence to the idea that FHM (and hence potentially typical migraine) can be described as a channelopathy (Ferrari MD 2006) (Figure 1.2). Cellular and knock-in mouse models show that causative FHM1 *CACNA1A* mutations result in a gain-of-function effect on channel activity (Ophoff, Terwindt et al. 1996, Tottene, Fellin et al. 2002). $\text{Ca}_v2.1$ channels are expressed on presynaptic nerve terminals and couple membrane depolarization with neurotransmitter release (Wu, Westenbroek et al. 1999). Using a knock-in mouse model, incorporating the R192Q FHM1 mutation, augmented excitatory neurotransmission has been shown (Tottene, Conti et al. 2009). FHM 3-associated mutations have given conflicting results. In heterologous expression systems, both gain and loss of function mutations have been described (Kahlig, Rhodes et al. 2008). Contrary to this, expression of the identified loss of function mutation in primary inhibitory interneurons rescues mutant channel current, sufficiently to induce an overall hyperexcitability phenotype (Cestele, Schiavon et al. 2013). In rodent CNS, $\text{Na}_v1.1$ is predominantly expressed by parvalbumin-positive inhibitory interneurons (Ogiwara, Miyamoto et al. 2007). How an enhancement of GABAergic neuron firing can contribute to migraine pathology is currently unclear. However, it has been speculated that excessive accumulation of extracellular GABA and K^+ could initiate CSD (Cestele, Schiavon et al. 2013).

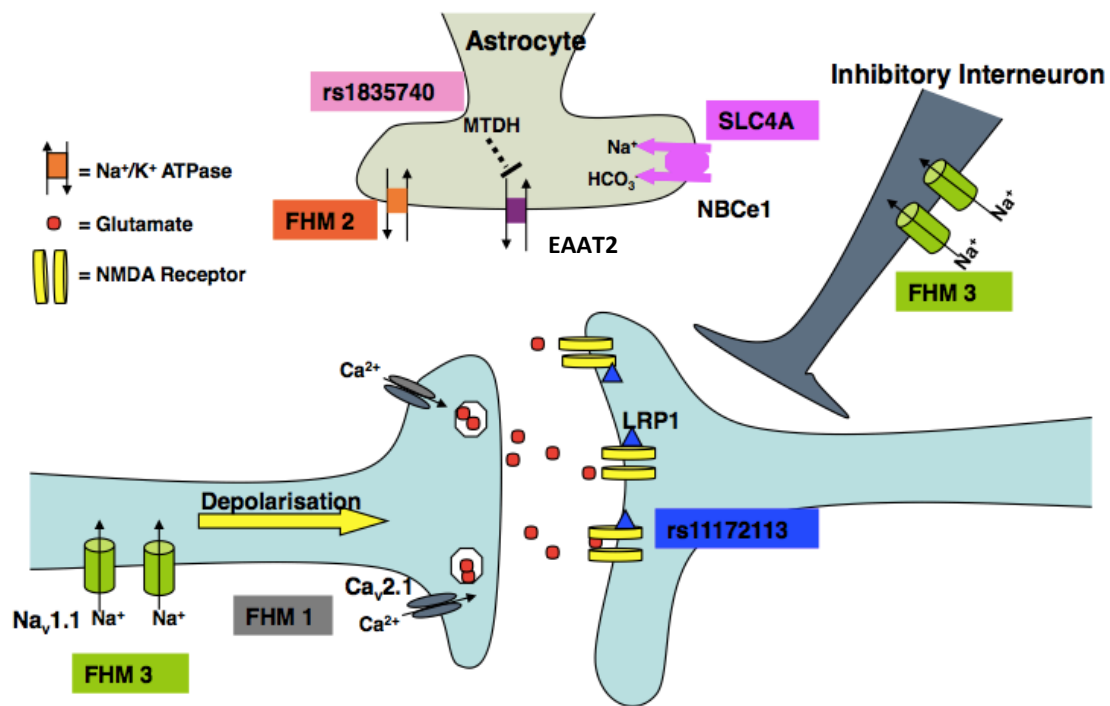


Figure 1.2 Migraine mutations affecting the central glutamate synapse. Increased Ca^{2+} influxes caused by familial hemiplegic migraine subtype-1 (FHM1)-associated mutations in $\text{Ca}_v2.1$ channels enhance glutamate release from presynaptic terminals. Loss of Na^+/K^+ ATPase function, as seen in FHM2, is proposed to indirectly reduce astrocyte uptake of glutamate, resulting in increased levels of the neurotransmitter in the synaptic cleft. FHM3-associated mutations increase firing of inhibitory interneurons. This may induce spreading depolarizations by releasing excessive GABA/K^+ into the extracellular space. Mutations in *SLC4A4* inhibit glia-mediated acid secretion and thus free *N*-methyl-D-aspartate (NMDA) receptors from proton-mediated inhibition. Activity of Excitatory Amino Acid Transporter 2 (EAAT2), the major glutamate transporter in the brain, is indirectly affected by up-regulation of the metadherin gene (*MTDH*), a likely consequence of a reported mutation in rs1835740. Low-density lipoprotein receptor-related protein 1 (*LRP1*) has a role in glutamate signaling and has been shown to directly modulate NMDA-dependent calcium currents *in vitro*.

The importance of glia is evident in FHM2; all studied mutations yield a 'loss-of-function' of the Na^+/K^+ ATPase pump (Segall, Mezzetti et al. 2005). These pumps are essential for the astrocyte-mediated uptake and clearance of neurotransmitters and K^+

ions in the synaptic cleft. It is presumed that reduced clearance will lead to enhanced neurotransmission. Neuronal excitation causes extracellular alkalosis, but this is buffered by acid secretion from glial cells. Such secretion is induced by glial cell depolarization and mediated by inward rectifying $\text{Na}^+ \text{HCO}_3^-$ co-transport (Chesler 2003). Mutations in *SLC4A4*, another glial transporter that has been reported to cause FHM in two sisters, cause defective trafficking of NBCe1 and reduced intracellular alkalization (Suzuki, Van Paesschen et al. 2010). Decreased capacities to buffer extracellular alkalosis could result in increased neuronal excitability, as *N*-methyl-D-aspartate (NMDA) receptors are sensitive to inhibition by protons.

While FHM and typical migraine overlap significantly, phenotypic differences do exist (Hansen, Thomsen et al. 2008) and to date, mutations in FHM genes have not been found to associate with typical migraine. Despite this, FHM serves as a highly valuable model of migraine. The usefulness of FHM genetic studies extends beyond simply identifying genes responsible for rare migraine subtypes. More importantly, they have provided mechanistic insights into the pathobiology of migraine in general, which seems likely to be a disease related to ionic disturbances with a resultant altered excitability of certain brain regions. Adopting this hypothesis may help prioritize findings, in the context of the large volume of data arising from genome-wide association studies and genome sequencing, by examining ionic homeostasis pathways.

1.3.3 Migraine as part of a broader syndrome

Migraine, both FHM and typical, often presents as part of a broader syndrome of disease. These conditions are usually attributable to highly penetrant mutations and offer an opportunity to investigate migraine-relevant pathways. To date, seven genes have been identified to cause syndromes of which migraine (FHM or MA/MO) is one component. *PRRT2*, encodes a transmembrane protein that associates with SNAP-25; mutations in *PRRT2* is causative for paroxysmal kinesigenic dyskinesia (PKD) and benign familial

infantile epilepsy (BFIE) (Riant, Roze et al. 2012). A mutation in *SLC1A3*, encoding the glial glutamate transporter EAAT1, has been identified in one patient who presented with alternating hemiplegia and migraine, in addition to episodic ataxia and seizures (Jen, Wan et al. 2005). *COL4A1*, encodes the type IV collagen alpha chain and mutations in this gene result in cerebral small vessel disease, porencephaly and infantile hemiparesis (Lanfranconi and Markus 2010). *NOTCH3* encodes a key-signaling molecule in neuronal development and mutations in this gene have been shown to underpin cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (Ceroni, Poloni et al. 2000). *TREX-1* encodes a DNA exonuclease which when mutated, causes hereditary vascular retinopathy (Stam, van den Maagdenberg et al. 2008) amongst other syndromes. Finally, casein kinase $\delta 1$ is a serine-threonine kinase associated with advanced sleep disturbances (Brennan, Bates et al. 2013) (Figure 1.3). Since migraine is only one part of each syndrome, this implies that these genetic defects lead to broader disruption of multiple pathways including those involved in migraine causation.

1.3.4 Genome-Wide Association Studies

Genome-Wide Association Studies (GWAS) allow for the un-biased interrogation of the genome on a large scale. Millions of SNPs can be compared between disease and control cohorts with the resultant analysis best suited to detect common disease variants, even those with low effect size. GWAS have been applied to migraine with varying success, with 8 associated variants identified to date (Figure 1.3).

The first GWAS used a relatively small European patient cohort (2,731 migraine cases) and identified one SNP that reached genome-wide significance (Anttila, Stefansson et al. 2010). Rs1835740 on chromosome 8q22.1 associated with migraine with a P value of 5.38×10^{-9} and odds ratios ranging from 1.21-1.33. Replication studies

confirmed the association in further European cohorts. The marker resides between two genes, *PGCP* and *MTDH*, both of which have been implicated in glutamatergic neurotransmission. Although minimal functional follow-up has been performed, the risk allele is associated with higher *MTDH* expression in lymphoblastoid cell lines. *MTDH* down-regulates EAAT2, a protein utilized by glia to clear glutamate from the synaptic cleft. It is hypothesized that higher levels of *MTDH* expression could therefore result in an increase of extracellular glutamate levels and thus promote a state of hyper-excitability in the CNS.

The second GWAS took a meta-analysis approach to six European cohorts and identified 32 SNPs associated with migraine (Ligthart, de Vries et al. 2011). Of particular interest from this study was a SNP in the nerve growth factor receptor gene. Despite the large study size, replication of all identified SNPs failed in 3-population based samples (from the Netherlands and Australia) leading the authors to postulate that the study lacked power due to the heterogeneity of their population-based samples. The samples used were obtained from patients diagnosed on the basis of short questionnaires, rather than a clinical diagnosis. This not only increases the probability of miss-diagnosis, but is also likely to generate bias for less severe phenotypes (and thus a lower predicted genetic risk). This study did however provide modest support for the association of the *MTDH* gene and migraine, yet failed to replicate the specific SNP association noted previously by Antilla, *et al.*, 2010 (rs1835740).

The largest migraine GWAS performed to date identified three SNPs: (rs2651899 [*PRDM16*], rs10166942 [*TRPM8*], and rs11172113 [*LRP1*]) (Chasman, Schurks et al. 2011). A plausible hypothesis linking *PRDM16* to migraine is lacking, however *TRPM8* is a cold-activated ion channel highly expressed in sensory neurons and with a large role in peripheral pain pathways (McKemy 2005). *LRP1* is expressed in a range of cells in the CNS (Bu, Maksymovitch et al. 1994) and *LRP1*^{-/-} mice present severe neurological defects (Liu, Trotter et al. 2010). The protein has been shown to modulate

NMDA-dependent intracellular signaling, suggestive for a role of LRP1 in controlling synaptic transmission (Nakajima, Kulik et al. 2013).

The most recent study replicated the *TRPM8* and *LRP1* loci but also identified two novel loci: *MEF2D* and *TGFB2R* (Freilinger, Anttila et al. 2012). *MEF2D* is a transcription factor that restricts the number of excitatory synapses in response to enhanced neuronal activity (Flavell, Cowan et al. 2006). *TGFB2R* is involved in cell proliferation and differentiation (Lin, Wang et al. 1992). A missense mutation in the gene has been associated with aortic dissection in a large multi-generational pedigree. Crucially, 11/14 mutation carriers also suffered from migraine (Law, Bunyan et al. 2006). This study identified two further, but minor associations with the genes *PHACTR* and *ASTN2*. While a functional link between *ASTN2* and migraine is unclear, the protein product of *PHACTR* controls synaptic morphology and activity (Greengard, Allen et al. 1999).

The majority of GWAS-identified SNPs fit well with the hypothesis born from earlier FHM studies: that migraine predisposition is at least partially due to a deregulation at the central synapse. However, it is also of note that some of the loci, *TRPM8* in particular, are more known for their role in peripheral pathways, arguing against the notion that peripheral pathways are redundant in migraine pathogenesis. Despite the undoubted utility of GWAS studies there exists two main weaknesses in their application. Firstly, the currently identified variants have odds ratios that range from 0.77-1.23. These variants are therefore present and well tolerated in the control group and thus can all be classified as having a low effect size. It is often desirable to perform functional follow-up studies on genetic variants as a way to gain insight into disease mechanisms. The low penetrance of GWAS-defined variants poses a significant challenge to this. Secondly, despite the large patient sizes in the documented studies (up to 23, 285) there is clearly a substantial amount of missing heritability. The effectiveness of GWAS is greatest in detecting disease-associated alleles that occur with

relatively high frequency. Thus, missing heritability can be partially explained by the inability of GWAS studies to detect rare disease variants, epistasis and epigenetic effects. (Eising, N et al. 2013). Progress in migraine genetics may therefore require further classical genetic studies in familial migraine and next generation sequencing approaches.

1.3.5 *KCNK18*

The candidate-based approach can be extremely powerful in detecting risk variants associated with disease, but requires *a priori* knowledge of disease mechanisms (Patnala, Clements et al. 2013). Our laboratory used this methodology to identify the first gene associated with typical migraine (Lafreniere, Cader et al. 2010). Using the knowledge base provided by FHM studies, a screen of 150 brain-expressed ion channel genes was undertaken in 110 unrelated migraine patients. In an attempt to improve the chance of identifying deleterious coding mutations, patients were selected on the basis of presenting with a strong family history of MA. A 2bp deletion in *KCNK18* (F139fsX24), which causes a premature truncation of the coded protein, was found in one proband. The *KCNK18* gene was sequenced in 16 family members of the proband, spanning several generations. F139fsX24 is inherited in a dominant fashion and revealed 100% penetrance and segregation of the mutation with MA. *KCNK18* encodes TRESK (TWIK-related spinal cord potassium channel), a K⁺ leak current channel and member of the two-pore K⁺ channel family. Given the apparent high penetrance of this mutation, characterizing protein function may reveal general disease mechanisms.

Despite the strong segregation of the *KCNK18* variant with disease phenotype, it would be counterintuitive to assume monogenic inheritance in a sporadic form of migraine. Further analysis of the original data was performed and a second genetic linkage peak [LOD score 2.8] was found with a segregating haplotype present in all affected individuals and two unaffected individuals. Exome sequencing with an average

100-fold coverage per exome, identified 2333 exonic SNPs and 104 exonic indels shared by all 3 individuals. The F139WfsX24 mutation was found in all 3 affected individuals but no other predicted deleterious variants in genes adjacent to *KCNK18*. At the second genetic locus, there were no indels and only 1 shared exonic polymorphism – a rare deleterious variant (G264A) in *SLC12A3*. We then confirmed that all affected individuals in the family carry the G264A mutation (*Cader et al, unpublished data*). *SLC12A3* encodes a Na⁺/Cl⁻ co-transporter (NCC) classically restricted to a role in the distal convoluted tubule of the kidney (Gamba 2009). Nothing is known about the role of NCC in migraine salient areas, but given the genetic association it is pertinent to investigate whether NCC could function as a modifier of migraine pathobiology.

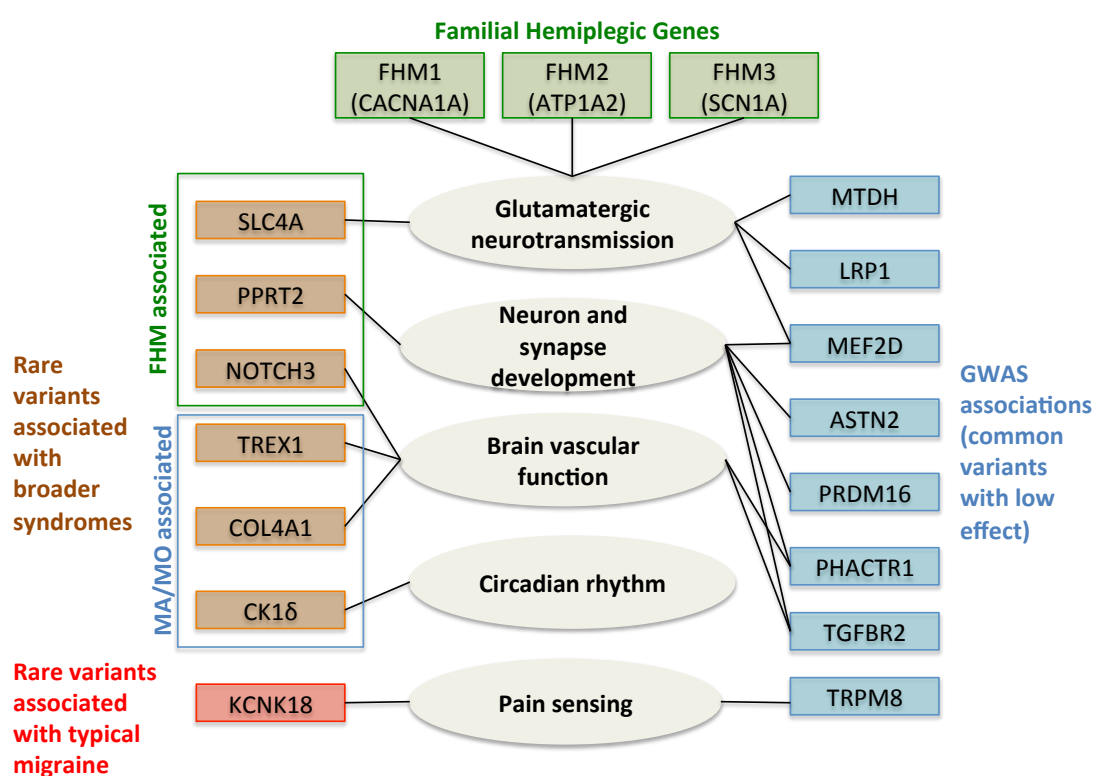


Figure 1.3 Genes and pathways involved in different forms of migraine. FHM genes: *CACNA1A*, *ATP1A2* and *SCN1A* are involved in the regulation of glutamatergic neurotransmission. Genes in which rare variants have been associated with migraine as part of a broader syndrome associate with both FHM and MA/MO. These variants are implicated in a multitude of pathways.

Overlapping with FHM, common migraine genes *MTDH*, *LRP1* and *MEF2D* also associate to glutamatergic neurotransmission. Additional common migraine genes implicate neuron and synapse development (*ASTN2*, *PRDM16*, *PHACTR1* and *TGFBR2*), brain vasculature function (*PHACTR1* and *TGFBR2*) and pain sensing (*TRPM8*) as further migraine-relevant pathways. A variant in *KCNK18* was the first to be associated with typical migraine and fits with the pain-sensing pathway.

1.3.6 Two-Pore Potassium (K_{2P}) channels

Although postulated by the constant field theory of Goldman, Hodgkin and Katz (Goldman 1943) the molecular basis for background K⁺ leak current went undefined for over half a century. However in the 1990's, with the identification of the TWIK (Tandem of pore domains in a weak inward rectifying K⁺ channel) channels came the first glimpse at the molecular pores through which the K⁺ leak flows (Lesage, Guillemare et al. 1996). Since then, 15 genes spanning 6 subfamilies have been identified and classified as members of the Tandem Two-Pore Domain super family of mammalian K⁺ channels- K_{2P} channels (Goldstein, Bayliss et al. 2005). While sequence homology between the different subfamilies is often low (Fink, Duprat et al. 1996), all share a common structural feature that is unique amongst K⁺ channels (Figure 1.4). Each subunit possesses four transmembrane domains and two pore-forming regions, generating dimers that conform to the classical tetrameric K⁺ pore (Piechotta, Rapedius et al. 2011). K_{2P} channels show only modest or no dependence on voltage, but can be heavily regulated by a range of endogenous factors (Maingret, Patel et al. 2000) and clinical compounds (Putzke, Hanley et al. 2007). Perhaps most crucial to their neuronal function, K_{2P} channels have a large impact on the intrinsic excitability of neurons. K_{2P} channels shape the duration, frequency, and amplitude of action potentials, and regulate neuronal excitability by modulating membrane resting potential and resistance (Goldstein, Bockenhauer et al. 2001). Indicative of a fundamental importance, genetic

orthologs are present in lower organisms (Goldstein, Price et al. 1996, de la Cruz, Levin et al. 2003). There is broad expression across the body (Reyes, Duprat et al. 1998, Hur, Choe et al. 2009, Buxton, Singer et al. 2010), suggesting that these channels play a role in a vast array of physiological processes, ranging from hearing (Holt, Asako et al. 2006) and pregnancy (Monaghan, Baker et al. 2011), to regulating the cardiac pacemaker (Joon Kim and Earm 2006) and the circadian rhythm (Enyedi and Czirjak 2010). K_{2P} channels are also widely distributed in the nervous system (Hervieu, Cluderay et al. 2001, Yamamoto, Kummer et al. 2002, Cadaveira-Mosquera, Perez et al. 2012). Research is now shedding light on the role of these channels in both the physiology and disease of the nervous system.

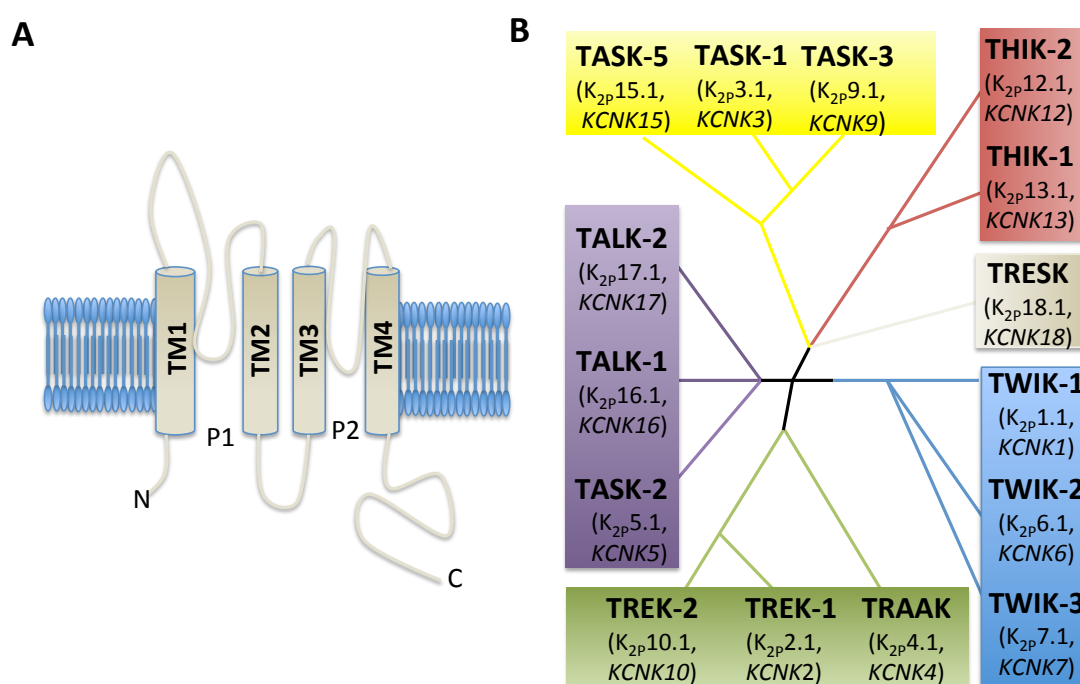


Figure 1.4 The K_{2P} family of K^+ channels. (A) The molecular structure of a K_{2P} subunit. Each subunit is composed of four transmembrane domains (TM1-4) and two pore-forming regions (P1-2). Two subunits form a dimer to create the tetrameric pore through which K^+ ions can flow. (B) Phylogenetic tree of the K_{2P} family. Six discrete subfamilies exist on the basis of the factors that modulate channel activity and their sequence homology. Naming is given as old

nomenclature (*bold text*) and current convention (K_{2P} X.1). Gene names are given in the form *KCNKX*.

1.3.7 TRESK function

TRESK expression is particularly enriched in sensory ganglia, and evidence is suggestive of a crucial role in peripheral pain pathways. Along with TREK-1 and TREK-2, TRESK contributes to >95% of the background K^+ conductance in DRG neurons (Alloui, Zimmermann et al. 2006) and by mRNA levels is the most abundant of the K_{2P} family in this tissue (Marsh, Acosta et al. 2012). TRESK functional knockout (KO) mice show enhanced DRG excitability (Dobler, Springauf et al. 2007) and application of the TRESK blocker hydroxy- α sanshool, increases activation of TRPV1 positive sensory neurons (Bautista, Sigal et al. 2008). In a peripheral nerve injury model, DRG taken from rats 3 weeks after sciatic nerve axotomy show an increased soma excitability that corresponds with a decrease in TRESK transcripts. *In vivo*, pharmacological and RNAi mediated inhibition of TRESK results in nocifensive behaviours and a decrease in the threshold to painful mechanical stimuli (Tulleuda, Cokic et al. 2011), although only a modest increase in thermal nociceptive sensitivity is observed in the knockout model (Dobler, Springauf et al. 2007). Similarly, intrathecal injection of adenovirus carrying the TRESK gene increased mRNA expression in the DRG and decreased sensitivity to pain in a rat model of neuropathic nerve injury (Zhou, Yang et al. 2013). TRESK is unique amongst ion channels in that it has a direct interaction with calcineurin (Czirjak and Enyedi 2006). In *Xenopus* oocytes, cyclosporine A, a calcineurin inhibitor, eliminates TRESK activation (Czirjak, Toth et al. 2004), and patients receiving cyclosporine A require higher doses of the anaesthetic isoflurane (a TRESK activator) (Niemann, Stabernack et al. 2002) to achieve appropriate levels of anaesthesia. TRESK may, therefore, play a key part in Calcineurin Inhibitor-Induced Pain Syndrome (CIPS), classified as severe pain in the lower limbs induced by immunosuppressive treatment following transplant. These

results suggest that TRESK plays an important physiological role in primary nociceptive pathways.

1.3.8 NCC function

The relevant function of NCC is much less clear than for TRESK. There are only a few examples of studies investigating NCC outside of the kidney (Bazzini, Vezzoli et al. 2005, Lauf, Chimote et al. 2008). Within the nephron, NCC has the function of reabsorbing Na⁺ and Cl⁻ ions from the tubular fluid (Gamba 2009) and no other, non-cannonical protein function has been described. Protein family members are expressed by neurons (Toyoda, Yamada et al. 2005) and other Na⁺/Cl⁻ co-transporters are now understood to be crucial for nociceptive pathway plasticity (Hasbargen, Ahmed et al. 2010). Despite this, no study to date has documented NCC presence or function in the nervous system. In support of a neuronal role, NCC is regulated by neuronal specific factors (Glover, Zuber et al. 2009). There is little overlap between TRESK and NCC pathways, but both are modulated by calcineurin signaling (although with opposing net effects). TRESK is activated by calcineurin (Czirjak, Toth et al. 2004), yet the activity of NCC is increased upon calcineurin inhibition (Hoorn, Walsh et al. 2011).

1.4 Induced Pluripotent Stem Cells to model neurological disorders

1.4.1 Current *in vitro* tools to study migraine

Animal models are relied upon heavily in research and have helped gain insight into the pathobiology of disease, from the cellular to the systems level. Such work however has its acknowledged pitfalls. *In vitro* and *in vivo* animal work relies upon the assumption that results can be extrapolated across species and can provide relevant information pertaining to the human condition. This assumption cannot be taken as a given, a fact best underlined by the consistently disappointing results when translating therapies

successful in animal models to human subjects (Hackam and Redelmeier 2006, Perel, Roberts et al. 2007). Access to live human tissue is scarce and often relies upon immortalized cell lines, especially in drug screening efforts. However, due to the process of immortalization it is questionable how accurately these cell types resemble native human cells. Both animal, and immortalized models are highly valuable in determining the functional impact of singular genetic factors through gain or loss of function experiments. However their usage in cases of complex hereditary may be less beneficial. Conditions such as migraine, where disease is based upon a complex multifactorial genetic background, and occurs in cells largely inaccessible to researchers, cannot be completely modelled by these standard cellular systems. Figure 1.5 details the disadvantages of traditional models.

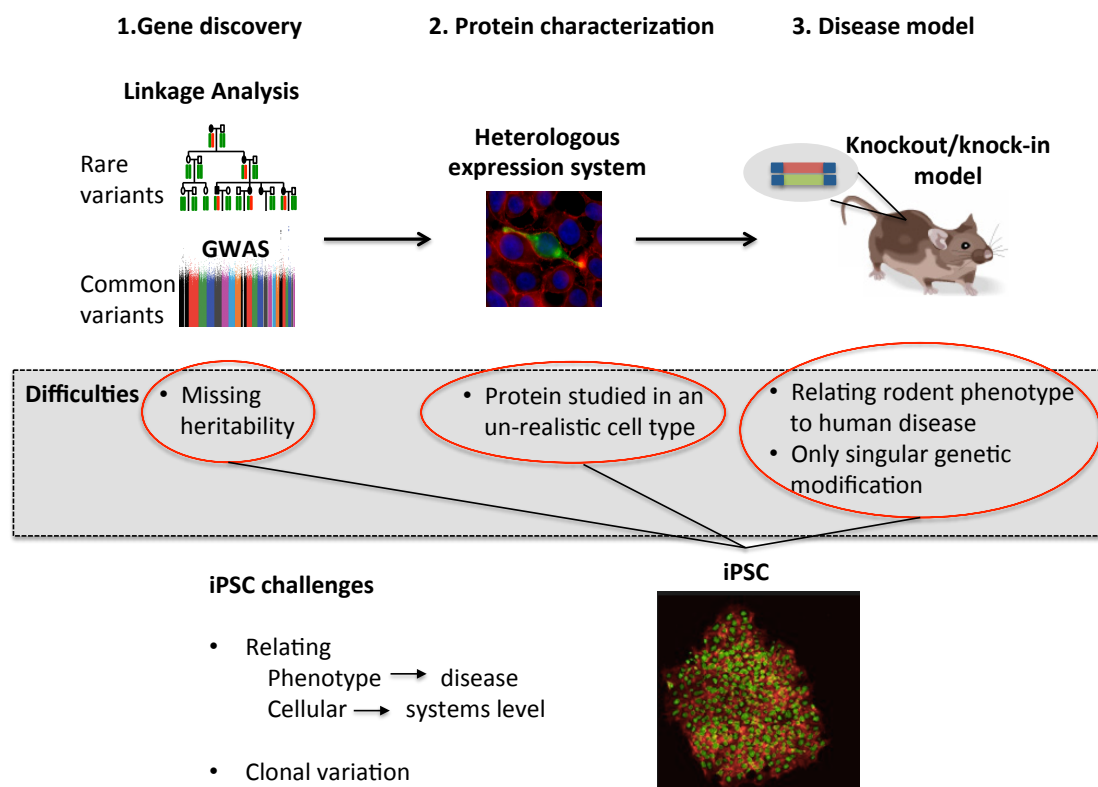


Figure 1.5. A standard functional genetics pipeline and its deficiencies. Classical linkage analysis or Genome Wide Association Studies (GWAS) first identifies disease-associated genes.

Linkage analysis efficiently identifies highly penetrant rare variants but the power of large-scale GWAS is required to identify common variants with low penetrance. However, despite complementary approaches many diseases still have missing genetic heritability. Identified genes can be assessed for protein function by overexpressing in immortalized cell lines and studying a multitude of cellular outcomes. This system is easy to use and amenable to genetic modifications, however it is clear that the protein under investigation is not functioning in the context of its *in vivo* environment. A more physiological system, and often the next step in the experimental strategy, is to generate a mouse with the gene of interest knocked-out, or the disease associated variant knocked-in. This provides a platform to study systems level consequences of gene expression, such as behavioural analysis. How well rodent phenotypes correlate with human disease and the similarity in function of rodent and human protein homologs, are significant issues to consider when evaluating results from such a model. Both overexpression and mouse models are limited in their capacity to study only a few genetic factors in combination. This fact reduces their appeal in terms of modelling polygenic diseases. iPSC technology offers promise to resolve many of the discussed challenges (circled red) and should be used at several points in the time line to complement traditional approaches.

1.4.2 Pluripotent cells as experimental models

Stem cell technology has been developed as a highly promising tool for basic science and regenerative medicine and could overcome some of the difficulties faced by traditional models. Embryonic stem cells (ESC) retain many stem cell properties in culture, namely the ability to expand indefinitely, self-renew and differentiate into specialized cellular progeny. As such, human ESCs have become an increasingly used tool in cellular biology in the decade and a half since they were first isolated (Thomson, Itskovitz-Eldor et al. 1998). They offer the potential to generate human cell types that were previously unattainable in cell culture conditions. In terms of disease modelling, ESCs can be derived from embryos known to carry genetic defects or can be genetically modified by a range of *in vitro* methods (Ding, Lee et al. 2013, Zhang, Rho et al. 2013). Differentiation

and interrogation of such models has been illuminating for disease pathogenesis across many systems (Lorincz 2005, van de Stolpe, van den Brink et al. 2005, Abdul Kadir, Ali et al. 2009, Niclis, Pinar et al. 2013). ESC technology negates the issue of species relevance, but fails to provide a system of genetic relevance to polygenic disorders where all disease-associated variants may not be known.

The reprogramming of somatic cells to an ESC-like state offers a second route for stem cell based disease modelling. Such efforts overcome not only the ethical considerations of ESC technology, but can also be used as a source of genetically relevant material. Somatic cells, traditionally skin fibroblasts (Takahashi, Tanabe et al. 2007), but also blood (Staerk, Dawlaty et al. 2010), urine (Zhou, Benda et al. 2012) and keratinocytes (Aasen, Raya et al. 2008), can be reprogrammed by forced expression of factors associated with pluripotency. Classically, virally mediated expression of the four transcription factors; OCT-4, c-MYC, SOX-2 and KLF-4, is enough to generate cells that can be expanded indefinitely in culture and differentiated into almost all cell types (Takahashi and Yamanaka 2006). Since the initial report in 2006, a multitude of reprogramming methods have been published in an attempt to increase efficiency, consistency and safety (Gonzalez, Boue et al. 2011). Reprogrammed cells are termed “Induced Pluripotent Stem Cells” (iPSC) and appear to very closely resemble ESC in terms of morphology, molecular identity and differentiation potential (Hu, Weick et al. 2010, Swistowski, Peng et al. 2010). iPSCs are capable of differentiating at comparable rates and efficiencies to ESCs and the range of cell lineages generated from iPSC is ever expanding. Specifically, many neuronal lineages such as motor neurons (Karumbayaram, Novitch et al. 2009), glutamatergic (of all six cortical layers) (Shi, Kirwan et al. 2012), GABAergic (Higurashi, Uchida et al. 2013), dopaminergic (Swistowski, Peng et al. 2010), sensory (Chambers, Qi et al. 2012) and retinal ganglion cells (Parameswaran, Balasubramanian et al. 2010), in addition to associated cell types such as astrocytes (Roybon, Lamas et al. 2013), oligodendrocytes (Wang, Bates et al.

2013) and Schwann cells (Kreitzer, Salomonis et al. 2013), have all been successfully derived. More recently it was demonstrated that it is possible to derive multicellular systems reminiscent of a developing brain (Lancaster, Renner et al. 2013). This expansion of differentiation capability has made investigation of previously unavailable human cell types of choice a reality.

As well as offering the ability to generate specific cell types, iPSC technology allows the investigation of cellular disease phenotypes. Fibroblasts taken from disease patients, once differentiated to the cell type of choice, retain the genetic background of disease. This allows cellular phenotypes to be compared between cells derived from healthy subjects and disease patients. Early studies focused upon monogenic conditions and investigating disease relevant phenotypes when the disease-associated variant was known (Chang, Zheng et al. 2011, Mitne-Neto, Machado-Costa et al. 2011, Jiang, Ren et al. 2012). Less genetically tractable, sporadic conditions have proven a greater challenge (Soldner, Hockemeyer et al. 2009, Almeida, Zhang et al. 2012), but it is heartening to see that examples of success are starting to emerge (Burkhardt, Martinez et al. 2013, Kondo, Asai et al. 2013).

The advantages of iPSC technology overlap significantly with the deficiencies in current migraine models. Neuroscience as a whole is continually hampered by a lack of relevant human tissue to study. In some neurological conditions post-mortem tissue analysis is highly informative. This however is not the case for migraine, as the condition is likely one associated with biochemical and biophysical changes, as opposed to morphological or degenerative changes that are amenable to post-mortem analysis. The latest imaging techniques are providing exciting data, with ever increasing spatial resolution, as to real-time neural activity during the different stages of a migraine attack. What is required is a system to explore the molecular and cellular processes that go hand-in-hand with these systems level changes. The predominant attraction of iPSC technology in relation to migraine modelling is the capturing of the whole genetic

landscape. Genetic association studies have failed to identify the variants responsible for the majority of migraine heritability. Thus it is impossible to totally model the disease by conventional means. iPSC taken from migraine patients and differentiated to mature cell types would contain every genetic contributor to disease for that patient. This makes the technology very attractive for use in the study of migraine. However it is also important to consider the limitations. iPSC technology will never be suitable to model complex physiological behaviour (Weir, Stein et al. 1989, Weir and Wale 2011). Further, if unknown environmental factors contribute substantially to disease causation, the *in vitro* model may never develop disease relevant abnormalities despite a predisposing genetic background. Emergent phenomenon may also be difficult to capture in single cell type cultures.

1.5 Aims of this work

My overarching aim for this thesis is to use functional studies of genetic variants to dissect mechanisms that may play a role in migraine pathogenesis.

The three principle aims are:

1. To assess the functional consequence of TRESK activity to a range of neuronal cell models and to understand how this function relates to disease.
2. To define whether there is a novel role for the thiazide-sensitive Na^+/Cl^- co-transporter (NCC) in neuronal physiology. This work is to support an unpublished observation associating a deleterious mutation with migraine.
3. To establish a humanised cell model of migraine by generating functional nociceptors from iPSCs. Second to this, I aim to compare migraine-derived

nociceptors to healthy controls in an attempt to define biophysical differences
that may be relevant for disease

2. Materials and Methods

2.1 General methods

2.1.1 RNA extraction and reverse transcription

RNA was extracted from cell pellets and frozen tissue by RNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. Cells were typically homogenized by prolonged vortex treatment, while tissue required QIAshredder homogenization (QIAGEN). Total RNA was purified from samples by RNeasy mini columns before elution in variable volumes of RNase-free H₂O. 2µg RNA was reverse transcribed to cDNA by MutiScribe reverse transcriptase (Life Technologies) following manufacturer's instructions. To overcome inhibitory effects of reverse transcription reagents to downstream applications, cDNA was diluted 1:5 in H₂O prior to use and stored at -20°C.

2.1.2 Reverse Transcription-PCR (RT-PCR)

1µl of sample cDNA (20ng/µl) was used as template for each 50µl PCR reaction with Taq DNA polymerase (Sigma-Aldrich). Primer sequences used for RT-PCR and their annealing temperatures (T_m) are shown in Table 2.1. Cycling conditions used: 95°C for 3 min, 30 x [95°C 30 sec, X°C 30 sec, 72°C 1 min], 72°C 10 min. Products were visualized on a 1% (w/v) agarose gel and base pair length estimated by comparison to 1kb/100bp standard DNA ladders (New England Biolabs).

