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T-type calcium channels in neurological disorders

Kalciové kanály typu T u neurologických poruch

Supervisor: Dr. Norbert Weiss

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List of abbreviations

ALS	Amyotrophic lateral sclerosis
ASD	Autism spectrum disorders
BK_{Ca}	Ca^{2+}-sensitive K^+ channels
BTB/POZ	Broad complex, tramtrack, bric-a-brac/poxvirus, and zinc finger
CACHD1	Calcium channel and chemotaxis receptor (cache) domain containing protein 1
CaM	Calmodulin
CAV-3	Caveolin-3
CNS	Central nervous system
CNX	Calnexin
DEE	Developmental and epileptic encephalopathy
DHP	Dihydropyridines
DRG	Dorsal root ganglia
ENaC	Epithelial sodium channel
ER	Endoplasmic reticulum
ExAC	Exome Aggregation Consortium
fALS	Familial Amyotrophic lateral sclerosis
GAERS	Genetic absence epilepsy rat from Strasburg
GoF	Gain-of-function
GPCRs	G protein-coupled receptors
HA	Hemagglutinin-tagged
HVA	High voltage activated
IGE	Idiopathic generalised epilepsies
IP3R	Inositol 1,4,5-trisphosphate receptor
LoF	Loss-of-function
LVA	Low voltage activated
N	Asparagine
NFAT	Calcineurin/nuclear factor of the activated T-cell
nRt	Reticularis thalami
PA	Primary aldosteronism
PKA	Protein kinase A
PKC	Protein kinase C

PTMs	Post-translational modifications
RACK1	Receptor for Activated C Kinase 1
RyR1	Ryanodine-sensitive Ca^{2+} release channels
sALS	Sporadic Amyotrophic lateral sclerosis
SARIs	Serotonin antagonist and reuptake inhibitors
SCA8	Spinocerebellar ataxia type 8
SCAMPs	Secretory carrier-associated membrane proteins
SH3	Src homology 3
STAC	SH3 and cysteine-rich domains
TRP	Transient receptor potential
UPS	Ubiquitin-proteasome system
VGCC	Voltage-Gated Calcium Channels
VWA	Von Willebrand Factor A
WD-repeat	Tryptophan-aspartate repeat

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1 Introduction

1.1 Voltage-gated Calcium Channels

The process of calcium influx, triggered by membrane depolarization, plays a critical role in initiating various cellular functions (W. A. Catterall 2011). This influx is controlled by Voltage-Gated Calcium Channels (VGCC), which are pivotal in regulating calcium entry across different membrane potential ranges, particularly in excitable cells. These channels act as vital molecular converters of electrical signals, enabling calcium to enter cells during action potentials or subthreshold depolarizations. In muscle cells (cardiac, skeletal, and smooth), VGCCs facilitate excitation-contraction coupling (Cooper, Soeller, and Cannell 2010), while in neurons, they are essential at presynaptic terminals for rapid neurotransmission (Reid et al. 2004; Cao et al. 2004) and play a role in synaptic plasticity by activating gene expression (Dolmetsch et al. 2001). Additionally, VGCCs initiate hormone secretion in endocrine cells and trigger calcium-dependent enzyme activities (Comunanza et al. 2010).

VGCCs are classified into several types based on their pharmacological and electrophysiological characteristics: T-, N-, L-, P/Q-, and R-types (William A. Catterall et al. 2005). Each type has distinct physiological functions. T-type (or LVA, low voltage activated) and HVA (high voltage activated) channels are the two subgroups (Gardoni 2008)(Bean, 1985 Tsien et al., 1988, 1991). T-type channels, which activate at potentials more negative than -40 mV, have low unitary conductance and rapid inactivation. They are crucial in regulating cellular excitability due to their activation near resting membrane potentials. In contrast, N-, L-, R-, P/Q-type channels, known as high voltage activated, function at more positive potentials. Although these channels have similar biophysical profiles, they can be differentiated by their responses to dihydropyridine agonists and antagonists and specific peptide inhibitors from various venoms (Doering and Zamponi 2005).

HVA calcium channels are multi-subunit complexes formed by a central pore-forming subunit $Ca_v\alpha1$, and auxiliary subunits $Ca_v\alpha2\delta$, $Ca_v\beta$, and $Ca_v\gamma$ (Fig. 1) (Curtis and Catterall 1984; 1986; Flockerzi et al. 1986; dHosey et al. 1987; Leung, Imagawa, and Campbell 1987; Takahashi et al. 1987). These subunits combine to create functional HVA calcium channel complexes, while a single pore-forming $Ca_v\alpha1$ subunit is typical for LVA channels. The $Ca_v\alpha1$ subunit is composed of four homologous domains (I to IV), connected by cytoplasmic

links, with each domain containing six membrane-spanning helices (S1-S6) and a pore-forming regions (P-loops). These cytoplasmic links provide sites for regulatory protein interactions and are potential targets for secondary messenger regulation (William A. Catterall et al. 2005; William A. Catterall 2000). The channel's major functional properties are determined by the pore-forming α1 subunit, which defines the calcium channel subtype, while other subunits modulate Ca_vα1 properties.

There are ten distinct calcium channel α1 subunits, classified into three families: Ca_v1, Ca_v2, and Ca_v3. The Ca_v3 family encodes T-type calcium channels (Perez-Reyes 2003), $Ca_v2.1$, $Ca_v2.2$, and $Ca_v2.3$ correspond to P/Q-type, N-type, and R-type channels, respectively, and the Ca_v1 family represents L-type calcium channels (William A. Catterall et al. 2005). Furthermore, four Ca_vβ subunit genes, four Ca_vα2δ subunit genes and nine γ subunit genes have been identified, each with several splice variants (Arikkath and Campbell 2003). Co-expression of Ca_vβ and Ca_vα2δ with the Ca_vα1 subunit can alter the channel's biophysical properties, affecting voltage-dependences, activation-inactivation rates, and enhancing Ca_vα1 subunit trafficking to the plasma membrane. However, the effect of Ca_vα2δ subunits is generally milder and more selective compared to Ca_vβ subunits (Arikkath and Campbell 2003; Bichet et al. 2000; Dolphin 2003; Yasuda et al. 2004). Ca_vβ also influences Cav channel biogenesis by regulating ubiquitination and preventing channel degradation (Altier et al. 2011). Of the nine γ subunit genes, only the γ1 subunit is linked to calcium channels, playing a specific role in their regulation. The remaining eight γ subunit genes have a different function; they encode transmembrane regulators of glutamate receptors. These receptors are crucial for initiating excitatory neurotransmission in the brain, a process fundamental to neural communication and various brain functions (Nicoll, Tomita, and Bredt 2006). Thus, auxiliary subunits refine VGCC gating and regulate their expression levels on the plasma membrane.

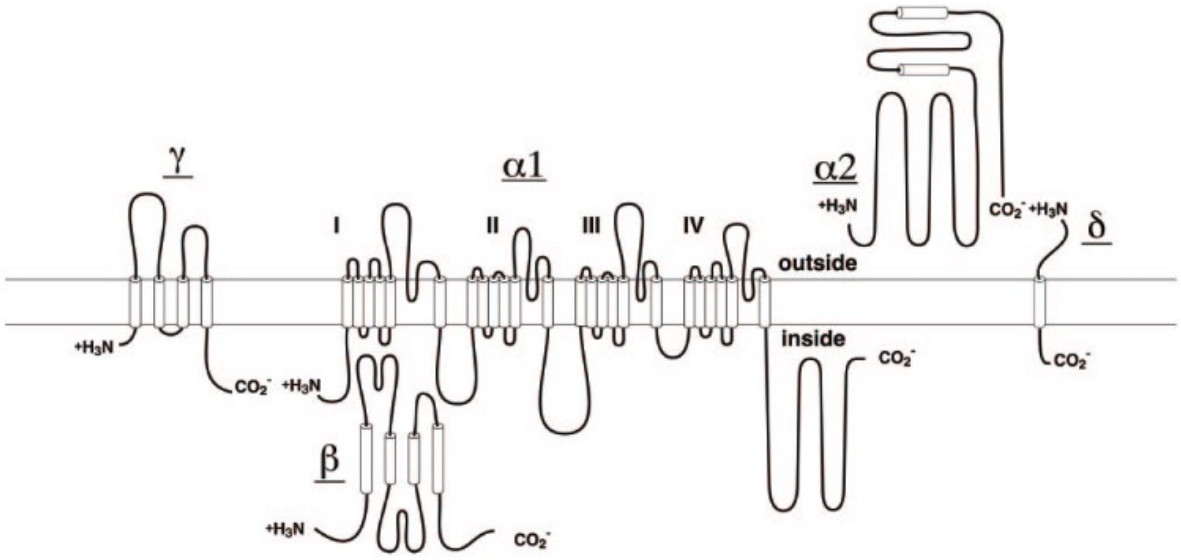

Figure 1 General structure of HVA channels including their auxiliary subunit distribution around the main α1 subunit (taken from (William A. Catterall et al. 2005)).