Target	Product (bp)	Forward Primer 5'-3'	Reverse Primer 5'-3'	T _m (°C)
<i>hPAX3</i>	481	AGCACCCCAATCAGATGA AG	TGTCTGGGTTGGAAGGA ATC	65
<i>hSOX9</i>	204	CGAAGATGGCCGAGAT	AGTGGGTAATGCGCTT	65
<i>hSOX10</i>	175	CCACCTGGCTCTCCTCTAA A	CTCAGCTCCACCTCCGAT AG	65
<i>hTRKA</i>	399	CACAGAGCTGGAGCAGTC	AGCGTGTAGTTGCCGTT	65

		AG	GTT	
<i>hBRN3A</i>	230	GCTTGAAAGGATGGCTCT	ATGTCCATGAACAGCAA	65
		TG	GCA	
<i>hGAPDH</i>	516	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAAT	55
			CC	
<i>hTRESK</i>	440	GACATCCTGGCAACCATCT	GCAGCTGCACAGGAAAT	65
		T	GTA	
<i>hER-α</i>	293	GCTTCGATGATGGGCTTA	ATGCGGAACCGAGATGA	55
		CTGA	TGT	
<i>hER-β</i>	393	TAGTGGTCCATCGCCAGT	GGGAGCCACCTTCACCA	55
		TAT	T	
<i>hNCC</i>	340	CTGACCTGCACTCCTTCCT	GACTTGACCTTGCCATT	60
		C	GGT	

Table 2.1. RT-PCR primer sequences

2.1.3 Sequencing and data analysis

mRNA for sequencing was first reverse transcribed to cDNA as previously described. cDNA was then cloned into pCR8/GW/TOPO vector (Life Technologies) following manufacturer's instructions. Single colonies of bacteria transformed with vector were grown, selected and expanded before DNA was extracted by Qiagen Miniprep Kit. Subsequent DNA was directly sequenced as per below. For genomic DNA (gDNA), 1µl of 10ng/µl gDNA was used as template for each 25µl PCR reaction with the high fidelity polymerase, Phusion Hot Start II (Thermo Scientific). Primer sequences used for PCR amplification and their annealing temperatures (T_m) are shown in Table 2.2. Cycling conditions used: 98°C for 1 min, 30 x [98°C 30 sec, X°C 30 sec, 72°C 1 min], 72°C 10 min. Products were visualized on a 1% (w/v) agarose gel and purified by Qiagen Gel Purification Kit, as per manufacturer's instructions. PCR amplified fragments were sequenced by Source Bioscience using the same primers as for the initial PCR amplification. Results were analyzed by SnapGene Viewer 2.4.3 and sequence alignment was performed by ClustalW multiple alignment tool.

Target	Product (bp)	Forward Primer 5'-3'	Reverse Primer 5'-3'	T_m (°C)
<i>mNCC</i> <i>exons 3-17</i>	1671	GTTGCATGCTCAACATC TGG	TGTTTCAGCCACTTGGTG TGT	65

<i>hKCNK18</i>	300	TTTAAAGCTTTTGGGGG GAAAAAGGG	GCGGGCTTGGGGTCCGG TTT	60
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Table 2.2. Primers used for PCR amplification and/or nucleotide sequencing

2.1.4 Qualitative RT-PCR (qPCR)

5µl of template cDNA (20ng/µl) was used for each 20µl PCR reaction. Reaction utilized SYBER Green chemistry (Applied Biosystems) and a StepOne Plus real-time PCR machine (Applied Biosystems). Primers were designed to span exon-exon boundaries of target genes to nullify amplification of genomic DNA. Each primer set was validated for efficiency by performing qPCR reactions on a set of serially diluted cDNA templates. Primer efficiency was calculated from the formula:

$$E = \left(10^{-\frac{1}{\text{slope}}} - 1 \right) \times 100$$

where slope represents the gradient of the line of linear regression fitted through the mean Ct values plotted against log₁₀ [cDNA concentration].

Only efficiencies ranging between 90-110% were deemed acceptable. Primer sets were monitored for primer dimerization and non-specific amplification through scrutiny of the melt curve analysis for additional peaks. All primer sequences and their relative efficiencies are detailed in Table 2.3.

Cycling conditions:

Step	Temperature	Duration	
Initial denaturation	95°C	10 min	
Denaturation	95°C	15 sec	x40 Cycles
Annealing/extension	60°C	1 min	

Table 2.3. Cycling conditions for Syber Green qPCR reactions

Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and Ribosomal protein L19 (*RPL19*) were used as reference genes for experiments on human and mouse tissues respectively. Ct values were corrected for primer efficiency using the following formula:

$$Ct_{corrected} = Ct_{measured} \times \frac{\log\left(\frac{2E}{100}\right)}{\log(2)}$$

Relative expression of target genes was calculated using the comparative Ct ($\Delta\Delta Ct$) method:

$$\Delta Ct = Ct_{target} - Ct_{reference}$$

$$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator}$$

$$Relative\ expression = 2^{-\Delta\Delta Ct}$$

At least 3 biological replicates, each with 3 technical replicates were used in all experiments.

Target	Forward Primer 5'-3'	Reverse Primer 5'-3'	Efficiency	Positive control
<i>mTRESK</i>	CTCTCTTCTCC GCTGTCGAG	AAGAGAGCGCTCAG GAAGG	105.9%	mTrigeminal Ganglion
<i>mRPL19</i>	AGCCTGTGACT GTCCATTCC	GGCAGTACCCTTCC TCTTCC	95.5%	N2A
<i>hGAPDH</i>	AGCCACATCGC TCAGACAC	GCCCAATACGACCA AATCT	95.4%	iPSC-sensory neuron D15
<i>hC-MET</i>	ACCTTTGATAT AACTGTTTACT TGTTGCA	GCTTTAGGGTGCCA GCATTTT	94.0%	iPS-sensory neuron D15
<i>hRUNX1</i>	CAAGACCCTGC CCATCGCTT	GTAGTTTTTCATCAT TGCCAGCC	104.4%	iPS-sensory neuron D15
<i>hTRESK</i>	GTGTTTCAGCAC	CCGTGAGAACGAGG	95.73%	hTRESK-AcGFP

CGTGGGCTA	AACATCA
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Table 2.4. qPCR primer sequences and amplification efficiency

2.1.5 Protein extraction

Dissected tissue was immediately placed in radioimmunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1% (v/v) sodium deoxycholate, 1% (v/v) Triton X-100, 0.1% (w/v) SDS) and 1.5% (v/v) protease inhibitor cocktail (Roche). Tissue was homogenized until solution appeared uniform by multiple rounds of gentle sonication, followed by ice cooling. Sample was centrifuged at 12,000rpm for 20 min (4°C) and supernatant transferred to a fresh epindorf. Protein concentration was quantified using Pierce BCA protein assay kit (Thermo Scientific) following the manufacturer's instructions. Proteins were diluted to a concentration of 1-5mg/ml and stored in single use aliquots at -80°C for future use.

2.1.6 Western blotting

Equal amounts of protein ($\approx 30\mu\text{g}$) from each sample were boiled for 10 min in Laemmli sample buffer (Bio-Rad) with 5% (v/v) β -mercaptoethanol. Proteins were separated alongside a protein standard ladder (GE Healthcare), by 10% (w/v) SDS polyacrylamide gel electrophoresis. After separation proteins were transferred to 0.2 μm nitrocellulose membranes (Millipore), which were then blocked for 1 h in 5% (w/v) milk powder in Tris-Buffered Saline-Tween 20 (0.1% (v/v) Tween-20 in TBS). Blocked membranes were incubated overnight (agitating at 4°C) with primary antibodies (α -NCC Millipore- Ab3553 (1:1000) and α - β -tubulin Abcam- ab15568 (1:5000)) diluted in 5% (w/v) milk TBST. The following day, primary antibody was washed off by 3x 10 min TBST washes. 1:10,000 (v/v) horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam) were diluted in 5% (w/v) milk TBST and incubated with membrane for 2hr at room temperature. Membranes were washed 3 times for 10 min in TBST before signal was detected using enhanced chemiluminescence reagents (Amersham) according to the manufacturer's instructions.

2.1.7 Immunocytochemistry (ICC)

Cells grown on coverslips were fixed with freshly prepared 4% (w/v) paraformaldehyde (PFA, Electron Microscopy Sciences) in PBS for 10 min. PFA was aspirated from the cells, which were then washed three times by excess PBS for 10 minutes. Cells were permeabilised/blocked for 1 h at RT in PBS containing 0.3% (v/v) Triton X-100, 0.05% (v/v) Tween-20 and 5% (v/v) Normal Goat Serum (Sigma Aldrich). Primary antibodies were diluted as detailed (Table 2.4) in blocking solution supplemented with 1% (w/v) Bovine Serum Albumin (BSA, Sigma Aldrich) and incubated overnight at 4°C. The following day cells were washed in excess PBS three times for 10 min, and incubated for 2 h with fluorophore-conjugated secondary antibodies (Life Technologies) diluted 1:1000 in antibody dilution buffer. Cells were washed in excess PBS three times for 10 min, mounted using Vectashield Mounting Medium with DAPI (Vector Laboratories), and imaged using a Zeiss LSM 510 META laser-scanning microscope. Isolectin B₄ (IB₄) binding was performed similarly to standard ICC. Primary antibody step was replaced by overnight incubation (4°C) with biotin-conjugated IB₄ (Sigma Aldrich) diluted 1:100 in blocking solution supplemented with 1% (w/v) BSA. Secondary antibody used for IB₄ labeling was Streptavidin, AlexaFluor 488 (Sigma Aldrich) handled in the standard way.

2.1.8 Immunohistochemistry (IHC)

For kidney and CNS protein localization studies, mice were perfused with 4%PFA in PBS. Brains were removed, post-fixed in the same fixative overnight at 4°C and embedded in 4% agarose. Serial 60µm sections were cut on a vibratome (Leica; VT1000S) and stored as free-floating sections. Fresh frozen TG tissue was cut serially in 13µm sections before hydration in PBS for 10 min. Tissue was fixed for 10 min in 4% PFA before 3 x 10 min PBS wash. All tissues were processed for antigen retrieval by 20 min in citrate buffer (10mM Sodium Citrate, (v/v) 0.05% Tween-20, pH 6.0) at 95°C,

before cooling to room temperature and washed twice in distilled H₂O. Sections were blocked in PBS containing 0.3% (v/v) Triton X-100, 0.05% (v/v) Tween-20 and 5% (v/v) Normal Goat Serum for 1hr under constant agitation. Sections were incubated overnight (4°C) with primary antibodies (Table 2.4) diluted in blocking solution containing 1% (w/v) BSA. Primary antibody was removed by 3 x 10 min PBS wash and replaced by secondary antibody, diluted in blocking solution containing 1% (w/v) BSA and maintained for 2hr at room temperature. For immunofluorescence studies, secondary antibodies used were Alexa-488/594/647 (Life Technologies-1:1000 concentration) and were washed off by 3 x 10 min PBS wash. Tissue was dried onto a glass slide, mounted using Vectashield Mounting Medium with DAPI and imaged using a Zeiss LSM 510 META laser-scanning microscope. Immunohistochemistry using a horseradish peroxidase detection system was used for some analysis. Here, secondary antibody was biotinylated Donkey anti-Rabbit IgG (Abcam), used at a 1:200 (v/v) concentration for 2hr. Biotin detection was performed using Vectorstain Elite ABC Kit (Vector Laboratories) followed by signal detection with DAB peroxidase substrate kit (Vector Laboratories) as per manufacturer's instructions. Sections were mounted on slides and dehydrated (80% EtOH 1min, 95% EtOH 1 min, 100% EtOH 1min, 100% EtOH 1 min, HistoChoice clearing agent 1 min -Sigma Aldrich). Images were acquired on a Zeiss Axioplan 2 microscope. Immunostaining with α -TRESK (Alomone Laboratories) was performed as above, but in the absence of Triton X-100 and Tween-20 throughout the whole process.

Antibody	Manufacturer/catalogue number	Dilution
Goat polyclonal α-mTRESK	Santa Cruz Biotech- sc-51240	1:100
Rabbit polyclonal α-RET	Alomone- ANT-025	1:50
Goat polyclonal α-hTRESK	Santa Cruz Biotech- sc-51238	1:100

Rabbit polyclonal α-TRPM8	Alomone- ACC-049	1:200
Rabbit polyclonal α-5-HT_{1D}	Alomone- ASR-023	1:100
Rabbit polyclonal α-Ca_v2.1	Alomone- ACC-001	1:500
Rabbit polyclonal α-VGLUT-1	Synaptic Systems	1: 500
Rabbit polyclonal α-NCC	Millipore- Ab3553	1:200
Goat polyclonal α-Nanog	Abcam- ab77095	1:100
Rabbit polyclonal α- III β-Tubulin	Covance- MMS-435P	1:500
Rabbit polyclonal α-Oct4	Abcam- ab19857	1:200
Mouse monoclonal α- AP2	Abcam- ab61	1:200
Rabbit polyclonal α-p75^{NTR}	Promega- G323A	1:100
Rabbit polyclonal α-Peripherin	Santa Cruz biotech- sc-7604	1:100
Mouse polyclonal α- Islet-1	DSHB- 39 4D5. s	1:100
Rabbit polyclonal α-P₂X₃	Alomone- APR-016	1:100
Mouse polyclonal α-Brn3A	Millipore- MAB1585	1:500
Mouse monoclonal α-CGRP	Abcam- ab81887	1:500

Table 2.5. List of primary antibodies used for ICC or IHC

2.1.9 Statistical analysis

All data sets were assessed for normal distribution by the Shapiro-Wilk test. When normally distributed, data was statistically analyzed using either an unpaired *t*- test with Welch's correction, a paired *t*- test, or a one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test. If the data did not pass normality testing, the non-parametric Mann-Whitney *U* test or Kruskal-Wallis test with Dunn's multiple comparison test were used. In the case of regression analysis, linear regression was performed and R² values calculated. Pearson's product-moment correlation coefficient (R) was calculated to quantify correlation. GraphPad Prism 6 and Excel software were used for all statistical analyses. Each figure highlights the specific statistical analysis applied to the described data sets.

2.2 Standard cell culture

2.2.1 Culture conditions

A variety of immortalized cell lines were used in this thesis; SH-SY5Y neuroblastoma, Human Embryonic Kidney (HEK) 293, U2OS (human osteosarcoma) and mouse Neuro-2A (N2A) cells. Common culturing conditions were routinely used to maintain all of these lines. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L D-glucose and pyruvate (Life Technologies) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Life Technologies) and 1% penicillin/streptomycin (PAA Laboratories). Cells were kept at 37°C in a 5% (v/v) CO₂ humidified atmosphere and sub-cultured every 4-7 days when $\approx$ 70-90% confluent.

2.2.2 Differentiation of SH-SY5Y

SH-SY5Y cells were differentiated to a neuronal-like state by prolonged retinoic acid treatment. 7 days prior to experiment SH-SY5Y (<P20) cells were passaged and plated at a density of 2×10^4 cells/well of a 24 well plate. Cells were cultured adherent to 13mm circular coverslips (VWR) and media was supplemented with 10 μ M retinoic acid (Sigma-Aldrich). Media was changed every 2-3 days to ensure optimum retinoic acid activity.

2.2.3 DNA transfection

Exogenous gene products were expressed in all cell lines by standard techniques. Cells were plated at least 24 h pre-transfection, such that by transfection they were $\approx$ 70-90% confluent. At this stage, cells were maintained in DMEM supplemented with (v/v) 10% FBS (no antibiotics). Cells were exposed to a cocktail of Lipofectamine 2000 (Life Technologies) and DNA (500ng/1.5 μ l Lipofectamine), diluted in Opti-Mem Reduced

Serum media (Life Technologies). Transfection cocktail was removed 12 h post application and cells were fed with routine culturing media. Experiments were performed 24-72 h post-transfection when robust expression was observed.

2.3 Mouse specific methods

2.3.1 Overview

Mouse handling and experiments were performed in accordance with the UK Home Office Animals (Scientific Procedures) Act (1986) and approved by the University of Oxford Ethical Review Panel.

2.3.2 Genotyping

This thesis contains data relating to two genetic ablation mouse models: KCNK18^{-/-} and SLC12A3^{-/-}. For genotyping, an ear punch biopsy was obtained from newborn pups that served as an identifier and source of DNA. Tissue biopsies were digested by 3µl of 20µg/ml Proteinase K (Roche) in 150µl DNA extraction buffer (100mM Tris-HCl [pH 7.5], 0.2% (w/v) SDS, 200 mM NaCl, 5mM EDTA [pH 8.0]) at 55 °C overnight. The next day, samples were vortexed thoroughly, followed by centrifugation at 16,000xg for 10 minutes to pellet non-digestible material. Supernatant was mixed with equal volumes of isopropanol and spun at 16,000xg for 10 minutes. DNA pellet was air-dried and resuspended in 50µl H₂O.

Genomic DNA was analyzed by PCR for the presence or absence of knockout alleles. SLC12A3^{-/-} mice were originally generated by the insertion of a neomycin cassette into exon 12. Wild-type genotyping primer pair amplified a region either side of naïve exon 12. Knockout primer pair used the same forward primer, but a reverse primer with sequence complimentary to the phosphoglycerate kinase promoter, which drives the neomycin gene (Figure 2.1) of the knockout cassette. KCNK18^{-/-} was generated by

VelociGene definitive null allele design (Valenzuela, Murphy et al. 2003). Wild-type genotyping primer pair amplified an intronic region of wild-type allele, while knockout primer pair targeted a region beginning in the LacZ cassette and ending in the 3' homology arm of the null allele (Figure 2.1).

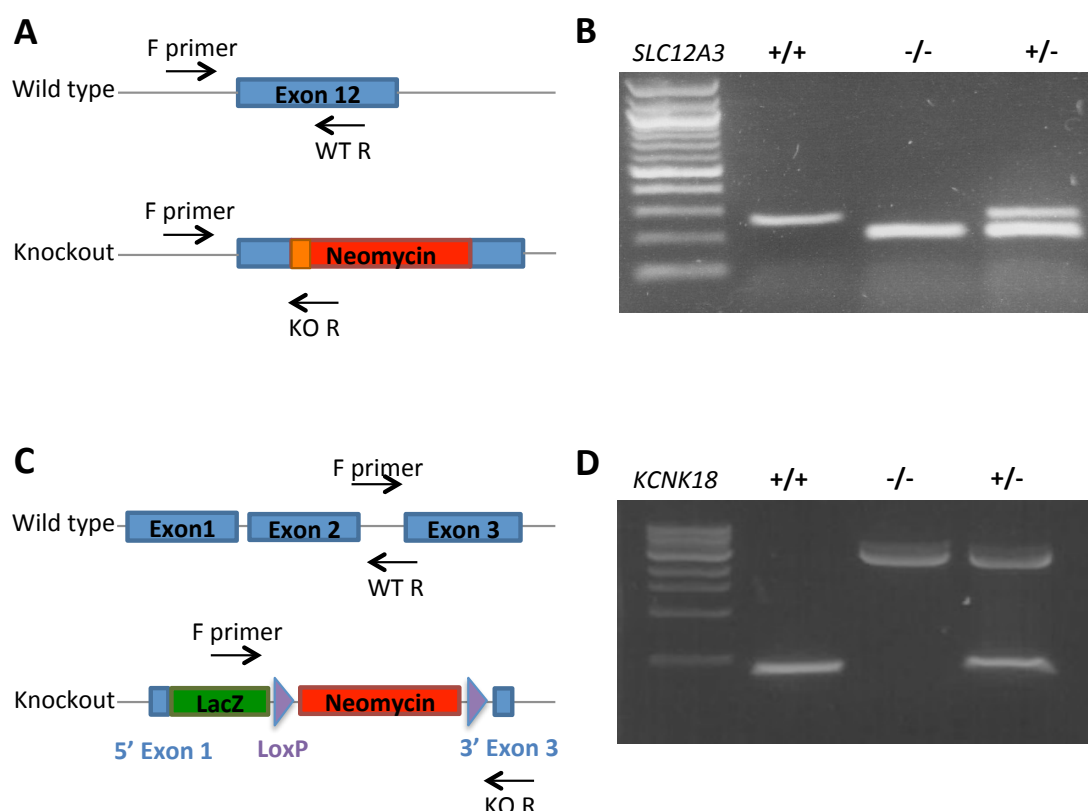


Figure 2.1 Allele structure and genotyping strategies for *SLC12A3* and *KCNK18* knockout mice. (A) Gene target strategy originally employed to ablate *SLC12A3*. Neomycin cassette was targeted to disrupt exon 12. (B) PCR products of genotyping reaction performed on mice of all three *SLC12A3* genotypes. Left lane represents 100bp reference ladder (New England Biolabs). (C) VelociGene targeting system ablates coding region of *KCNK18* by inserting a LacZ-neomycin cassette into the majority of the coding region of *KCNK18*. (D) PCR products of genotyping reaction performed on mice of all three *KCNK18* genotypes. Left lane represents 1kb reference ladder (New England Biolabs).

All PCR reactions were performed using 1 µl gDNA template in a 20µl *Taq* polymerase reaction.

Cycling conditions:

Step	Temperature	Duration	
Initial denaturation	95°C	2 min	
Denaturation	95°C	30 sec	x30 Cycles
Annealing	X°C	30 sec	
Extension	72°C	1 min	
Final extension	72°C	10 min	

Table 2.6 Genotyping PCR conditions

PCR products were analyzed on a 1% (w/v) agarose gel and genotype determined (Figure 2.1).

Target	Product (bp)	Forward Primer 5'-3'	Reverse Primer 5'-3'	T _m (C)
<i>Wild type KCNK18</i>	433	ACAGGCAGGGTGATAAGG TG	GCATGGCTGAGTGATTTC CT	65
<i>Knockout KCNK18</i>	3218	TTTCCATATGGGGATTGG TG	TTTGGCCCTCAAGTAGGT T	65
<i>Wild type SLC12A3</i>	265	AGGGTCAAGGGCACGGTT GGC	GGTAAAGGGAGCGGGTCC GAGG	70
<i>Knockout SLC12A3</i>	188	AGGGTCAAGGGCACGGTT GGC	GCATGCTCCAGACTGCCTT G	70

Table 2.7 Genotyping primer sequences

2.3.3 Primary culture

Mouse TG neuron cultures were prepared as outlined previously (Malin, Davis et al. 2007). Postnatal mice (P5-20) were sacrificed and TG rapidly excised into ice-cold HBSS without Ca²⁺/Mg²⁺ (Life Technologies). Ganglia were diced into small pieces and incubated in Papain (20U/ml- Worthington Biochemical) solution for 20 min at 37°C. Further enzymatic dissociation involved a second 20-minute incubation in Dispase type II/Collagenase solution (Worthington Biochemical). Cells were washed to remove

residual enzyme and mechanically dissociated through a fire-polished glass pipette. Cells were plated onto laminin/poly-D lysine (BD Biosciences) treated coverslips in DMEM-F12 media (Life Technologies) supplemented with 10% (v/v) FBS and 1% (v/v) P/S. Cells used for voltage-clamp experiments were cultured devoid of growth factors but for all other assays, plating media contained 50ng/ml mouse Nerve Growth Factor (NGF- PeproTech) and 10ng/ml Glial-Derived Neurotrophic Factor (GDNF- PeproTech). Culture media was replaced every 3 days over the course of the experiment.

2.4 Induced Pluripotent Stem Cell culture

2.4.1 Culture conditions

All iPSC clones (Table 2.8) were routinely maintained in mTESR1 (Stem Cell Technology) on hESC-qualified matrigel (BD Biosciences) coated wells. Cells were enzymatically passaged every 5-7 days and plated at 2.5×10^5 cells/ml in the presence of $10 \mu\text{M}$ Y-27632 (Merck Millipore). iPSC lines were analyzed for genome integrity every 5 passages by Illumina Human CytoSNP-12v2.1 array. DNA was extracted from a cell pellet of 2×10^6 cells, by PureLink Genomic DNA kit (Life Technologies). The Oxford Genomics Centre carried out sample array and analysis. Only lines exhibiting less than five genome abnormalities and no aberrant differentiation were taken forward for neural induction.

Clone name	Disease vs Control	Gender	Age	Reprogramming method
<i>AH017-3</i>	Control	Female	67	Nakanishi
<i>AH017-7</i>	Control	Female	67	Nakanishi
<i>NHDF-1</i>	Control	Female	44	Retrovirus

<i>JL-3</i>	Disease	Female	64	Nakanishi
<i>SH-4</i>	Disease	Female	62	Nakanishi
<i>DD-6</i>	Disease	Female	65	Nakanishi

Table 2.8 Details of iPS subject clones

2.4.2 Sensory neuron induction

Sensory neuron differentiation was performed similarly to a published protocol (Chambers, Qi et al. 2012) and an overview is given in Figure 2.2. iPSC were rendered to single cells by enzymatic passage and plated on matrigel-coated coverslips at a density of 30,000cells/cm². At this point, cells were maintained in MEF-conditioned knockout serum replacement (KSR) media (see below) supplemented with 10μM Y-27632 and 10ng/ml bFGF (Miltenyi Biotec Ltd). Media was changed daily until 40-50% confluence was achieved (24 – 48 h). To initiate neuronal induction, media was replaced with KSR containing 100nM LDN-193189 (Stemgent) and 10μM SB431542 (Tocris Bioscience). This time-point was denoted as day 0. Media was changed daily with LDN-193189 and SB431542 added on days 0-5. 3μM CHIR99021 (Tocris Bioscience), 10μM SU5402 (Sigma-Aldrich) and 10μM DAPT (Sigma-Aldrich) specify the sensory lineage and were added on days 2-10. N2 media was added in increasing increments every other day from day 4, until completely replacing KSR on day 10. Cells were only passaged during days 1-10 if the cell line had accelerated growth properties. On day 10, cells were passaged onto new matrigel-coated coverslips at a density of 1.5x10⁵ cells/cm² in N2 supplemented with 50ng/ml NGF (PeproTech), 20ng/ml BDNF (PeproTech) and 20ng/ml GDNF (PeproTech). After this point, cells were fed every 2-3 days. For longer-term cultures, cells were treated on day 14 with 4μM Cytosine β-D-arabinofuranoside (Sigma Aldrich) to eliminate proliferating progenitor cells and mouse laminin I (1μg/ml- R&D Systems) was added weekly to the media to enhance cell adherence.

KSR medium was prepared as follows: 81% (v/v) Knockout DMEM (Life Technologies), 15% Knockout Serum Replacement (Life Technologies), 1% non-essential amino acids (PAA Laboratories), 1% GlutaMAX (Life Technologies), 1% penicillin/streptomycin (PAA Laboratories) and 0.1mM β -mercaptoethanol (Life Technologies).

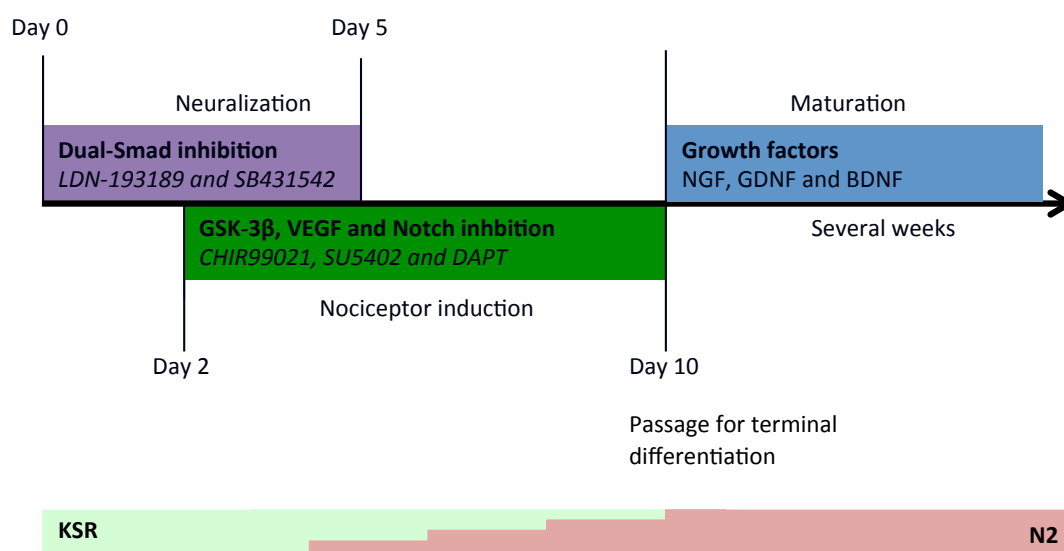


Figure 2.2. Workflow of sensory neuron induction

N2 medium was prepared as follows: 95% (v/v) Neurobasal (Life Technologies), 1% N2 supplement (Life Technologies), 2% B-27 supplement minus Vitamin A (Life Technologies), 1% GlutaMAX and 1% penicillin/streptomycin

MEF-conditioned KSR media was prepared by seeding murine embryonic fibroblasts in 0.1% gelatin (Sigma-Aldrich) coated flasks with MEF-medium (90% (v/v) DMEM (Life Technologies) and 10% (v/v) FBS (Life Technologies)). 24 hours after seeding, media was replaced with KSR media. Media was harvested every day and replaced with fresh KSR. MEF-conditioned KSR was collected for 9 days and frozen at -20°C for storage.

2.5 Electrophysiology

2.5.1. Solutions

Whole-cell patch clamp recordings were undertaken at room temperature with cells bathed in solution containing:

<u>Immortalized cells</u> (HEK 293, U2OS and SH-SY5Y)	<u>Primary/iPSC-neurons</u>
--	------------------------------------

<i>125 mM NaCl</i>	<i>140 mM NaCl</i>
<i>4 mM KCl</i>	<i>4.7 mM KCl</i>
<i>2 mM CaCl₂</i>	<i>2.5 mM CaCl₂</i>
<i>1.2 mM MgSO₄</i>	<i>1.2 mM MgCl₂</i>
<i>10 mM Glucose</i>	<i>10 mM Glucose</i>
<i>10 mM HEPES.</i>	<i>10 mM HEPES.</i>

Osmolarity was adjusted to 320 mOsm and pH adjusted to 7.3 with NaOH. Patch pipettes of 3–6 MΩ tip resistance were pulled from filamental borosilicate glass capillaries (1.2 mm outer diameter, 0.69 mm inner diameter; Harvard Apparatus, USA), using a horizontal puller (Sutter P-97) and filled with an internal solution containing:

<u>Immortalized cells</u> (HEK 293, U2OS and SH-SY5Y)	<u>Primary/iPSC-neurons</u>
--	------------------------------------

<i>140mM KCl</i>	<i>130mM KCl</i>
<i>4mM NaCl</i>	<i>4.7mM KCl</i>
<i>0.02mM CaCl₂</i>	<i>0.5mM EGTA</i>
<i>0.8mM EGTA</i>	<i>1mM MgCl₂</i>
<i>2mM MgCl₂</i>	<i>5mM MgATP</i>
<i>4mM MgATP</i>	<i>10mM HEPES.</i>

Osmolarity was adjusted to 305 mOsm and pH adjusted to 7.3 with KOH. All chemicals

were purchased from Sigma Aldrich.

2.5.2 Whole cell recordings

Recordings were made using an Axopatch 1D amplifier (Molecular Devices, USA) and data acquired using WinWCP Strathclyde Whole Cell Analysis software (V.3.9.7; University of Strathclyde, UK). The digitized data were analyzed with pClamp9 (Axon Instruments, Inc., USA). Upon breakthrough, cells were left dormant for 1-3 min before protocols were run to allow for intracellular solution dialysis. For I/V curves, a current-voltage protocol was applied using 300-ms pulses from -80 mV to $+50$ mV in 10 mV increments from a holding potential of -80 mV. P/4 leak subtraction was used to account for passive seal and membrane leak. Control pulses were given in a potential range unlikely to elicit significant TRESK-mediated leak. Several minutes after breakthrough, resting membrane potential (RMP) was noted in current clamp bridge mode. During the course of recordings R_{access} and RMP were noted and recordings were abandoned if values deviated by $\pm 20\%$. Current clamp was used for all measures of excitability and in this mode cells were maintained at a holding level of -60 mV. For analysis of rheobase, short (50ms) injections of depolarizing current were increased incrementally until an action potential was fired. The current required, beyond that to hold the cell at -60 mV, was defined as the rheobase. For repetitive firing assays, long pulses (500ms) of supra-threshold current ($2 \times$ rheobase) were applied and number of action potential events counted as an average of five repeat pulses. Input resistance (R_{input}) was calculated from the voltage change induced by a 10 pA hyperpolarizing pulse at -60 mV. Action potential (AP) amplitude was measured as the voltage change from holding potential to the peak of the action potential. After-hyperpolarization was defined as the peak negative voltage deflection (relative to the potential induced by the current injection) directly following an action potential. AP duration was measured as the time between points of half-maximal amplitude of AP trace. Spontaneous firing was recorded in current clamp mode while zero current was inputted. All drugs used are

detailed in Table 2.9.

Drug	Source	Stock solution	Working concentration
Tetrapentylammonium Bromide (TPA)	Sigma Aldrich (241970)	Fresh-ACSF solution*	500nM
Phorbol-12-myristate-13 acetate (PMA)	Sigma Aldrich (P8139)	10mM-DMSO	100nM
5-chloroquinolin-8-ol (CQ)	Dr. Paul Wright MRCT	Fresh- DMSO	10nM-100µM
Tetradotoxin (TTX) citrate	Tocris (1069)	1mM- dH ₂ O	500nM
Hydrochlorothiazide (HCTZ)	Sigma Aldrich (H4759)	Fresh-DMSO	100µM
α,β Methylene ATP	Sigma Aldrich (M6517)	30mM DMSO	30µM
A 803467	Tocris (2976)	10mM- DMSO	500nM
17-β Estradiol	Sigma Aldrich (E2758)	10mM DMSO	5µM

Table 2.9 Drug solutions. *Working solution of drug was made up fresh on the day of experiment

3. The role of TRESK in neuronal physiology

3.1 Introduction

3.1.1 Genetic associations require functional validation

A deleterious mutation in *KCNK18* (F139fsWx24), the gene encoding TRESK, lies within a genome-wide significant linkage region and segregates perfectly with MA in one multigenerational family (Lafreniere, Cader et al. 2010) (Figure 3.1). Outwith the family, frame-shift mutations in TRESK have been reported in MA patients, but these studies have not been published in any detail (Lafreniere and Rouleau 2011). The paucity of further MA families with TRESK mutations and due to the candidate-based approach taken to discover the frame-shift mutation, there exists doubt on the causality of the mutation to migraine pathogenesis (Eising, de Vries et al. 2013).

In support of this notion, disease-associating variants in *KCNK18* have not been identified in recent GWAS studies. However, GWAS studies in migraine are not powered to detect rare variants (Ward and Kellis 2012). Sequencing studies can identify rare variants and in follow up studies on a cohort of several hundred-migraine patients, two deleterious *KCNK18* variants were found that result in a complete loss of channel function (Andres-Enguix, Shang et al. 2012). A34V was found in one migraine proband, while C110R was present in both migraine and control subjects (Lafreniere, Cader et al. 2010). These results suggest that loss of TRESK activity can be tolerated and indeed, is not causative of disease. Intriguingly, a recent report suggests that the C110R variant has different effects to the frame-shift mutation in TG neurons (Guo, Liu et al. 2014), although as an over-expression heterologous study, this should be interpreted with caution.

Mutations in *KCNK18* clearly have a complex and uncertain relationship with migraine. It is possible that the frame-shift mutation is a chance association and that another gene in the same linkage region is causally related to migraine. It is also plausible that other modifier genes significantly alter the penetrance of deleterious *KCNK18* variants. Irrespective, TRESK remains of considerable interest in migraine given its prominent physiological role in sensory ganglion neuron excitability. To better establish the relevance of TRESK in migraine, functional studies offer an alternative way forward. Testing the hypothesis that a given protein has a role in disease-relevant pathways is not only profitable for supporting/challenging genetic association, but also affords the opportunity to define novel information about the protein. It is with these two objectives in mind, that this chapter attempts to build upon current knowledge of TRESK function.

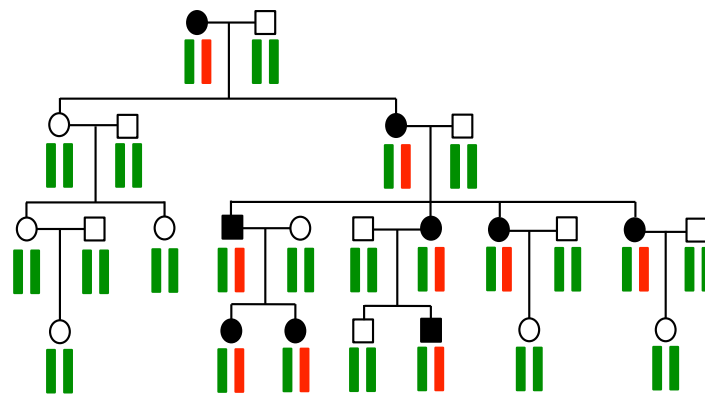


Figure 3.1 Genetic association of F139WfsX24 *KCNK18* with MA. Family members of original proband had the coding region of *KCNK18* sequenced. Black symbols represent patients with a history of MA, with white representing healthy controls. Wild-type *KCNK18* allele is colored green and F139WfsX24 *KCNK18* red. Figure modified from *Lanfreniere et al 2010* (Lanfreniere, Cader et al. 2010).

3.1.2 Fundamental properties of TRESK

TRESK was first cloned in 2003 from mouse spinal cord and found to have two pore-forming, and four transmembrane domains (Sano, Inamura et al. 2003). This structural confirmation is evolutionarily conserved across K_{2P} channels (Enyedi and Czirjak 2010). Despite this, TRESK demonstrates low amino acid homology with other K_{2P} channels (13.5-19.7%) and represents a distinct subtype within the K_{2P} phylogenetic tree (Sano, Inamura et al. 2003). Background K^+ conductance hyperpolarizes the resting membrane potential towards the reversal potential for K^+ , and also counteracts depolarizing stimuli. As such, K_{2P} channels are considered important in controlling the initiation, shape and duration of the action potential (Enyedi and Czirjak 2010). The majority of K_{2P} family members conform to classical definitions of leak conductance in that they are independent of voltage, non-inactivating and non- or weakly rectifying. Expression of TRESK in heterologous systems induces a voltage-independent, and only slightly outwardly rectifying K^+ current (Sano, Inamura et al. 2003). Although tissue expression data is somewhat variable between species and independent studies, TRESK is consistently found in sensory ganglia (Kang and Kim 2006, Yoo, Liu et al. 2009). In mouse, expression can be detected by embryonic day 15.5 and continues into adulthood (Lafreniere and Rouleau 2011). Expression of TRESK in human sensory neurons has also been documented (Lafreniere, Cader et al. 2010). Further to this, TRESK has been detected in autonomic structures such as stellate ganglion and the paravertebral sympathetic chain (Lafreniere, Cader et al. 2010). A systematic characterization of expression is of integral importance for future studies of pharmacological interventions and for understanding results from behavioral models.

3.1.3 Migraine: a disorder of hyper-excitability neurons?

Functional studies investigating FHM have shaped our thinking of migraine pathophysiology. FHM-associated proteins have been proposed to play a role in the

central glutamate synapse (Figure 1.2) and there is direct evidence for FHM1 mutations causing increased excitatory neurotransmission in pyramidal cells (Tottene, Conti et al. 2009). . These findings have led to the idea that migraine is a disorder associated with hyper-excitability of cortical areas and in which excitatory/inhibitory influences are unbalanced (Pietrobon and Moskowitz 2013).

Hyper-excitability may not be confined to cortical regions; activation of spinal trigeminal nucleus increases in the period leading up to an attack and the intensity of the given activation can be used to predict the next attack (Stankewitz, Aderjan et al. 2011). Whilst the excitability changes of the cortex may be complex, depending on the final net effect on excitatory-inhibitory balance, hyper-excitability of trigeminal neurons should have a simpler relationship to migraine since TG neuron activation is considered likely a central feature of migraine headache (Olesen, Burstein et al. 2009).

K_{2P} channels have a documented role in modulating neuronal excitability (Deng, Poudel et al. 2007). The K⁺ leak they conduct acts as a hyperpolarizing force to counteract depolarizing stimuli. The same is true of TRESK activity. Loss of TRESK channel activity, with the loss of this opposing effect, has in a variety of conditions been associated with enhanced dorsal root ganglion excitability and increased activation of *in vivo* pain pathways (Dobler, Springauf et al. 2007, Tulleuda, Cokic et al. 2011). Hence, pre-existing functional evidence suggests that it is plausible that loss of TRESK activity may also play a role in increasing the excitability of migraine-specific pain pathways.

3.1.4 Effective pharmacological toolkit for research and therapy

Pharmacological approaches are vital for effective studies of protein function and can lead to drug discovery. As is common for all the K_{2P} channels, pharmacological methods for specific inhibition of TRESK are currently lacking. TRESK is not inhibited by the classical K⁺ channel blockers tetraethylammonium (TEA) and 4-aminopyridine (4-AP), but is sensitive to Ba²⁺ and quinidine (Kang, Han et al. 2004). Alpha-hydroxy-sanshool

(Bautista, Sigal et al. 2008), a derivative IBA (Isobutylalkenyl amide) (Tulleuda, Cokic et al. 2011) and lamotrigine (Kang, Kim et al. 2008), potentially inhibit TRESK but also other K_{2P} channels (Bautista, Sigal et al. 2008). Despite the documented effects on other channels, these inhibitors are used as the primary tool to delineate TRESK function (Tulleuda, Cokic et al. 2011); these non-selective effects will impact the validity of conclusions drawn from their use.

While specific inhibitors would greatly aid basic research, novel activators may prove profitable as therapeutic agents. Activation of Gq-coupled receptors augments TRESK current by increasing cytoplasmic Ca^{2+} levels. Ca^{2+} -dependent regulation is mediated by a unique, direct interaction of TRESK with calcineurin (Czirjak, Toth et al. 2004). Histamine, which binds to Gq-coupled H1 receptors, can elevate TRESK currents 2-fold in *Xenopus* Oocytes (Dobler, Springauf et al. 2007). Stimulation of protein kinase C by phorbol 12-myristate-13-acetate (PMA) can elevate TRESK currents by 3-fold in a calcineurin-independent manner (Rahm, Gierten et al. 2012). With regard to exogenous agents, volatile anesthetics activate TRESK (Liu, Au et al. 2004) as well as other K_{2P} channels (Patel, Honore et al. 1999). The diversity of inhibitors/activators of TRESK offer promise that it will be possible to define specific pharmacology for the channel. This is of utmost importance for both the study and therapeutic targeting of TRESK.

3.1.5 Mouse models of TRESK

To date, two mouse models of TRESK ablation have been generated. Clear from both models is that under normal conditions TRESK is not critical for survival. Homozygous animals are viable and neither study observed overt phenotypes as a consequence of gene ablation. Deletion of exon 3 leads to animals with a minor increase in sensitivity to Isoflurane and a 20% increase in thermal sensitivity to pain. Interestingly, TRESK^{-/-} mice have a higher mortality rate following anesthetic testing (Chae, Zhang et al. 2010), which suggests that TRESK may have vital functions that are not observable at rest. DRG

neurons from mice with a functional TRESK knockout (induced by substitution of glycine for arginine at position 339) have a 27% reduction in background conductance and exhibit enhanced excitability. TRESK^{-/-} neurons exhibit lower threshold for activation, reduced action potential duration, and moderately higher amplitudes of after-hyperpolarization (Dobler, Springauf et al. 2007). These studies illuminate some of the properties of TRESK function however, with specific reference to TRESK function in migraine, the properties of TRESK^{-/-} trigeminal neurons have not been assessed.

3.2 Aims of Chapter 3

The aim of Chapter 3 is to build upon previous knowledge of TRESK physiology and further define its role in pain pathways. To this end it has several sub-aims:

- 1) Assess the basic role of wild-type TRESK, and migraine-associated variants in neuronal physiology
- 2) Investigate novel exogenous modulators of TRESK activity
- 3) Characterize CNS TRESK expression

3.3 Results

3.3.1 TRESK induces a prominent outward current in SH-SY5Y cells

Human TRESK acts to conduct K⁺ efflux, however the influence of this on the biophysical properties of humanized neuronal cells has never been studied. I sought to generate a humanized cell-based system to assess the consequence of expression of different hTRESK variants. Wild-type (WT), F139fsWx24 and C110R hTRESK-GFP (Gift, Dr. S Tucker) were transiently transfected into SH-SY5Y cells. The SH-SY5Y line is a cell line of human neuroblastoma origin, that replicates some features of human neurons

(Kovalevich and Langford 2013). Localization studies revealed that unlike WT protein, F139fsWx24 and C110R TRESK were not transported to the plasma membrane (Figure 3.2 A). Work performed by collaborators suggests that the protein products are retained in the ER (F139fsWx24) and Golgi (C110R) (data not shown). Whole-cell voltage-clamp recordings offer the ability to directly record ionic currents in response to changes in membrane potential. Control (GFP transfected) SH-SY5Y cells exhibit a small outward current in response to incremental voltage steps from -80mV to +30mV (Figure 3.2 B), indicative of a low level of endogenous ion channels. This current was unchanged in cells expressing either F139fsWx24 or C110R TRESK (Figure 3.2 B and C). These results are consistent with the localization studies and previous reports that both variants are non-functional (Andres-Enguix, Shang et al. 2012). WT-expressing cells demonstrated >5-fold increase in outward current compared to control (Figure 3.2 B and C). Control subtracted current was weakly outwardly rectifying and had not reversed by -80mV, suggestive of the current being carried by K^+ ions (predicted $V_{eq}K^+ = -90.68mV$). These findings demonstrate that transfected WT-hTRESK successfully traffics to the plasma membrane of SH-SY5Y cells and is capable of conducting K^+ current efflux. Furthermore, these data support previous reports that neither variant (F139fsWx24 or C110R) has any residual channel activity, however does not rule out the possibility that subunits could conduct current if properly trafficked to the plasma membrane.

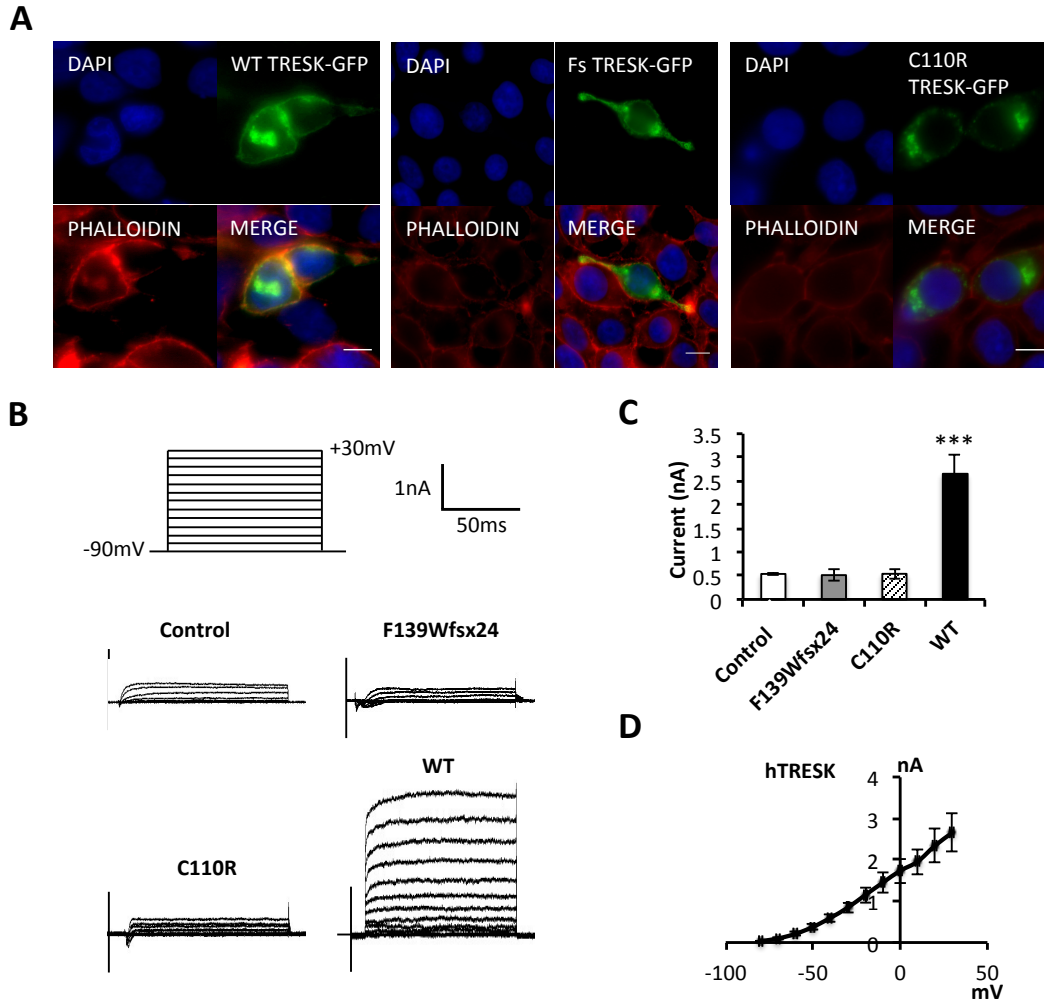


Figure 3.2 Transient overexpression of hTRESK induces a large outward current.

Constructs harboring WT-hTRESK and F139Wfsx24 and C110R forms of hTRESK were transiently overexpressed in SH-SY5Y cells. **(A)** GFP tagged WT-hTRESK has membranous localization 48hrs post-transfection. F139Wfsx24 and C110R reside in the cytoplasm and fail to reach the membrane. Cells were stained with phalloidin (red) to mark F-actin skeleton. **(B)** Representative traces of currents recorded from cells in the whole-cell voltage clamp mode. Currents were induced by depolarizing the membrane in 10mV increments, from a holding potential of -90mV to +30mV. **(C)** Total outward current, as measured at the end of a 300ms voltage step to +30mV. **(D)** TRESK mediated outward current. Current-voltage relationship for current measured in WT-TRESK cells subtracted by background current (control). *** $P < 0.001$; Kruskal-Wallis test, Dunn's multiple comparison test; results represent mean $\pm$ s.e.m of at least seven cells per group.

3.3.2 TRESK expression hyperpolarizes the cell membrane and reduces excitability.

I wanted to understand the consequence of an enhanced TRESK current to the behaviour of neurons. A key hallmark of neurons is their ability to fire action potentials in response to depolarizing stimuli. Unlike most immortalized lines, SH-SY5Y cells can be differentiated to the extent that they are capable of firing action potentials (Tosetti, Taglietti et al. 1998). Whole-cell current clamp allows the investigator to input current to a cell and measure associated membrane potential changes. I first verified that SH-SY5Y cells could fire action potentials in response to current injection. Cells were treated for 7 days with 10 μ M retinoic acid to enhance neuronal features prior to recording. Short (50ms) current injections of 5pA increments were applied to the cells until an action potential was generated (Figure 3.3 A). Action potentials could be abolished in the presence of 500nM TTX (Figure 3.3 B). The rheobase (the minimal current amplitude required to generate an action potential) is a common measure of membrane excitability. Expression of WT-TRESK, but not F139fsWx24 or C110R, significantly increased the rheobase (Figure 3.3 B). K_{2P} channels are open at all voltages and so could be expected to have a large impact on the resting membrane potential (RMP) (Enyedi and Czirjak 2010). WT-TRESK expression markedly hyperpolarizes the cell membrane and moves the RMP towards the reversal potential for K^+ (Figure 3.3 D). Hence, exogenous TRESK renders cells resistant to firing and alters their steady state biophysical properties.

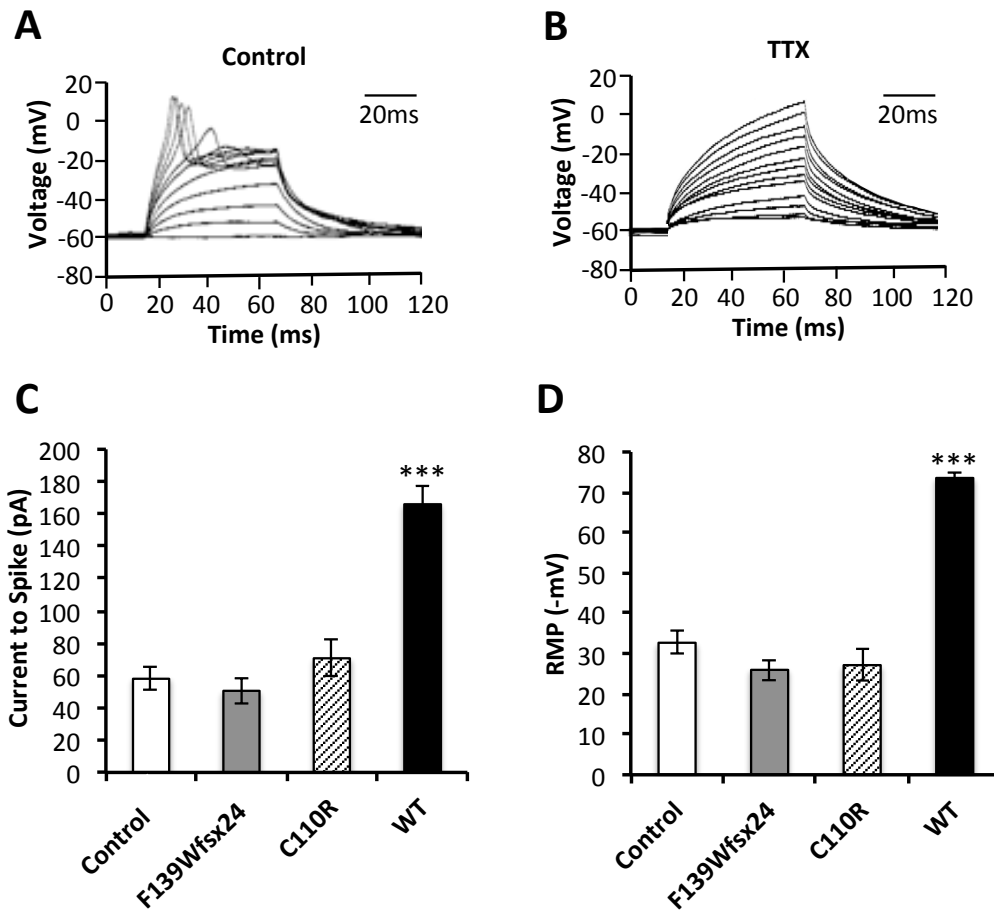


Figure 3.3 TRESK over-expression dampens neuronal excitability. Whole-cell current clamp recordings from differentiated SH-SY5Y cells. **(A)** Representative trace of an action potential generated by increasing increments of depolarizing current. **(B)** Action potentials could not be generated in the presence of 500nM TTX. **(C)** Rheobase current of cells expressing GFP (control), F139Wfsx24, C110R or WT TRESK, 48 hours following transfection. **(D)** Resting membrane potential of transfected groups. *** $P < 0.001$, ** $P < 0.01$; One-way ANNOVA, Bonferroni's's multiple comparison test; results represent mean $\pm$ s.e.m of at least six cells per group.

3.3.3 Low levels of TRESK are sufficient to dampen excitability

Transient overexpression can lead to exogenous protein levels far higher than are ever observed endogenously, and therefore can result in observations not relevant to normal physiology. I sought to perform analogous experiments as above, but with more moderate levels of TRESK expression. To this end, stable hTRESK-GFP (WT and

F139fsWx24) SH-SY5Y lines were made (initial work carried out by Dr. Jinghuan Li, Cader lab) utilizing a tetracycline-inducible expression system (Figure 3.4 A). Under normal culture conditions, cells do not express the gene of interest, but transcription can be initiated upon addition of doxycycline to the culture medium. Following FACS sorting of transduced cells; I generated lines of which 100% of the population expressed TRESK upon doxycycline treatment. The level of this expression was far below that of transient transfection experiments. WT-TRESK-GFP expressing lines exhibited a modestly enhanced outward current in comparison to control and F139fsWx24-GFP lines (Figure 3.4 B). Induced current was approximately 6-fold lower than that observed under transient transfection conditions. Despite the lower levels of exogenous TRESK, similar cellular phenotypes were still observed. RMP was hyperpolarized (Figure 3.4 C) and rheobase increased (Figure 3.4 D) in WT-TRESK-GFP cells. These results indicate that even low levels of TRESK have measurable effects on the biophysical properties of a neuron and acts to make a cell less excitable.

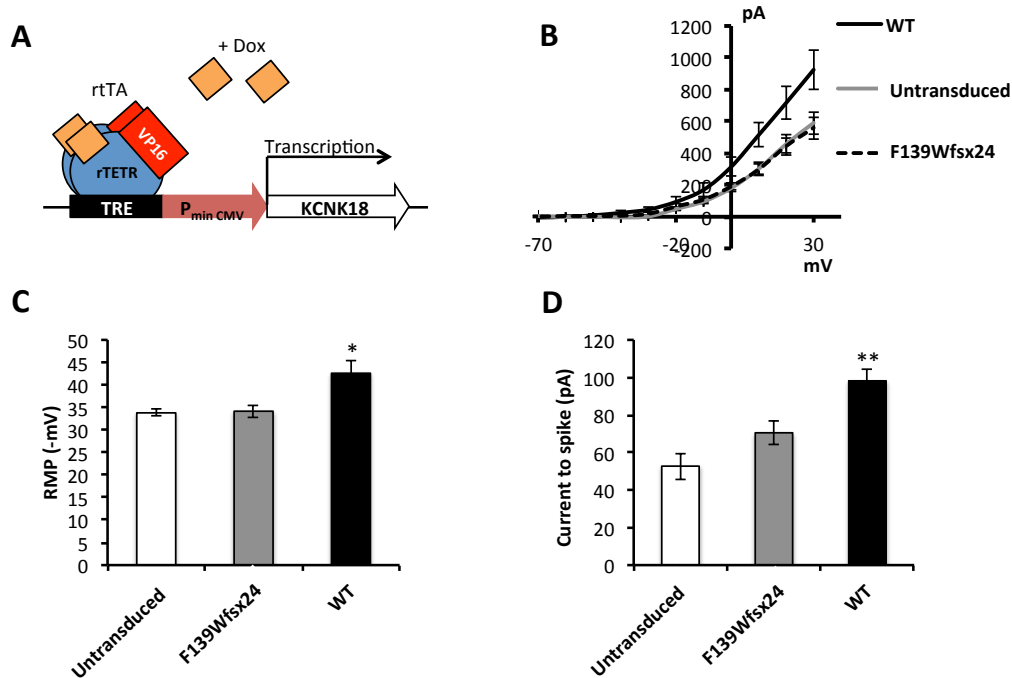


Figure 3.4 Low levels of TRESK induce a hypo-excitable phenotype. (A) Schematic detailing tetracycline-inducible expression system. Under basal condition the E. Coli “reverse” Tet-repressor protein (rTETR) is silent. In the presence of doxycycline, rTETR binds the tetracycline response element (TRE) and activates expression of the transgene (TRESK-GFP). **(B)** Current-voltage relationships for stable transduced lines expressing low levels of WT-TRESK or F139Wfsx24. **(C)** Resting membrane potential and **(D)** rheobase of transduced lines. ***P<0.01, *P<0.05; One-way ANNOVA, Bonferroni’s’s multiple comparison test; results represent mean± s.e.m of at least fifteen cells per group.*

3.3.4 Novel pharmacology targeting TRESK activity

Pharmacological modulators of K_{2P} channels are lacking; making it difficult to assess function of distinct channels. Specific modulators of TRESK would be highly valuable for basic research and could form the basis of future drug development.

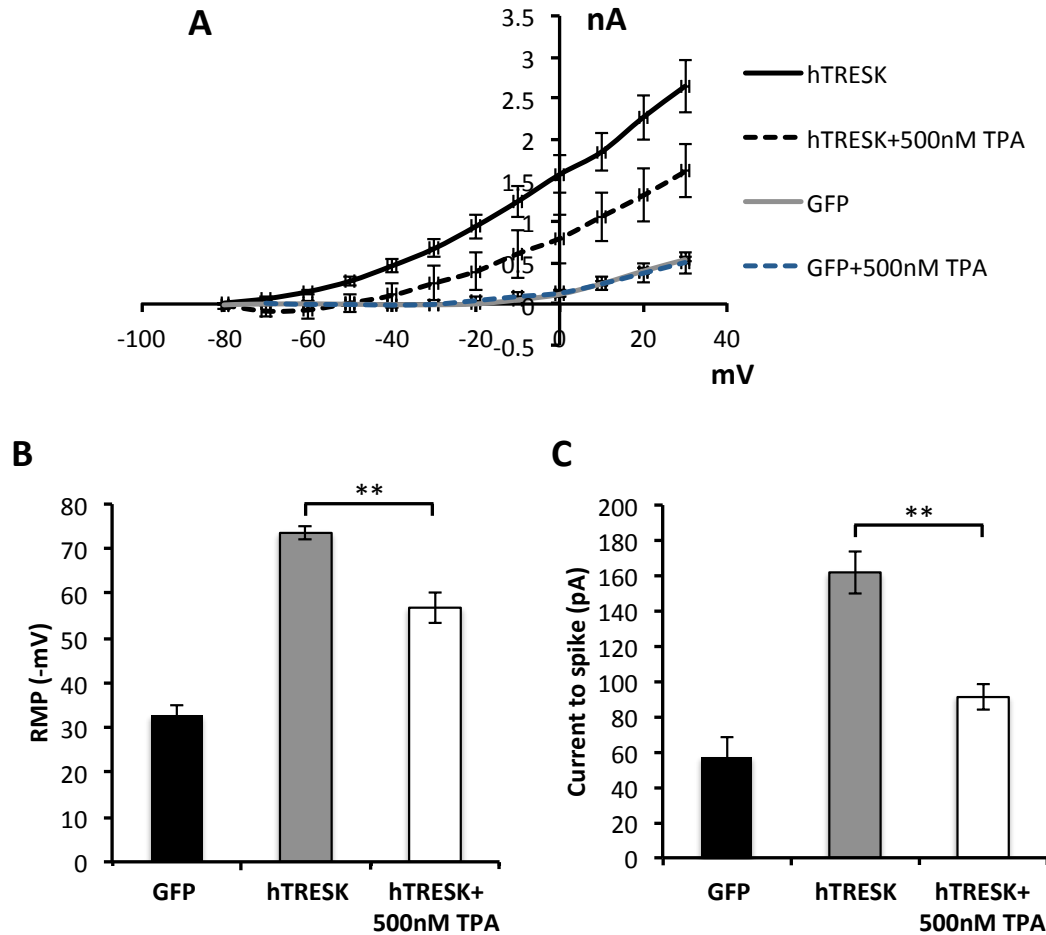


Figure 3.5 Tetrapentylammonium (TPA) inhibits TRESK activity. Patch clamp recordings of transiently transfected SH-SY5Y cells. **(A)** Current-voltage relationship of hTRESK-GFP and GFP cells, with or without bath application of 500nM TPA. **(B)** Effect of TPA on RMP, $**P<0.01$; Kruskal-Wallis test, Dunn's multiple comparison test. **(C)** Rheobase current across the groups. $**P<0.01$; One-way ANNOVA, Bonferroni's's multiple comparison test; results represent mean $\pm$ s.e.m of at least five cells per group.

TRESK activity is inhibited by a range of endogenous (Braun, Nemcsics et al. 2011) and exogenous factors (Bautista, Sigal et al. 2008); however these also have effects on other pathways and channels (Bautista, Sigal et al. 2008). At low concentrations (500nM), tetrapentylammonium (TPA) is suggested to specifically inhibit TRESK, while having minimal effect on other K_{2P} channels (personal communications Dr. S Tucker). I wished to investigate whether TPA was able to inhibit TRESK activity and if so, whether it could

be used to rescue the excitability phenotypes observed in the over-expression model. Externally applied TPA partially (41-52%) inhibited TRESK current, but had no effect on outward current of naïve SH-SY5Y cells (Figure 3.5 A) indicating that its actions are mediated through TRESK inhibition. TPA application brought excitability phenotypes towards control levels; depolarized the RMP (Figure 3.5B), and reduced the rheobase current (Figure 3.5 C) of WT-TRESK expressing cells. These results show that TPA can inhibit human TRESK and that by doing so, can alleviate phenotypes associated with TRESK activity.

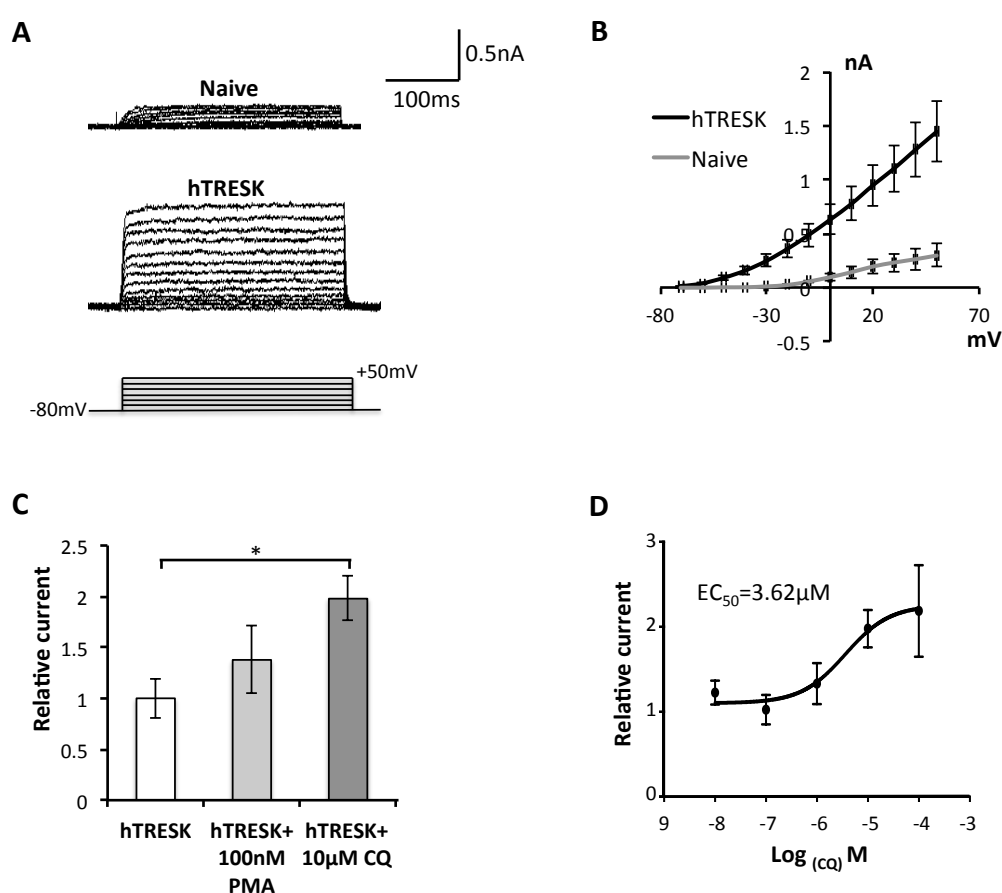


Figure 3.6 5-Chloroquinolin-8-ol (CQ) potently activates human TRESK. Patch clamp recordings of U2OS cells virally transduced with hTRESK. **(A)** Representative current traces obtained from hTRESK expressing or un-transduced (naïve) U2OS cells, depolarized by incremental voltage steps. **(B)** Current-voltage relationship for naïve (n=7) and hTRESK (n=17) U2OS cells. **(C)** Normalized currents in the presence of TRESK activators. Currents were evoked

by a voltage step from -80mV to +50mV and values were taken at the end of the 300ms pulse (n≥ 5 cells per group.) **(D)** Dose response curve for CQ activation of TRESK, normalized to vehicle-treated control (n≥ 4 cells per group.) * $P < 0.05$; One-way ANNOVA, Bonferroni's's multiple comparison test; results represent mean $\pm$ s.e.m

3.3.5 5-chloroquinolin-8-ol potently augments TRESK current

Modulators that up-regulate TRESK function would be powerful research tools. In addition, specific TRESK activators would be exciting therapeutic agents. As part of a drug discovery programme, and in collaboration with MRC Technology, we developed a screening method to identify novel TRESK activators. The drug 5-Chloroquinolin-8-ol (CQ) was found to enhance TRESK activity 2-fold, but have no effect on other K⁺ channels tested (Paul D. Wright 2013). These data were obtained using an indirect method for measuring TRESK activity (FluxOR K⁺ Ion Channel Assay, Life Technologies) and so direct validation by patch clamp analysis of compound pharmacology was sought. U2OS cells transduced with human TRESK (kind gift Dr. Paul Wright) exhibited a marked outward current that was not present in naïve cells lacking TRESK (Figure 3.6 A and B). This current could be abolished by treatment with 500nM TPA and enhanced by treatment with the TRESK activator, PMA (100nM) (Rahm, Gierten et al. 2012) (Figure 3.6 C). These results confirmed that the current was TRESK-mediated. CQ activates TRESK in a concentration-dependent fashion with a maximal activation $\approx$ 2-fold above control conductance (Figure 3.6 C and D). CQ activity had an EC₅₀ of 3.62 μ M, consistent with that measured independently by thallium flux (3.8 μ M) (Paul D. Wright 2013). These data support the original thallium flux findings and highlights CQ as a promising compound for increasing basal TRESK activity.

3.3.4 TRESK in sensory neurons

TRESK function has been studied most extensively in peripheral sensory neurons. Overexpression (Zhou, Yao et al. 2012), pharmacological inhibition (Tulleuda, Cokic et

al. 2011) and genetic ablation studies (Dobler, Springauf et al. 2007) have illustrated the role TRESK plays in murine DRG neurons. The TG is often considered analogous to the DRG, however differences do exist (Ambalavanar, Moritani et al. 2005). To date, TRESK has only been studied in TG neurons via overexpression experiments wherein exogenous TRESK currents are in significant excess of endogenous levels (Guo and Cao 2014). In an attempt to interrogate its role in a more physiological context, I wished to study the consequence of reduction of endogenous TRESK current to TG neuron physiology. To achieve this I utilized primary murine trigeminal cultures, in conjunction with pharmacology and a novel transgenic model.

Gender differences in animal models of pain are an accepted phenomenon (Greenspan, Craft et al. 2007) and the incidence of migraine is far higher in woman than men (Lipton and Stewart 1997). As a prelude to patch clamp studies, I confirmed levels of TRESK mRNA in TG tissue were similar between male and female animals (Figure 3.7). This rationale was used to justify the use of tissue from both sexes for all subsequent studies.

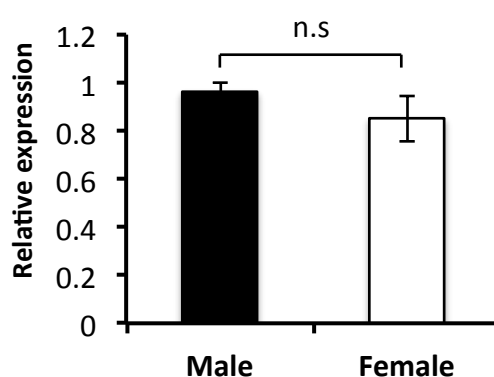


Figure 3.7 TRESK expression between the genders. Levels of TRESK mRNA are equivalent in male and female adult mice. mRNA was extracted from sensory ganglia and pooled per animal. TRESK mRNA was quantified by qPCR using Rpl19 levels as an internal control. *Un-paired student's t test, results represent mean ± s.e.m of six animals per group.*

Pharmacological inhibition results in a transient reduction in protein activity that can be informative with respect to function, but it is important to consider selectivity and efficacy when analyzing such data. In contrast, genetic ablation results in a permanent and total loss of protein function. The disadvantage of permanent loss of protein is that compensatory mechanisms may develop and hide relevant cellular phenotypes. It is for these reasons that I used both pharmacology and a genetic ablation model to investigate TRESK function in TG neurons. Knockout animals were generated by the trans-NIH Knock-Out Mouse Project (KOMP) and obtained from the KOMP Repository (www.komp.org). The VelociGene targeting system (Valenzuela, Murphy et al. 2003) ablates *KCNK18* by replacing the majority of the coding region with a LacZ-neomycin cassette (Figure 3.8 A). Homozygous animals express no TRESK mRNA in tissue where expression is normally high (Figure 3.8 B). These results confirm that the *KCNK18* gene has successfully been ablated from homozygous animals.

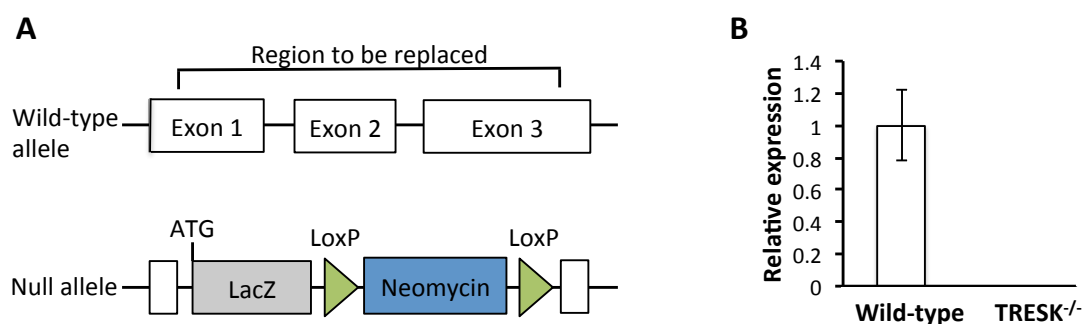


Figure 3.8 TRESK knockout mouse model. (A) *KCNK18* is ablated by the insertion of a LacZ-neomycin cassette with homology arms targeting the 5' of exon 1 and the 3' of exon 3. (B) TRESK mRNA is abundant in DRG tissue of wild-type animals, but absent in tissue from TRESK^{-/-} littermates. Results represent mean ± s.e.m of three animals per group.

3.3.5 Voltage-clamp recordings from trigeminal neurons

In comparison to immortalized lines, primary neurons offer the opportunity to study the contribution of a protein of interest within the context of other endogenous ion channels/transporters. Primary TG neurons were subjected to whole-cell voltage-clamp recordings within 48 hours of dissociation from adult mice. Cells of $<30\mu\text{M}$ diameter and with limited dendrites were chosen for analysis to enrich for nociceptors and to reduce experimental error due to space clamp issues. TRESK is expressed homogenously across sensory neuron subtypes (Dobler, Springauf et al. 2007) and so specific sub-populations of small/medium TG neurons were not exclusively targeted. A previously documented protocol was used to dissect TRESK currents from other ionic conductance (Dobler, Springauf et al. 2007, Liu, Xiao et al. 2013). Neurons were held at -60mV before depolarization to -25mV for 300ms and then the potential was ramped to -135mV over 550ms (Figure 3.9 A). Outward current was measured at the end of the 300ms step to minimize the contribution from high-voltage activated Ca^{2+} or fast TTX-resistant Na^{+} channels (Dobler, Springauf et al. 2007). At -25mV there is minimal activation of voltage-gated K^{+} currents (Dobler, Springauf et al. 2007) and $1\mu\text{M}$ TTX was bath applied to suppress voltage-gated Na^{+} channels. This protocol however does not distinguish TRESK current from some channel currents, such as those mediated by other $\text{K}_{2\text{P}}$ channels. Naïve cells exhibit an outward current that is reduced by 24.5% in the presence of 500nM TPA (Figure 3.9 B). Inhibition was largely reversed by drug wash-off, indicating that the reduction was due to the reversible pharmacological actions of TPA (Figure 3.9 A). Current rundown is a common occurrence during whole-cell recordings (Hattori, Murakami et al. 2003) and must be considered when performing multiple measurements of a cell over time. I therefore assessed the magnitude of rundown to the standing outward current. In the absence of pharmacology, outward current was reduced by 6.7% (Figure 3.9B), 20 minutes after achieving the whole-cell configuration (the time-point at which TPA inhibition was measured). TPA did not

significantly reduce the outward current of TRESK^{-/-} neurons (Figure 3.9 B) beyond the natural current rundown, suggesting that the effect of TPA on WT neurons outward K⁺ current is due to a selective action on TRESK.

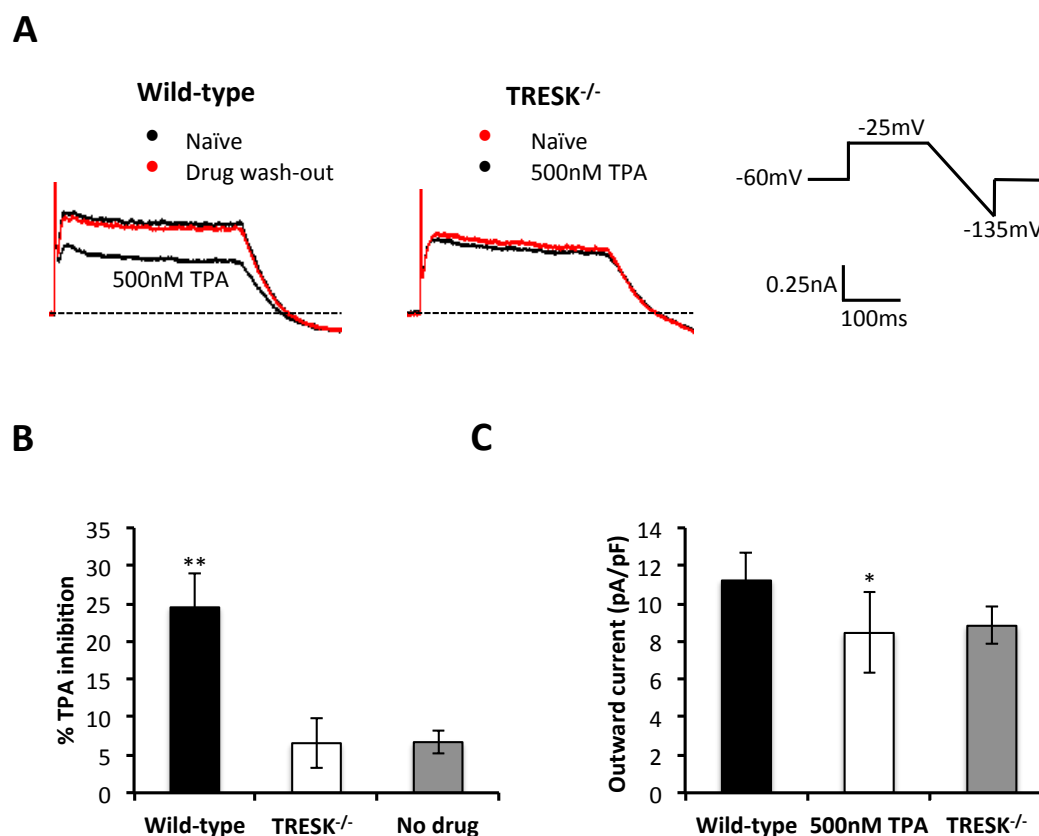


Figure 3.9 TPA sensitive outward current in TG neurons. (A) Representative traces of the outward current induced by prolonged depolarization to -25mV. In wild-type neurons, this current was inhibited by perfusion for 20 minutes of 500nM TPA. Upon drug wash off, current returned towards basal levels. TPA had only a minor effect on TRESK^{-/-} neurons. **(B)** Quantification of the reduction in outward current following TPA treatment ($n \geq 7$ cells per group). **(C)** Total outward current present in neurons with and without TRESK activity. Outward current was normalized for cell capacitance to reflect the current density ($n \geq 12$ cells per group). * $P < 0.05$, ** $P < 0.01$; Comparison between wild-type and TPA treated groups was performed by a paired students *t*-test. Comparison between wild type and TRESK^{-/-} groups was performed by unpaired students *t*-test; results represent mean $\pm$ s.e.m of at least three independent experiments.

Total outward current was reduced in TPA-treated neurons, as well as TRESK^{-/-} neurons, compared to wild type. This reduction was 24.7 and 21.3% respectively, and is of a similar magnitude to the TRESK contribution in DRG neurons (Dobler, Springauf et al. 2007). The difference between wild type and TRESK^{-/-} neurons did not reach statistical significance, most likely due to the heterogeneity of the cell population. No difference was observed between TPA-treated wild-type neurons and TRESK^{-/-} neurons. As previous data suggests that TPA partially blocks TRESK (Figure 3.5), this finding would suggest a degree of current compensation following genetic ablation of TRESK (Figure 3.9C).

3.3.6 Passive membrane properties of trigeminal neurons

In the SH-SY5Y model, overexpression of TRESK induced robust phenotypes associated with altered membrane properties. I investigated the basal cell membrane properties in the different groups of TG neurons. Wild-type neurons exhibited properties consistent with those observed in the literature (Table 3.1) (Cabanes, Lopez de Armentia et al. 2002). TPA treatment induced a modest depolarization of the membrane potential but RMP was unaltered in TRESK^{-/-} neurons. Input resistance (R_{input}) is a measure of the number of open channels in the membrane, and determines how much the membrane potential changes in response to a flow of current. R_{input} was unaltered in TRESK^{-/-} neurons but increased in wild-type neurons following TPA treatment (Table 3.1). These results are consistent with loss of TRESK activity. They also indicate that changes may occur in TRESK^{-/-} neurons with respect to other ion channels open at RMP to compensate for alterations in these key cellular parameters. This suggestion supports the voltage-clamp data.

Group	RMP (mV)	R _{input} (MΩ)	Capacitance (pF)
Wild-type	-53.75±0.98	269.99±37.62	51.40±6.97
500nM TPA	-48.55±2.43*	312.98±91.29**	49.67±15.60
TRESK ^{-/-}	-50.00±2.49	233.4573±26.47	52.75±9.31

Table 3.1 Passive properties of trigeminal neurons. * $P<0.05$, ** $P<0.01$; paired student's *t* test; results represent mean $\pm$ s.e.m of at least three independent experiments ($n \geq 9$ cells per group).