1.1.1 HVA Distribution and Function

HVA channels make up the majority of VGCCs and play pivotal roles in many physiological processes. L-types which are represented by Ca_v1 family are divided into four subtypes. Ca_v1 channels play a pivotal role in linking the depolarization of the plasma membrane to various cellular responses, demonstrated in processes like excitation-contraction coupling in muscle, excitation-transcription in nerve and muscle, and excitation-secretion coupling in endocrine cells and ribbon synapses (Armstrong, Bezanilla, and Horowicz 1972; Numa et al. 1990; Coetzee 1988; Liao et al. 2004; Tippens et al. 2008; Fabiato 1983; Bers 2002; Kollmar et al. 1997; Braun et al. 2009; Eliasson et al. 2008; Brandt, Striessnig, and Moser 2003; Mangoni et al. 2003; H.F. Vandael, Marcantoni, and Carbone 2015; Barnes and Kelly 2002).

L-type calcium channels, or Ca_v1 channels, are categorized into different subtypes based on their molecular structure and functional properties. These subtypes are $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$, and $Ca_v1.4$ (W. A. Catterall 2011). $Ca_v1.1$ is distributed primarily in skeletal muscles where it is essential for excitation-contraction coupling in skeletal muscle fibres. It plays a crucial role in translating the electrical signal into muscle contraction (Armstrong, Bezanilla, and Horowicz 1972). $Ca_v1.1$ channels in the transverse tubules interact directly with ryanodine-sensitive Ca^{2+} release channels (RyR1) in the sarcoplasmic reticulum, as shown in

high-resolution electron microscopy (Block et al. 1988). This interaction and the voltage-driven changes in the voltage-sensing domains of $Ca_v1.1$ channels directly activate RyR1 (Numa et al. 1990). $Ca_v1.2$ channels are distributed in cardiac muscle cells, smooth muscle cells, neurons, and endocrine cells. In cardiac muscles, it is key in initiating cardiac muscle contraction. Here, Ca^{2+} entry through $Ca_v1.2$ channels triggers RyR2 activation, leading to Ca^{2+}-induced Ca^{2+}-release, actomyosin activation, and contraction (Fabiato 1983; Bers 2002). $Ca_v1.2$ channels also play a role in setting the duration of the cardiac action potential, thereby influencing the heart rate and rhythm. They contribute to the plateau phase of the cardiac action potential, which is crucial for the rhythmic and coordinated contraction of the heart (Coetzee 1988). In smooth muscle cells, especially those lining blood vessels, $Ca_v1.2$ channels are involved in regulating vascular tone. Their activation leads to calcium influx, which promotes muscle contraction, thus influencing blood vessel diameter and blood pressure (Liao et al. 2004). In neurons, $Ca_v1.2$ channels are critical for the release of neurotransmitters (Kollmar et al. 1997). They are activated during the depolarization phase of the neuronal action potential, triggering the influx of calcium, which is a key signal for neurotransmitter vesicles to fuse with the cell membrane and release their contents (Tippens et al. 2008). These channels influence neuronal excitability and are involved in synaptic plasticity, a fundamental mechanism for learning and memory, regulating gene transcription (Tippens et al. 2008; Berger and Bartsch 2014). $Ca_v1.2$ channels are present in certain endocrine cells, such as pancreatic beta cells (Braun et al. 2009). The calcium influx through these channels triggers the release of hormones, like insulin, which is critical for glucose homeostasis (Eliasson et al. 2008). $Ca_v1.3$ is distribute in neurons, endocrine cells, and some types of smooth muscle cells. $Ca_v1.3$ channels are predominantly expressed in the cochlea's inner hair cells. They are essential for normal inner hair cell development and synaptic transmission, making them crucial for proper hearing (Brandt, Striessnig, and Moser 2003). It plays a role in neurotransmitter release in the nervous system and is involved in pacemaking activities in the heart and certain neurons (Mangoni et al. 2003; H.F. Vandael, Marcantoni, and Carbone 2015). In chromaffin cells, it contributes to catecholamine secretion (H.F. Vandael, Marcantoni, and Carbone 2015). $Ca_v1.4$ are distributed in the retina where they are critical for normal visual processing. It is involved in the phototransduction pathway in the retina by initiating exocytosis of neurotransmitters (Barnes and Kelly 2002).

The $Ca_v2.1$ channels, are a vital component of the nervous system. These channels are extensively distributed throughout the brain, particularly in the presynaptic terminals and somatodendritic membranes (Ludwig, Flockerzi, and Hofmann 1997; Dolphin and Lee 2020). Their primary role is in mediating neurotransmitter release, which is crucial for effective communication between neurons. $Ca_v2.1$ channels are key players in several neurological processes such as postsynaptic integration, neuroplasticity, neural excitability, and gene transcription, showcasing their wide-ranging influence on brain function (Ludwig, Flockerzi, and Hofmann 1997; Im, Me, and Bp 1992; R et al. 1992; Sutton et al. 1999; Hoxha et al. 2018; Folacci et al. 2023; Alehabib et al. 2021).

In the central nervous system, $Ca_v2.1$ channels are broadly expressed and are particularly prominent on the cell bodies and dendrites of cerebellar Purkinje cells and granule cells of the cerebellum (Ludwig, Flockerzi, and Hofmann 1997). This strategic positioning underscores their critical role in controlling the release of neurotransmitters (Sutton et al. 1999). These neurons in the cerebellum exhibit spontaneous firing, and the $Ca_v2.1$ channels contribute significantly to their regular pacemaking activity (Hoxha et al. 2018). This function is essential for maintaining the rhythmic firing patterns of these neurons, which is critical for their role in coordinating movement and balance (Folacci et al. 2023).This specific distribution highlights their importance in the cerebellar functioning, which plays a key role in motor coordination and balance (Alehabib et al. 2021).

$Ca_v2.2$ channels, also known as N-type calcium channels, are predominantly found in neuronal tissues and are characterized by their sensitivity to ω-conotoxins, a group of toxins isolated from marine cone snails (Reynolds et al. 1986). These channels are mainly localized on nerve terminals and dendrites, as well as in neuroendocrine cells, highlighting their importance in neuronal communication and hormone regulation (Westenbroek et al. 1992; Westenbroek, Hoskins, and Catterall 1998; Nowycky, Fox, and Tsien 1985).

$Ca_v2.2$ channels are essential mediators in the release of neurotransmitters and the transmission of sensory information from peripheral to central sites in the nervous system (Nowycky, Fox, and Tsien 1985). This function is particularly significant in sensory neurons, where $Ca_v2.2$ channels are highly expressed (Nowycky, Fox, and Tsien 1985). As the main presynaptic VGCCs, they are pivotal in controlling the flow of calcium ions, which is crucial

for neurotransmitter release and sensory signal transmission (Weber et al. 2010; Bourinet et al. 2014).

$Ca_V2.3$ channels, also known as R-type calcium channels, are primarily found in apical dendritic and spines of neurons throughout the central nervous system (CNS) (Sabatini and Svoboda 2000). They are defined by their resistance to blockers of L-type, N-type, and P/Q-type calcium channels, while they are sensitive to SNX-482, a peptide component of spider venom (Murakami et al. 2004). They play a variety of roles in synaptic plasticity, cerebellar function, nociception, and morphine tolerance (Metz et al. 2005; Tai, Kuzmiski, and MacVicar 2006; Kubota et al. 2001; Breustedt et al. 2003; Dietrich et al. 2003; Yokoyama et al. 2004; Osanai et al. 2006; Saegusa et al. 2000). They contribute significantly to synaptic transduction and aid in the release of neurotransmitters (Metz et al. 2005; Tai, Kuzmiski, and MacVicar 2006; Breustedt et al. 2003; Dietrich et al. 2003). They also play an important role in Purkinje cells aiding in spike generation (Osanai et al. 2006). This helps in motor coordination and learning (Kubota et al. 2001; Osanai et al. 2006). They have also been connected to pain transduction, especially in the activity of morphine (Yokoyama et al. 2004; Saegusa et al. 2000).

1.1.2 T-type calcium channels

1.1.2.1 Initial description and cloning

T-type calcium channels are a subset of VGCCs that activate at lower voltage thresholds. Their initial identification dates back to 1975, thanks to Hagiwara and team's research on starfish eggs (Hagiwara, Ozawa, and Sand 1975). The first detection of neuronal T-type calcium channels was in Purkinje and inferior olivary neurons (Regan 1991). T-type channels are now know to be ubiquitously expressed throughout the body, found in nervous tissues, the heart, kidneys, smooth muscle, reproductive cells, and several glands (Kostyuk, Shuba, and Savchenko 1988; Regan 1991; McKay et al. 2006; Cribbs et al. 1998a; Talley et al. 1999; Vassort, Talavera, and Alvarez 2006; Fry, Sui, and Wu 2006; Beam and Knudson 1988; Nilius and Droogmans 2001; Gu et al. 1999; Marcantoni et al. 2008; S.-N. Yang and Berggren 2006; Biagi and Enyeart 1991; Asem, Qin, and Rane 2002; Jagannathan, Publicover, and Barratt 2002).