3.3.7 Firing properties of trigeminal neurons with reduced TRESK current

The firing properties of primary neurons recapitulate *in vivo* behavior to a greater extent than immortalized cell lines e.g. action potential morphology and the ability to fire repetitively. I wished to investigate whether loss of TRESK current induced firing phenotypes in this more physiological setting. To do this, I investigated similar parameters as those detailed for SH-SY5Y cells, but extended the analysis to more complex traits of action potential morphology and firing. Rheobase current was decreased to a similar extent upon 500nM TPA treatment and in TRESK^{-/-} neurons, compared to control (Figure 3.10 A and B). TG neurons have the capacity to fire multiple action potentials in response to a sustained, depolarizing stimuli. A long (500ms) pulse of current with amplitude 2-fold the measured rheobase generated an average of 2.4 spikes in wild-type cells. This appeared to be enhanced by TPA treatment and in TRESK^{-/-} neurons, but differences failed to reach significance (Figure 3.10 C and D). Previous analysis of TRESK^{-/-} DRG neurons reported subtle changes in action potential morphology (reduced action potential duration and increased after-hyperpolarization amplitude), hypothesized to be due to compensatory mechanisms (Dobler, Springauf et al. 2007). No difference between control and TPA treated/TRESK^{-/-} neurons was observed for any of these parameters. Nevertheless my findings extend the importance of TRESK to parameters of cellular excitability, in a more physiological setting.

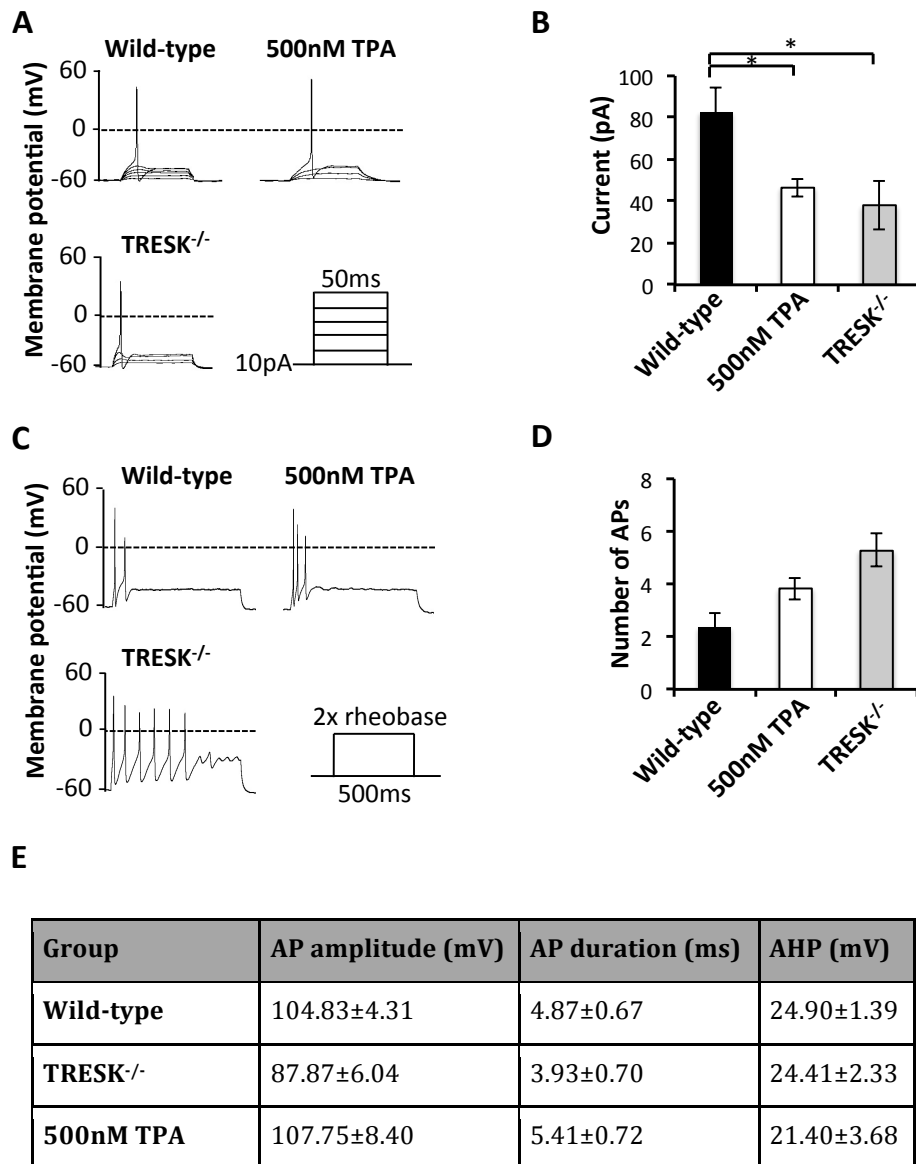


Figure 3.10 Current clamp recordings in neurons from wild type, TPA-treated and TRESK^{-/-} neurons. **(A)** Cells were depolarized with increasing current of 10pA increments from a holding potential of -60mV. Rheobase was assessed as the minimum current required to elicit an action potential. Panels show representative images from a wild-type neuron, the same neuron 20 minutes following exposure to 500nM TPA, and a TRESK^{-/-} neuron. **(B)** Quantification of rheobase across the different groups. **(C)** Cells were depolarized by a prolonged stimulus (500ms) corresponding to double their previously assessed rheobase. Stimulus was applied 5 times and the average number of action potential generated per stimulus calculated. Panels show representative images from a wild-type neuron, the same neuron 20 minutes following exposure to 500nM TPA and a TRESK^{-/-} neuron. **(D)** Quantification of repetitive firing across the groups.

(E) Action potential characteristics analyzed from traces obtained in A. $*P<0.05$; One-way ANNOVA, Bonferroni's's multiple comparison test; results represent mean $\pm$ s.e.m of at least three independent experiments ($n \geq 9$ cells per group).

3.3.8 TRESK expression throughout the CNS

Data relating to the expression of TRESK expression in tissues/neuronal regions is conflicting, with differences reported across species and between studies (Sano, Inamura et al. 2003, Yoo, Liu et al. 2009). Reliable expression data would be of great value for understanding TRESK physiology and its relevance to migraine. The field is challenged by a lack of specific antibodies capable of recognizing TRESK epitopes. One previous study of rat CNS found TRESK protein expression to be abundant in most brain regions (Yoo, Liu et al. 2009), contradicting other studies that found low levels/absence of TRESK mRNA in brain tissue (Sano, Inamura et al. 2003).

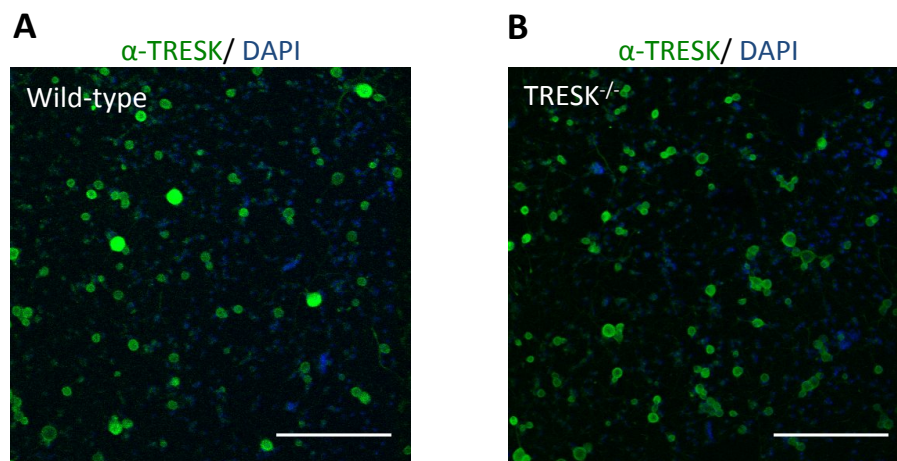


Figure 3.11 Non-specific binding of TRESK antibody. TG neurons derived from **(A)** wild type and **(B)** TRESK^{-/-} tissue stained with α -TRESK (Santa Cruz sc-51240). Scale bars represent 50 μ m.

Using TRESK^{-/-} neurons I sought to determine the specificity of the antibody used by Yoo *et al.* Under analogous conditions to those reported, α -TRESK Ab produced a positive

signal that was comparable in wild type and TRESK^{-/-} neurons (Figure 3.11 A and B), undermining the specificity of this antibody. I wished to map TRESK expression using an alternative approach. The null allele present in TRESK^{-/-} mice contains a reporter system, with the LacZ transgene under transcriptional control of the endogenous *KCNK18* promoter (Figure 3.8).

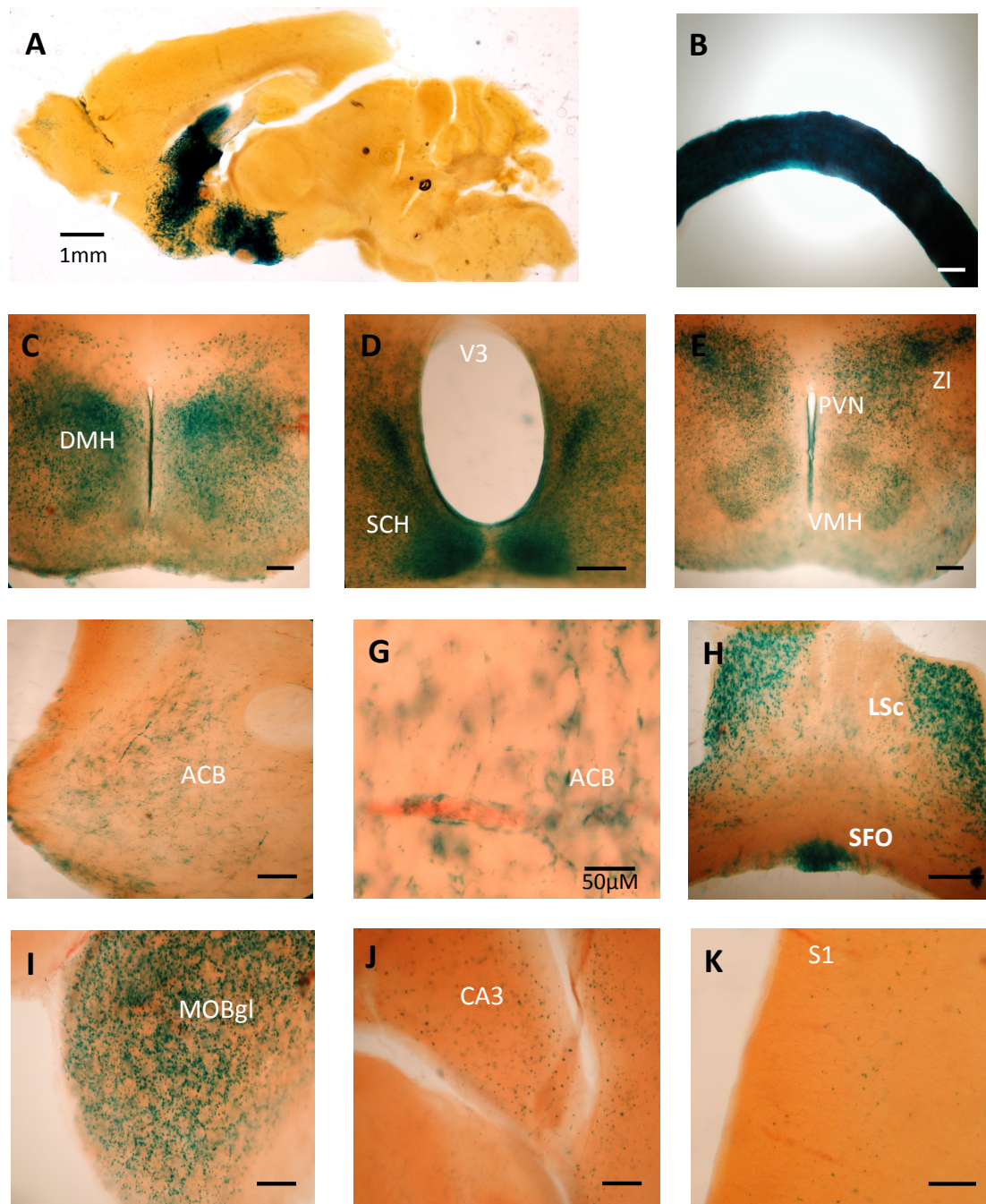


Figure 3.12. *KCNK18* promoter activity in *TRESK*^{-/-} CNS. Brains harvested from homozygous knockouts were sectioned at 300µm and probed for regions of LacZ expression by X-gal staining. **(A)** Low magnification sagittal section, illustrating β -galactosidase activity is not uniform throughout the CNS and localizes to specific brain regions. **(B)** Low magnification image of optic nerve taken in the sagittal plane. **(C-K)** Coronal sections of regions positive for LacZ expression as identified by a serial screen of whole brain. Scale bars represent 200µm unless otherwise stated. Abbreviations; DMH, dorsomedial hypothalamic nucleus; SCH, superchiasmatic nucleus;

ZI, zona incerta; PVN paraventricular nucleus; VMH, ventromedial hypothalamic nucleus; ACB, nucleus accumbens; MOBgl, main olfactory bulb, glomerular layer; LSc, lateral septal nucleus; SFO, subfornical organ; CA3, field CA3 of hippocampus; S1, primary somatosensory cortex.

As a proxy for TRESK expression, I investigated the location of β -galactosidase activity in TRESK^{-/-} CNS. Thick (300 μ M) sagittal sections indicated relatively restricted expression of LacZ, predominantly clustered around the hypothalamus and striatum (Figure 3.12 A). Optic nerve demonstrated high levels of LacZ activity (Figure 3.12 B). Serial coronal sections supported this finding with multiple nuclei of the hypothalamus (Figure 3.12 C-E) and regions of the striatum (Figure 3.12 F-H) exhibiting robust β -galactosidase activity. Higher magnification imaging of positive regions in the nucleus accumbens revealed staining adjacent to blood vessels (Figure 3.12 G). This was not seen in other brain regions. Additional LacZ expression was noted throughout the olfactory bulb (Figure 3.12 I) and hippocampus (Figure 3.12 J). Primary somatosensory cortex was positive for LacZ expression, however signal was detected in far fewer cells relative to other areas (Figure 3.12 K). Data presented here contradicts previous assessments of CNS TRESK expression and identifies regions that could link TRESK dysfunction to primary phases of a migraine attack.

3.4 Discussion

A deleterious mutation in *KCNK18* associates with migraine with aura (Lafreniere, Cader et al. 2010), however the cellular mechanisms, and indeed the neuronal population on which the mutation has its disease-modifying effect, are unknown. Key to answering these questions is a good understanding of TRESK physiology under normal conditions. TRESK is a protein with a documented role in sensory pain pathways (Tulleuda, Cokic et

al. 2011) yet its direct contribution to neuronal behavior has mainly been investigated indirectly, or by the use of non-selective pharmacology. Further, while TRESK is known to be highly enriched in sensory ganglia (Braun, Nemcsics et al. 2011), its expression within other migraine-salient areas has not been satisfactorily resolved. In this chapter, I have detailed the significant contribution of TRESK to cellular excitability. Affected parameters are robust across a range of cellular models, from overexpression in immortalized lines, to inhibition/inactivation of endogenous protein in primary neurons. These models have also been utilized to screen novel modulators of TRESK activity, which may prove beneficial for future studies and even drug discovery projects. Finally, CNS TRESK expression has been systematically mapped to specific brain regions. Such data will support future investigation of TRESK function in relation to migraine pathogenesis.

3.4.1 Fundamental properties of TRESK current

Heterologous expression in immortalized lines is a system commonly used to interrogate the basic properties of ion channel function. This is due to the simplicity and consistency of sample preparation, but also the ease in which specific currents can be recorded without the challenge of distinguishing exogenous from endogenous conductance. SH-SY5Y cells offer the benefit of being easy to culture and manipulate, yet also display biophysical characteristics of neurons when differentiated (Tosetti, Taglietti et al. 1998). Hence, they offer the possibility to interrogate basic channel function, but also to perform rudimentary investigations of neuronal firing. In previous studies, TRESK is documented as an outwardly rectifying K⁺ channel that polarizes the cell membrane (Sano, Inamura et al. 2003). Transient overexpression of WT hTRESK in SH-SY5Y cells generated a current consistent with these observations. In this study, neither the C110R nor F139Wfsx24 TRESK variants were able to transport to the membrane and conduct current (Figure 3.2). This poses the intriguing question of why one loss-of-

function variant associates with migraine, and another does not? Recent evidence has suggested that F139Wfsx24 may have a larger inhibitory effect on wild-type channel subunits than C110R (Liu, Xiao et al. 2013), however care must be taken when analyzing direct interactions between mouse and human forms of TRESK protein. Given that migraine is thought of as a polygenic disorder, perhaps a profitable approach may be to look for epistatic interactions with other gene variants that may precipitate disease.

Consistent with previous reports, I found that overexpression of TRESK hyperpolarized neurons and made them resistant to firing an action potential (Figure 3.3). This held true even when TRESK was introduced to the cell at a much lower level (Figure 3.4). This fits well with the clinical picture of a deleterious variant in TRESK associating with migraine, a disorder considered to be based upon altered excitability of the nervous system (Aurora and Wilkinson 2007).

3.4.2 Novel TRESK pharmacology

Studies of protein function are hindered by a lack of selective agonists and/or antagonists. I have shown that at 500nM TPA can effectively block TRESK currents while having minimal effects on other background conductance (Figures 3.5 and 3.9). However, it should be noted that these studies were performed at room temperature, yet several K_{2P} channels are known to be more active at 37°C (Maingret, Lauritzen et al. 2000, Kang, Choe et al. 2005). To rigorously assess drug selectivity it would be necessary to screen TPA on TRESK^{-/-} neurons at this temperature. However, TPA presents as a useful research tool under suitable experimental conditions and should be used in conjunction with classical TRESK blockers that are known to have non-specific actions (Bautista, Sigal et al. 2008).

TRESK is an attractive analgesic drug target. Up-regulating channel activity could act as a molecular brake on primary afferent activation. This action would be in

the periphery, negating the need for compounds to cross the blood brain barrier. Expression in peripheral tissue appears relatively restricted to sensory ganglia, although there is some evidence of expression in other neuronal (Cadaveira-Mosquera, Perez et al. 2012) and non-neuronal tissue (Kang, Mariash et al. 2004). Multiple agents are currently known to both inhibit (Lennertz, Tsunozaki et al. 2010) and activate (Liu, Au et al. 2004) TRESK, albeit in a non-selective manner. This chapter describes a TRESK agonist, CQ, which was discovered in a high-throughput screen of TRESK activity. Patch clamp analysis directly confirmed a 2-fold current activation induced by CQ (Figure 3.6). These data validate TRESK as a druggable target, justifying further screening for additional agonists, or medicinal chemistry approaches to improve CQ selectivity and efficacy. Such therapeutics could have diverse applicability across a spectrum of pain-related disorders, including migraine.

3.4.3 TRESK modulates the properties of trigeminal neurons

TRESK is highly expressed in the TG, which in turn is a body of cells implicated in transducing the headache pain of migraine (Pietrobon and Striessnig 2003). I have shown that TRESK represents 20-30% of the background current in TG neurons (Figure 3.9). At the mRNA level, TRESK is the most abundant K_{2P} channel in sensory neurons (Marsh, Acosta et al. 2012). It has also been shown that TRESK expression is decreased following cell dissociation (Tulleuda, Cokic et al. 2011) and therefore, estimates may undervalue its contribution *in vivo*. Loss of TRESK in TG neurons mirrors the effects of overexpression, i.e. depolarization of RMP and a decrease in rheobase (Table 3.1 and Figure 3.10). Indicative of its importance, multiple migraine candidate genes have been shown to contribute to the function of the TGVS (McKemy 2005, Hullugundi, Ansuini et al. 2014). It can be postulated that any reduction in the activation threshold of trigeminal nociceptors could lead to aberrant or sustained firing, which in turn would feed forward to second order neurons and generate the perception of headache pain.

Hence the finding that TRESK has substantial control over TG neuron excitability has important relevance to understanding migraine pathogenesis.

3.4.4 TRESK in the CNS

Migraine is a complex brain disorder whose origins are unlikely to be in the periphery. TRESK may or may not have a role in the early, CNS-intrinsic events of a migraine attack. As a first step to answer this question, TRESK expression within the CNS must be characterized. Using our reporter mouse line, I have shown that TRESK expression is not CNS-wide as previously described (Yoo, Liu et al. 2009). Of particular note, TRESK was extensively distributed in the hypothalamus (and hypothalamus-associated regions such as the lateral septal nucleus (Figure 3.12)), a region heavily implicated in migraine. PET studies have observed hypothalamic activation during spontaneous migraine attacks (Denuelle, Fabre et al. 2007) and many of the features of migraine could be attributed to hypothalamic dysfunction (Alstadhaug 2009). While expression appears low in cortical regions, robust expression is seen in the hippocampus (Figure 3.12). The hippocampus acts on hypothalamic regions to modify the stress response and is implicated in pain processing (Liu and Chen 2009). Direct links between hippocampus dysfunction and migraine have been made by recent imaging studies (Moulton, Becerra et al. 2011).

I have taken the approach of analyzing regions of *KCNK18* promoter activity. Thus, findings must be tempered with the detail that I do not describe TRESK protein presence, but rather the likely transcriptional activity of the *KCNK18* locus. Data from the Allen Brain Atlas does not support the presence of *KCNK18* transcripts in mouse CNS. However this is concluded from a single *in situ* experiment, relying upon only one set of anti-sense probes. Further evaluation of TRESK mRNA presence in highlighted brain regions should be performed.

Future efforts should specify the precise cell types that possess TRESK current and investigate the functional role this may have in the relevant regions. For example, it

would be interesting to investigate whether TRESK^{-/-} mice have a circadian rhythm dysfunction, or if CSD propagation/initiation is different in these animals compared to control.

Data presented in this chapter serve to highlight the function of TRESK as a “molecular brake” on neuronal excitability. This property is particularly prominent in trigeminal neurons, however expression of the channel is also seen in CNS regions that warrant further investigation. Using TRESK dysfunction as a model, it may be possible to investigate common pathways of migraine pathogenesis. Although TRESK variants may underlie only a minor proportion of migraineurs, it still presents as a highly promising drug target. Efforts to develop novel therapeutics to up-regulate channel activity could create novel opportunities for pain management and migraine treatment.

4. Investigating the association of *SLC12A3* with migraine

4.1 Introduction

While monogenic forms of migraine do exist: a multifactorial heredity model best explains common migraine. The mode of migraine inheritance is most compatible with multiple genetic susceptibility factors, interacting with numerous environmental cues, combining to result in recurring migraine attacks (Russell, Iselius et al. 1995). The F139Wfsx24 *KCNK18* variant appears to show 100% penetrance in our pedigree population (Lafreniere, Cader et al. 2010), apparently suggesting that this rare variant represents a monogenic form of migraine. Contrary to this, the C110R *KCNK18* variant that also results in a total loss of TRESK function, is found in both migraine and healthy control cases (Andres-Enguix, Shang et al. 2012). There can be two plausible explanations for this observation. Firstly, the two variants may have differing impacts on cell physiology. This argument has been discussed previously (Chapter 3, Discussion 3.3.1) but does not exclude the second hypothesis. Due to the knowledge that migraine is a polygenic disorder, it can be speculated that other genetic loci outside of *KCNK18* may play a role in disease pathogenesis. Such genetic factors could act in a “double hit” manner, acting in concert with loss of TRESK activity, or alternatively such loci could act at a different point in the disease cascade, in a different cell type and/or tissue. The latter would be an attractive way of explaining why loss of TRESK, with its principle role in the TG, associates with a whole-brain disorder like migraine.

During the original linkage analysis only one locus was identified with a LOD score >3.0. This region contained the *KCNK18* frame-shift mutation. A second linkage peak (LOD score 2.8) was subsequently defined with a segregating haplotype present in all affected individuals, but also two unaffected individuals. One deleterious mutation

(G264A) was defined in this region and it occurred in the gene *SLC12A3* (Cader *et al*, unpublished data, Figure 4.1).

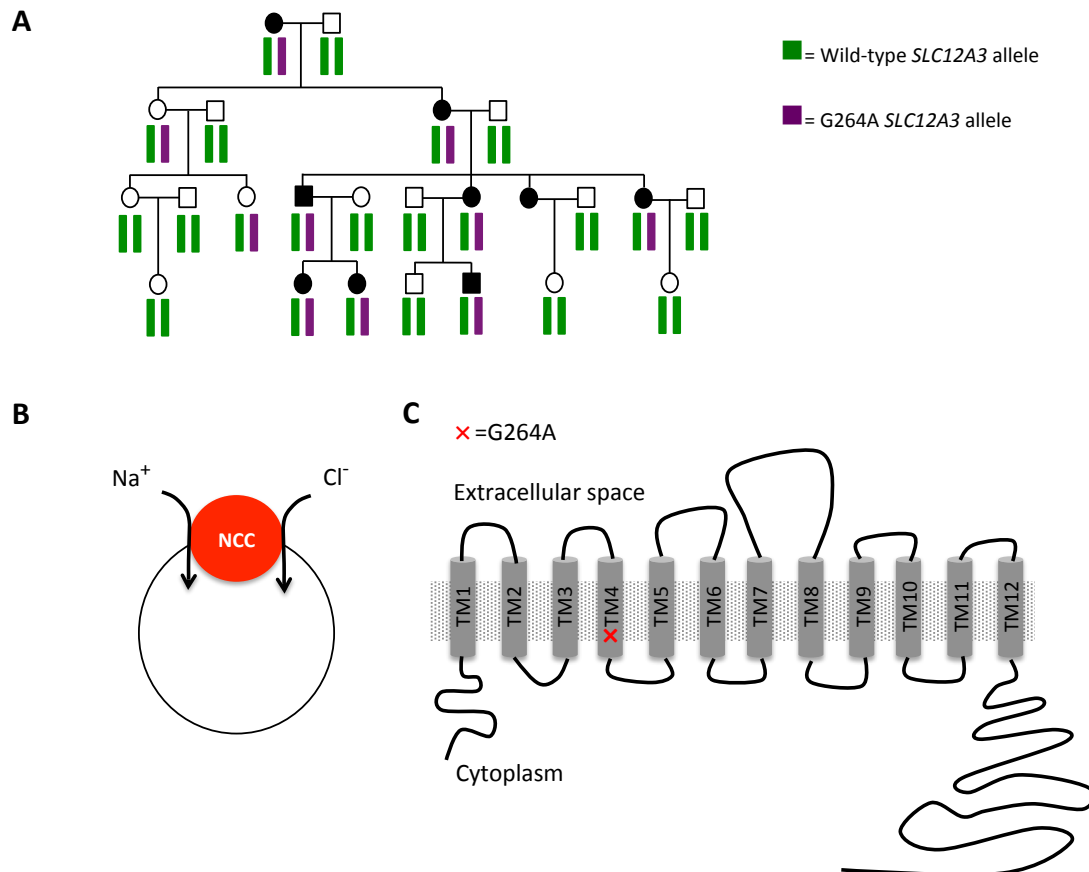


Figure 4.1 The SNP polymorphism, G246A segregates with MA in one pedigree. (A) All carriers of the F139fsWx24 *KCNK18* mutation also harbour a mutation in *SLC12A3*. SNP (G246A) is also present in two unaffected individuals. *SLC12A3* encodes for the thiazide-sensitive Na^+/Cl^- co-transporter, NCC. **(B)** NCC transports one Na^+ ion into the cell along with one Cl^- ion. The net flow of charge is zero and defines the transporter as electroneutral. **(C)** Predicted structure of human NCC, including 12 transmembrane domains (TM1-12). Migraine-associated G264A represents a substitution of alanine for glycine at amino acid 264 in the 4th transmembrane domain. The mutation causes a 50% loss of transporter function (Moreno, Tovar-Palacio *et al.* 2004).

SLC12A3 belongs to the *SLC12* family of electro-neutral cation/ Cl^- co-transporters (Gamba 2009). The gene encodes a transporter (NCC) that inwardly translocates Cl^- along with Na^+ with 1:1 stoichiometry (Figure 4.1 B). NCC is a protein of 1,002-1,028 amino acid residues and has a prominent role in reabsorbing Na^+ and Cl^- from the tubular fluid into cells of the distal convoluted tubule of the nephron (Gamba 2009). Traditionally, NCC expression has been thought to be restricted to the kidney (Mastroianni, De Fusco et al. 1996), however evidence for expression in other tissues is now emerging (Bazzini, Vezzoli et al. 2005, Dvorak, De Joussineau et al. 2007). Despite this, no past observations supportive of a role in migraine salient tissues have been made.

Deleterious mutations in *SLC12A3* cause Gitelman's syndrome- an autosomal recessive disorder characterized by hypokalemic metabolic alkalosis, hypocalciuria, hypomagnesaemia, and arterial hypotension (Simon, Nelson-Williams et al. 1996). In the G246A variant, a highly conserved glycine residue at position 264 is replaced by alanine (Figure 4.1 C). Previous *in vitro* investigations associate the variant with a partial loss-of-function phenotype. G264A causes a 50% reduction in $^{22}\text{Na}^+$ uptake but an increase in transporter affinity for extracellular Cl^- and thiazides (Moreno, Tovar-Palacio et al. 2004). These results are consistent with a reduction of intrinsic co-transporter activity and diminution of Na^+/Cl^- translocation. To date, G264A has not been associated with Gitelman's syndrome and there is no history of tubular acidosis in the affected family. All subjects have normal blood pressure, serum and urinary electrolytes including: Na^+ , K^+ , Mg^{2+} and HCO_3^- (Cader et al, unpublished data).

Cl^- /cation co-transporters are electro-neutral. Despite this, some have been heavily implicated in neuronal physiology and spiking behaviour. KCC2 and NKCC1 have both been shown to modulate the excitability of a range of neuronal cell types (Zhu, Polley et al. 2008, Sipila, Huttu et al. 2009, Seja, Schonewille et al. 2012), including trigeminal neurons (Schobel, Radtke et al. 2012). Both proteins have relevance to

seizure and pain pathways. NKCC1 inhibition suppresses epileptiform activity in hippocampal slices (Dzhala, Talos et al. 2005), while down-regulation of KCC2 increases the susceptibility of immature rats to seizure (Huang, McMahon et al. 2012). KCC2 and NKCC1 are both present in spinal nociceptive neurons and expression changes under acute/chronic pain conditions have been noted (Cramer, Baggott et al. 2008, Miletic and Miletic 2008). Furthermore, such changes have been shown to have functional effects on pain behaviour (Hasbargen, Ahmed et al. 2010, Austin and Delpire 2011).

4.2 Aims of Chapter 4

Chapter 4 is concerned with providing functional information to support or refute the hypothesis that a deleterious variant in *SLC12A3* is causative for migraine. To this end, the chapter has two sub-aims:

- 1) Document expression of NCC in migraine-salient tissue.
- 2) Investigate whether NCC has a role in neuronal physiology.

4.3 Results

4.3.1 NCC expression in migraine-salient regions

NCC function has rarely been investigated outside of the kidney and has never been studied for a neuronal role. I wished to investigate whether NCC expression was detectable in regions of the nervous system implicated in migraine. To confirm antibody specificity, immunohistochemistry was performed on mouse kidney sections from wild type and NCC^{-/-} mice (characterized and detailed in Results 4.3.6 and Figure 4.10). α -NCC produced a strong and specific signal localized to discrete regions of the kidney (Figure 4.2 A). The staining profile was consistent with expression in the distal convoluted tubules of the nephron. No signal was detected in kidney tissue of NCC^{-/-}

mice (Figure 4.2 A). Higher magnification images revealed apical localization of antibody binding (Figure 4.2 B). These results are consistent with previous NCC localization studies (Loffing, Vallon et al. 2004). By western blot, α -NCC detects a single band at ~190kDa (Figure 4.2 C) that is absent when probing lysate from NCC^{-/-} kidney (Figure 4.2 C). Human and mouse NCC share 89% amino acid sequence homology. To confirm that α -NCC also recognizes the human form of the protein, human NCC (hNCC) tagged with GFP was expressed in N2A cells, followed by immunocytochemistry. α -NCC stained GFP positive cells with 100% overlap (Figure 4.2 D), indicating that the antibody was compatible with both human and mouse NCC detection. These results gave me confidence in the use of the antibody for all future studies.

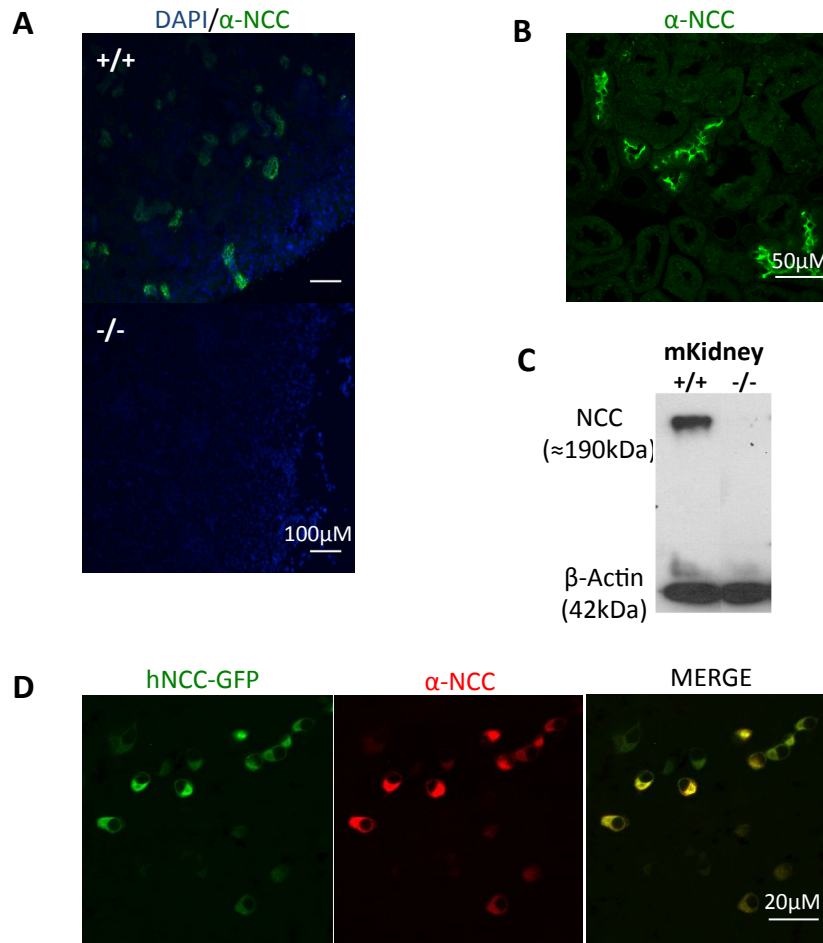


Figure 4.2 Validation of primary antibody against NCC. **(A)** Renal cortex of wild type and NCC^{-/-} mice immunostained with rabbit α-NCC (Abcam- AB3553), followed by incubation with Alexa 488-conjugated goat anti-rabbit IgG. **(B)** Zoomed image of NCC in wild type kidney showing expected apical localization of the target epitope. **(C)** Kidney lysate from wild type and NCC^{-/-} mice subjected to SDS-PAGE analysis and probed with α-NCC and α-β-actin. Incubation with HRP-coupled α-rabbit IgG followed by ECL detection results in discreet bands of molecular weight ≈42kDa and ≈190kDa. These bands represent the expected size of β-actin and NCC respectively. No band was observed at 190kDa when NCC^{-/-} kidney lysate was probed. **(D)** N2A cells overexpressing hNCC-GFP immunostained with α-NCC, followed by incubation with Alexa 594-conjugated goat α-rabbit IgG.

As a first step to investigate NCC expression in neuronal tissue, crude lysates of mouse kidney, occipital cortex, whole brainstem and TG were probed by Western blot.

Brainstem and TG showed strong bands, while occipital cortex was marked by a low level of NCC protein (Figure 4.3 A). TG lysate exhibited the highest level of expression and so further studies were performed. RT-PCR confirmed the presence of NCC mRNA in rat TG (Figure 4.3 B) and immunohistochemistry of mouse TG revealed neuronal staining of all sized trigeminal neurons (Figure 4.3 C).

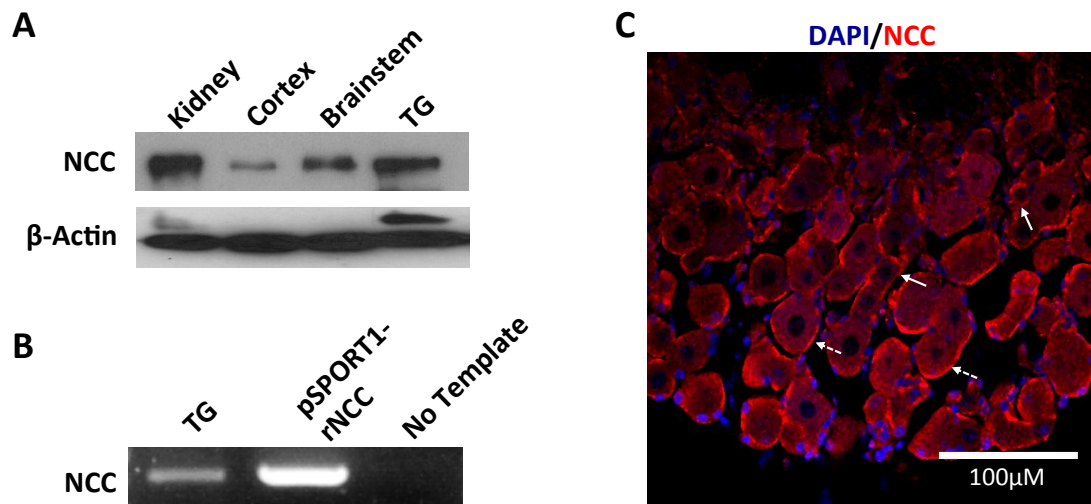


Figure 4.3 NCC expression in migraine-salient tissue. (A) Presence of NCC protein in regions classically associated with migraine. Whole tissue lysates of mouse kidney, occipital cortex, brainstem and TG were probed for NCC expression by Western blot analysis. Levels of β -actin served as a loading control. **(B)** Confirmation of NCC mRNA in rat TG using RT-PCR and exon-spanning primers specific to NCC. Test template was 10ng/ μ l cDNA derived from rat TG mRNA. Rat NCC expression construct (pSPORT1-rNCC) and no template control, were run in parallel as positive and negative controls respectively. **(C)** Whole tissue immunostaining of mouse TG with α -NCC, followed by incubation with Alexa 594-conjugated goat α -rabbit IgG. Membranous signal is apparent in all neuronal cell bodies, including large diameter (>30 μ M, dashed arrows) and medium/small diameter (<30 μ M, solid arrows) neurons.

4.3.2 NCC throughout the CNS

The observation that NCC could be detected by western blot in multiple CNS areas, led to the desire to comprehensively localize protein expression. Serial coronal sections of 60 μ M thickness were generated from the whole brain of P56 male mice. Sections were subsequently probed by immunohistochemistry to identify regions of protein expression. Two areas within the hypothalamus showed a robust and clear positive signal. These regions were the paraventricular (PVN) (Figure 4.4 B) and supraoptic nucleus (SON) (Figure 4.4 C). Staining suggests that NCC is present on the cell body and neurites of neurons in these regions. No other brain areas were identified to have signal above background.

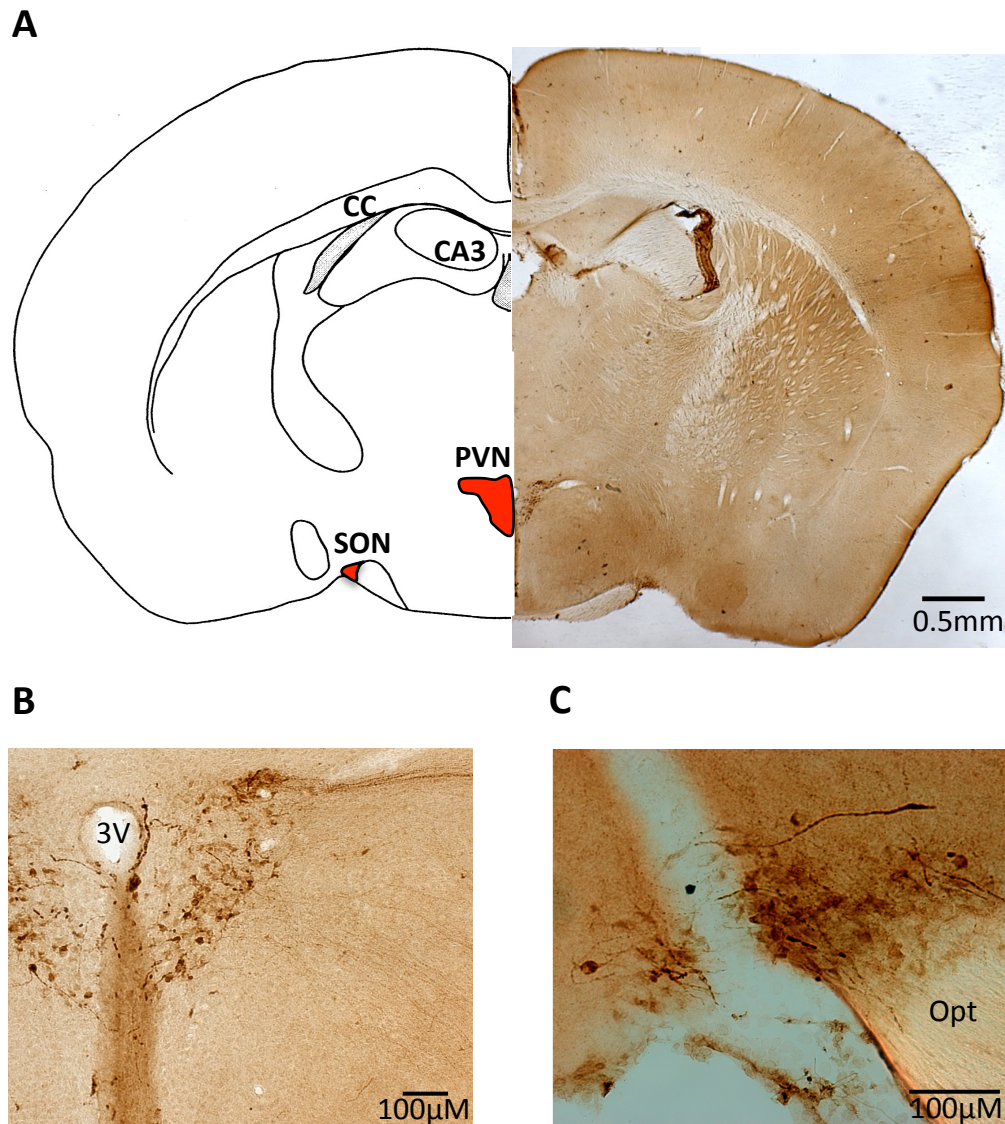


Figure 4.4 Spatial expression of NCC throughout adult mouse CNS. Serial sections representing the whole brain were probed for NCC expression. **(A)** Immunoreactivity was not widespread, but was noted in two discrete hypothalamic regions. Higher magnification images of **(B)** PVN and **(C)** SON revealed robust staining of cell body and projections. *CC*, corpus callosum; *CA3*, field CA3 of hippocampus; *SON*, supraoptic nucleus; *PVN*, paraventricular nucleus; *3V*, third cerebroventricle; *Opt*, optic tract.

Immunohistochemistry provides good spatial resolution of expression, but does not convey quantitative information. To compare relative expression levels across tissues, mRNA was extracted from key regions highlighted in previous studies: kidney,

brainstem, TG, DRG, PVN and SON. Hypothalamic samples were a kind gift of Dr. Mike Greenwood, University of Bristol. NCC mRNA was readily detectable in neuronal tissues, however levels were roughly 100-fold lower than found in kidney. Expression was highest in the sensory ganglia compared to other neural tissue (Figure 4.5). These results represent a novel finding that NCC mRNA and protein is present in key neuronal populations.

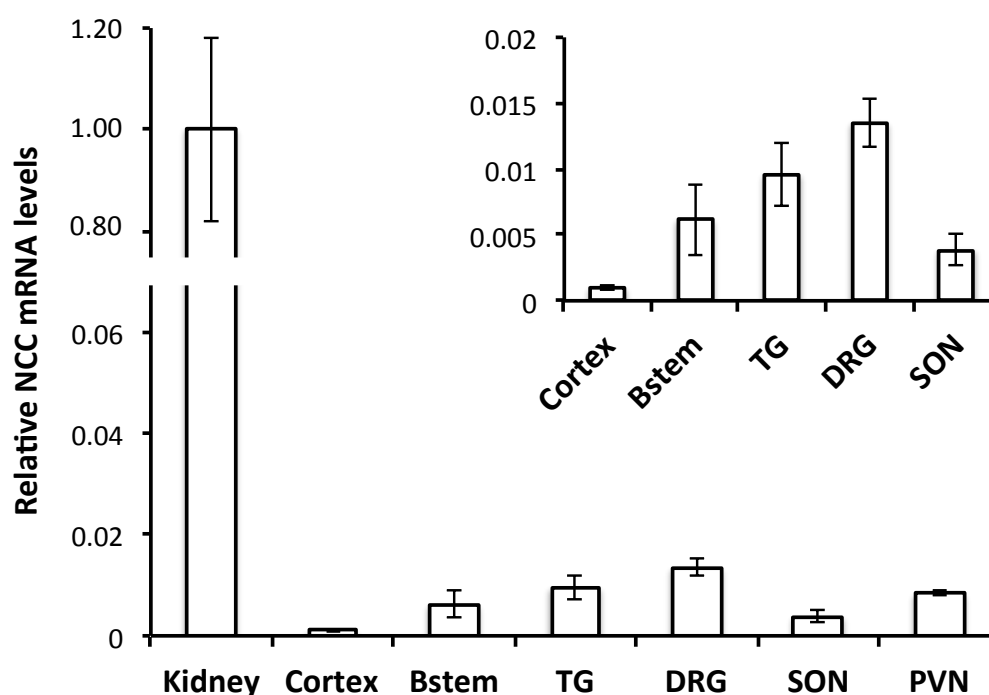


Figure 4.5 Quantification of NCC mRNA in neuronal tissue. mRNA was harvested from adult male Sprague Dawley rat tissue and reverse transcribed to cDNA. Expression is normalised to the housekeeping gene RPL19 and results are depicted relative to the kidney. *Bstem*, whole brainstem. Results represent the mean $\pm$ s.e.m of 3 independent experiments.

4.3.3 NCC overexpression does not alter firing properties of SH-SY5Y cells

NCC imports one positive charge (Na^+) along with one negative charge (Cl^-) and hence, is an electroneutral transporter. Despite being electroneutral, there are transporters that have profound effects on neuronal physiology (Mercado, Mount et al. 2004, Cooper,

Saxena et al. 2005). Given its expression in neuronal tissues, I wanted to define whether NCC has a modulatory role on neuronal properties. hNCC-GFP was expressed in differentiated SH-SY5Y cells and exhibited localization that was consistent with proper trafficking to the plasma membrane (Figure 4.6 A). 48hrs following transfection, biophysical parameters were assessed by whole-cell patch clamp. To ascertain if hNCC induced any gross changes in membrane currents, a basic voltage-clamp protocol ramping the potential from -80mV to +30mV in 10mV increments was used. Maximum inward and outward current for each depolarizing pulse were plotted as a function of membrane potential. hNCC did not affect inward or outward voltage-activated current (Figure 4.6 B and C). At hyperpolarized potentials there existed a consistent inward current that was potentiated by hNCC expression (Figure 4.6 C). With regard to firing properties, there was no difference in RMP or rheobase between hNCC-GFP and control (GFP-empty vector expressing) cells (Figure 4.6 D and E).

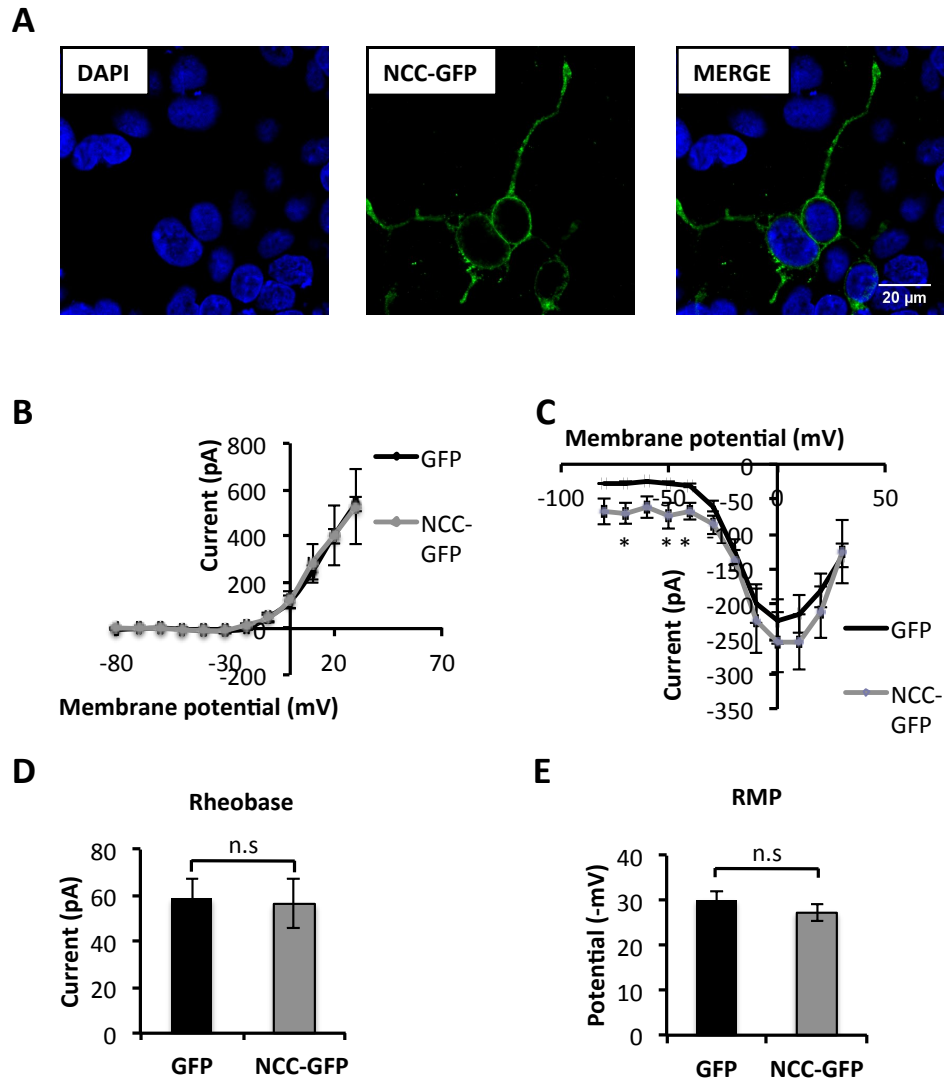


Figure 4.6 NCC overexpression induces no notable excitability phenotype in SH-SY5Y cells.

hNCC-GFP was overexpressed in differentiated SH-SY5Y cells and biophysical parameters measured in whole-cell mode. **(A)** Immunofluorescence of SH-SY5Y cells, 48 hours post-transfection with NCC-GFP. Localisation of fluorescence is consistent with proper membrane trafficking of NCC. **(B)** Voltage clamp protocols were performed to step membrane potential from -80mV to +30mV in 10mV increments. Outward currents were measured at the end of the depolarization step. **(C)** Inward currents were measured at the beginning of each depolarization step and noted as the peak current recorded. **(D)** Rheobase current of cells expressing hNCC-GFP or GFP empty vector (control). Increasing 5pA increments were applied to cells and rheobase determined as the minimal amount of current required to generate an action potential. **(E)** RMP

as recorded in current-clamp bridge mode. $*P<0.05$, $N.s P>0.05$; Unpaired Student's *t*-test; results represent mean $\pm$ s.e.m ($n \geq 7$ cells per group).

4.3.4 HCTZ alters the excitability of trigeminal neurons

SH-SY5Y cells do not express the full repertoire of channels/transporters that are present in primary neurons. Nor do they allow for detailed investigation of firing properties, as they are only capable of rudimentary action potentials and cannot spike repetitively. Due to the observation of high expression of NCC in the TG, I performed whole-cell recordings from TG neurons while applying pharmacological inhibition of NCC activity. The specific inhibitor (1944, de Jong, Willems et al. 2003, Hebert, Mount et al. 2004, Hunter, Craigie et al. 2014)- hydrochlorothiazide (HCTZ) was used to inactivate NCC. HCTZ treatment did not significantly alter passive membrane properties (*RMP*, Vehicle=52.13 $\pm$ 1.1mV, 100 μ M HCTZ=53.39 $\pm$ 1.5mV; *R_{input}*, Vehicle=287.65 $\pm$ 29.3M Ω , 100 μ M HCTZ=375.48 $\pm$ 39.29M Ω ; *Capacitance*, Vehicle=20.95 $\pm$ 1.5pF, 100 μ M HCTZ=28.01 $\pm$ 3.5pF).

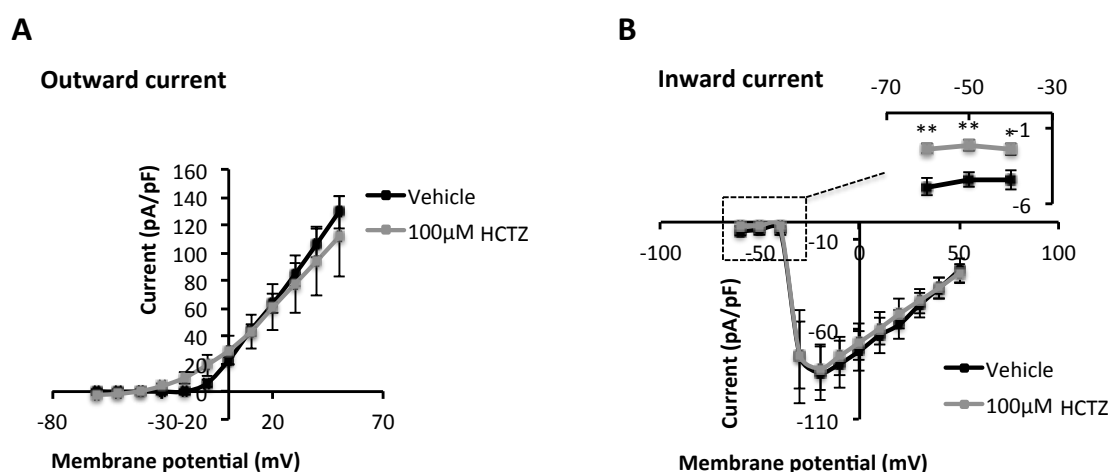


Figure 4.7 HCTZ does not alter gross voltage-activated currents. Whole-cell voltage-clamp recordings of mouse TG neurons exposed to vehicle (0.01%DMSO) or 100 μ M HCTZ 1-3 hours prior to recording. Membrane potential was ramped from a holding

level of -60mV to +50mV in 10mV increments. **(A)** Outward current was measured as the maximal positive current observed during each 500ms step. **(B)** Inward current was measured as the maximal negative current observed during each 500ms step. Voltage-gated currents were noted from potentials of -30mV. Inset shows enlarged image of currents recorded at hyperpolarized potentials. **P<0.05, **P<0.01; Unpaired Student's t-test; results represent mean± s.e.m (n≥ 14 cells per group).*

Voltage-clamp recordings of neurons were used to define whether drug treatment altered ionic conductance. While it would be very challenging to isolate each current in turn, it is possible to assess gross currents. Potential was ramped from -60mV to +50mV and currents divided into those that represent net outward, or inward flow of current. Voltage-activated currents were present from potentials of -30mV and were unaltered between vehicle and drug treated groups (Figure 4.7 A and B). As was noted for SH-SY5Y cells there were significant differences in current in response to potential changes at hyperpolarized values. 100μM HCTZ reduced the maximal inward current observed between potentials of -60mV and -30mV (Figure 4.7 B). These results support the SH-SY5Y data in concluding that while voltage-activated currents are unaffected, net inward current present at hyperpolarized potentials is reduced by transporter inhibition.

To examine measures of excitability, groups were assessed for their rheobase, action potential morphology and response to prolonged depolarization. Rheobase was unaltered between groups (Figure 4.8 A), as was action potential amplitude (Figure 4.8 B), after-hyperpolarization (Figure 4.8 C) and duration (Figure 4.8 D). In response to prolonged (500ms) depolarization, HCTZ treated cells fired 2-fold more action potentials than vehicle treated (Figure 4.8 E and F). As such, while most electrical parameters are unaltered by HCTZ treatment, neurons have a greater propensity to fire repetitive action potentials in response to sustained depolarization.

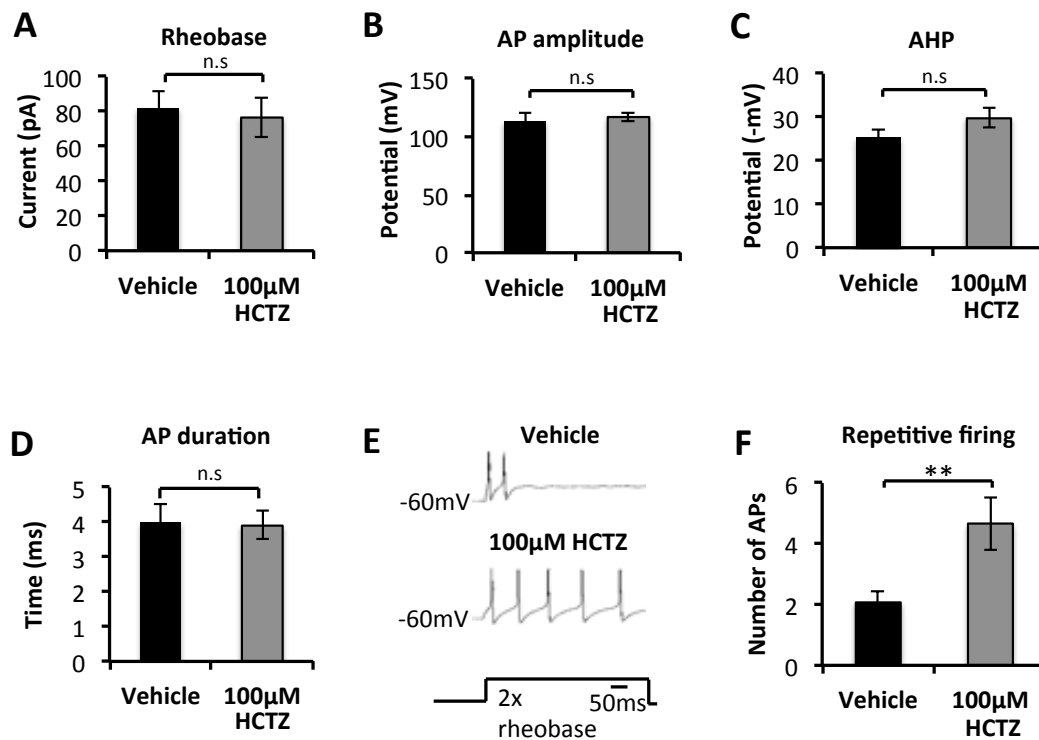


Figure 4.8. HCTZ alters the spiking properties of TG neurons. Whole-cell current-clamp recordings of TG neurons exposed to vehicle (0.01%DMSO) or 100µM HCTZ 1-3 hours prior to recording. **(A)** Rheobase current was defined as the minimal current required to elicit an action potential. **(B)** Action potential (AP) amplitude was measured as the maximal positive potential change during the action potential. **(C)** After-hyperpolarization (AHP) was defined as the negative potential deflection relative to the holding level, observed directly following an action potential. **(D)** AP duration was calculated as the time between the points of half-maximal amplitude of the action potential. **(E)** Representative traces of cells stimulated by current 2-fold the previously defined rheobase for 500ms. **(F)** Quantification of mean number of action potentials generated in response to prolonged depolarization. *N.s* $P>0.05$, *Unpaired Students t-test* $**P<0.01$; *Mann-Whitney U test*; results represent mean $\pm$ s.e.m ($n \geq 14$ cells per group).

4.3.5 NCC inhibition does not impact on cell size

Cell swelling can increase the excitability of neurons (Rosen and Andrew 1990). As the movement of Na^+ and Cl^- ions by NCC likely increases H_2O entry to the cell, I

hypothesized that the effect of NCC inhibition on excitability may be mediated through changes in cell volume. I assessed whether HCTZ altered the cell volume of TG neurons. As a proxy for cell volume, the soma surface area was analyzed in dissociated cultures treated with 100 μ M HCTZ. Calcein AM is a cell permeant dye that strongly fluoresces upon interaction with intracellular esterases. I used this reagent to aid in visualizing soma size. Under analogous conditions to those used for patch clamp analysis, no significant difference in cell area was noted between vehicle and drug treated groups (Figure 4.9). These results argue against cell shrinkage as a mechanism for enhanced excitability.

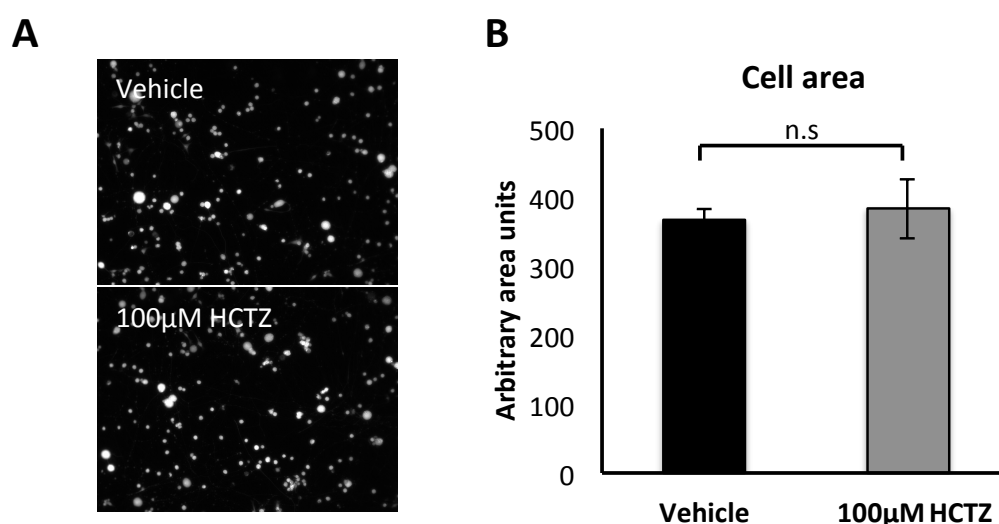


Figure 4.9 HCTZ does not alter soma size. Murine trigeminal neurons were cultured in similar conditions as those for patch clamp analysis. Groups were treated with 100 μ M HCTZ or vehicle for 1 hour prior to analysis. Cells were loaded with 5 μ M Calcein AM for 30 minutes before fluorescent microscopy was used to capture multiple images per well. Images were analyzed for cell size using ImageJ analysis software. **(A)** Representative images of vehicle and drug treated cultures. **(B)** Quantification of cell size did not show a difference between vehicle and drug treated groups. *N.s* $P > 0.05$; *Unpaired Student's t-test*; results represent mean $\pm$ s.e.m cell size per group (3 independent experiments with at least 5 images per experiment)

4.3.6 Characterisation of NCC^{-/-} mouse

Despite the documented specificity of HCTZ (de Jong, Willems et al. 2003, Hebert, Mount et al. 2004, Hunter, Craigie et al. 2014), non-canonical actions of pharmacological agents must always be considered when interpreting data. Furthermore, HCTZ may not enact a complete block of NCC protein activity, allowing residual function to mask subtle phenotypes. To add support to the evidence garnered by pharmacological inhibition, I wished to study the properties of TG neurons derived from mice lacking the *SLC12A3* locus.

Previously generated and characterized NCC^{-/-} mice were imported (kindly provided by Dr. Jan Loffing, University of Zurich (Loffing, Vallon et al. 2004)) and a colony established. The knockout model was originally generated by inserting a neomycin cassette into exon 12 of *SLC12A3*. Resultant mice lack NCC mRNA and protein in kidney tissue (Schultheis, Lorenz et al. 1998). I developed a genotyping strategy for the colony based upon PCR of genomic DNA and two sets of primers which recognized either; naïve exon 12, or the junction between the 3' of exon 12 and the beginning of the neomycin cassette (Methods: Figure 2.1). Ear clips taken from individual mice were used to clearly define genotype and hence reduce the chance of miss-identification.

Western blot analysis of tissue lysates was performed to confirm the lack of NCC protein in knockout mice. The band corresponding to NCC was not present in kidney samples from knockout animals (Figure 4.10 A). However, bands remained for neuronal tissue: occipital cortex, brainstem and TG. Neuronal lysates were taken from the same animal as the kidney samples and the results replicated on a second set of samples in an independent experiment. Neuronal bands appear at the same molecular weight to kidney, but are not diminished in knockout animals (Figure 4.10 A). To confirm the apparent protein expression in knockout tissues, immunostaining of NCC^{-/-} TG was performed. NCC^{-/-} sections probed with α -NCC demonstrated a comparable signal to wild-type sections (Figure 4.3 C and 4.10 B).

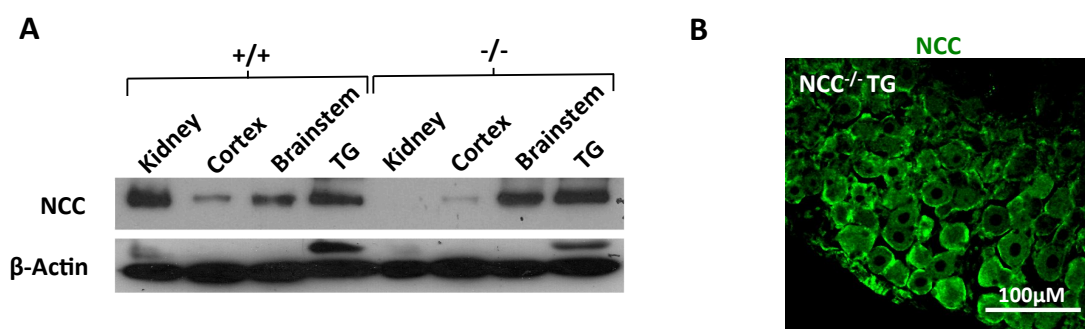


Figure 4.10. Continued presence of NCC protein in tissue from knockout mouse. (A) Western blot analysis of tissue lysates derived from wild type and NCC^{-/-} littermates. Levels of β -actin served as a loading control. **(B)** Whole tissue immunostaining of NCC^{-/-} mouse TG with α -NCC, followed by incubation with Alexa 488-conjugated goat α -rabbit IgG. Membranous signal is apparent in all neuronal cell bodies.

These results could best be explained by non-specific antibody-antigen interactions. Four of the five proteins with the highest amino acid homology to NCC (NKCC1- 53% homology, NKCC2- 50%, KCC3- 28% and KCC1- 28%) are abundantly expressed in the kidney (Liapis, Nag et al. 1998, Wall, Fischer et al. 2001, Liu, Xie et al. 2011, Melo, Cruz-Rangel et al. 2013). As such, they are unlikely to represent the neuronal band observed by western blot, as they would also be present in NCC^{-/-} kidney samples. KCC2 shares 27% amino acid sequence homology with NCC and uniquely, expression is confined to neurons (Williams, Sharp et al. 1999). The predicted molecular weight of KCC2 is 123.6kDa and it is typically detected at ~140kDa in neuronal tissue (Williams, Sharp et al. 1999, Jaenisch, Witte et al. 2010). Hence it is unlikely that the neuronal band can be attributed to KCC2 detection.

I investigated whether NCC mRNA was absent from knockout tissues. RT-PCR analysis of tissue-derived cDNA, yielded a band of a size consistent with the presence of NCC mRNA (exons 3-17). NCC mRNA was detected in kidney, brainstem and TG of wild

type mouse. NCC was absent in kidney mRNA but still present in brainstem and trigeminal tissues of knockout animals (Figure 4.11 C). The band corresponding to NCC mRNA in TG of *NCC*^{-/-} mice was purified, cloned into pCR8/GW/TOPO vector and sequenced. The fragment contained all expected exons from 3-17 and was consistent with the wild type sequence of *SLC12A3* (Figure 4.11 D). No detection of the neomycin cassette was made. These results suggest that in neuronal tissue of *NCC*^{-/-} mice, NCC is transcribed in the wild type form and is translated into protein. It is assumed that resultant protein has full activity and thus the *NCC*^{-/-} mouse is not suitable for the study of NCC function in neurons.

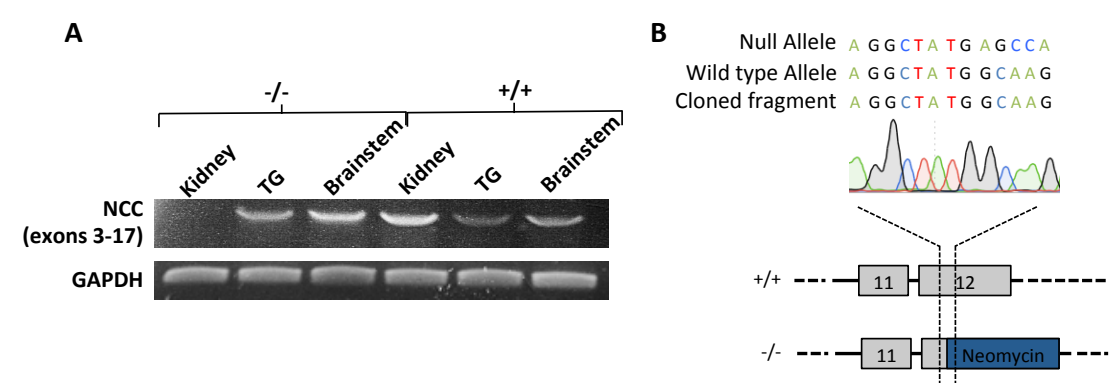


Figure 4.11 NCC mRNA is detectable in neuronal tissue of *NCC*^{-/-} animals. (A) RT-PCR analysis amplifying exons 3-17 of mouse NCC transcripts. mRNA was extracted from wild type and *NCC*^{-/-} tissues and reversed transcribed to cDNA, for subsequent PCR amplification. **(B)** Sequences of mRNA fragment amplified from *NCC*^{-/-} TG. Cloned sequence aligned 100% with wild type NCC with no detectable neomycin cassette.

4.4 Discussion

Identifying and performing functional follow up studies of disease-associated genetic variants offers significant potential in understanding migraine pathogenesis. The association of G264A *SLC12A3* with MA, is to date confined to one family. As such, functional work also serves the purpose of supporting an association that is unlikely to be replicated in future genetic studies. This is particularly pertinent in the case of *SLC12A3* as current literature provides little support for such an association. There is scant work detailing NCC function in tissues other than the kidney. In this chapter I have shown that expression of NCC is not restricted to the nephron, but is also present in migraine-salient neuronal tissues. More than this, expression appears to impact on the basic firing properties of neurons.

4.4.1 NCC expression in migraine-salient tissue

NCC is expressed in peripheral and central nervous tissue (Figures 4.2-4.5). These findings are contradictory to current literature suggesting a near total specificity of NCC for the kidney (Mastroianni, De Fusco et al. 1996). Data presented in this chapter is based on an antibody that has been validated (Schultheis, Lorenz et al. 1998) and extensively relied upon in the literature (Richardson, Rafiqi et al. 2008, Cui, Huang et al. 2011, Ledeganck, Boulet et al. 2011). Additionally, NCC mRNA was detected in all tissues found to stain positive in protein-based studies. One explanation for the contradiction between this work and previous studies, could be the spatial resolution of past experiments. Whole brain (and never peripheral nervous tissue) was previously analyzed and given the apparent region specific localization of NCC, this expression may go undetected in a larger pool of mRNA/protein.

In the periphery NCC is prevalent in sensory ganglia (Figures 4.3 and 4.5)-clusters of nerve cell bodies, which contain primary nociceptive afferents. There is a significant body of work detailing the functional expression of other Cl⁻ co-transporters in sensory neurons. NKCC1 and KCC2 are expressed by trigeminal neurons (Toyoda,

Yamada et al. 2005) and modulate excitability under normal (Zhu, Polley et al. 2008, Sipila, Huttu et al. 2009) and disease conditions (Schobel, Radtke et al. 2012, Gagnon, Bergeron et al. 2013). Due to the role of TRESK in TG neurons (Chapter 3), it is tempting to speculate that the sensory ganglia could be a tissue in which NCC and TRESK functions combine to affect physiology.

NCC is also present in hypothalamic nuclei; namely the PVN and SON (Figure 4.4). Both of these nuclei are heavily involved in water retention, with many neurons projecting directly to the posterior pituitary where they release vasopressin into the general circulation (Yang, Bertram et al. 2001). Under conditions of dehydration, vasopressin affects a variety of processes in the nephron, including up-regulation of NCC activity (Pedersen, Hofmeister et al. 2010), to increase water retention. From an evolutionary point of view, it is thus perhaps not surprising that we find NCC in structures outside of the kidney, but with still a large role to be play in kidney physiology. PVN and SON have also been implicated in descending pain modulation. Electrical stimulation of both nuclei alters pain threshold in rat (Yang, Chen et al. 2008, Yang, Yang et al. 2008) and NTG administration results in robust c-Fos activity in PVN and SON (Greco, Mangione et al. 2011). Tracing studies have shown that PVN neurons send descending projections to laminae I and outer II of Sp5C, and the superior salivatory nucleus (SSN) (Abdallah, Artola et al. 2013, Robert, Bourgeais et al. 2013). Microinjection of GABA_A agonists onto PVN neurons reduces basal and evoked responses of neurons in Sp5C (Robert, Bourgeais et al. 2013), suggesting that PVN modulation is primarily excitatory. The SSN controls the parasympathetic outflow to cephalic and ocular/nasal structures via the sphenopalantine ganglion (SPG). Activation of the SPG is known to induce plasma protein extravasation, vasodilation and local inflammatory factor release (Delepine and Aubineau 1997) that can activate meningeal nociceptors. As such, the structures in which NCC expression has been documented likely play a significant role in modulating the headache phase of migraine.

4.4.2 Neuronal function of NCC

Co-transporter channels are present in neurons and can affect excitability. The main mode of influence of Cl⁻ transporters is to alter the Cl⁻ reversal potential, to subsequently modify the strength and direction of GABA_A-induced currents (Rivera, Li et al. 2002, Yamada, Okabe et al. 2004). Adult sensory neurons are distinct in that [Cl⁻]_i is maintained high enough to shift the Cl⁻ reversal potential depolarized relative to RMP (Pieraut, Laurent-Matha et al. 2007, Rocha-Gonzalez, Mao et al. 2008). This renders GABA_A activation excitatory rather than inhibitory (De Groat, Lalley et al. 1972, Levy 1977). In this context, it is clear to explain the hyper-excitability phenotypes induced by up-regulation of NKCC1 (mediates Cl⁻ influx) and down-regulation of KCC2 (mediates Cl⁻ efflux) (Pfeffer, Stein et al. 2009, Seja, Schonewille et al. 2012). Data presented in this chapter shows that loss of NCC activity (and thus a presumed reduction in [Cl⁻]_i) leads to a hyper-excitability phenotype in sensory neurons (Figures 4.8). However it is important to consider that my data were generated in a cell autonomous fashion, in which NCC exerts its effects in the context of dissociated cultures, free of GABAergic influence. In this respect, it is interesting to note that while NKCC1 is believed to be an agent whose activity promotes hyper-excitability, prolonged pharmacological inhibition or genetic ablation renders neurons intrinsically more excitable. *Sipilla et al 2009* found that if they recorded from pyramidal cells of NKCC1^{-/-} mice in the presence of blockers of ionotropic glutamatergic and GABAergic transmission, spiking frequency was six-fold higher than that of wild-type neurons. In addition, this increase in intrinsic excitability could be achieved by prolonged (2-3h) exposure to the NKCC1 blocker, bumetanide (Sipila, Huttu et al. 2009). The authors postulate a compensatory mechanism that induces autonomous cell changes that would counter the systems level increase in GABAergic induced hyperpolarization.

The mechanisms involved in this phenomenon may be similar to those acting in the context of NCC. While no gross conductance changes were seen to be induced by NCC activity, a low level inward current was noted to be enhanced in SH-SY5Y cells upon overexpression of NCC (Figure 4.6) and reduced in TG neurons when NCC was inhibited (Figure 4.7). The identity of the ionic flow that this current represents is unclear. It is also unclear whether this current has any role in the firing phenotypes observed in primary neurons. In this regard, it is interesting to note that neurons wherein Cl⁻ leak currents have been abolished, are hyper-excitable and fire more action potentials in response to a supra-threshold current stimulus (Ratte and Prescott 2011). One of the few studies to look at NCC outside of the kidney explores the transporters function in the small intestine. Strong evidence for expression in rat small intestine is presented that marries well with clinical data, illustrating a significant role for NCC in Ca²⁺ homeostasis (Simon, Nelson-Williams et al. 1996). HCTZ induces a transient influx of Ca²⁺ in human HT-29 cells (Bazzini, Vezzoli et al. 2005). It would be pertinent to investigate whether HCTZ has similar actions in sensory neurons, as Ca²⁺ influx would be an appealing candidate as an initiator of signalling cascades that could subsequently lead to hyper-excitability.

4.4.3 Incomplete knockout of NCC

To determine a definitive mechanism for the observed hyper-excitability phenotype, it would be necessary to carefully dissect various currents. Trigeminal neurons lacking NCC protein would significantly aid this effort by circumventing the use of potentially non-specific pharmacology. In this chapter, I have described the characterization of a previously generated (Schultheis, Lorenz et al. 1998) and extensively used NCC knockout mouse model (Loffing, Vallon et al. 2004, Soleimani, Barone et al. 2012). The absence of NCC was confirmed in kidney tissue by Western blot, immunohistochemistry and RT-PCR (Figures 4.2, 4.9 and 4.10). However, using these same assays, NCC was

detected at both the mRNA and protein level in several neuronal tissues of knockout animals (Figure 4.10 and 4.11). These results could best be explained by non-specificity of detection assays. This is however unlikely, due to the validation undertaken in kidney tissue and the stringent specificity of techniques such as RT-PCR. Intriguingly, when NCC mRNA from NCC^{-/-} neuronal tissue was cloned and sequenced, the sequence corresponded to wild-type NCC, devoid of the ablation cassette (Figure 4.11 B). Such results are challenging to explain, but are most likely due to neuron-specific splicing events.

In summary, in this chapter I have shown NCC expression outwith the kidney and specifically in neuronal tissue that can be linked with migraine pathogenesis. Furthermore it has been demonstrated that NCC activity modulates primary neuron firing, such that loss of transporter function results in a hyper-excitability phenotype. This work provides functional evidence to support an association of a deleterious mutation in *SLC12A3* with migraine. It also serves to highlight the need to further investigate the novel role of NCC in neuronal physiology. Such work could have implications for both the understanding of migraine and in understanding the side effects of current therapies targeting NCC.

5. A cellular model for the study of migraine

5.1 Introduction

iPSC technology holds promise as a platform to both model disease and provide an *in vitro* substrate for drug screening efforts. Key to fulfilling this potential is the ability to efficiently and consistently derive a mature cell type of choice that is suitable for downstream assays. Migraine is a whole-brain disorder, and as such there are multiple cell types one might wish to access. Pertinent cell types will include sensory neurons, endothelial cells, astrocytes and a plethora of CNS neurons. Differentiation protocols have been described for some of these lineages (Chambers, Qi et al. 2012, Emdad, D'Souza et al. 2012, Lippmann, Azarin et al. 2012, Shi, Kirwan et al. 2012). Due to the prominent role of the TGVS in migraine, patient-derived iPSC modelling of primary afferents of the sensory ganglia could be of great benefit to our understanding of disease. While a protocol for the derivation of trigeminal neurons has only been published recently (Dincer, Piao et al. 2013) (after work for this chapter), success was first had in generating dorsal root ganglion neurons (Chambers, Qi et al. 2012).

To successfully utilize stem cell models it is key to understand the analogous endogenous developmental pathways to be recapitulated. *In vivo*, sensory neurons arise from the neural crest (NC) (Marmigere and Ernfors 2007). Situated at the border between ectoderm and neural plate, the neural crest is a transient embryonic structure that in vertebrates, gives rise to most of the PNS and other non-neural cell types (smooth muscle cells, melanocytes, craniofacial cartilage etc.). Multipotent neural crest cells are born of dorsal neural tube cells, which under the influence of Bone Morphogenetic Protein (BMP) and WNT signalling have undergone an epithelial to mesenchymal transition (Garcia-Castro, Marcelle et al. 2002). During an extensive period of neural crest migration, sensory neurogenesis takes place in successive waves.