The late 20th century, especially with the cloning of T-type channels by Perez-Reyes in the 1990s, offered deeper insights into the biophysical attributes and presence of these channels

in both central and peripheral nervous systems (Cribbs et al. 1998; J.-H. Lee et al. 1999; Perez-Reyes et al. 1998). Importantly, these channels were found to be integral in modulating neuronal network dynamics.

1.1.2.2 Molecular structure and biophysical properties

For mammals, these three T-type channel types—$Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$—are directed by individual genes: *CACNA1G* (chromosome 17), *CACNA1H* (chromosome 16), and *CACNA1I* (chromosome 22) (Cribbs et al. 1998) (Figure 2A). Further studies on these channels also revealed several splice variants, adding to T-type channels' molecular and functional diversity (Zhong et al. 2006; Latour et al. 2004; Murbartián, Arias, and Perez-Reyes 2004). Despite all three channel types operating at hyperpolarized potentials, their inactivation speeds differ, serving as a signature of their specific molecular make-up (Perez-Reyes and Schneider 1994).

T-type channels differ from other VGCCs; they don't merge with auxiliary subunits but stand alone with only the central Ca_v3 core subunit. T-type channels have a membrane structure comparable to other members of the VGCC family. Recent cryo-electron microscopy studies of $Ca_v3.1$ and $Ca_v3.3$ channels have offered significant understanding into the molecular processes of channel operation and the effects of drugs (Zhao et al. 2019; L. He et al. 2022). The Ca_v3 core comprises four homologous domains (DI to DIV), each consisting of six transmembrane helices (S1 to S6). These domains are connected by intracellular loops. A central pore is made up of four loops (p-loops) connecting the S5 and S6 segments of each domain, with four essential acidic residues ensuring calcium selectivity. The S4 segments, enriched with charged residues, play a role in voltage detection (Jurkovicova-Tarabova et al. 2018). These components, together with the amino and carboxy termini, create a molecular centre for protein interactions. This influences the expression and regulation of the channel at the plasma membrane (Weiss and Zamponi 2023) (Figure 2B).

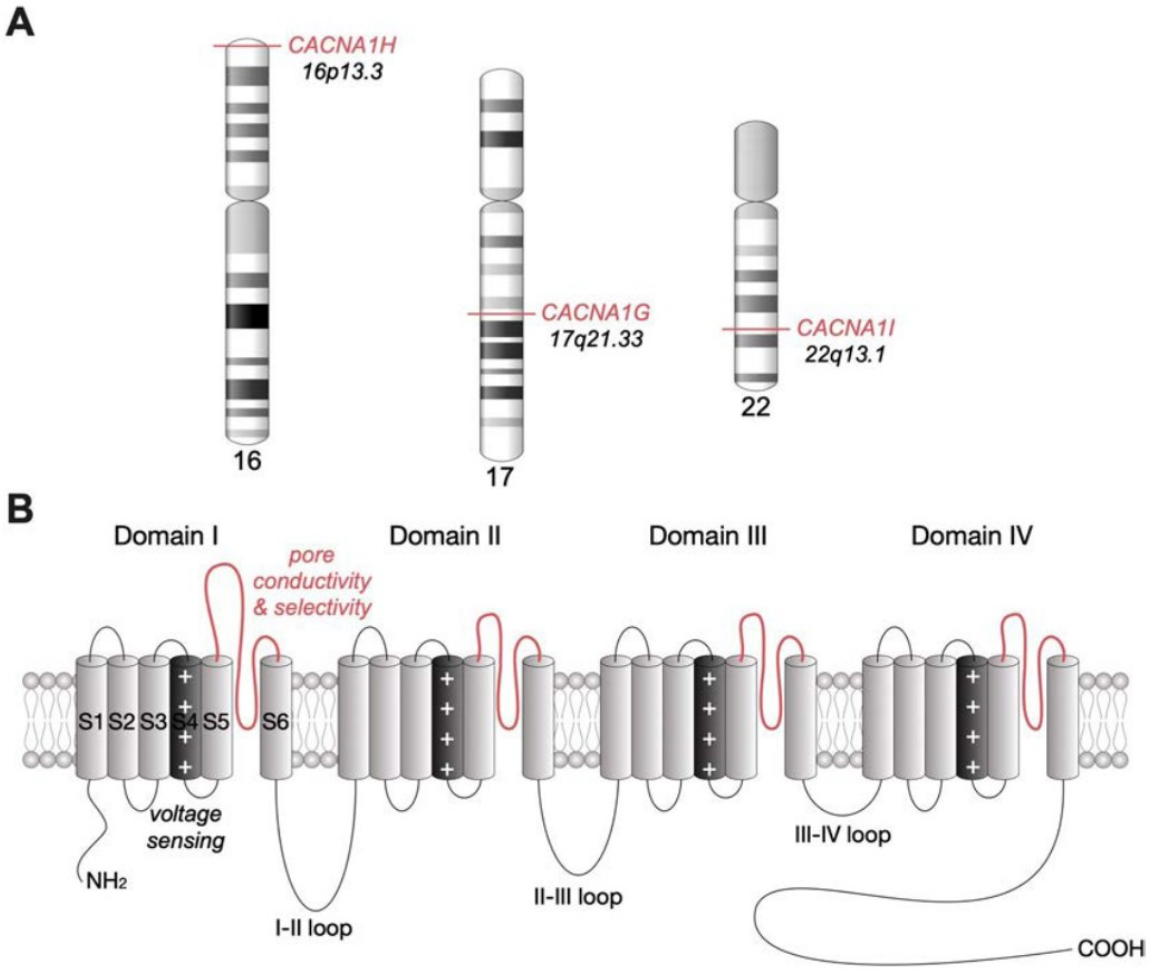

Figure 2 Cav3 channel location in human chromosomes and their membrane structure (taken from (Weiss and Zamponi 2020)). A: The three chromosomes and the specific genes encoding for $Ca_v3.1$, $Ca_v3.2$, $Ca_v3.3$. B: Ca_v3 secondary structure in the membrane and their main structures integral for gating.

These channels have unique biophysical properties compared to HVA VGCCs. They open in response to smaller depolarizations compared to other types of voltage-gated calcium channels. This allows them to be activated at more negative membrane potentials compared to HVA, typically in the range of -60 to -50 mV (Chemin et al. 2002). Due to this, they play a key role in controlling neuronal activity, by enhancing subthreshold excitatory postsynaptic potentials, and propagating electrical impulses to the cell body (Crandall, Govindaiah, and Cox 2010). These channels typically produce transient currents, hence T-type (transient), which means they open briefly and then quickly inactivate compared to HVA channels (J R Huguenard 1996). This rapid inactivation contributes to the short-lived nature of the calcium influx.

T-type channels also exhibit both voltage-dependent and calcium-dependent inactivation in which an increase in submembrane Ca^{2+} induces a significant decrease in T-type current amplitude due to a hyperpolarizing shift in the steady-state inactivation (Cazade et al. 2017). These channels can also recover from inactivation relatively quickly compared to HVA channels. A key characteristic of T-type channels is their ability to produce calcium spikes during membrane hyperpolarization, leading to rebound burst firing (D. Kim et al. 2001). Interestingly, these channels also have overlapping voltage-activated and inactivated states. This maintains a “window current”, facilitating passive calcium influx at resting membrane potentials (S. R. Williams et al. 1997).

During a typical neuronal action potential, T-type channels are activated in the initial depolarization phase, provided the membrane potential was sufficiently hyperpolarized beforehand. However, the primary calcium conductance through these channels happens during repolarization, as cells rapidly return to resting potential, mainly due to voltage-gated potassium channel activation (Llinás, Steinberg, and Walton 1981; McCobb and Beam 1991). This occurs because T-type channel kinetics are relatively slow compared to the action potential duration, keeping many channels open during repolarization. As the cell moves further from the calcium equilibrium potential (generally around +10 to +40 mV), the calcium conductance increases, creating a "tail current" that then deactivates (McCobb and Beam 1991).

1.1.2.3 Tissue distribution and physiological functions

These channels are prevalent in both the central and peripheral nervous systems, playing a crucial role in modulating neuronal activities (Fig. 3). For instance, in thalamic and hippocampal neurons, their activation at low membrane potentials amplifies subthreshold excitatory potentials and aids signal transmission to the cell body (Crandall, Govindaiah, and Cox 2010). Furthermore, their expression in the axon initial segment allows them to regulate the timing and generation of action potentials in axons (Bender and Trussell 2009). In thalamocortical circuits, the ability of T-types to aid in rebound burst firing contributes to spike-and-wave discharges seen in conditions like absence epilepsy (D. Kim et al. 2001). In thalamocortical neurons, the ability of T-type channels to maintain a "window current" is

essential for neuronal oscillations during sleep rhythms (S. R. Williams et al. 1997; Hughes et al. 2002).