The precise genetic cascades occurring during these waves gives rise to the three subtypes of sensory neuron: mechanoreceptors, proprioceptors and nociceptors. Within nociceptors, there are two distinct populations; peptidergic neurons that express neuropeptides such as CGRP and substance P, and non-peptidergic neurons that are devoid of neuropeptides and instead express the purinergic receptor P2X₃ (Burnstock 2000). Both populations originate from a common precursor expressing Runt-related transcription factor 1 (RUNX1) and neurotrophic tyrosine receptor kinase-A (TrkA) (Marmigere, Montelius et al. 2006). Terminal differentiation occurs post-natally in the mouse. Peptidergic neurons lose RUNX1 expression but maintain TrkA, rendering them responsive to NGF signalling. In reverse, non-peptidergic neurons retain RUNX1 expression but TrkA is diminished (Chen, Broom et al. 2006). Continued RUNX1 expression induces activation of common glial-derived neurotrophic factor family ligand receptor, Ret (Franck, Stenqvist et al. 2011) (Figure 5.1).

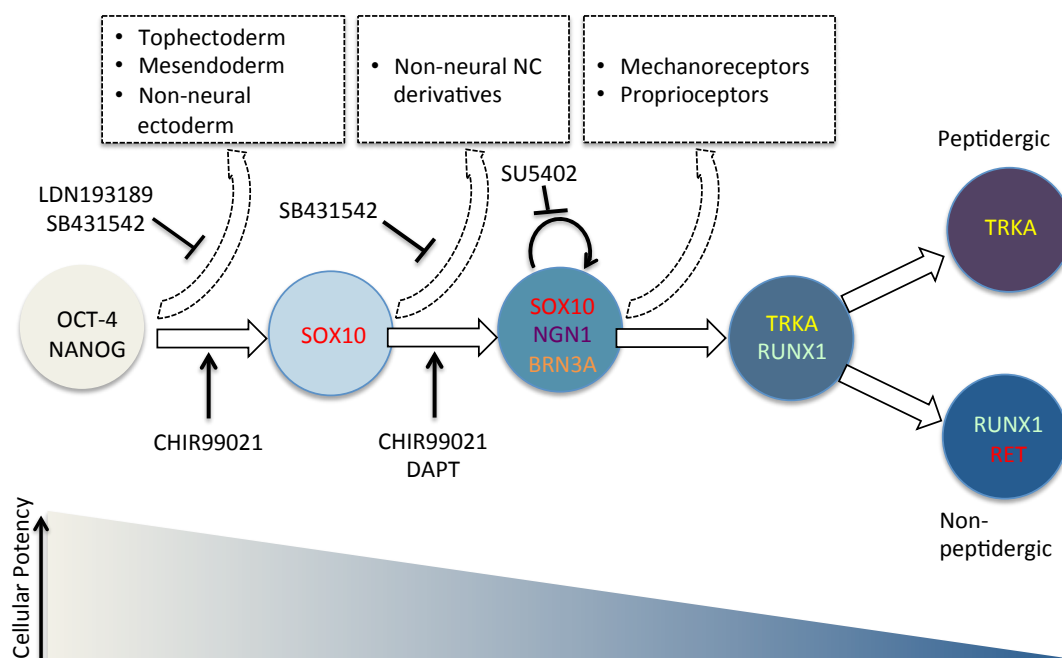


Figure 5.1 Sensory neurogenesis and likely points of action of small molecule inhibitors.

Pluripotent cells can differentiate into cells of all three of the germ layers. Dual-SMAD inhibition by the small molecules- SB431542 (TGF- β antagonist) and LDN193189 (BMP antagonist)

restricts differentiation to the neuroectoderm. Early in development, NC cells arise at the border of the neural plate and epidermis (Garcia-Castro, Marcelle et al. 2002) and induction is dependent on both WNT signaling and BMP inhibition (Kleber, Lee et al. 2005). Hence simultaneous LDN193189 and CHIR99021 (potent inhibitor of glycogen synthase kinase 3) will help direct cells towards the NC fate. SOX10 is vital for both the formation and maintenance of the NC (Kelsh 2006) and expression retains multipotency and inhibits neuronal differentiation (Kim, Lo et al. 2003). This transient SOX10⁺ population spans the pre-migratory and migratory stage of NC development and it is expression of neuregulins that determine sensory fate. High levels of neuregulin 2 (Ngn 2) biases SOX10⁺ progenitor cells down the sensory lineage and high enough levels causes sensory commitment and expression of neuregulin 1 (Ngn1), as well as post-migratory markers such as Brn3a. WNT activation has a key role in stabilizing neuregulins, acting therefore as another likely site of CHIR99021 action. FGF signaling arrests NC cells in the proliferative state and must be inactivated for neuronal differentiation to occur (Murphy, Reid et al. 1994). SU5402 has a range of inhibitory actions on multiple pathways including the FGF signaling pathway (Sun, Tran et al. 1999). Likely, one effect is to help induce terminal neuronal differentiation of proliferating progenitor cells. DAPT is a γ -secretase inhibitor with indirect inhibitory actions on Notch signaling. Notch signaling blocks neuronal differentiation and this is most likely the site of DAPT action. Sensory precursors commit to the nociceptive lineage through expression of RUNX1 over RUNX3 (mechano- and proprioceptive neurons) before making a final fate decision between peptidergic (TrkA⁺) or non-peptidergic (Ret⁺) identity.

Multiple groups have been successful in generating ES and iPSC-derived NC populations *in vitro* (Lee, Kim et al. 2007, Motohashi, Aoki et al. 2007, Pomp, Brokhman et al. 2008, Liu, Spusta et al. 2012, Wang, Tang et al. 2012). These have been shown to have the capacity to differentiate into multiple lineages including PNS lineages (sensory neurons and Schwann cells) and mesenchymal lineages (chondrogenic, smooth muscle, adipogenic and osteogenic cells) (Lee, Kim et al. 2007). Using a combination of five specific small molecule inhibitors, *Chambers et al* have shown rapid and efficient production of sensory neurons from iPSC. Within 10 days of culture, the population

contained >75% neurons which co-expressed sensory markers such as Brn3a (Chambers, Qi et al. 2012). Further maturation of these cells revealed expression and functional properties consistent with human nociceptors: such as functional TTX-resistant currents and response to noxious stimuli ATP and capsaicin. The timescale for this *in vitro* process is considerably shorter than *in vivo*, and suggests that targeting of specific signalling pathways with small molecules may be able to overcome a main hurdle of iPSC technology, namely the lengthy culturing conditions.

5.2 Aims of Chapter 5

The overarching aim of this chapter is to develop an iPSC cellular model for the study of migraine. To this end, this chapter is devoted to:

- 1) The optimisation of protocols (based on *Chambers et al 2012*) for the differentiation of nociceptive neurons (akin to those in the dorsal root ganglion) and to characterise these cells both in terms of expression and function.
- 2) To assess the relevance of derived cells to the investigation of migraine-associated gene products.

5.3 Results

5.3.1 Induction of a NC intermediate population

Using an iPSC line derived from a healthy subject, (AH017-7, kindly provided by Dr. Sally Cowley) I cultured cells as per the published protocol (Chambers, Qi et al. 2012) with the aim of generating sensory neurons. Before differentiation was initiated, cells were assessed for DNA integrity and expression of pluripotency markers. CytoSNP (Illumina) karyotyping revealed normal chromosomal structure and detected only two regions of over-representation (Figure 5.2 A). CytoSNP utilizes an array of 300,000 SNP markers and so has higher resolution, even when compared to high-resolution G-banding (10Kb vs 3Mb) (Shaffer and Bejjani 2004, Dussailant, Serrano et al. 2012). We determined a line to pass karyotype quality control if 5 or less over/under-representations were detected and if these regions were <1Mb in size. Affected regions were further assessed for the presence of genes with known roles in sensory neuronal physiology. Applying these criteria, AH017-7 line was deemed to have passed quality control. Cells showing no morphological signs of aberrant differentiation and exhibiting uniform OCT-4/NANOG expression were they taken forward for differentiation (Figure 5.2 B). Cells were seeded at a low density and cultured with small molecule inhibitors at the appropriate times.

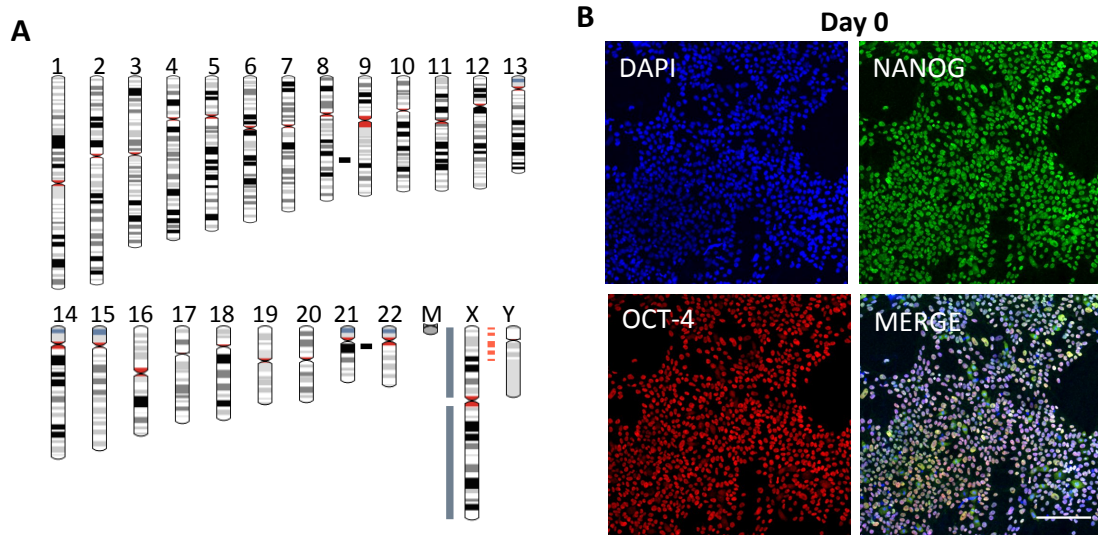


Figure 5.2 AH017-7 is a karyotypically normal pluripotent stem cell line. (A) Karyogram of AH017-7 DNA. Karyotyping reveals acceptable DNA integrity with only two (black bands parallel to chromosome) instances of over-representation. Cells were used for differentiation experiments within 5 passages of this stage. **(B)** Cells homogenously stain positive for pluripotency markers OCT-4 and NANOG. *Scale bars represent 200μm*

When applying differentiation protocols it is important to track temporal development of the cell population, to confirm that they represent the terminal cell type you wish to study. OCT-4 expression was reduced to a very low level in all cells by day 10 suggesting efficient, whole-population loss of pluripotency (Figure 5.3 A). Expression of key NC genes was documented through early time-points of differentiation. Paired box 3 (Pax3) is known to both initiate NC specification from early embryonic lineages, but also to drive migration and differentiation of NC derivatives (Milet, Maczkowiak et al. 2013). In our system, Pax3 is activated by day 5 and remains present up to day 15 (Figure 5.3 B). Group E Sox genes (Sox8, Sox9 and Sox10) are highly expressed in the prospective NC (Cheung and Briscoe 2003). Sox9 expression precedes the appearance of NC pre-migratory markers and biases NC formation over CNS neuronal differentiation (Cheung and Briscoe 2003, Lee, Aoki et al. 2004). Up-regulation of Sox9 as early as day 2 is

observed, with a peak at day 9 (Figure 5.3 B). Sox10 is the central regulator of NC development. It is involved in the formation and maintenance of multipotency of the NC (Kelsh 2006). Low expression of Sox10 is observed by day 2, but this is up regulated to higher levels and a peak at day 9 (Figure 5.3 B). Cells are passaged at day 10 to allow for accelerated maturation. The decrease in Sox9/10 levels post day 10 is consistent with a loss of multipotency and lineage restriction. To determine whether NC induction was population wide or restricted to a subset of cells, ICC was performed at different time-points. The transcription factor, AP2- α , is integral to NC development: loss of the protein results in defects of NC development (Schorle, Meier et al. 1996) and forced expression of AP2- α is sufficient for NC induction in frog embryos (Luo, Matsuo-Takasaki et al. 2002). AP2- α is rapidly induced by day 3 and continues to increase to day 6. By day 6 >95% of cells stained positive for nuclear AP2- α (Figure 5.3 C). Another marker of NC, p75 neurotrophin receptor (p75^{NTR}), is also found present in a large percentage of the population by day 8 (Figure 5.3 D). Such results illustrate that under our culturing conditions, pluripotent cells are driven down the NC lineage in an accelerated and homogenous fashion.

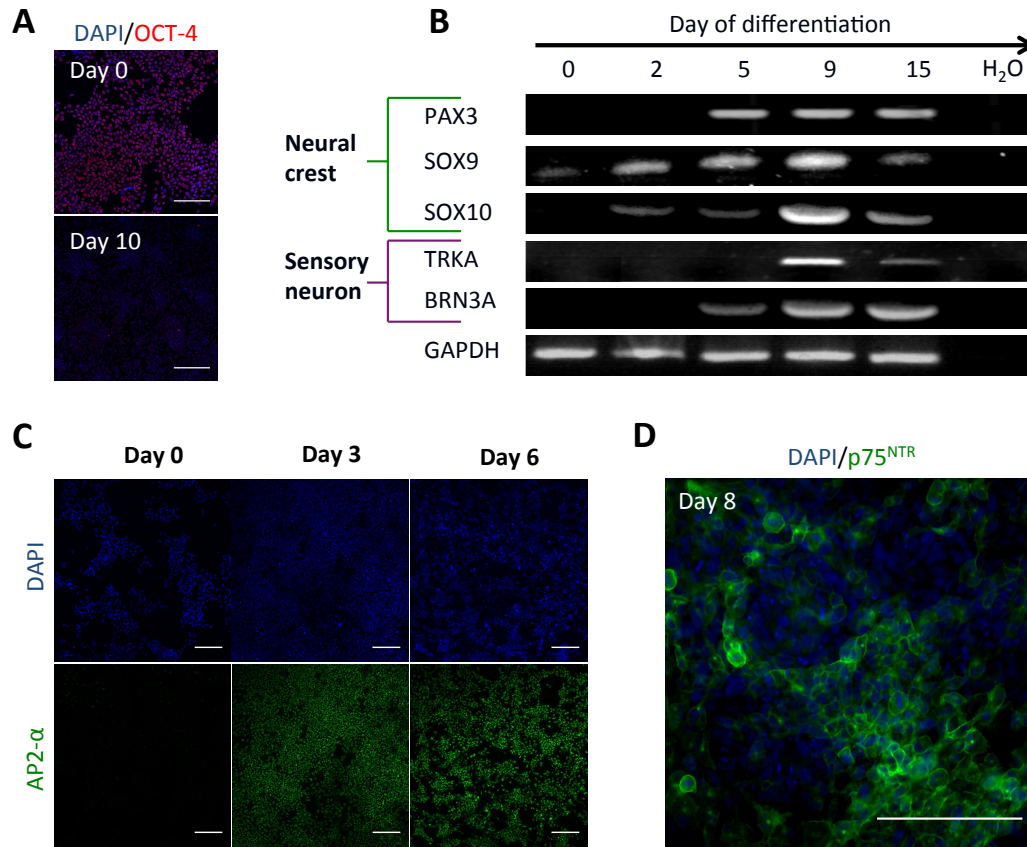


Figure 5.3 Small molecule inhibitors rapidly induce expression of NC markers. (A) Expression of the pluripotency marker OCT-4 at day 0 (pre-differentiation) and day 10, showing a reduction in expression during this time. **(B)** RT-PCR analysis of mRNA extracted from cultures at progressing time-points. Transcripts for NC/sensory neuron identity were detected with increasing levels during differentiation. GAPDH serves as an internal control. **(C)** Time-course of expression of the NC marker, AP2-α. **(D)** Supporting evidence for NC identity with high expression of p75^{NTR} by day 8 in culture. Scale bars represent 200μM

5.3.2 Functional sensory neurons after 15 days

NC cells give rise to multiple lineages: both neuronal and non-neuronal. By Day 13, cells exhibit preliminary processes that resemble neurites. Staining for the pan-neuronal marker β-Tubulin III (Tuj I) reveals that $39.6 \pm 8.3\%$ (mean $\pm$ s.e.m efficiency, derived from three independent experiments) of cells have converted to the neuronal fate. More

specifically, >90% of Tuj I⁺ cells co-express the sensory markers Brn3a and Islet-1 (Figure 5.4 A and B). Co-expression of Brn3a and Islet-1 is common across all sensory subtypes (Anderson 1999, Fedtsova, Perris et al. 2003): mechanoreceptors, proprioceptors and nociceptors. Subsets can be distinguished by their specific expression of neurotrophic factors (Marmigere and Ernfors 2007). Cells up-regulate TrkA by day 9 (Figure 5.3 B) suggestive of a nociceptive lineage (Fang, Djouhri et al. 2005). Peripherin is a type III intermediary filament protein expressed by neurons of the PNS and associated with unmyelinated (C-fiber) and thinly myelinated (A- δ fiber) nociceptors (Rose, Lunardi et al. 2013). Low-level, but robust co-expression with Brn3a was noted by day 15 (Figure 5.4 C). For any workable neuron based model, cells must be able to respond to depolarizing stimuli by firing an action potential. Patch clamp analysis of day 15 neurons was performed in current-clamp mode. Increasing increments of depolarizing current was injected until cells fired an action potential. Even at this early stage, cells were capable of action potential generation (Figure 5.4 D). However, neurons had a depolarized resting membrane potential and a low density of ionic currents in response to depolarizing potentials, indicating that neurons were still immature (Figure 5.4 E). This was particularly evident in relation to voltage-activated inward currents (Figure 5.4 F). In sum, day 15 neurons were functional and showed signs of restriction to the nociceptor lineage, but were at too immature a stage to do meaningful biophysical assessments.

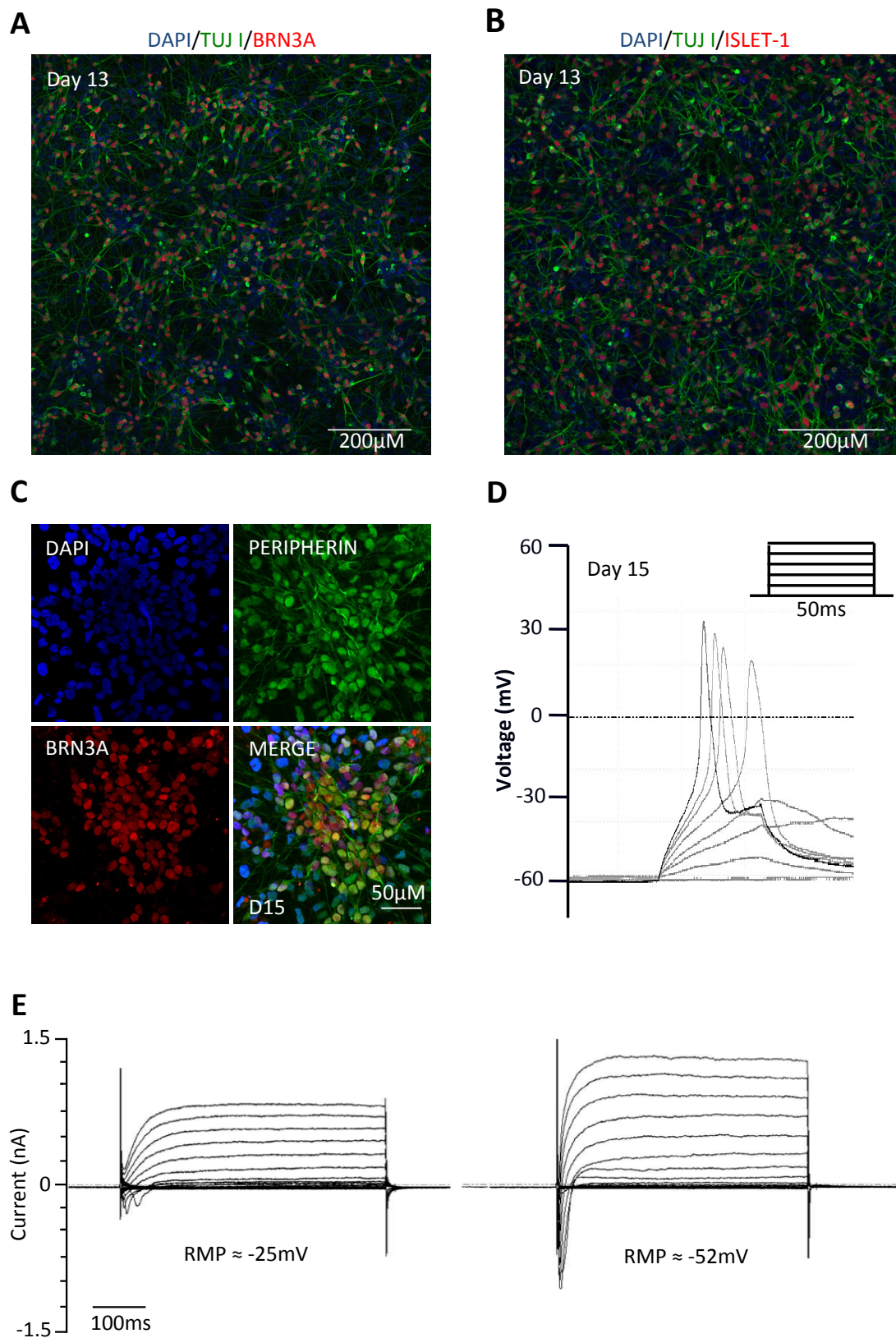


Figure 5.4 Efficient generation of functional sensory neurons within 15 days of culture. (A) By day 13, the majority of cells co-express the neuronal marker β -tubulin III, with the prototypical sensory marker Brn3a. **(B)** β -tubulin III⁺ cells are found also positive for a second

sensory marker, Islet-1. **(C)** Robust expression of peripherin is noted in Brn3a neurons at day 15. **(D)** Patch clamp recording from a day 15 neuron illustrating that neurons can illicit an action potential in response to increasing increments of depolarizing current. **(E)** Representative traces of currents evoked by serial potential steps (-80mV to +30mV in 10mV increments) of a day 15 neuron (*left trace*) and a post-natal mouse DRG neuron (*right trace*) of similar capacitance. Resting membrane potentials represent an approximation of values from cell groups assessed.

5.3.3 Maturation of iPSC-nociceptors takes several weeks

Estimates suggest that during human development nociceptors emerge after 30-50 days (Kitao, Robertson et al. 1996). Thus it is not surprising that after only 15 days in culture, iPSC-nociceptors lack complete maturity in terms of electrophysiological function. Cells were further maintained in a cocktail of neurotrophic factors until day 50, in an attempt to enhance maturation. If cells were challenged with an anti-mitotic agent (4 μ M Cytosine β -D-arabinofuranoside) at day 14 of culture, cultures were almost entirely homogenous for sensory neurons at day 50 (Figure 5.5). Likely due to the high levels of growth factor, neurons exhibited extensive neurites and organized into clusters reminiscent of ganglionic structures (Figure 5.5).

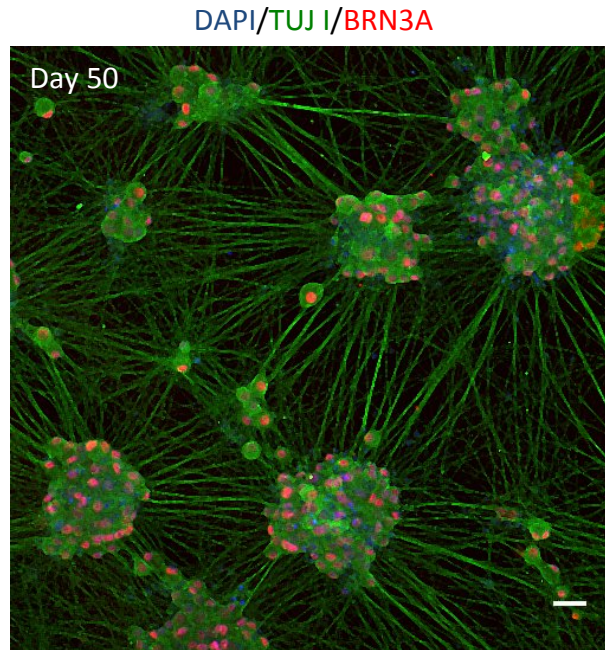


Figure 5.5 Culture maturation. Immunocytochemistry of 50-day old cultures reveals an almost entirely homogenous population of sensory neurons. Neuron bodies can be seen clustering in “ganglion-like” structures. *Scale bar represents 50μm.*

Late stage neurons displayed more physiological firing behaviour in response to depolarization. A “shoulder” during the falling phase of an action potential is associated with C-fibre nociceptors (Lopez de Armentia, Cabanes et al. 2000): a phenomenon that has been attributed to voltage-activated TTX-resistant Na^+ channels and high voltage-activated Ca^{2+} currents that are not totally inactivated during the falling phase (Blair and Bean 2002). 25% (9/36) of cells tested exhibited a shoulder during the falling phase of action potentials (Figure 5.6 A) and mean action potential duration was 3.2 ± 0.3 ms (mean $\pm$ s.e.m duration, derived from three independent experiments). This duration is consistent with previous recordings of small-diameter DRG neurons (Blair and Bean 2002). During post-natal maturation, sensory neurons develop the ability to fire trains of action potentials (Cho, Furness et al. 2011). In our culture 17% (13/53) of neurons fired multiple action potentials in response to a prolonged current stimulus (Figure 5.6 B). Repetitive firing was never observed in day 15 cultures and mirrors maturation of

this function that has been observed in other iPSC-neuron cultures (Shi, Kirwan et al. 2012). $\text{Na}_v1.8$ is selectively expressed in both rodent and human nociceptors (Dib-Hajj, Tyrrell et al. 1999) and has been implicated in mediating inflammatory pain (Porreca, Lai et al. 1999). Using a voltage-clamp protocol, an inward current was evoked when cells were stepped from -60mV to 0mV. This current was only partially blocked by TTX (2/5 cells), suggesting the presence of TTX-resistant Na^+ or voltage-gated Ca^{2+} currents. Inward current were further blocked by the $\text{Na}_v1.8$ -specific inhibitor- A803467 (Figure 5.6 C). $\text{Na}_v1.8$ is selectively expressed by nociceptive sensory neurons (Dib-Hajj, Tyrrell et al. 1999) and as such these results indicate that nociceptor-specific currents are present in a proportion of our neurons. It is not clear whether cells in which inward current was completely abolished by TTX treatment represent a mature population of non- $\text{Na}_v1.8$ expressing neurons, or cells at an earlier developmental stage that will eventually express $\text{Na}_v1.8$. To gain insight into how accurately iPSC-derived neurons recapitulate primary neurons, electrophysiological properties were compared between day 50 iPSC-derived nociceptors and a heterogeneous population of small/medium diameter DRG neurons, acutely dissociated from P12-20 mice. Resting membrane potential and rheobase were comparable between the two groups. iPSC-neurons had larger capacitance values, likely a consequence of the elongated culture and the development of significant neurites. iPSC-neurons also have reduced input resistance; however these values are within the reported range for sensory neurons (Ma, Greenquist et al. 2006). These results are consistent with neurons being at a mature stage after 50 days of culture.

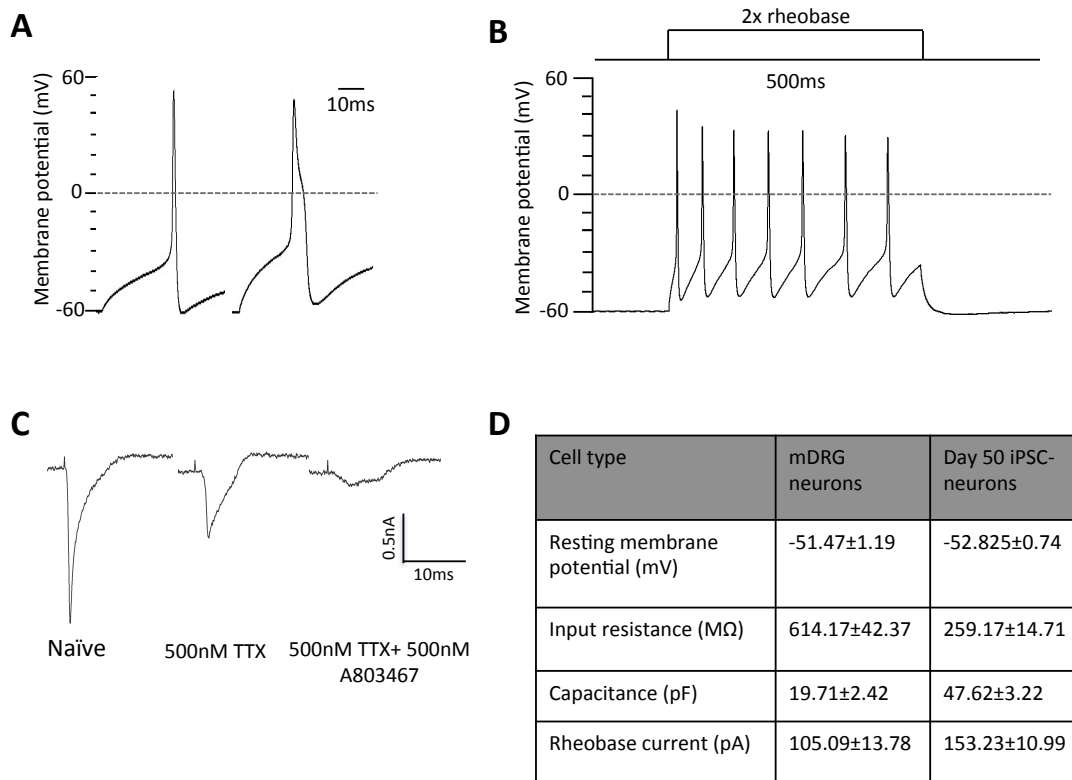


Figure 5.6 Cells require several weeks in culture to develop into a mature biophysical state. **(A)** Action potential morphology of day 50 neurons. Following multiple weeks cultured with growth factors, a proportion of neurons display action potential morphology typical of *in vivo* nociceptors (note “shoulder” during falling phase of *right trace*). **(B)** A train of action potentials generated in a day 50 neuron by a 500ms current injection of 2x rheobase amplitude. **(C)** Example voltage-clamp recording showing the presence of both TTX-sensitive and TTX-resistant Na^+ currents, where the $\text{Na}_v1.8$ blocker A-803467 further blocks the TTX-resistant component. **(D)** Biophysical properties comparing day 50 neurons and adult mouse DRG neurons. *Results represent mean $\pm$ s.e.m of at least 6 cells per group.*

5.3.4 Non-peptidergic identity of iPSC-nociceptors

Nociceptors can be sub-classified as peptidergic (neuropeptide containing) or non-peptidergic (devoid of neuropeptides and binding IB4) (Zylka, Rice et al. 2005). The two classes have been shown to have distinct neurophysiology (Stucky and Lewin 1999, Cavanaugh, Chesler et al. 2011) and differing roles in pain pathways (Cavanaugh, Lee et

al. 2009). Hence, I sought to define which population the iPSC-nociceptors most resembled. RUNX1 expression specifies nociceptors during sensory neurogenesis and maintained expression drives the formation of TrkA⁻ non-peptidergic neurons. Conversely, loss of RUNX1 activity leads to sustained TrkA expression and a peptidergic phenotype (Marmigere and Ernfors 2007). I sought to document RUNX1 expression throughout the first 24 days of nociceptor induction to gain insight into the early processes of fate decision. Expression was initiated by day 9 and following an initial decrease, expression increased to maturity (Figure 5.7 A). C-Met activity, in concert with NGF signalling, is required for extinction of RUNX1 activity and proper peptidergic differentiation that results in activation of neuropeptides (Gascon, Gaillard et al. 2010). c-Met expression saw a peak at day 13 before a sharp reduction back to pre-differentiation levels by day 15 (Figure 5.7 A). The peak of c-Met expression correlated with the transient decrease in RUNX1 levels, which is consistent with the notion that c-Met and RUNX1 orchestrate mutually antagonistic pathways (Gascon, Gaillard et al. 2010) during sensory neurogenesis. A sustained RUNX1, and only transient c-Met expression profile is consistent with non-peptidergic differentiation. In harmony with this, TrkA levels drop from a peak at day 9 to day 15 (Figure 5.3 B).

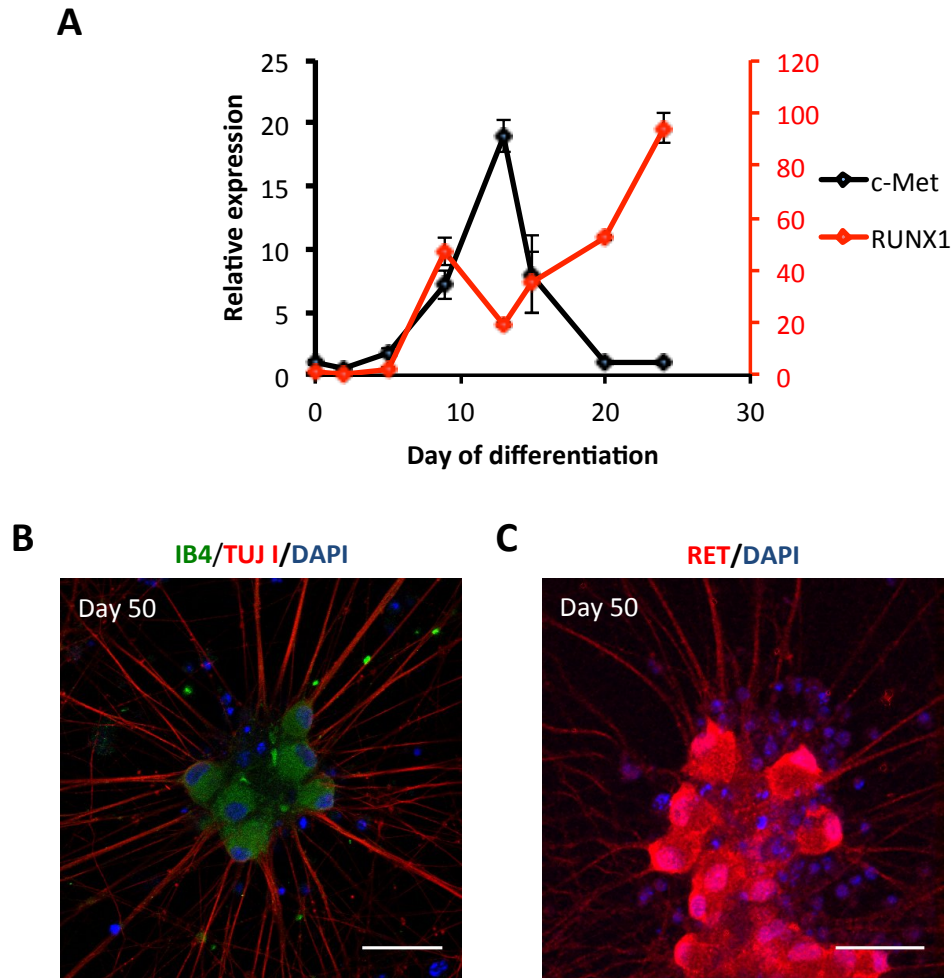


Figure 5.7 Sensory neurons can be sub-classified as non-peptidergic. (A) Expression profile of key peptidergic vs. non-peptidergic transcription factors; c-Met and Runx1. Quantitative PCR analysis throughout the first 24 days of culture shows an expected pattern of expression for a non-peptidergic population. **(B)** Neuron cell bodies are positive for binding to isolectin IB4 **(C)** and **(C)** Ret. *Results represent mean $\pm$ s.e.m of a single experiment with technical triplicates*

In support of the developmental profile, mature neurons bound isolectin IB4 (Figure 5.7 B) but had no detectable CGRP expression. Further, neurons stained positive for common glial-derived neurotrophic factor family ligand receptor, Ret (Figure 5.7 C)- another non-peptidergic marker (Franck, Stenqvist et al. 2011). Functionally, non-peptidergic sensory neurons can be identified by the expression of P2X₃ receptors that are activated by ATP (Zylka 2005). Over 50% of Tuj I⁺ neurons expressed P2X₃R by day 20 in culture (Figure 5.8 A). By day 35, the majority of neurons tested (4/5) responded

to topical application of 30 μ M Methylene-ATP. Drug application induced an inward current that was either fast or slowly inactivating (Figure 5.8 B). In contrast, no neurons responded to application of the classical peptidergic agonist, capsaicin (Cavanaugh, Chesler et al. 2011). These data illustrate that cells follow a developmental pathway consistent with non-peptidergic differentiation and result in neurons with functionality consistent with this phenotype.

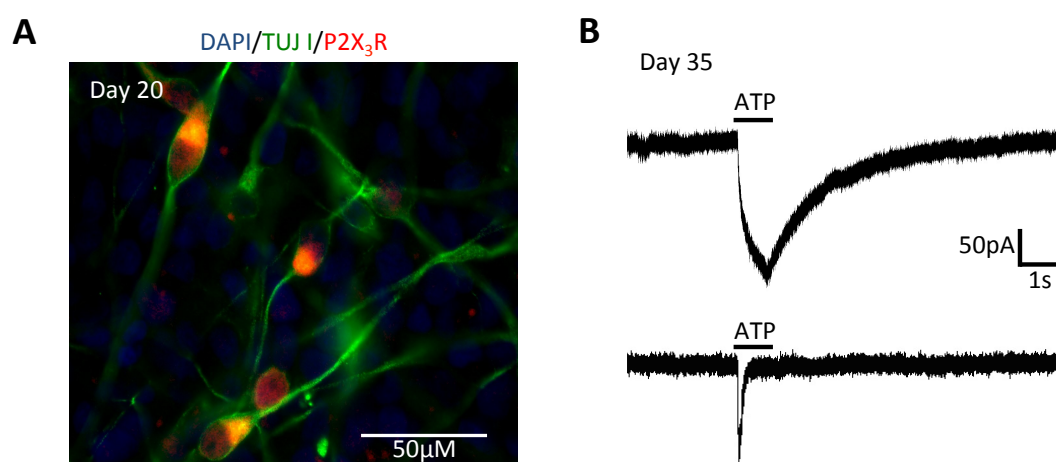


Figure 5.8 Neurons possess functional purinergic receptors. (A) A sub-population of day 20 iPSC-neurons stain positive for P2X₃ receptors. **(B)** The majority of cells tested responded to focal application of ATP, by inducing an inward current. Representative traces of slowly (*top*) and fast (*bottom*) inactivating currents. Cells were held at -60mV and drug was transiently applied (1s) directly to the cell surface.

5.3.5 iPSC-derived neurons express TRESK and NCC

The rationale behind establishing the described cellular system is to have a physiological model in which to study the functional effects of migraine-associated *KCNK18* and *SLC12A3* variants. Hence, it was imperative to assess whether iPSC-derived neurons express TRESK and NCC. By day 50, mRNA transcripts for both genes could be detected, with TRESK amplification generating high, and NCC low, levels of product (Figure 5.9 A). I wished to document the temporal pattern of TRESK expression to define a time at

which the channel would likely have a significant role in neuronal behaviour. Transcript levels peaked at day 5, before returning to baseline and then steadily increasing until maturity (Figure 5.9 B). The finding of a spike in TRESK expression during early stages of differentiation is of interest, as K_{2P} channels are postulated to play a role in neuronal development (Ramadoss, Lunde et al. 2008, Kim, Cho et al. 2012). Cells at day 50 stained positive for TRESK (Figure 5.9 C), but did not support NCC protein expression (data not shown). α -NCC recognizes human NCC (Figure 4.2 D) and so these results would suggest that either: protein levels are too low to detect, or that there is a disconnect between presence of mRNA and protein.