T-type channels also play a key role in neurotransmitter release at synapses via Ca^{2+}-dependent vesicle binding to the plasma membrane (Fig.3)(For a full review see 101). This is primarily in GABAergic and glutamatergic synapses. For example, in hippocampal perisomatic targeting interneurons, $Ca_v3.1$ plays a role in release of GABA with the aid of release of Ca^{2+} from internal stores (Tang et al. 2011). In cortical neurons, presynaptic $Ca_v3.2$ aids the release of glutamate into the synaptic cleft (Z. Huang et al. 2011). Furthermore in dopaminergic neurons, activation of T-type channels controls dopamine release from the soma (Y. Kim et al. 2007).

T-type calcium channels also play a crucial role in various neurodevelopmental processes, including the growth of axons and dendrites, as well as neuronal migration. These channels are key in initiating localized calcium oscillations, which are vital for the expression of guidance molecules in developing neurons. For example, in chick embryonic motor neurons, these oscillations are essential for axonal migration (S. Wang, Polo-Parada, and Landmesser 2009). Furthermore, T-type channels are involved in TNFα reverse signalling-induced axonal growth in sympathetic neurons (Kisiswa et al. 2017).

In addition to these roles, T-type channels have been implicated in the regulation of other types of calcium channels. For instance, a study found that disrupting $Ca_v3.2$ through oligonucleotide based knockdown or pharmacological inhibition in neuroblastoma NG108-15 cells altered the expression of HVA calcium channels as these cells differentiated (Chemin, Nargeot, and Lory 2002). This interplay was further evidenced by (Nagasawa et al. 2009), who showed that hydrogen sulphite-induced neurite outgrowth and the expression of HVA channels in NG108-15 cells depended on the activation of T-type channels. This process is believed to involve Src kinase-dependent signalling pathways (Tarui et al. 2010). T-type channels are also crucial in maintaining the viability of neuronal progenitor cells (J.-W. Kim et al. 2018). These finding suggests that T-type channels might play a significant role in neurodevelopmental or neurodegenerative disorders, offering a new perspective on their importance in the nervous system.

T-type channels are also found in other tissues apart from the nervous system contributing to specific cellular functions. For example, $Ca_v3.1$ help regulate cardiac pacemaking in sinoatrial and atrioventricular nodes (Fig. 3)(Mesirca, Torrente, and Mangoni 2014). In vascular smooth muscles, $Ca_v3.1$ and $Ca_v3.2$ help control vascular tone (Cazade et al. 2014; El-Rahman et al. 2013), possibly due to their interaction with Ca^{2+}-sensitive K^+ channels (BK_{Ca}) which are key mediators in smooth muscle relaxation (Brenner et al. 2000; M. T. Nelson and Quayle 1995). Although absent in adult skeletal muscle, $Ca_v3.2$ is involved in the development of embryonic fibers, aiding in the maturation of myoblasts (Fig. 3)(Berthier et al. 2002; Bijlenga et al. 2000).

T-type channels are significant in hormone secretion, with $Ca_v3.2$ channels aiding in the release of aldosterone and catecholamines from the adrenal cortex and chromaffin cells (Mahapatra et al. 2012; T. Yang et al. 2020), and facilitating insulin secretion in pancreatic β-cells (Fig. 3)(Barghouth et al. 2022). They are also found in non-excitable tissues, such as immune cells, where Cav3.1 may activate T lymphocytes (H. Wang et al. 2016), and in blood cells, $Ca_v3.2$ contributes to platelet activation and arterial thrombosis (Fig. 3)(Tamang et al. 2022; Weiss 2022). $Ca_v3.2$ channels also play roles in sperm function, aiding in the acrosome reaction during fertilisation (Escoffier et al. 2007), and chondrogenesis in tracheal cartilage (S.-S. Lin et al. 2014).

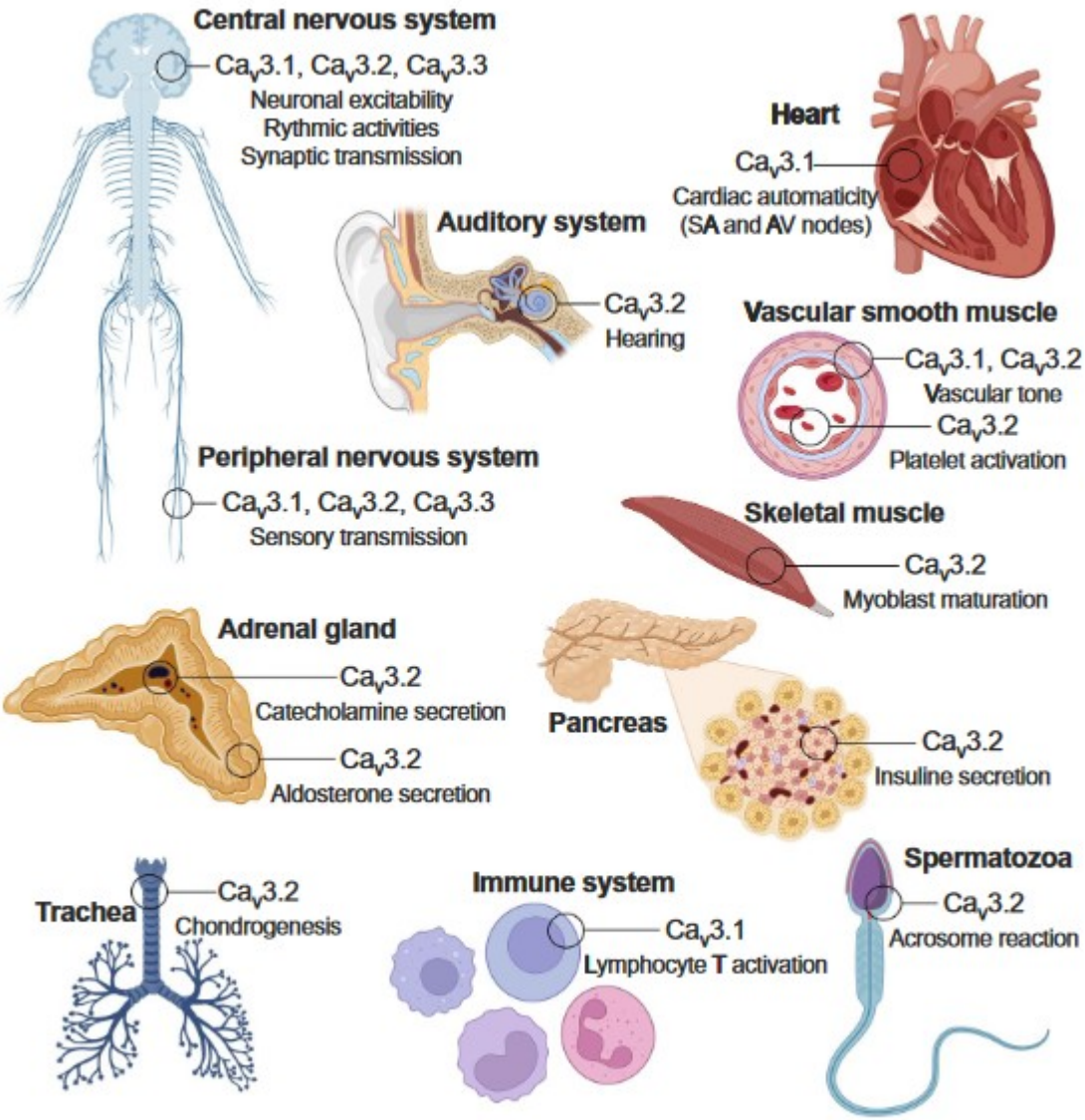

Figure 3 T-type channel tissue distribution and physiological function (taken from (Weiss and Zamponi 2023).

In line with T-type channel functions, $Ca_V3.1$ deficient mice demonstrate a variety of physiological changes. These include increased sensitivity to visceral pain in established rodent pain models, which is thought to be due to alterations in the way pain is processed in the thalamus, where they are abundantly expressed (D. Kim et al. 2001). Additionally, these mice exhibit bradycardia (Mangoni et al. 2006), which makes sense considering their role in cardiac pacemaking and vascular tone (Mesirca, Torrente, and Mangoni 2014; Cazade et al. 2014; El-Rahman et al. 2013). Remarkably, they show resistance to conditions such as

experimentally induced epilepsy, which likely due to their role in burst firing in hippocampal and thalamic neurons (D. Kim et al. 2001; Sakkaki et al. 2016). They are also resistant to high-fat diets (Uebele et al. 2009), which is surprising considering evidence for disturbed sleep patterns and decreased slow wave sleep in mice lacking $Ca_v3.1$ (J. Lee, Kim, and Shin 2004). In humans a lack of sleep has been found to correlate with obesity and other metabolic disorders (Taheri 2006; S. R. Patel et al. 2006; Cizza, Skarulis, and Mignot 2005). This may outline the fact that rodent models are not a perfect representation of human physiology.

In contrast, mice lacking $Ca_v3.2$ channels present a different set of physiological alterations. These include an increased level of anxiety and difficulties with memory (Gangarossa et al. 2014). It is difficult to identify the reason for an increase in anxiety due to the complex nature of this mental state. However, the deficits in memory could be due to the expression of $Ca_v3.2$ in the hippocampus and amygdala where it is a key regulator of neurotransmitter release (Carbone, Calorio, and Vandael 2014), which is important for long-term potentiation of memory formation (Korchounov and Ziemann 2011) which has already been associated with block of T-type channels (Ly et al. 2013). They also have an increased hearing threshold, which is evidenced by their expression in the auditory system (Lundt et al. 2019). Furthermore, these mice show reduced sensitivity to pain (S. Choi et al. 2007) which likely due to their expression in DRGs which are integral for processing peripheral pain (Talley et al. 2000). $Ca_v3.2$ deficient mice also show a reduced ability for vascular smooth muscle contraction (C.-C. Chen et al. 2003), possibly due to their interaction with BK_{Ca} channels which are activated from the influx of Ca^{2+} from $Ca_v3.2$ (Brenner et al. 2000; M. T. Nelson and Quayle 1995). Furthermore, $Ca_v3.2$ deficient mice had narrow and elliptically shaped tracheas (S.-S. Lin et al. 2014). This is due to their expression in mesenchymal cells where they essential for differentiation into chondrocytes through interaction with calcineurin/nuclear factor of the activated T-cell (NFAT) signalling pathway (Matta et al. 2008; Shao, Alicknavitch, and Farach-Carson 2005).
Lastly, mice deficient in $Ca_v3.3$ channels exhibit disturbances in sleep rhythmogenesis due to their significantly less rebound bursting in nucleus reticularis thalami (nRt)(Talley et al. 1999; Astori et al. 2011; Pellegrini et al. 2016), a crucial generator of oscillatory bursts which produce sleep spindles (Steriade 2006). They also have an increased sensitivity to anaesthesia induced by isoflurane, which is also thought to be due to the reduced activity of nRt (Timic Stamenic et al. 2020). This is due to nRT important regulation of the thalamus and sensory

cortex due to their abundant inhibitory GABAergic neurons composition (Mc Alonan and Brown 2002; Pinault 2004).

Mutations in genes encoding T-type channels have been associated with several human disorders, including epilepsy, hypertension, autism, amyotrophic lateral sclerosis, and schizophrenia.

These channels are essential in shaping neuronal activities, such as low-threshold calcium spikes and burst firing. This is controlled by the unique biophysical properties of T-type channels, which will be described in this thesis. However, for a full in-depth review see (Cain and Snutch 2010).

T-type channel dysfunction has been linked to many chronic conditions, including hyperaldosteronism, various epileptic disorders, cancer, certain pain conditions, and motor defects (Talley et al. 2000; Heinzen et al. 2007; Meis, Biella, and Pape 1996; Scholl et al. 2015; Khosravani and Zamponi 2006; M. Nelson, Todorovic, and Perez-Reyes 2006; Jagodic et al. 2007; D. Wang et al. 2006). Emerging studies also hint at T-type channels' roles in other health issues. Some mutations in the *CACNA1H* gene have been linked to autism, with these mutations affecting the activity of $Ca_V3.2$ channels (Splawski et al. 2006). A significant number of autism patients also have epilepsy (Splawski et al. 2006). There's a possibility that these gene mutations might impact brain development, potentially contributing to autism's onset. Moreover, T-type calcium channel blockers show potential as treatments for conditions like schizophrenia and essential tremor (Uslaner et al. 2012).

To sum up, T-type calcium channels have unique features that play pivotal roles in various cellular activities, and when they malfunction, it can result in neurological issues. Their activity is adjusted by several cellular mechanisms, and they have emerged as important targets for potential drug therapies (Belardetti and Zamponi 2008; Bergson et al. 2011; Weiss 2019).

1.1.2.4 Regulation

T-type calcium channels, critical to neuronal and non-neuronal cell activity, have an intricate regulation system. Their involvement in numerous physiological and pathological processes is modulated by a variety of endogenous and exogenous factors, going through a number of regulatory processes. These will be explored below.

1.1.2.4.1 Regulation by interacting proteins

KLHL1

KLHL1, a brain-specific member of the actin-binding protein family. It is characterized by its BTB/POZ (broad complex, tramtrack, bric-a-brac/poxvirus, and zinc finger) domains and actin-binding Kelch domains, playing a key role in neuronal structure and function (Shi et al. 2019). This protein is crucial in organizing actin, impacting neurite formation and dendritic spine head dynamics (S. Jiang et al. 2007). Its significance is highlighted in the context of spinocerebellar ataxia type 8 (SCA8), where CTG trinucleotide repeat expansions in the KLHL1 gene's untranslated antisense RNA are implicated (Nemes, Benzow, and Koob 2000). This genetic link is corroborated by the phenotype observed in KLHL1 knockout mice, which mirrors SCA8 (Y. He et al. 2006).

KLHL1's influence extends to the regulation of VGCCs. It interacts with $Ca_v2.1$ and $Ca_v3.2$ channels, both in recombinant and native systems (K. A. Aromolaran et al. 2007; 2010; Kelly A. Aromolaran et al. 2009). In HEK293 cells, KLHL1 enhances calcium conductance when co-expressed with $Ca_v2.1$ (K. A. Aromolaran et al. 2007). Particularly notable is KLHL1's specific interaction with $Ca_v3.2$ channels, boosting their plasma membrane expression through facilitating their endosomal recycling. This specificity is underscored by the lack of regulation on $Ca_v3.1$ and $Ca_v3.3$ channels by KLHL1 (K. A. Aromolaran et al. 2010; Kelly A. Aromolaran et al. 2009).

Further studies employing shRNA-mediated knockdown of KLHL1 in cultured hippocampal neurons demonstrate a decrease in the expression levels of $Ca_v2.1$ and $Ca_v3.2$, accompanied by reductions in HVA and LVA calcium currents, and a decrease in postsynaptic currents (Perissinotti et al. 2014). These effects are partially echoed in hippocampal neurons from KLHL1 knockout mice, where a decrease in $Ca_v3.2$ channels is somewhat offset by an increase in $Ca_v3.1$ channel expression (Perissinotti et al. 2015).

Spectrin α/β and ankyrin B

Spectrin proteins, found on the inner side of the plasma membrane, interact closely with the actin cytoskeleton. They are crucial for maintaining membrane stability and structure, and they play a key role in anchoring and grouping membrane proteins like ion channels and receptors. This is done through their connection with ankyrins, which act as a bridge between the membrane proteins and the spectrin-actin based membrane cytoskeleton (refer to (Machnicka et al. 2014; Stevens and Rasband 2022) for more details). For instance, the clustering of voltage-gated sodium channels at the axon initial segment and nodes of Ranvier, important for fast and effective action potential propagation, depends on their interaction with the actin-based cytoskeleton (Ho et al. 2014; Komada and Soriano 2002; Liu, Stevens, et al. 2020; Liu, Seo, et al. 2020). In a similar vein, T-type channels such as $Ca_v3.1$ and $Ca_v3.2$ have been shown to interact with various cytoskeletal proteins like spectrin α_I, spectrin β_{II}, spectrin β_{III}, and ankyrin B. This interaction occurs through a specific α-helical stretch of charged amino acids in the channels' proximal carboxy-terminal region (Garcia-Caballero et al. 2018). Removing this binding site reduces the channels' functional expression in tsA-201 cells and their mobility in re-expressed cultured hippocampal neurons. Additionally, reducing spectrin α_I and ankyrin B levels in hippocampal neurons leads to lower native $Ca_v3.1$ and $Ca_v3.2$ channel expression. This finding is consistent with other studies showing that ankyrin B's binding to $Ca_v2.1$ and $Ca_v2.2$ channels, through the cytoplasmic loop between repeats II and III, is vital for the channels' correct positioning on the cell surface (C. S. W. Choi et al. 2019; Kline et al. 2014). Similarly, ankyrin B's attachment to the distal part of the carboxy-terminal domain of $Ca_v1.3$ is necessary for the channel's proper localization in atrial myocytes (Cunha et al. 2011). Therefore, the attachment of cytoskeletal proteins to VGCCs, through various channel-specific molecular features, appears to be a common regulatory mechanism for ensuring the channels are correctly positioned on the cell surface.

STAC1

STAC (Src homology three (SH3) and cysteine-rich domains) proteins, consisting of STAC1, STAC2, and STAC3 isoforms, are a small group of adaptor proteins. Their role was unclear for many years, but they are now recognized as important regulators of VGCCs. STAC3, primarily found in skeletal muscles (B. R. Nelson et al. 2013), is crucial in the excitation-contraction coupling process. It does this by interacting with and influencing the expression of $Ca_v1.1$ channels, playing a significant part in muscle function (see reviews (Flucher and

Campiglio 2019; Rufenach and Van Petegem 2021)). In contrast, STAC1 and STAC2 are mainly located in nerve cells [146], such as dorsal root ganglia (DRG) neurons. They are involved in defining specific types of nociceptors (Legha et al. 2010).