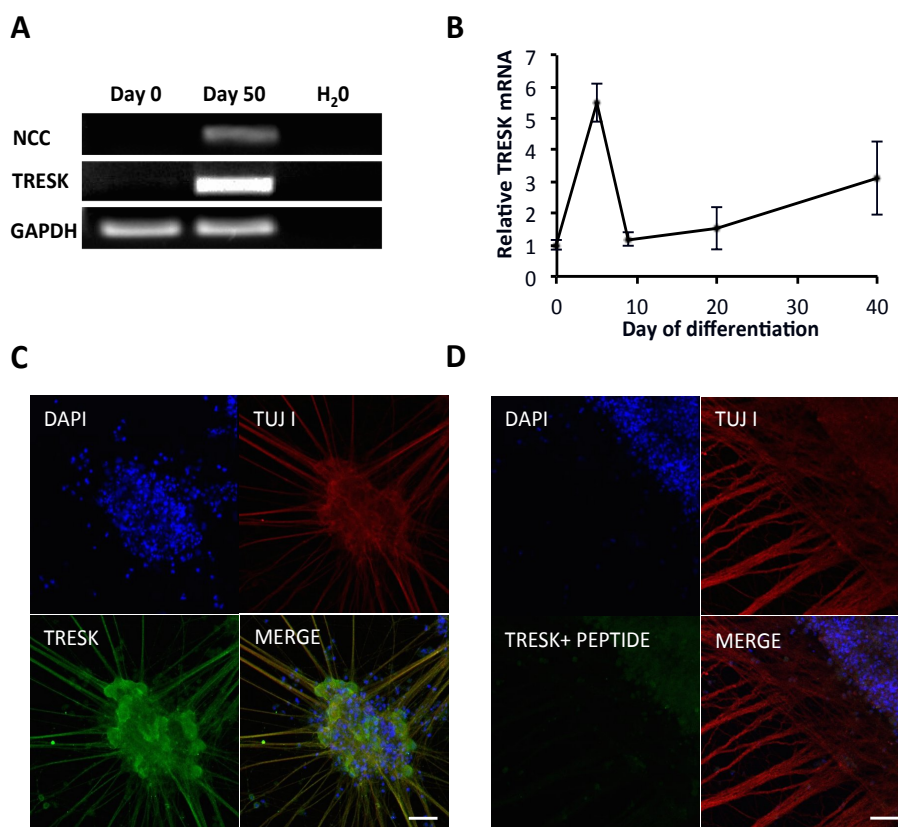


Figure 5.9 TRESK and NCC expression in 50-day old iPSC nociceptors. (A) RT-PCR detection of TRESK and NCC mRNA. GAPDH serves as an internal control. **(B)** Quantitative analysis (qPCR) of TRESK expression during differentiation. **(C)** Cells stained positive for TRESK (α -human TRESK, Santa Cruz Biotechnology 51238). **(D)** No signal was detected when TRESK antibody was

pre-incubated with a blocking peptide (Santa Cruz Biotechnology 51238 P). *Results represent mean $\pm$ s.e.m of a single experiment with technical triplicates*

In whole-cell voltage clamp mode, and in response to membrane depolarization, neurons exhibited an outward current of similar magnitude to mouse primary neurons (Figure 3.9 A and 5.10 A). Application of 500nM TPA reduced the amplitude of this current by 17.31%, although challenges with maintaining a viable patch precluded the testing of many neurons. These results illustrate the presence of functional TRESK channels by day 50 of differentiation, and along with the maturity data- suggest that this may be a profitable time-point at which to assess neuron physiology.

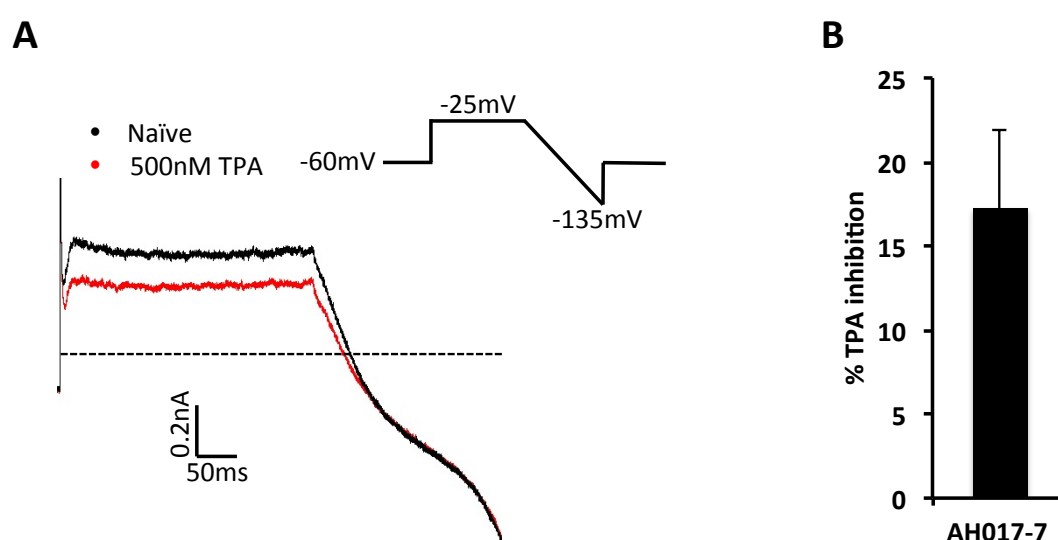


Figure 5.10 Functional TRESK currents in iPSC-neurons. (A) Representative trace of current induced by prolonged depolarization to -25mV; followed by ramping to -135mV at 0.2mV/ms. Current was inhibited by perfusion for 20 minutes of 500nM TPA. **(B)** Quantification of the reduction in outward current, as measured at the end of the depolarizing step, following TPA treatment. *Results represent mean $\pm$ s.e.m of four cells*

5.3.6 Relevance to other migraine-associated proteins

The system described should be of great use for interrogating the peripheral aspect of migraine pathophysiology. Multiple GWAS-discovered and FHM-associated genetic variants have been shown to have a role in peripheral sensory neurons. I wished to define whether key migraine-associated proteins were present in mature iPSC-nociceptors, to assess the broader applicability of this system to model migraine biology. TRPM8 is a cold-sensitive ion channel (McKemy 2005) and variants have shown a protective effect in migraine (Chasman, Schurks et al. 2011). iPSC-derived neurons uniformly express this channel (Figure 5.11 A). Triptans are the gold standard medication for migraine treatment and are thought to confer their efficacy partially through binding to 5-HT_{1D} receptors of primary afferents (Tepper, Rapoport et al. 2002). 5-HT_{1D} was found expressed by a majority of neurons (Figure 5.11 B). Ca_v2.1 is a high voltage-activated Ca²⁺ channel and gain of function mutations lead to FHM1 and enhanced excitability of TG neurons (Hullugundi, Ansuini et al. 2014). Significant staining of neuronal cell bodies was seen by day 50 (Figure 5.11 C). Consistent with what is observed in native tissue, Ca_v2.1 was highly localized to discrete puncta (Wheeler, Groth et al. 2012). One role of Ca_v2.1 is to couple depolarization with neurotransmitter release and so it is interesting to observe enriched staining at neurite endings in addition to the cell body (Figure 5.11 D). All FHM-associated and multiple GWAS-discovered genes have a proposed function at the glutamatergic synapse (Figure 1.3). iPSC-derived neurons express the glutamate transporter VGLUT-1 (Figure 5.11 E), suggesting that they may be a suitable model for investigating glutamate transmission. Hence, I have shown that the neurons generated have an expression profile that would allow investigation of other migraine-associated proteins.

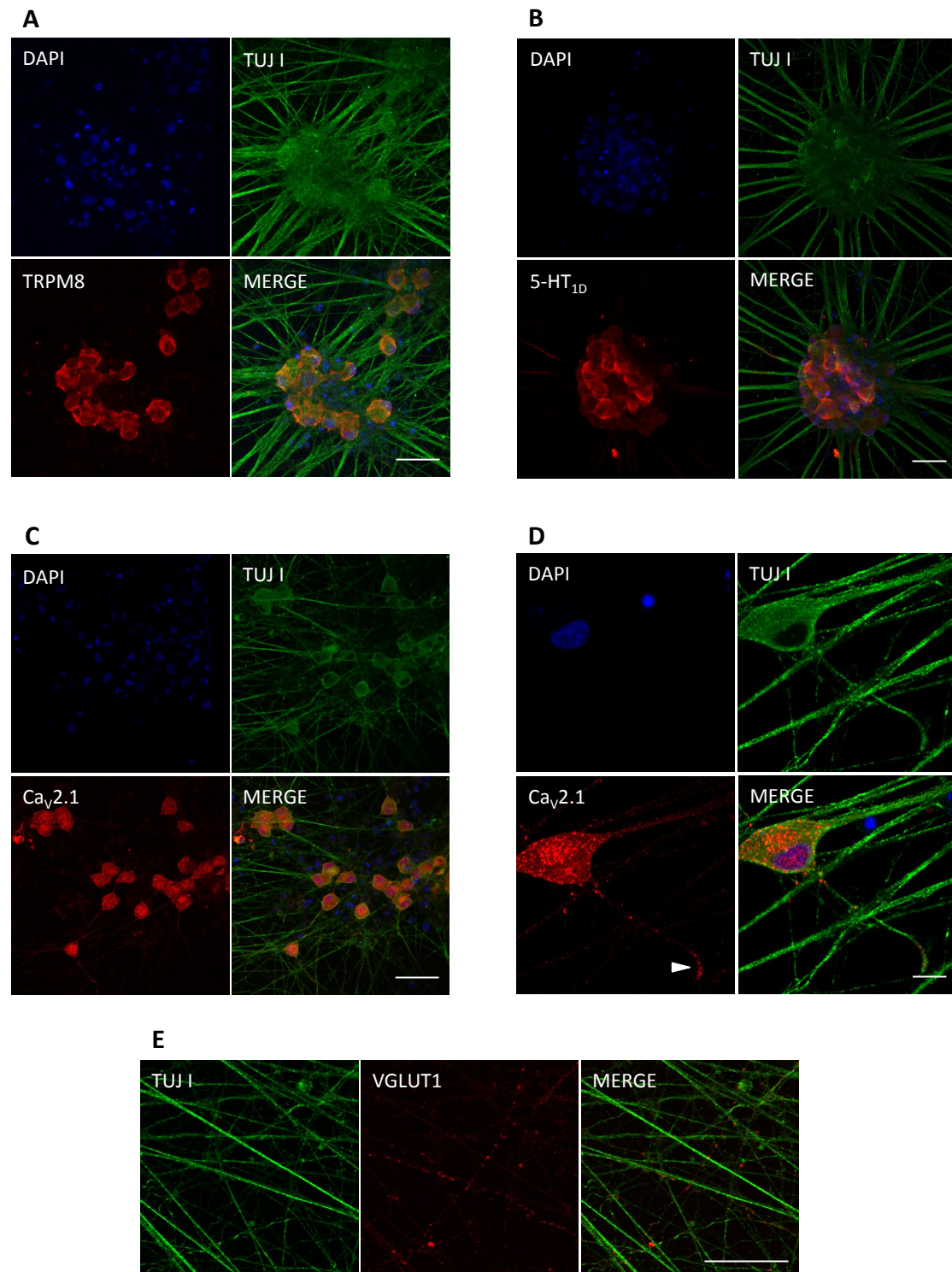


Figure 5.11 Expression of migraine relevant proteins. Immunocytochemistry was performed on day 50 neurons to assess their relevance to migraine-associated genes. **(A)** Expression of the cold-sensitive ion channel TRPM8. **(B)** Neurons express the serotonin receptor 5-HT_{1D} predominantly on the cell body. **(C)** High levels of Ca_v2.1. **(D)** Higher magnification image revealed punctate localization of Ca_v2.1 protein, predominantly localized to the cell body and

neurite endings (*white arrow*). *Scale bar represents 10 μ M.* **(E)** Expression of the glutamate transporter V-Glut 1 is seen in the majority of neurites. *Unless otherwise stated, scale bar represents 50 μ M.*

5.3.7 iPSC-derived neurons offer advantages over traditional models

A significant advantage of iPSC technology over traditional cell models is that terminal cell types are of human origin. This is particularly pertinent when investigating the function of genes that have substantial genomic sequence differences between species. TRESK presents as a likely candidate for regulation by estrogen due to the higher incidence of migraine in females, but also due to previously documented effects of estrogen on expression of other K_{2P} channels (Alvarez-Baron, Jonsson et al. 2011). Using Dragon ERE (Estrogen Response Element) 3.0 software, I identified a region 5767-5783bp upstream of exon 1 of human *KCNK18* that had a sequence likely to act as an ERE. The sequence differed to the classical ERE motif by 2bp. The motif was present in the same region of mouse DNA but differed by 3bp (Figure 5.12 A). 17- β estradiol failed to modulate TRESK expression when applied for 24hrs onto mouse DRG cultures (Figure 5.12 B). *In vitro* and *in vivo*, sensory neurons express both estrogen receptor alpha (ER- α) and beta (ER- β) (Patrone, Andersson et al. 1999) and activation of these receptors results in a variety of pain-related expression changes (Chaban and Micevych 2005, Hu, Wang et al. 2012). By day 50 of differentiation, mRNA was detectable for both receptors in iPSC-neuron cultures (Figure 5.12 C), illustrating their utility for estrogen-associated expression studies. Due to the higher stringency to which the human sequence matches the ERE motif, I investigated whether estrogen affected TRESK expression in these cells. 5 μ M 17- β estradiol caused a 3-fold reduction in TRESK levels (Figure 5.12 C). These results illustrate that estrogen treatment modulates human, but not mouse, TRESK transcription. As such, these data serve as an example of the advantage of iPSC-derived sensory neurons over traditional primary culture models.

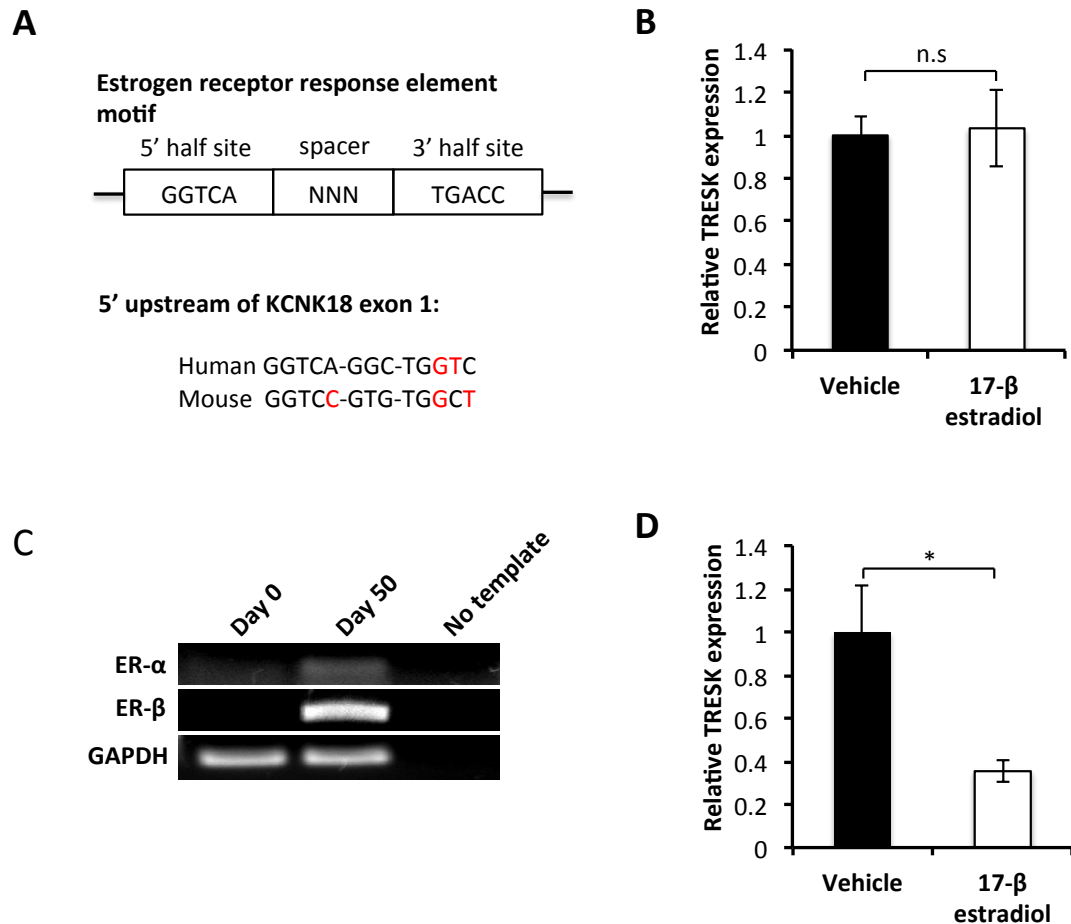


Figure 5.12 17-β estradiol regulates human, but not mouse TRESK transcription. (A) Estrogen Response Element (ERE) motif and likely ERE sequences in region upstream of human and mouse *KCNK18* exon 1. Mouse sequence harbours more (3- red bases) base pair mismatches to motif sequence than human sequence. (B) Dissociated neuron cultures taken from adult mouse DRG tissue were cultured with vehicle or 5μM 17-β estradiol for 24hrs before mRNA was extracted and relative TRESK levels quantified by qPCR. Rpl19 served as an internal control. (C) RT-PCR for the detection of ER-α/β receptor mRNA in day 50 iPSC-nociceptor cultures. (D) Day 50 neurons were exposed to vehicle or 5μM 17-β estradiol for 24hrs before mRNA was extracted and relative TRESK levels quantified by qPCR. GAPDH served as an internal control. * $P < 0.05$, Unpaired Student's *t*-test, results represent mean $\pm$ s.e.m of four independent experiments

5.4 Discussion

Polygenic disorders like migraine are arguably over simplified when modeled by single genetic variants studied in isolation and within traditional cell models. Key components of an optimal cell model are to have the relevant human cell type, incorporating all disease modifying genetic factors. iPSCs derived from migraine patients and re-differentiated to mature and migraine-relevant cell types, offer the potential to fulfill these criteria. In this chapter, I have detailed the generation of sensory neurons from a control iPSC clone, as proof of concept for creating such a disease model. Neurons were produced quickly and matured to perform functions expected of endogenous nociceptors. Furthermore, cells were shown to have direct applicability for the study of a variety of migraine-associated proteins. Optimized protocols can be applied to patient clones in order to compare and define differences between migraine-derived and control iPSC- neurons. These differences should be illuminating for understanding the cellular basis of migraine.

A cocktail of small molecules efficiently induce neuronal differentiation. Cells pass through intermediary developmental stages (Figure 5.3) before committing to the sensory neuron lineage. Brn3a⁺/Islet-1⁺ neurons appear within 13 days of culture, and even at this early time-point express specialized proteins that define terminal nociceptors (Figure 5.4). We can be confident in the sensory identity as while both transcription factors have been found in other cell types (Nadal-Nicolas, Jimenez-Lopez et al. 2009, Kobayashi, Nakano et al. 2011), their co-expression appears specific for sensory neurons (Dykes, Tempest et al. 2011). However, these neurons are not fully mature at this time-point- as evident by a markedly depolarized membrane potential and a low density of voltage-gated ionic currents (Figure 5.4). The interplay between RUNX1 and c-Met expression would also seem to suggest that neurons are still yet to fully commit to their terminal lineage. It could be approximated that day 15 in culture

resembles the post-migratory population observed at E10.5-11.5 during mouse development (Marmigere and Ernfors 2007). It takes several weeks of further culture to produce neurons with mature electrical properties that also respond appropriately to nociceptive stimuli (Figure 5.6). Hence the differentiation process *in vitro* seems to proceed at a similar rate to *in vivo* estimates (Kitao, Robertson et al. 1996). Nevertheless, this differentiation scheme is much accelerated in comparison to other iPSC-derived neuronal protocols (Valensi-Kurtz, Lefler et al. 2010, Shi, Kirwan et al. 2012).

For the study of migraine, it is an acknowledged drawback that this protocol produces sensory neurons akin to DRG, rather than TG neurons. While the two are often used as surrogates, there have been subtle differences documented (Ambalavanar, Moritani et al. 2005, Blentic, Chambers et al. 2011). We can be confident that the described protocol produce DRG neurons due to the developmental pathways followed through differentiation (Figure 5.3). While a proportion of TG neurons arise from the neural crest, the majority have their origins in the cranial placode (D'Amico-Martel and Noden 1983), a specialized region of the ectoderm. While not available at the time of work in this chapter was being under-taken, a newly published protocol has for the first time demonstrated the differentiation of sensory neurons from the cranial-placode (Dincer, Piao et al. 2013). Extensive functional analysis was not performed, but it would be informative to study differences between the physiology of the two resultant terminal cell types. If differentiation schemes were comparable in terms of efficiency and consistency, it would be hard to argue that the latter protocol would not be the more relevant for migraine study.

Neurons were found to express a multitude of migraine-relevant proteins (Figure 5.11) and as such, the system should have broad applicability to migraine research. Predominantly, iPSC technology is used to compare patient and control derived cells and look for disease relevant phenotypes. This can be extremely useful and

should be done for cells derived from a raft of migraine cohorts (e.g. FHM, MA, MO etc.). Outwith of these studies, cells can be thought of as analogous to rodent primary neurons, without the concern of translating results from rodent to human. This concern is particularly pertinent when considering TRESK, a protein with significant disparity between rodent and human homologues (Keshavaprasad, Liu et al. 2005). In this chapter, I have shown one example of the dangers of relying solely on studies in mouse neurons (Figure 5.12).

Perhaps the most exciting avenue for this system is in drug screening efforts. iPSC-neurons have successfully been used for this purpose for a range of neurological disorders (Egawa, Kitaoka et al. 2012, Tiscornia, Vivas et al. 2013, Yu, Swaroop et al. 2014). The approach is attractive because iPSC-derived neurons represent a humanized model, which more readily recapitulates somatic cell types than immortalized lines. High throughput screening can be performed to narrow in on candidate compounds, before assessing efficacy *in vivo*. This may improve the success rate of drug development and reduce reliance on animal models.

In this chapter I have detailed the successful generation of an iPSC-based system to interrogate migraine relevant cellular phenotypes. Cells accurately recapitulate what we know about nociceptors *in vivo* and can be cultured long-term, as a highly pure and homogenous population. This represents the first stem cell model of migraine and opens up exciting possibilities to improve our understanding of disease pathobiology at the cellular level.

6. Migraine-iPSC disease modelling

6.1 Introduction

iPSC technology has opened the door for many experimental studies that were previously not possible. Research into the pathological mechanisms of human disease and the development of therapeutics to counter such, has been hindered by a lack of predictive disease models that faithfully recapitulate humanized cells *in vitro*. Ideally, models would incorporate disease-modifying genetic variants and so it is not trivial that the genetic architecture of many neurological disorders is poorly understood (Keller, Saad et al. 2012, Eising, de Vries et al. 2013, Ridge, Mukherjee et al. 2013). Disease modeling using iPSC technology offers an approach to tackle some of these challenges. Somatic cells ultimately generated from patient tissue, can be compared to that derived from healthy subjects. Identifying disease relevant cellular phenotypes can advance our understanding of pathological mechanisms, but then can also serve as an excellent platform to screen phenotype-modifying compounds. This logic has been applied to define cellular traits for multiple disorders of the nervous system (Mitne-Neto, Machado-Costa et al. 2011, Chung, Khurana et al. 2013, Donnelly, Zhang et al. 2013, Ryan, Dolatabadi et al. 2013). We are now beginning to see these traits act as phenotypic outcomes in the screening of novel therapeutics (Egawa, Kitaoka et al. 2012, Yu, Swaroop et al. 2014).

Migraine presents as an attractive disease to perform similar studies. At present, the majority of *in vitro* systems are confounded by the use of rodent tissue, which may not accurately replicate human analogues. Furthermore, while genetic association studies are providing some candidate genes, there still exists a large proportion of missing heritability (Eising, de Vries et al. 2013). This heritability may never be fully defined. The question then is: How do you manipulate the function of a gene in a cellular

or animal model, if you do not know the gene to target in the first instance? iPSC disease modeling overcomes this issue by incorporating the genetic background of patients, with all the disease modifying variants (identified or not), into a malleable model.

Proper patient selection is crucial for successful iPSC experiments. Monogenic forms of disease are likely to exhibit more tractable phenotypes, however many neurological disorders are predominantly polygenic in nature. This is true in the case of migraine, wherein FHM is the standard molecular model, but clinically FHM patients account for less than 0.1% of migraine sufferers (Thomsen, Eriksen et al. 2002, Smitherman, Burch et al. 2013). The MA pedigree described earlier in this thesis (harboring *KCNK18* and *SLC12A3* mutations) offers an opportunity to study factors relevant for typical migraine, but also exemplifies a situation where we have identified disease-modifying variants. Additionally, given the dominant negative inheritance of deleterious *KCNK18*, we can be optimistic that loss of TRESK function has a large effect size, which may translate into an observable cellular phenotype. For this reason, and as NCC protein/function could not be verified in iPSC-derived nociceptors, work in this chapter will revolve around interrogating likely TRESK-mediated phenotypes. Heterogeneity is inherent with iPSC work and is caused by variation between patients, clones from the same patient and even between different differentiation batches. This means care must be taken when prescribing differences between cell populations as disease relevant and not simply noise from the experimental setup. For this reason it is beneficial to have prior hypotheses about specific cellular phenotypes that may be affected in disease clones. Previous work (Chapter 3) aids in this regard as it highlights electrical excitability as a hallmark of TRESK dysfunction that marries well with *a priori* knowledge of migraine. For these reasons, I believe that iPSC derived from our MA pedigree, represent an excellent starting point to investigate differences at the cellular level between migraineur sensory neurons and those that recapitulate the cells of a healthy individual.

6.2 Aims of Chapter 6

Work presented in this chapter is concerned with the characterization of a set of healthy and migraine patient-derived iPSC lines. To this end, two principle goals were established:

- 1) To define the utility of a set of iPSC lines to be used in future comparative studies.
- 2) To perform a pilot comparison between healthy patient iPSC-derived sensory neurons and their disease counterparts.

6.3 Results

6.3.1 Characterization of a set of migraine and control iPSC lines

Proper patient selection is a vital step in iPSC studies. Variation in reprogramming efficiencies have been noted when working with somatic cells from old and young donors (Li, Collado et al. 2009, Cheng, Ito et al. 2011), cells with different genetic backgrounds (Schnabel, Abratte et al. 2012) and from different donor cell types (Kim, Doi et al. 2010, Bar-Nur, Russ et al. 2011). For this reason we wished to standardize iPSC generation as far as possible and use donors matched closely for age and gender. Fibroblasts were taken from three sisters of the original MA pedigree and reprogrammed to iPSC. As part of a collaboration, Dr. Sally Cowley (University of Oxford) undertook all reprogramming. Migraine-derived iPSC were named *838*, *839* and *840* (Ages: 65, 64 and 62 years old respectively). As controls, we selected the lines *AH017-7*, *AH017-3* and *NHDF-1*. These lines were generated from female healthy patients of a similar age to the disease lines. Disease lines and *AH017* clones were reprogrammed using the Nakanishi SeVdp vector Sendai system (Nishimura, Sano et al. 2011, Nishimura, Kaneko et al. 2013), while *NHDF-1* is a previously generated and

described line reprogrammed by retroviral delivery of pluripotency genes (Hartfield, Yamasaki-Mann et al. 2014). Details of all lines and their reprogramming are listed in Chapter 2, Materials and Methods: Table 2.5.

The process of reprogramming somatic cells to the iPSC fate can induce instability in the genome (Luo, Kawakatsu et al. 2014). Alterations can affect gene function and therefore can lead to artificial cellular phenotypes. To assess genome integrity all lines were karyotyped using a CytoSNP (Illumina) array. Lines passed quality control by meeting criteria set out in Chapter 5 (Figure 6.1 A and B).

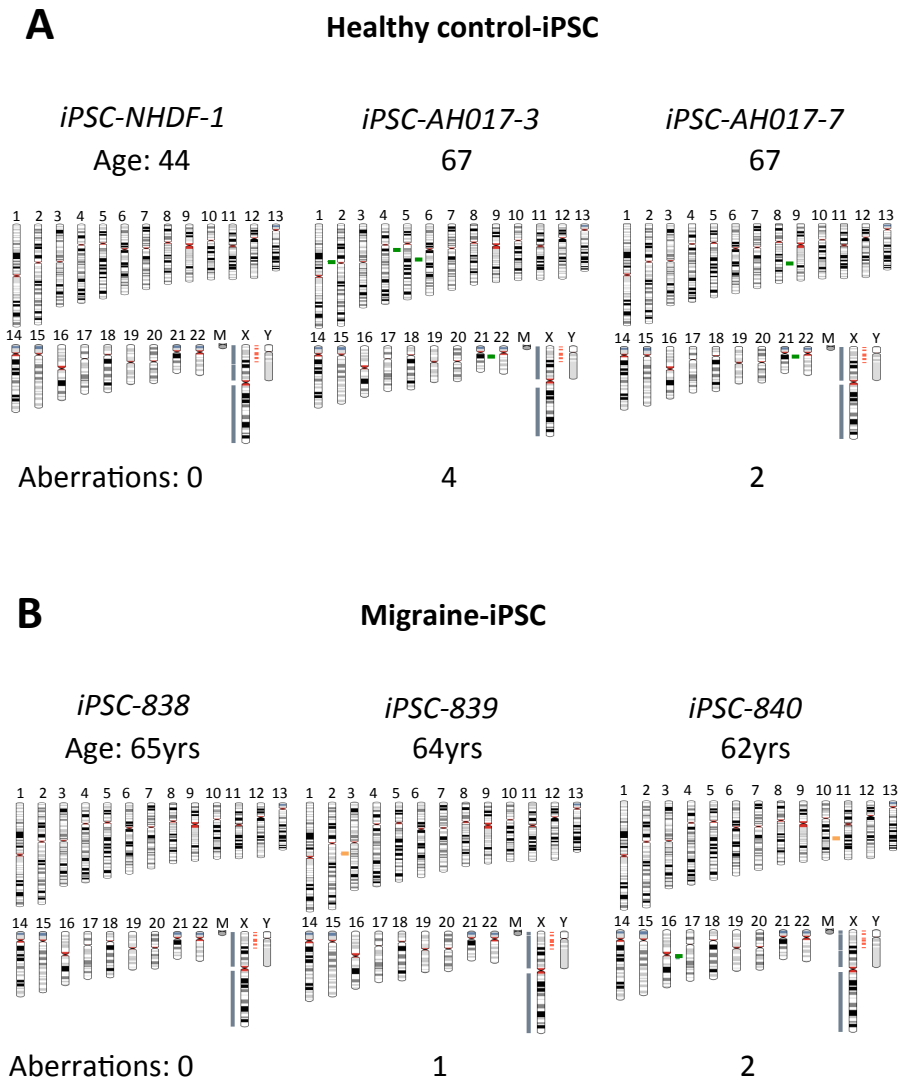


Figure 6.1 Karyotype analysis of iPSC derived from healthy control and migraine patient dermal fibroblasts. (A) Control data set includes *NHDF-1* and two clones from the *AH017*

donor: *AH017-7* and *AH017-3*. **(B)** Migraine lines derive from three MA patients from the original F139fsWx24 *KCNK18* pedigree. A green band to the left of the affected chromosome denotes increased, and orange denotes decreased, detection of SNPs. Any line possessing less than five genetic aberrations was deemed acceptable for future studies.

Lines were assessed for pluripotency by the expression of the transcription factors OCT-4 and NANOG (Figure 6.2). Robust expression of these factors in >95% of the population, in addition to a normal morphology were prerequisites for initiating differentiation. It was very uncommon to observe lines with loss of pluripotency markers, but when this did occur it presented in cultures that had been passaged multiple times. For this reason, efforts were made to retain lines in the iPSC state pre-differentiation for only a short duration of time (<5 passages).

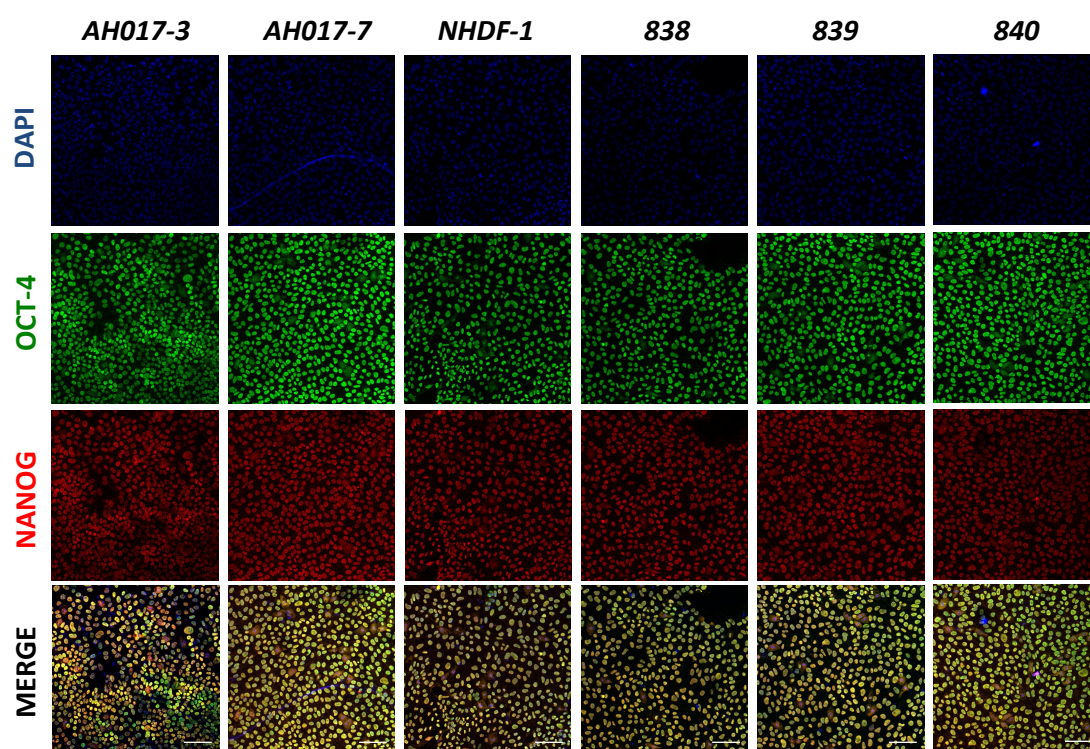


Figure 6.2 Pluripotency of control and migraine iPSC lines. Before differentiation each line was assessed for pluripotency by immunoreactivity for the prototypical pluripotency markers: OCT-4 (*green*) and NANOG (*red*). Scale bars represent 100 μ M.

To ensure that disease lines carried the F139fsWx24 *KCNK18* variant, iPSC DNA was genotyped. As expected, 838, 839 and 840-iPSC were heterozygous for a CT deletion at position c.414-415 of *KCNK18* (F139fsWx24), while AH017 clones and NHDF-1 were homozygous for the wild-type *KCNK18* sequence of this region (Figure 6.3). These results represent the quality control of both sets of iPSC and confirmed that the disease-associated genetic variant segregates in the correct groups.

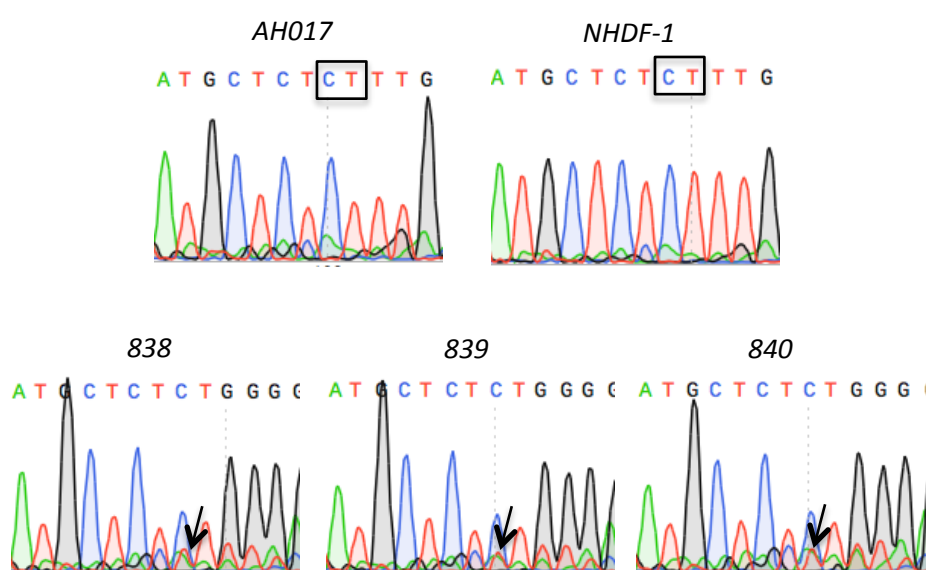


Figure 6.3 Patient lines possess F139fsWx24 allele. A fragment of *KCNK18* was PCR amplified from template DNA derived from control (AH017-7 and NHDF-1) and migraine (838, 839 and 840) iPSC lines. Sequencing of *KCNK18*-amplified products demonstrated the expected wild-type sequence for control lines, but a CT deletion at position c.414-415 in one allele of disease-lines. This represents the previously documented F139fsWx24 variant. Black box labels CT at position c.414-415 in wild-type sequence, while arrows point to additional sequencing peaks consistent with CT deletion in one allele.

6.3.2 Ability of lines to generate functional sensory neurons

It is common for iPSC clones to vary in their propensity to differentiate into certain cell types. I wished to assess the ability of each clone to produce sensory neurons. iPSC were differentiated to an early neuronal stage (day 13) as per the protocol described in Chapter 5, before counting for the proportion of TUJI⁺/BRN3A⁺ cells. There was no difference in differentiation efficiency between lines (16.7%-41%), although there was a trend for *AH017-3* to produce a lower proportion of sensory neurons (Figure 6.4 A and B). The moderately reduced efficiency of this clone to produce the desired cell type was mirrored by an increase in proliferating cells of unknown lineage. Proliferating cells maintained to mature stages of culture (day 50+), despite several treatments with anti-mitotic agents (Figure 6.4 C). The presence of proliferating cells has been documented to dramatically affect neuron maturation and gene expression (personal communications Dr. James Bilsland, *Pfizer*). For these reasons *AH017-3* was deemed unacceptable for comparisons and was not used in future studies.

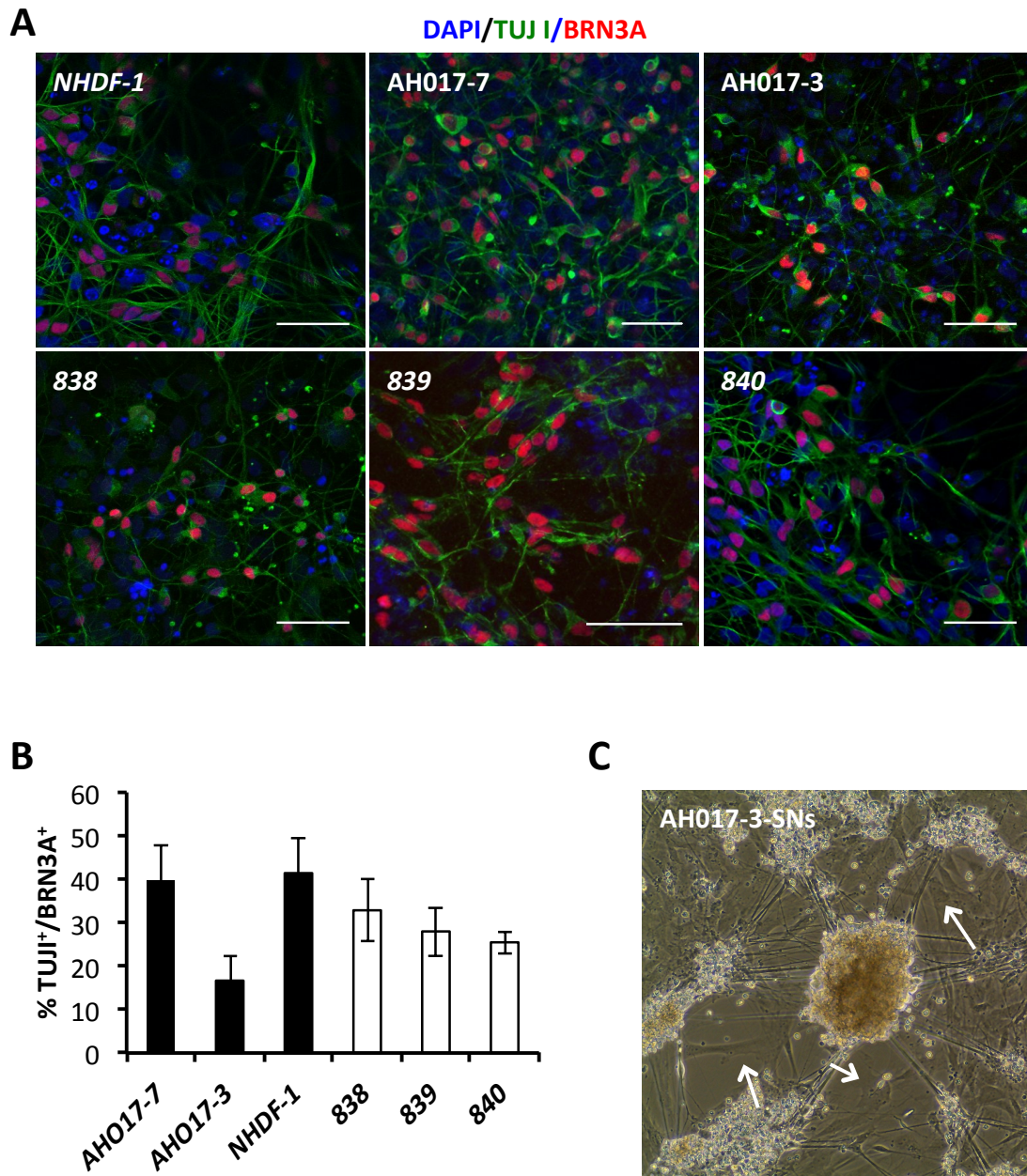


Figure 6.4 Clone efficiency of sensory neuron induction. Lines were assessed for sensory neuron induction by counting the proportion of TUJ1/BRN3A co-expressing cells at day 13. **(A)** Representative images illustrating presence of neuronal (TUJ1) and sensory (BRN3A) markers. **(B)** Quantification of differentiation efficiency across all cell lines. **(C)** Brightfield image of day 50 *AH017-3* sensory neuron culture illustrating the presence of cells with a non-neuronal morphology. Contaminating cells form a continuous layer on top of which the neurons grow. Isolated cells are marked with a white arrow to illustrate morphology. *One-way ANNOVA*,

Bonferroni's multiple comparison test; results represent mean $\pm$ s.e.m of three independent experiments.