Research has shown that STAC1 forms a complex with $Ca_v3.2$ channels by binding to the distal area of the cytoplasmic amino-terminal domain of $Ca_v3.2$. This interaction boosts $Ca_v3.2$'s presence in the plasma membrane, leading to increased T-type currents without altering the channel's gating characteristics (Rzhepetskyy, Lazniewska, Proft, et al. 2016). STAC proteins also influence neuronal $Ca_v1.2$ channels. However, unlike with $Ca_v3.2$, the STAC's interaction with $Ca_v1.2$ mainly involves the IQ domain within $Ca_v1.2$'s carboxy-terminal region and disrupts the channel's calcium-dependent inactivation (Campiglio et al. 2018; Polster et al. 2018).

The full physiological impact of these interactions is still being studied. Notably, Stac2 was identified as the most upregulated gene in DRGs in a rodent model of nerve injury-induced chronic pain (K. E. Stephens et al. 2019). Given the known role of $Ca_v3.2$ channels in pain (Cai et al. 2021), it's plausible that STAC2's enhancement of $Ca_v3.2$ function could contribute to the sensitization of peripheral nociceptive neurons. This hypothesis is significant as it may reveal a potential mechanism in the development of chronic pain, warranting further investigation.

RACK1

RACK1 (Receptor for Activated C Kinase 1) is a scaffold protein that is part of the tryptophan-aspartate repeat (WD-repeat) family. Initially known for its link with the activated form of protein kinase C (PKC) βII, RACK1 has emerged as a key molecular junction, binding to a variety of signalling proteins. This binding affects numerous aspects of these proteins, including their movement within the cell, activity, interactions with other proteins, and stability (for a detailed review, see (Adams, Ron, and Kiely 2011)). As a result, several ion channels, such as the inositol 1,4,5-trisphosphate receptor (IP3R) (Patterson et al. 2004), the transient receptor potential (TRP) Pkd2L1 channel (J. Yang et al. 2012), and the BK_{Ca} channel (Isacson et al. 2007), have been found to interact with RACK1. This interaction leads to changes in their functional expression.

A prime example of RACK1's role in regulating ion channels is its essential role in the formation of the multi-channel complex Orai1-STIM1-TRPC3-RACK1-IP3R. This complex allows for precise control of intracellular calcium levels in response to various channel agonists (Woodard et al. 2010). Studies have shown that RACK1 forms a molecular complex with $Ca_v3.2$, binding to the cytoplasmic linker between repeats II and III, and the carboxy-terminal region of the channel (Gandini et al. 2022). Intriguingly, when RACK1 is co-expressed with $Ca_v3.2$ in tsA-201 cells, there is a decrease in the channel's expression in the plasma membrane. However, this effect is negated in the presence of PKCβII, suggesting an interaction between RACK1 and PKCβII in regulating $Ca_v3.2$ channels.

Although the exact mechanisms behind this regulation are not fully understood, another study reported PKC-dependent phosphorylation of $Ca_v3.2$ at serine residues (positions 1144 and 2188 in the human channel), which align with the sites of RACK1/$Ca_v3.2$ interaction, enhances the channel's surface expression (Gaifullina et al. 2019). Therefore, it's conceivable that PKC might increase $Ca_v3.2$'s surface expression by mitigating the inhibitory effect of RACK1.

SNARE

Syntaxin 1A, a member of the syntaxin superfamily, plays a pivotal role in the docking of synaptic vesicles with the presynaptic plasma membrane in neurons. It is recognized not only for this role but also as a regulator of presynaptic calcium entry. This regulatory function involves interactions with neuronal $Ca_v2.1$ and $Ca_v2.2$ channels, as highlighted in various studies (for a comprehensive review, see (Weiss and Zamponi 2012)). The regulation mainly occurs through the binding of synaptic proteins to the "synprint" domain of these channels, located in the cytoplasmic loop between repeats II and III. These interactions are essential for facilitating neurotransmitter release.

Interestingly, research has shown that T-type channels, known for supporting fast and low-threshold exocytosis (Carabelli, Marcantoni, Comunanza, and Carbone 2007; Carabelli, Marcantoni, Comunanza, De Luca, et al. 2007; Ivanov and Calabrese 2000; Pan et al. 2001), might also interact with synaptic proteins involved in vesicular release (Weiss and Zamponi 2013). In particular, syntaxin 1A has been found to interact with the carboxy-terminal domain of $Ca_v3.2$ (Weiss et al. 2012). This interaction, while not altering $Ca_v3.2$'s expression level in

the plasma membrane of tsA-201 cells, significantly modifies the channel's gating properties. It causes a shift in the voltage dependence of inactivation, and to some extent activation, towards more hyperpolarized membrane potentials. Similar effects have been observed with $Ca_v3.1$ and $Ca_v3.3$ channels, although their interactions with syntaxin 1A require further investigation.

The regulation of $Ca_v3.2$ by syntaxin 1A is particularly noteworthy because it is negated when SNAP-25, which also binds to the carboxy-terminal domain of $Ca_v3.2$, is co-expressed (Weiss et al. 2012). This cross-talk is reminiscent of the combined effects of syntaxin 1A and SNAP-25 on $Ca_v2.1$ and $Ca_v2.2$ channels. Significantly, this interaction between syntaxin 1A and $Ca_v3.2$ has been shown to support T-type channel-mediated exocytosis in chromaffin cells (Carabelli, Marcantoni, Comunanza, and Carbone 2007), suggesting that T-type channels, despite having different molecular binding sites, engage with the vesicular release machinery in a manner akin to Ca_v2 channels. This finding provides insight into the complex interactions and regulatory mechanisms within the neuronal signalling and vesicular release systems.

Calnexin

Calnexin (CNX) is an endoplasmic reticulum (ER) chaperone protein known for its role in aiding the folding and quality control of newly synthesized glycoproteins before they proceed through the secretory pathway. This function is crucial, as most plasma membrane proteins, including ion channels, are glycosylated at their extracellular domains (Kozlov and Gehring 2020; Lazniewska and Weiss 2017; 2014). T-type calcium channels such as $Ca_v3.2$ are among these glycosylated proteins (Orestes et al. 2013; Weiss et al. 2013).

Studies have identified a molecular complex formed between CNX and $Ca_v3.2$, hinging on the interaction between the cytosolic carboxy-terminal region of CNX and the cytosolic domain of $Ca_v3.2$ that links repeats III and IV (Proft et al. 2017). This interaction predominantly limits the exit of $Ca_v3.2$ from the ER. When CNX is co-expressed with $Ca_v3.2$ in tsA-201 cells, there is a notable decrease in the channel's surface expression. This regulatory mechanism is particularly significant in $Ca_v3.2$ channels that include exon 25, which encodes a small portion of the III-IV linker. This observation suggests that CNX's influence on $Ca_v3.2$ is delicately modulated through alternative splicing of the channel.

In the context of neurological disorders, the GAERS mutation found in a rodent model of absence epilepsy, which involves replacing an arginine with a proline within the III-IV linker of $Ca_v3.2$, disrupts the CNX/$Ca_v3.2$ interaction. This disruption leads to an increased presence of the channel in the plasma membrane. This change is likely a contributing factor to the heightened T-type currents observed in reticular thalamic neurons derived from GAERS, which are thought to be central to the development of seizures in this model (Tsakiridou et al. 1995).

It is also noteworthy that similar to what has been observed with $Ca_v3.2$ channels, co-expression of CNX with $Ca_v3.1$ and $Ca_v3.3$ channels in tsA-201 cells results in a comparable downregulation of T-type conductance. However, the specific details of CNX's interaction with $Ca_v3.1$ and $Ca_v3.3$ channels remain to be fully elucidated (Proft et al. 2017).

CACHD1

Early research suggested that the plasma membrane expression of T-type channels could be influenced by known VGCC ancillary subunits $Ca_v\beta$ and $Ca_v\alpha2\delta$ (Dubel et al. 2004), possibly via low-affinity interactions (Thompson et al. 2011; Bae, Suh, and Lee 2010). However, these interactions and their regulatory effects have been a subject of debate (Arteaga-Tlecuitl et al. 2018; Leuranguer et al. 1998). This leaves the precise mechanisms through which $Ca_v\beta$ and $Ca_v\alpha2\delta$ might affect T-type channels somewhat unclear.

In contrast to this uncertainty, a recent discovery has brought to light CACHD1 (calcium channel and chemotaxis receptor (cache) domain containing protein 1) as a novel modulator of Ca_v3 channels, with functional similarities to $Ca_v\alpha2\delta$ (Cottrell et al. 2018; G. J. Stephens and Cottrell 2019). Despite not sharing significant sequence identity with traditional $Ca_v\alpha2\delta$ proteins, CACHD1 possesses a characteristic von Willebrand Factor A (VWA) domain, similar to $Ca_v\alpha2\delta$, along with a bacterial chemosensory-like cache domain.

CACHD1 is particularly abundant in brain regions such as the thalamus, hippocampus, and cerebellum, which also have a high prevalence of T-type channels. When co-expressed with $Ca_v3.1$ channels in tsA-201 cells, CACHD1 forms a molecular complex that increases the membrane expression of all three T-type channel members (Cottrell et al. 2018). Beyond just

boosting their presence in the plasma membrane, CACHD1 also seems to alter the biophysical properties of $Ca_v3.1$, notably increasing the channel's open probability. As a result, the overexpression of CACHD1 in cultured hippocampal neurons enhances T-type channel-mediated neuronal excitability (Cottrell et al. 2018).

A key distinction of CACHD1, compared to $Ca_v\alpha2\delta$ members which are known to exclusively affect HVA calcium channels, is its broader influence. CACHD1 not only impacts $Ca_v3.x$ channels but also has been demonstrated to interact with $Ca_v2.2$ channels. In the case of $Ca_v2.2$, CACHD1 enhances their expression at the cell surface by stabilizing these channels in the plasma membrane (Dahimene et al. 2018).

Caveolins

Caveolins are a small family of membrane proteins known for their association with Caveolae, a specialized type of lipid rafts. These proteins are crucial for forming and maintaining Caveolae and also act as scaffolding proteins within the Caveolar membrane, organizing and concentrating signalling molecules (for an in-depth review, see (T. M. Williams and Lisanti 2004)). There are three mammalian Caveolins, of which Caveolin-3 (CAV-3) is primarily found in striated and smooth muscles, where it interacts with a range of ion channels, including $Ca_v1.2$ channels (Bryant et al. 2014).

CAV-3 also interacts with both recombinant and native $Ca_v3.1$ and $Ca_v3.2$ channels in ventricular myocytes (Markandeya et al. 2011). When CAV-3 is co-expressed in HEK293 cells, there is a significant decrease in conductance in cells expressing $Ca_v3.2$, but not in those expressing $Ca_v3.1$. This reduction occurs without altering the surface expression of the channel. Furthermore, overexpressing CAV-3 inhibits $Ca_v3.2$ currents in isolated ventricular myocytes. Conversely, knocking down CAV-3 in ventricular myocytes doesn’t seem to affect T-type channels directly but does abolish their potentiation by protein kinase A (PKA).

Calmodulin

Calmodulin (CaM) is a highly versatile calcium-binding protein known for modulating the activity of numerous effector proteins via calcium signalling. Its role in regulating VGCCs has been the subject of extensive study. CaM is associated with almost all HVA calcium

channels, evidencing its integral role in the calcium channel complex (for detailed reviews, refer to (Ben-Johny and Yue 2014; S. R. Lee et al. 2015)).

CaM has also been shown to bind to the carboxy-terminal region of recombinant and native $Ca_v3.1$ channels (Asmara et al. 2017). Upon activation of $Ca_v3.1$ at rest the influx of calcium leads to the dissociation of CaM from the channel. This dissociation triggers the activation and phosphorylation of αCaMKII (calcium/calmodulin-dependent protein kinase II).

Furthermore, it has been reported that all three T-type channel isoforms interact with CaM. The high-affinity binding site for CaM has been identified as the proximal region of the cytoplasmic linker connecting repeats I and II, termed the "gating brake" (Chemin et al. 2017). Intriguingly, when the gating brake peptide is introduced into HEK293 cells expressing $Ca_v3.2$, it results in a hyperpolarizing shift in the voltage dependence of activation and inactivation of the channel, and faster gating kinetics.

Though it is not known whether these gating effects are due to the direct dissociation of CaM from the channel (an allosteric modulation) or if they are the result of a feedback loop activated by CaM following its dissociation from the channel. This feedback loop could potentially involve the activation of CaMKII (Barrett et al. 2000; Lu et al. 1994; Welsby et al. 2003; Wolfe et al. 2002).

G-protein $\beta_2\gamma_2$

VGCCs are significantly modulated by a wide array of heterotrimeric G protein-coupled receptors (GPCRs) (Waard et al. 1997). The activation of these receptors leads to the exchange of GDP for GTP on the Gα subunit, resulting in a structural change within the Gαβγ complex. This reconfiguration causes the complex to dissociate into two separate, active signalling substances: the Gβγ dimer and the GTP-bound Gα.

Both the Gβγ dimer and the Gα-GTP initiate a variety of signalling pathways. These include the activation of protein kinases which phosphorylate specific proteins, such as ion channels. However, the Gβγ dimer is also known for its ability to directly associate with neuronal VGCCs, especially $Ca_v2.1$ and $Ca_v2.2$ channels (Waard et al. 1997; Zamponi et al. 1997).

This binding occurs through a G protein-binding pocket created by specific molecular components of the channel, leading to a substantial inhibition of the channel's activity.

This inhibition is described as voltage-dependent because it can be experimentally reversed by strong membrane depolarization or, under physiological conditions, by a series of action potentials. These action potentials are thought to cause the temporary dissociation of the Gβγ dimer from the channel. This mechanism is a key aspect of how GPCRs regulate VGCCs and is extensively reviewed in literature (J. Huang and Zamponi 2017; Proft and Weiss 2015).

One significant physiological outcome of this regulation is its role in how opioids enact their effects (Weiss and Zamponi 2021). Opioids, by activating their respective GPCRs, may indirectly modulate VGCC activity through the Gβγ dimer's interaction with the channels (Wolfe et al. 2002; Lledo et al. 1992; Marchetti, Carbone, and Lux 1986; P. J. Williams, MacVicar, and Pittman 1990; DePuy et al. 2006; Hu et al. 2009). Understanding this complex interaction between GPCRs, G proteins, and VGCCs is vital in comprehending the broader implications of GPCR-mediated signalling in neuronal function and pharmacology.

WWP1/2 and USP5

The ubiquitin-proteasome system (UPS) plays a crucial role in the regulation of VGCCs, particularly in controlling their degradation by aiding in the removal of these channels from the cell membrane. This process is detailed in review (Felix and Weiss 2017). The mechanism begins with the binding of a ubiquitin ligase to the channel, followed by the addition of ubiquitin moieties to the channel. These ubiquitin tags signal the polyubiquitinated channels for degradation by the proteasome system.

Specifically, ubiquitin ligases WWP1 and WWP2, as well as the ubiquitin hydrolase USP5, bind with the cytoplasmic region between repeats III and IV of $Ca_v3.2$ channels. This interaction regulates both the ubiquitination and the expression levels of $Ca_v3.2$ in the plasma membrane (García-Caballero et al. 2014). Interestingly, USP5 is upregulated in numerous pain conditions, leading to decreased ubiquitination of $Ca_v3.2$. This reduced ubiquitination results in an increased surface expression of the channel, which contributes to pain.

Experimental approaches have explored inhibiting the $Ca_v3.2$/USP5 interaction as a potential pain treatment strategy. These methods have produced analgesic effects in a variety of preclinical rodent models of pain (García-Caballero et al. 2014; Gadotti et al. 2015; Garcia-Caballero et al. 2022; 2016). Furthermore, the interaction between $Ca_v3.2$ and USP5 is known to diminish following the SUMOylation of USP5 (Garcia-Caballero, Zhang, et al. 2019). Targeting this SUMOylation process may present another promising path for developing pain therapeutics.

ENaC

T-type channels, particularly $Ca_v3.2$, have been found to form a signalling complex with the epithelial sodium channel (ENaC). ENaC channels are primarily known for their role in the reabsorption of sodium ions in the kidney. However, they are also expressed in the central and peripheral nervous system, where their function is less defined (for a detailed review, see (Giraldez, Dominguez, and Rosa 2013)). ENaC is composed of three homologous subunits: α, β, and γ. An association between $Ca_v3.2$ and the β- and γ-subunits of ENaC has been discovered (Garcia-Caballero, Gandini, et al. 2019). This interaction suggests a more complex role for both sets of channels than previously understood. Additionally, when αβγENaC and $Ca_v3.2$ are co-expressed in tsA-201 cells, there is an increase in the expression of both channels at the plasma membrane. This observation implies that there might be a co-trafficking mechanism at play between ENaC and $Ca_v3.2$ channels, indicating a synergistic relationship in their membrane localization and function (Garcia-Caballero, Gandini, et al. 2019).

However, the physiological significance of this interaction between T-type channels and ENaC in the nervous system remains an open area for exploration. Given the crucial roles of both channel types in various physiological processes, understanding this relationship could provide valuable insights into their joint contributions to cellular and systemic functions, particularly in the context of nervous system signalling and ion homeostasis.

1.1.2.4.2 Regulation by post-translational modifications

Post-translational modifications (PTMs) are crucial in regulating the function of many proteins, including T-type calcium channels. Understanding the PTMs of T-type channels is essential for comprehending their regulation and function in different cellular contexts. This has been well reviewed in (Iftinca and Zamponi 2009; Chemin, Traboulsie, and Lory 2006; Huc et al. 2009; Yuan Zhang et al. 2013).

Phosphorylation is an integral PTM of T-type channels involving different kinases that target specific amino acid residues on T-type channels. For example, phosphorylation by PKA usually occurs on serine or threonine residues, while tyrosine kinases target tyrosine residues (Park et al. 2006; J.-A. Kim et al. 2006). Phosphorylation can lead to changes in channel conductance, opening and closing kinetics, and sensitivity to voltage changes. This can result in altered calcium influx, impacting cellular excitability and signalling. For example, phosphorylation by PKC or PKA can modulate the gating properties of these channels, changing the voltage-dependence of activation and inactivation and augmenting T-type currents (J.-A. Kim et al. 2006; Dong et al. 2020; Blesneac et al. 2015).