In addition to expression studies, I wanted to investigate if all clones could produce functioning neurons by day 50 of culture. Neurons derived from all clones exhibited substantial currents in response to potential steps (Figure 6.5 A), including voltage-gated inward currents that are critical for action potential generation. Additionally, all cells identified to have a neuronal morphology, were capable of generating a singular action potential in response to depolarizing current. This was true for all healthy and migraine patient derived lines (Figure 6.5 B).

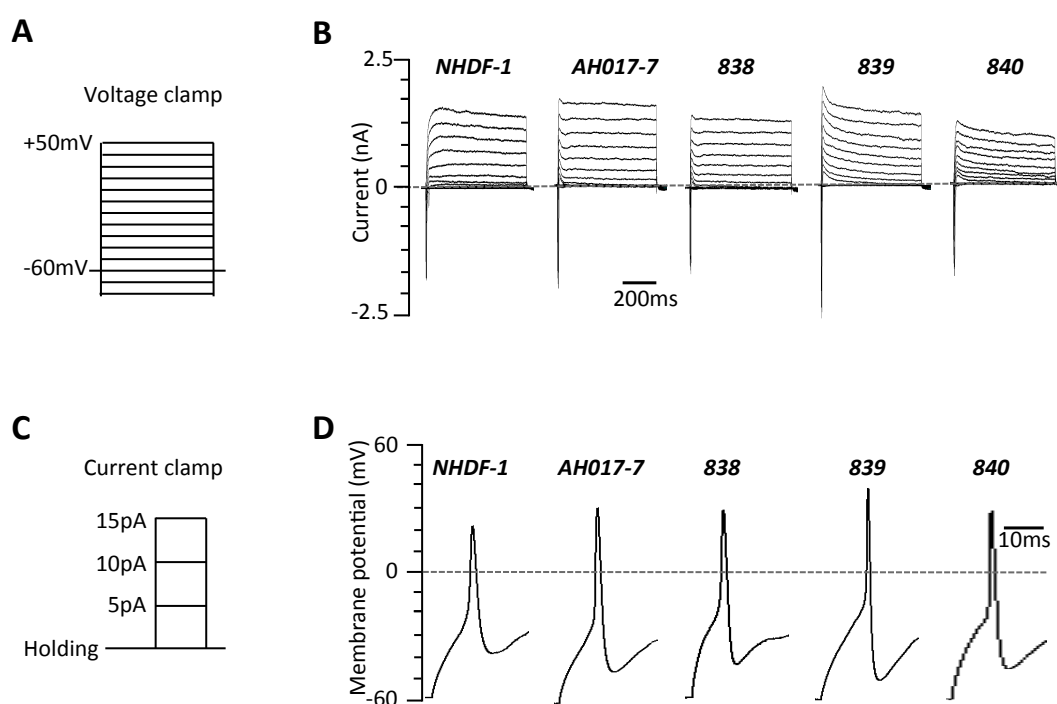


Figure 6.5 Generated sensory neurons are functional (A) Illustration of voltage-clamp protocol used to assess membrane currents. Cells were stepped from -80mV to +50mV in 10mV steps of 500ms duration. **(B)** Representative voltage-clamp traces from all line sensory neurons (SNs) at day 50 of maturation. **(C)** Illustration of current clamp protocol used to initiate an action potential. Neurons were held at -60mV before being depolarized by increasing current injection. **(D)** Action potentials could be induced in all line-SNs with a neuronal morphology.

6.3.3 Spontaneous activity in 840-SNs

Spontaneous firing of neurons has been documented for multiple iPSC-derived neuronal lineages (Zhang, Pak et al. 2013, Naujock, Stanslowsky et al. 2014, Stanslowsky, Haase et al. 2014) and is often used as a proxy of maturation (Shi, Kirwan et al. 2012). A small percentage (<5%) of sensory neurons in culture will exhibit spontaneous activity (SA) (Ma and LaMotte 2005). The proportion of cells with SA is dramatically increased when taken from animal models of nerve injury (Hu, Song et al. 2001, Ma and LaMotte 2005). No evidence for the presence of SA was seen in recordings for *AH017-7*, *NHDF-1*, *838* or *839-SNs* (total of 69 recordings). Preliminary assessment of *840-SNs* indicated that cells from this line exhibited spontaneous firing (3/7- 42.9%) (Figure 6.6 B). While enhanced spontaneous activity is associated with hyper-excitability, it is not believed that this phenotype is disease relevant, however it serves as an example as to the difficulties faced when investigating a heterogeneous set of cell lines.

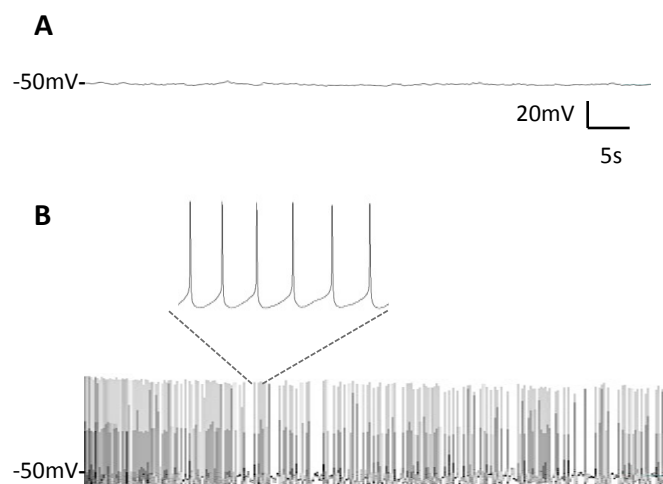


Figure 6.6 SA in the migraine line, 840-SNs. All cells analyzed in whole-cell current clamp mode were assessed for SA. Potential deflections were monitored for 60 seconds while holding current was 0pA. No cells from *AH017-7-SNs*, *NHDF-1-SNs*, *838-SNs* or *839-SNs* groups displayed any signs of spontaneous firing activity. **(A)** Representative trace of a day 50 *AH017-7-SN*

membrane potential over a 60 second period. **(B)** Trace from a day 50 *840-SN* exhibiting spontaneous firing.

6.3.4 838-SNs have reduced TRESK currents compared to *AH017-7-SNs*

Due to the labour intensive approach of patch clamp recordings and in an attempt to define a finite list of cellular phenotypes of which to assess for all lines, I performed a pilot comparison of one healthy and one migraine-derived iPSC line. *AH017-7* and *838* were chosen for their ease of handling and assessed for biophysical properties. *I/V* relationships were plotted for both groups to investigate if the crude membrane currents were equivalent. Outward and inward current showed marked similarity between the two groups, with no significant difference seen in either component (Figure 6.7 A). It is presently unknown to what extent F139fsWx24 *KCNK18* negatively impact upon wild-type TRESK subunit current. Using the voltage clamp protocol previously described to isolate TRESK current, day 50 neurons exhibit an outward current roughly equitable to dissociated rodent cultures (Chapter 3: Figure 3.9). Total current was significantly smaller in *838-SNs* vs. *AH017-7-SNs* (Figure 6.7 B). 500nM TPA reduced the outward current by 17.3% in *AH017-7-SNs* but by only 4.66% in *838-SNs*. It is however acknowledged that data is derived from a low number of neurons. Nevertheless, it supports the notion that *838-SNs* have a reduced TRESK current, but appear similar to *AH017-7-SNs* with regards to overall conductance.

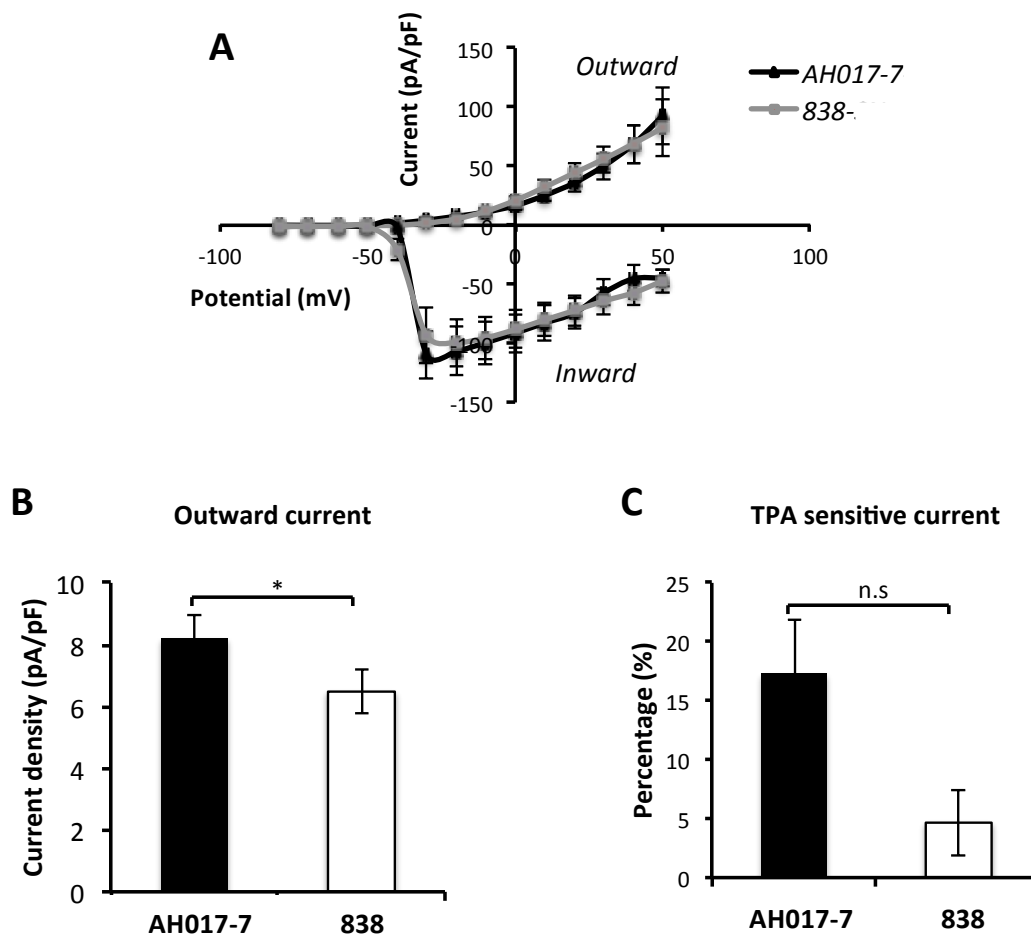


Figure 6.7 Active and passive membrane currents of AH017-7-SNs and 838-SNs. Voltage-clamp recordings of day 50 SNs illustrating voltage-activated and background currents. **(A)** Membrane potential was ramped from -80mV to +50mV in 10mV increments. Outward current was measured at the end of each 500ms voltage step, while inward current was measured as the maximum negative current inflection during the step. **(B)** Protocols were used to limit the interference of voltage-gated currents to outward current measurements. Cells were stepped from -80mV to -25mV and outward current recorded. * $P < 0.05$; un-paired Student's *t*-test; results represent mean $\pm$ s.e.m of three independent experiments (838-SNs $n=28$, AH017-7 $n=27$). **(C)** A small number of neurons from each group (838-SNs $n=5$, AH017-7 $n=4$) were assessed for outward current before and after 15 minute application of 500nM TPA. Results represent percentage inhibition caused by TPA application.

6.3.5 838-SNs exhibit features of hyper-excitability compared to AH017-7-SNs

Many genetic variants associated with migraine have been linked with increased neuronal activation (Weir and Cader 2011). Indeed, in Chapters 3 and 4, I have showed that loss of TRESK and NCC activity contributes to greater excitability of rodent sensory neurons. As a way of identifying disease relevant cellular phenotypes, I tested the hypothesis that migraine derived nociceptors are more excitable than control. Capacitance was equal among groups (Figure 6.8 A) indicating that there was no gross difference in cell size. The R_{input} of a cell is determined by the permeability of the cell membrane to current. It is an important parameter in controlling neuronal excitability as it determines the degree of polarization in response to a given stimulus. 838-SNs had a mean R_{input} significantly higher than AH017-7-SNs (Figure 6.8 B). Additionally, 838-SNs had a depolarized membrane at rest compared to AH017-7-SNs (Figure 6.8 C). Such passive properties would intimate that 838-SNs could be hypersensitive to activation and are consistent with previous findings of a reduced background K^+ current.

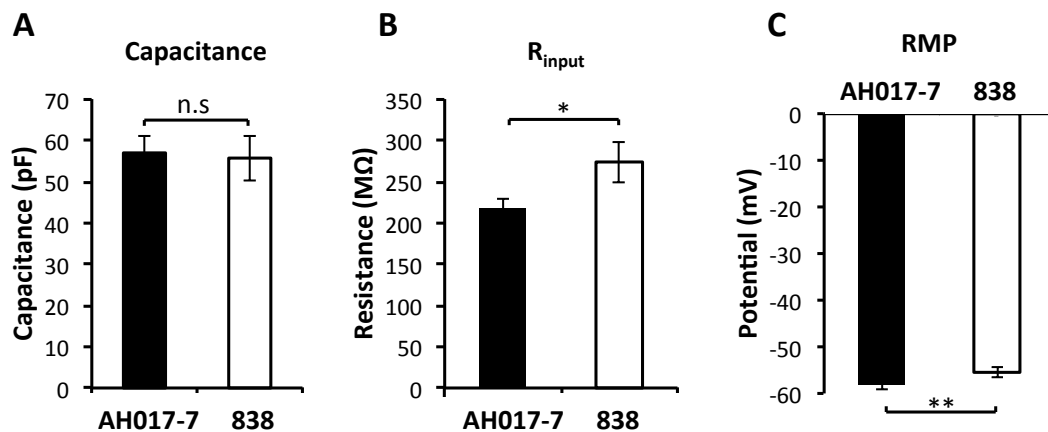


Figure 6.8 Passive membrane properties of 838 and AH017-7-SNs. **(A)** Capacitance is similar between lines. **(B)** R_{input} was calculated by measuring the membrane potential change induced by a 20pA hyperpolarizing pulse, while cells were held at -60mV in current clamp mode. AH017-7-SNs had a reduced resistance at this potential. **(C)** RMP values were determined in bridge mode

and showed a modest depolarization of *838-SNs* compared with *AH017-7-SNs*. $*P<0.05$, $**P<0.01$; un-paired Student's *t*-test; results represent mean $\pm$ s.e.m (*838-SNs* $n=26$, *AH017-7* $n=24$).

The most robust function found in previous studies of TRESK activity has been in determining the rheobase of neurons (Chapter 3). *838-SNs* had a lower rheobase than *AH017-SNs* (Figures 6.9 A and B). This assessment was made after pooling data together from three independent experiments. When data is presented for each experiment it is clear that this trend is consistent, but absolute values do vary noticeably (Figure 6.9 C). This is shown to illustrate the challenge posed by intra-experimental variation and the need for large data sets to conclude robust cellular phenotypes. Loss of TRESK and NCC leads to an increase in firing frequency following supra-threshold current injection (Figures 3.9 D and 4.8 F). The average number of APs following such a stimulus was not different between the two lines, although a larger proportion of *838-SNs* (9/27- 33.33%) fired multiple APs compared to *AH017-SNs* (4/26- 15.38%) (Figure 6.9 D). These data are consistent with the notion that *838-SNs* are more excitable than *A017-7-SNs*.

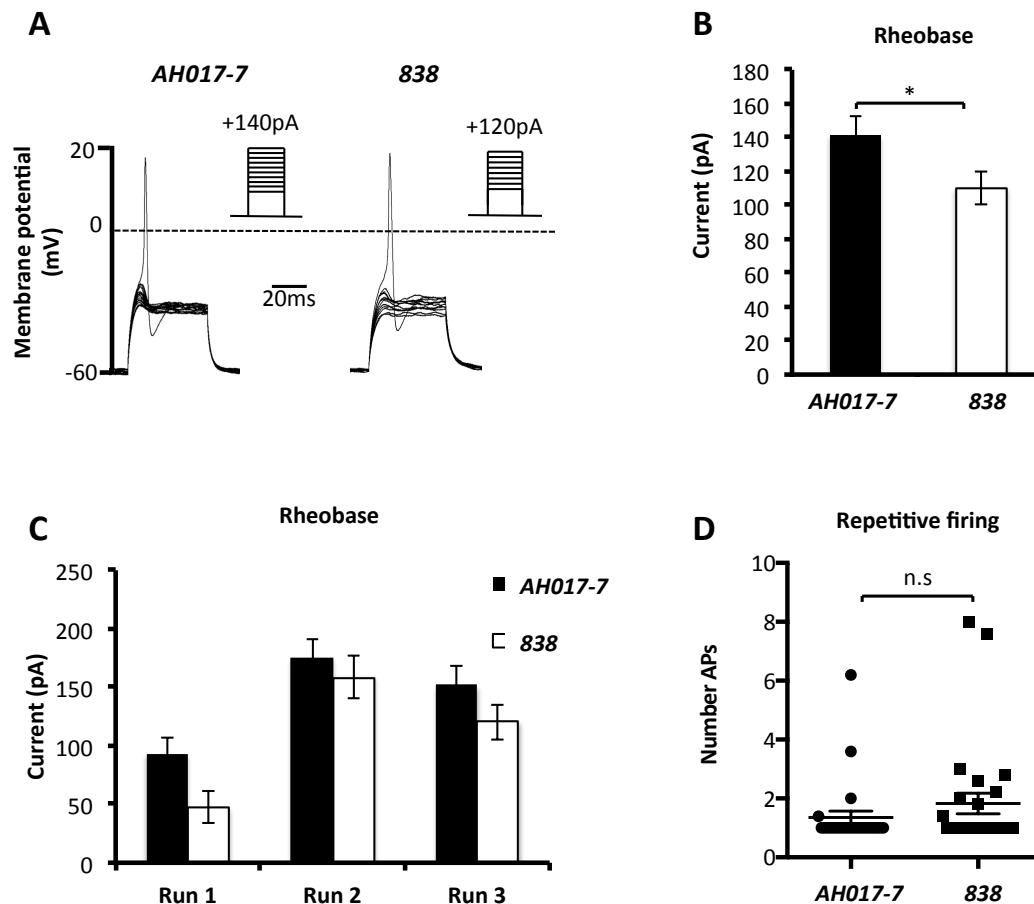


Figure 6.9 Migraine line has a reduced rheobase current compared to control. (A) Action potential firing by day 50 SNs. Cells were held at -60mV in current clamp mode and depolarized with incremental current. Traces illustrate representative neurons from the two groups and the minimum current required to induce firing. **(B)** Quantification of rheobase values comparing the two groups. **(C)** Average rheobase values derived from sequential differentiations of AH017-7 and 838 in parallel. **(D)** Dot plot graph showing the number of action potentials generated in response to a 500ms supra-threshold current injection. * $P < 0.05$; un-paired Student's *t*-test; results represent mean $\pm$ s.e.m (838 $n=27$, AH017-7 $n=26$).

6.4 Discussion

iPSC comparison studies are providing insights into the molecular underpinnings of neurological disorders, but to date this technique has not been applied to migraine. The MA pedigree described represents an excellent source of patient tissue, as although

family members clinically exhibit typical migraine, rare variants with high effect size have been associated with disease. This is a unique situation in the field. In this chapter, I have performed the basic characterization necessary for iPSC lines from three family members of the MA pedigree and control lines matched for age and gender. All lines were deemed to have passed quality control at the pluripotent stage, but not all were able to generate sensory neurons equally (Figure 6.4). Before large-scale comparative studies (between multiple lines from each group), hypotheses must be formed as to which cellular phenotypes to assay. To this end, I have performed a robust comparison of the biophysical properties of mature sensory neurons derived from one disease and one control line. The migraine-derived line was shown hyper-excitable relative to control. Genetic variants implicated in these cells have been extensively studied in other systems, in previous chapters. The cellular phenotypes described in those systems upon altering protein activity, are consistent with data presented in this chapter.

Heterogeneity of iPSC preparations represents a significant challenge. Reprogramming efficiency of patient lines was not assessed, but a deficiency to differentiate solely down the sensory lineage was noted for one clone (*AH017-3*). iPSC for this clone appeared normal, although karyotype analysis showed the greatest number of genetic abnormalities out of all lines (Figure 6.1). It is difficult to interpret why differentiation potential is different, especially when comparing two clones from the same donor. It would be of interest to search regions over/under represented in the CytoSNP array for genes with known roles in sensory neurogenesis. Variation was also noted for the *840* line, despite the fact that it passed both the iPSC stage quality control and demonstrated equivalent differentiation capacity to other lines. The existence of SA in a proportion of *840*-SNs (Figure 6.6 B), when none was observed during any other recording is potentially problematic. It may be the case that future studies need to carefully choose only a subset of lines from large pools that conform to strict parameters. The final form of variation noted was between different differentiation

runs. Features such as rheobase, varied markedly intra-clonally despite best effort to keep conditions consistent. The list of variables that could contribute to this is numerous, however the challenge of regulating the phenomena is of vital importance. One resolution may be to genetically integrate reporter markers that signal the maturation state of individual cells. This would allow for the targeting of only those cells that were at a specific developmental time-point, e.g. expression of TTX-resistant Na⁺ channels, for assay. This could, reduce intra-clonal differences but may also go some way to account for variation in differentiation rates between lines.

It is hoped that cellular phenotypes observed in iPSC disease modelling are reminiscent of processes occurring in patients. Once matured to SNs, the migraine-derived line demonstrated features of hyper-excitability. Passive properties such as R_{input} and RMP were altered (Figure 6.8) as expected of a neuron that is more susceptible to firing. Indeed, direct assessments of firing properties support this notion (Figure 6.9). Patient-derived SNs fired an action potential in response to a depolarizing current that was less than that required for control SNs. Our hypothesis is that the reduced rheobase level is primarily due to diminished TRESK channel activity as caused by the frame shift mutation. Outward current (of which TRESK represents a significant fraction) was reduced in hyper-excitabile cells that harbour the loss of function allele (Figure 6.7). The association of increased excitability and reduced outward current is supportive of prescribing the observed excitability phenotype with loss of TRESK activity; however further studies are required to fully establish the link. In the first instance, compounds that enhance TRESK current (Figure 3.6) could be used to see if they can rescue the excitability phenotype. Isogenic control lines are increasingly being utilized to link cellular phenotypes with specific genetic variants (Ananiev, Williams et al. 2011, Fong, Wang et al. 2013, Ryan, Dolatabadi et al. 2013). Precise control of DNA sequence can be achieved by technologies such as CRISPR (Hou, Zhang et al. 2013). It would be

rewarding to correct the F139fsWx24 mutation in patient lines to see whether phenotypes disappear, fully or partially.

The phenotypic data presented in this chapter must be confirmed in a large set of disease and control clones. The lines characterized herein will serve as a good foundation for such future work. If confirmed by future studies, the cellular phenotype described would be of importance to our understanding of migraine. It serves to underline the hypothesis that hyper-excitability neurons do play a part in pathogenesis and reveals a critical role for TRESK irrespective of any genetic association (Eising, de Vries et al. 2013). In future, cellular phenotypes like the ones described, could act as robust assay outputs in therapeutic compound screening.

7. Conclusions and future directions

During my DPhil studies I have investigated the functional implications of migraine-associated genetic variants to neuronal physiology. Work focusing on two proteins, TRESK and NCC, has been centered on their involvement in the excitability of sensory neurons. As such, a large effort has been made to develop new and improved models to study sensory neurons *in vitro*. The specific variants investigated in this thesis are not likely to contribute substantially to the incidence of migraine and so I will now reflect on the significance of my results and discuss how they can be built upon to improve our understanding of disease pathogenesis.

7.1 Altered excitability is a hallmark of migraine

My work identified that both TRESK and NCC have modulatory roles in neuronal activity, which can be generalized to say that loss of either leads to hyper-excitable neurons. These data fit well with previous hypotheses prescribing neuronal hyperactivity as a causative agent during a migraine attack.

It is clear that the migraine brain differs in its state of excitability compared to those of healthy subjects. Despite this, the previous dogma of a purely hyper-excitable phenotype (Aurora and Wilkinson 2007) is no longer fully supported (Coppola, Pierelli et al. 2007). Migraineurs are more sensitive to uncomfortable visual stimuli (Marcus and Soso 1989) and exhibit a range of abnormal responses at the level of cortical evoked potentials (Diener, Scholz et al. 1989, Schoenen 1996, Shibata, Osawa et al. 1998), which can be normalized by medication (Nyrke, Kangasniemi et al. 1984, Diener, Scholz et al. 1989). Transcranial Magnetic Stimulation (TMS) studies have shown decreased threshold for observing phosphenes (the experience of light, in the absence of luminous stimulation) in both MA and MO patients (Mulleners, Chronicle et al. 2001). However,

other studies have demonstrated lower visually evoked-potential amplitudes- data that would seem counter to the notion of hyper-excitability (Wang, Schoenen et al. 1995, Ozkul and Uckardes 2002). Instead, current opinion is now based upon the idea that there is cortical impairment in the maintenance of proper excitatory/inhibitory balance (Vecchia and Pietrobon 2012). Reduced evoked-potential habituation to a range of repetitive sensory stimuli has consistently been reported (Wang, Schoenen et al. 1995, Ozkul and Uckardes 2002, Ambrosini, Rossi et al. 2003) implicating both excitatory and inhibitory mechanisms (Coppola, Pierelli et al. 2007). Whilst the excitability changes of the cortex may be complex, and dependent on the final net effect on excitatory-inhibitory balance, hyper-excitability of trigeminal neurons should have a simpler relationship to migraine since TGVS activation is considered a central feature of migraine headache (Olesen, Burstein et al. 2009).

At the genetic level, many associations have supported the concept of aberrant hyper-responsiveness. All three FHM-associated genes have a hypothetical role at the central glutamate synapse (Figure 1.2). Indeed, variants have been shown to augment cortical spreading depression in animal models (van den Maagdenberg, Pietrobon et al. 2004, Leo, Gherardini et al. 2011). Outside of FHM, many rare and GWAS-discovered variants fit functionally into the common pathway of glutamatergic neurotransmission (Figure 1.3). Thus, the finding that TRESK and NCC control neuronal excitability has two implications: (I) It provides functional evidence to support the genetic data, illustrating an association between deleterious variants in these two genes and MA and (II), adds weight to the above notion that migraine is indeed linked to aberrant excitability of the nervous system.

It is imperative to translate the cellular studies detailed in Chapter 3 and 4 into the *in vivo* setting. Dissociated cell cultures only allow for the investigation of one type of cell, un-modulated by the inhibitory/excitatory systems that are in place *in vivo*. This has been clearly demonstrated as a critical factor in FHM sub-types, where the effect of a

variant is highly dependent on cellular context (Benarroch 2005, Vecchia, Tottene et al. 2014). Future studies should consider more complex culture systems and *ex vivo* preparations to bridge between dissociated cultures and *in vivo* pathophysiology. It will also be important to utilize rodent models of migraine. For example, it could be studied whether TRESK or NCC inhibition increases TGVS transmission following GTN administration, or alternatively, whether TRESK/NCC knockout animals have heightened susceptibility to CSD triggering.

7.2 Brain regions implicated in migraine

Clinical presentation would strongly argue for central dysfunction as the initiator of a migraine attack. A proposed cascade then links this dysfunction, possibly via a CSD-like event, to activation of peripheral afferents. These in turn convey a now nociceptive signal, to higher brain regions to generate the perception of pain (Figure 1.1).

How do TRESK and NCC fit in with this model? Does their prominent role in sensory neuron physiology suggest that dysfunction in trigeminal afferents can initiate an attack, rather than simply convey the headache phase? It is also conceivable that under normal circumstances, peripheral nociceptor thresholds are a check against frequent central or environmental migraine initiators, but that only in the context of genetic defects reducing TGVS thresholds, does this gate keeping capacity fail. The high expression and described modulatory function of both, lends support to the notion that peripheral input is an important aspect of migraine. In this regard, they join the list of FHM/GWAS variants for which peripheral function is considered likely to be of importance to their disease-modifying effect (McKemy 2005, Nair, Simonetti et al. 2010). Despite this, both TRESK and NCC show restrictive but intriguing CNS expression. The high expression of TRESK across hypothalamic nuclei and the co-presence of NCC in some discreet nuclei highlight this structure as a region of particular interest.

Body homeostasis is largely orchestrated by the hypothalamus, which releases the effector hormones responsible for food/water intake, temperature regulation, sexual behaviour, circadian cycles and emotional responses (Alstadhaug 2009). The structure is also intimately involved in the central processing of a painful experience. Hypothalamic nuclei have efferent and afferent connections with other brain regions more classically associated with pain processing (Murphy, Rizvi et al. 1999, Jiang and Behbehani 2001). Many hypothalamic neurons contain analgesic agents, such that hypothalamic stimulation alleviates pain in animal models (Carstens 1986). Activation of several nuclei has been observed in animal models of the headache phase. The Paraventricular nucleus (PVN) has shown activation upon dura mater stimulation (Malick, Jakubowski et al. 2001) while the Supraoptic nucleus (SON) is similarly activated following stimulation of the superior sagittal sinus (SSS) (Benjamin, Levy et al. 2004). More recently, PVN projection neurons were found to modulate the activity of Sp5C neurons in the rat (Robert, Bourgeois et al. 2013). In this context it is clear to see how NCC function could contribute to the headache phase, in addition to activation of primary afferents.

Beyond a modulatory role in pain processing, the hypothalamus has also been linked with earlier events in an attack. Premonitory symptoms in migraine include sleep disturbances and changes in arousal, mood, food craving and thirst (Blau 1980, Dalkvist, Ekbom et al. 1984, Goder, Fritzer et al. 2001, Giffin, Ruggiero et al. 2003). With its extensive role in the limbic system, such symptoms could nicely be explained by a dysfunction in the hypothalamus. In support, a recent PET study showed activation of posterolateral hypothalamus during the premonitory phase of induced migraine attacks (Maniyar, Sprenger et al. 2014)

Many migraine triggers converge on the hypothalamus, most notably bright lights, sleep disturbance and stress. The seemingly high expression of TRESK in the optic nerve and hypothalamic regions involved in processing incoming visual signals is

therefore of great interest, as are centers regulating sleep and circadian entrainment. It is also noteworthy that members of the MA pedigree with the deleterious *KCNK18* allele list bright light as a strong migraine trigger. Parasomnias are more prevalent in the migraine population (Dahmen, Kasten et al. 2003, Calhoun, Ford et al. 2006) and sleep is a common abortive strategy (Wilkinson 1994). Furthermore, disturbed sleep is reported to trigger migraine attacks in most sufferers (Kelman 2007). The superchiasmatic nucleus (SCN) is the master regulator of circadian rhythms and is well placed at the interface between sleep disturbance and migraine. While qualitative assessments of expression level is not possible with the β -galactosidase staining reported; qualitatively, the SCN appeared to possess the highest density of LacZ⁺ (and therefore TRESK⁺) neurons of all hypothalamic nuclei. TRESK is not the first migraine-associated factor linked with the SCN. Mutations in the gene encoding casein kinase I δ (CKI δ) have been found to co-segregate in families with migraine and advance sleep phase (Brennan, Bates et al. 2013). This core clock gene has a well-documented role in circadian rhythm control in the SCN. Therefore it would be of great interest to assess the circadian phenotype of TRESK^{-/-} mice. This work would also go some way to help explain how a deleterious variant in *KCNK18*, with its sole role described in the periphery, could segregate with a whole-brain disorder like migraine.

The work detailed in this thesis does not investigate the function of either TRESK or NCC in CNS regions. The restrictive nature of the patterns, do however suggest that these proteins may have important CNS physiological roles. It is plausible that TRESK would play a largely similar role in most neuronal systems i.e. membrane hyperpolarization and setting of the rheobase current. The K⁺ reversal potential is far enough removed from physiological membrane potentials as to assume that current would always represent a hyperpolarizing force. This does not necessitate a net hyper- or hypo-excitability effect at the systems level, as that would be dependent on whether function was predominant in excitatory or inhibitory populations. Less clear is the role

NCC would have in CNS neurons. Data provided in this thesis does not implicate a specific mechanism of how protein activity influences neuronal excitability. If for example, the effect was mediated by Cl⁻, the consequence of NCC activity may be completely reversed considering the stark differences in Cl⁻ handling between sensory neurons and adult CNS neurons. These questions can only be assessed by performing patch clamp recordings from CNS regions with specific pharmacological manipulation or in the context of knockout animal models.

In sum, the specific expression of TRESK and NCC in rodent CNS provides an exciting avenue to pursue future work with these proteins. Defining where, and in what context they influence systems level function could be highly illuminating for broader migraine understanding.

7.3 Molecular models for the study of migraine

Migraine represents a challenging disorder to replicate experimentally. The proposed cascade of an attack involves several critical structures, both in the periphery and brain, integrating a wide range of cell types. Furthermore, one of the defining features of migraine is the clinical heterogeneity of patient presentations. Severity is hugely disparate between those that suffer infrequent and mild attacks, and those that experience chronic, recurrent and debilitating episodes. Symptoms outwith the headache also vary hugely- the presence or absence of the aura is a classical example of this. Finally, there are a range of triggers, encompassing most forms of environmental stimuli, which appear relatively specific to different patient populations. How then is it best to model such a diverse condition?

Most likely as a consequence of the “whole brain” nature of the disorder, current models of migraine are predominantly focused at the systems level. These typically consist of either human brain imaging, or *in vivo* animal models that try and recapitulate different aspects of the disease. The development of high-resolution neuroimaging has

been an immense resource for migraine research. PET scanning was the first experimental paradigm to illustrate a clear role for central mechanisms during attack initiation (Weiller, May et al. 1995). Further, BOLD imaging provides some of the best evidence linking a CSD-like event in patients to the aura (Olesen, Friberg et al. 1990). Functional imaging will likely remain at the forefront of defining brain regions involved in attack pathogenesis. Despite this, imaging in migraine has its drawbacks and limitations. Methodologically, it is very hard to coordinate scanning time with phase of attack. This has meant that most imaging studies have been performed during the transition from ictal to interictal state, but data relating to the premonitory or headache phase is scant (May 2013). Furthermore, the functional resolution, while excellent for defining target structures, gives no insight into specific cellular or molecular targets for clinical applications.

With respect to drug discovery, animal models currently represent the most promising avenue. *In vivo* setups, such as TGVS stimulation or CSD studies have importantly shown many of the downstream effects, primarily in terms of activation of specific brain regions, as expected from human imaging studies (Akerman, Holland et al. 2013). Critically, animal models offer the opportunity to more expansively modulate systems pharmacologically and therefore represent a primary assay in drug development. Stimulation of the TG was a key assay in the development of triptans (Akerman, Holland et al. 2013) and TGVS activation, as well as CSD propensity, continue to act as valid screens to test new compounds (Levy, Zhang et al. 2008, Hoffmann, Suprongsinchai et al. 2012, Holland, Akerman et al. 2012). However, such studies are technically challenging and both time and resource intensive, such that they will never be able to act as high throughput drug screening tools.

iPSC models will not provide a solution to all the needs of migraine research however, they could redefine the current *in vitro* toolkit and complement work carried out *in vivo*. iPSC work is placed at the boundary between genetics and functional studies

and so can be used to translate genetic findings into cellular outcomes, but also presents as an attractive drug-screening platform. There is a huge diversity of cellular phenotypes that one could study using this approach. In this thesis, I have focused primarily on basal biophysical properties of iPSC-derived neurons. However, other migraine surrogates could be investigated: neurotransmitter release, synaptic strength, expression analysis etc. Further, assessing phenotypes under conditions thought to be relevant to pathogenesis may also be fruitful e.g. nociceptors in the presence of sensitizing inflammatory factors or prophylactics.

Key to success will be the proper harnessing of differentiation protocols to generate exactly the cell type required. Sensory neurons in general and nociceptors specifically, are heterogeneous. They can be sub-classified on the basis of size, degree of myelination and repertoire of expressed receptors, channels and neuropeptides. Ablation studies have shown that distinct subsets have discrete functions in transducing pain. One classification relies upon the presence or absence of neuropeptides such as CGRP and Substance P. Ablation of non-peptidergic (devoid of neuropeptides) neurons by targeting MrgprD⁺ expressing cells with Diphtheria toxin, results in reduced mechanical sensitivity (Cavanaugh, Lee et al. 2009). In contrast, selective ablation of TRPV1⁺ peptidergic fibers leads to a loss of responsiveness to noxious heat (Cavanaugh, Lee et al. 2009). Little is known about which subgroup of nociceptors is more relevant for migraine headache; however CGRP has a clear role in migraine, with CGRP receptor antagonism at the forefront of developing therapeutics (Villalon and Olesen 2009). iPSC-derived nociceptors develop (Figure 5.7), express relevant proteins (Figure 5.7) and respond functionally (Figure 5.8) in accordance with a non-peptidergic phenotype. Specifically, an acknowledged drawback of the described system is that no CGRP expression was seen. It may be that the cocktail of small molecules used very precisely restrict neurons to the non-peptidergic lineage. Alternatively, marginal changes in timings, inhibitor withdrawal, or growth factor treatment may be sufficient to alter

differentiation and generate peptidergic neurons. Key to future experiments exploring this issue is the successful extinction of the master regulator of nociceptor differentiation, RUNX1. *In vivo*, extinction of RUNX1 is pivotal for peptidergic differentiation, but *in vitro*, I documented sustained RUNX1 expression (Figure 5.7). With respect to this, siRNA or available antagonists to RUNX1 (Cunningham, Finckbeiner et al. 2012) may be profitable avenues of investigation.

With the described utility of iPSC, one outstanding question would be- what cell type(s) should be generated and investigated? I have chosen primary afferents but with respect to migraine, there is a vast array of cell types that it could be profitable to produce. These include: glial, vascular and a whole host of CNS neurons. While it would be counterproductive to try and recapitulate whole systems at this level, discreet system units consisting of multiple cell types could be extremely useful. For example, a model could be envisaged composed of cortical neurons, astrocytes and endothelial cells cultured and organized to function as an *in vitro* blood brain barrier surrogate. An alternative approach could consist of co-cultures of iPSC-derived primary and secondary nociceptive neurons for the exploration of peripheral and central sensitization. These types of co-cultures will help answer questions that are beyond the scope of single cell type cultures.

In conclusion, this thesis has been directed towards using rare genetic variants to gain insight into generalities of migraine pathobiology. The molecular and cellular mechanisms underlying migraine are relatively under-studied and subsequently less is known about pathogenesis than for many other neurological disorders. However, the field is at the forefront of new discoveries, largely thanks to powerful genetic studies. It is hoped that my work will help to provide context when trying to understand how and where variants have their disease-modifying effect. Furthermore, effort should be made to follow up initial studies targeting the proteins pharmacologically. New genetic associations, in combination with the newly available iPSC models generated in this

thesis, may together lead to successful drug screening programmes. Triptans were approved for clinical use over 20 years ago, however the knowledge base of migraine research has expanded hugely since then. Our attention should be focused on developing the next “Gold-standard” therapeutic to help those migraine sufferers still blighted by the condition.

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