Asparagine (N)-linked glycosylation plays a crucial role in the functionality of $Ca_v3.2$ channels, a type of T-type calcium channel. This glycosylation involves the addition of carbohydrate chains to asparagine residues and is essential for the proper surface expression, stability, and gating mechanisms of these channels (Weiss et al. 2013; Ondacova et al. 2016). N-glycosylation also enhances the surface expression of $Ca_v3.2$ in response to glucose (Lazniewska et al. 2016). This glucose-dependent potentiation of $Ca_v3.2$ may have significant implications for the development of painful diabetic neuropathy, a common complication of diabetes.

Supporting this link, experimental evidence has shown that pharmacological interference with N-glycosylation can have therapeutic effects in diabetic neuropathy (Orestes et al. 2013). They discovered that disrupting N-glycosylation in a rodent model of diabetes led to a reduction in T-type currents and, importantly, alleviated neuropathic pain. This suggests that the modulation of N-glycosylation of $Ca_v3.2$ channels could be a potential target for the treatment of neuropathic pain, especially in the context of diabetes.

The regulation of T-type channels, particularly $Ca_v3.2$, extends beyond glycosylation and involves the UPS, which is crucial in controlling protein degradation and turnover in cells (Felix and Weiss 2017). The ubiquitin ligase WWP1 and the ubiquitin protease USP5 have been identified as key players in managing the density of $Ca_v3.2$ channels in the plasma membrane, especially in primary nociceptive fibres and spinal cord neurons. This was demonstrated in a study by (García-Caballero et al. 2014), which highlighted the significant role of these proteins in pain modulation. These in vivo experiments disrupted the interaction between $Ca_v3.2$ and USP5 showing remarkable outcomes in terms of pain management. Specifically, interfering with the $Ca_v3.2$/USP5 complex led to significant analgesic effects in various rodent models of inflammatory and neuropathic pain. This finding suggests that the UPS, through the actions of WWP1 and USP5, directly influences the availability and function of $Ca_v3.2$ channels on the cell surface, thereby affecting pain signalling pathways.

These insights into the regulation of T-type channels by the UPS offer potential therapeutic avenues. By targeting specific components of the UPS, such as WWP1 and USP5, it may be possible to develop novel pain management strategies, especially for conditions like inflammatory and neuropathic pain, where conventional treatments are often inadequate.

T-type calcium channels, especially $Ca_v3.2$, are also influenced by redox conditions. Reducing agents, such as the naturally occurring amino acid L-cysteine, have been found to amplify T-type currents in nociceptive neurons, leading to hyperalgesia due to heightened neuronal excitability (Todorovic et al. 2001; Michael T. Nelson et al. 2005). This effect is particularly pronounced with $Ca_v3.2$ channels and has been observed in various neuron types, including nociceptive and reticular thalamic neurons (Michael T. Nelson et al. 2005; Joksovic et al. 2006).

In contrast, oxidizing agents act as inhibitors of $Ca_v3.2$ channels, illustrating the channels' sensitivity to changes in the cellular redox environment (Joksovic et al. 2007). Notably, external application of compounds like L-nitrosocysteine and ascorbate can suppress T-type calcium channel activity and decrease the burst firing of reticular thalamic neurons (Joksovic et al. 2007; Michael T. Nelson et al. 2007). The action of ascorbate is unique in that it oxidizes a specific histidine residue (His191) in $Ca_v3.2$, altering channel function.

L-cysteine's role is also significant in relation to $Ca_v3.2$ channels. It counteracts the inhibitory effect of zinc ions, which normally bind to extracellular histidine residues on the channel, thus enhancing channel activity (M. T. Nelson et al. 2007). Zinc not only inhibits $Ca_v3.2$ but also significantly slows $Ca_v3.3$ tail currents, leading to increased activity of $Ca_v3.3$ channels during action potential bursts (Traboulsie et al. 2007). This modulation by zinc has implications for epileptic activity, suggesting a role in seizure-related disorders (Cataldi et al. 2007).

Additionally, lead ions have been shown to stimulate T-type calcium channel activity, in contrast to zinc (Yan et al. 2008). This stimulation by lead is linked to the release of calcium from internal stores, mediated by receptors like inositol trisphosphate and ryanodine, particularly affecting hippocampal pyramidal neurons.

Overall, these findings emphasize the complex regulation of T-type calcium channels by various redox agents, including endogenous compounds like L-cysteine and external elements like zinc and lead. This regulation is crucial for understanding the channels' roles in pathological conditions such as pain and epilepsy, highlighting their significance in neural excitability and related disorders.

1.1.2.5 Channelopathies

T-type calcium channels are central in governing neuronal activities under normal physiological states, such as in sleep rhythms. However, their involvement extends far beyond just regular physiological functions. Recent research paints a broader picture, highlighting the role of T-type channels in various pathophysiological conditions.

1.1.2.5.1 CACNA1H channelopathies

Primary Aldosteronism: Primary aldosteronism (PA) is a condition wherein the adrenal glands produce too much aldosterone, leading to hypertension and low potassium levels. Aldosterone is a hormone that helps manage sodium and potassium levels in the blood, and its excessive secretion can result in high blood pressure and other complications (Ganguly

1998). T-type calcium channels, specifically $Ca_v3.2$, play a role in the secretion of aldosterone from the adrenal zona glomerulosa. The adrenal zona glomerulosa is the outermost layer of the adrenal cortex and is primarily responsible for producing aldosterone. In situ hybridisation studies, combined with functional and pharmacological analyses, have identified $Ca_v3.2$ as the predominant channel isoform responsible for the T-type current associated with aldosterone secretion (Schrier et al. 2001).

With advances in genetic sequencing, researchers have been able to delve deeper into the genetic underpinnings of diseases. Whole exome sequencing of PA patients led to the identification of several mutations in the *CACNA1H* gene, which encodes for the $Ca_v3.2$ channel. Although these genetic variants don't consistently manifest in all carriers, they often are associated with the presence of PA (Scholl et al. 2015; Daniil et al. 2016). When researchers expressed these $Ca_v3.2$ variants in a laboratory setting (specifically HEK-293 cells), they observed a gain-of-function in channel activity. Furthermore, there's an increased release of aldosterone in certain adrenal cell lines that produce aldosterone and express these $Ca_v3.2$ variants. This might be due to a direct boost in aldosterone release or potentially an increase in aldosterone production since cells with these channel variants also exhibited elevated levels of genes linked to aldosterone metabolism (Daniil et al. 2016; Reimer et al. 2016).

Interestingly, while mutations in another related gene, *CACNA1D*, are linked to both severe neurodevelopmental issues and endocrine disorders, mutations in *CACNA1H* associated with PA don't seem to present these additional health concerns (Flanagan et al. 2017; Scholl et al. 2013). This suggests a more specific or isolated impact of *CACNA1H* mutations on the adrenal system, emphasising the need for specialised medical interventions for patients with different genetic backgrounds.

Epilepsy: T-type channels are closely associated with the onset and progression of generalised seizures in both humans and animals. Mutations in $Ca_v3.2$ channels have been linked to childhood absence epilepsy and other idiopathic generalised epilepsies (Zhong et al. 2006; Khosravani and Zamponi 2006; M. Nelson, Todorovic, and Perez-Reyes 2006; Eckle et al. 2014; Powell et al. 2009; Cain et al. 2018; J. Liang et al. 2007; Heron et al. 2007; 2004; Peloquin et al. 2006; Vitko et al. 2005; Yi Zhang et al. 2004; Y. Chen et al. 2003; Tsakiridou

et al. 1995). The functional ramifications of these mutations, however, can be subtle and at times even elusive when assessed in recombinant channel expression in heterologous systems (for a full review of these subtle changes see 84).

T-type channels are crucial for the thalamocortical circuitry and are implicated in the spike-and-wave discharges seen during absence seizures (Khosravani and Zamponi 2006; Cain et al. 2018; John R. Huguenard 2002; J. R. Huguenard and McCormick 1992; Destexhe et al. 1998; D. Kim et al. 2001; J. R. Huguenard and Prince 1992). Rodent models have shown enhanced thalamic T-type currents in cases of absence epilepsy (Tsakiridou et al. 1995; Yi Zhang et al. 2002; 2004). Additionally, several drugs that block T-type channels have proven effective against absence seizures in humans (Zamponi 2016; Capovilla et al. 1999; Mattson et al. 1978; Kwan et al. 2015).

Over 200 genetic variants in the human *CACNA1H* gene, linked to T-type channels, have been identified in people with various epilepsy syndromes that fall under the idiopathic generalised epilepsies (IGE) category (Y. Chen et al. 2003; Heron et al. 2007; 2004; Jianmin Liang et al. 2006; J. Liang et al. 2007; Chourasia et al. 2019). Although many of these variants can also be found in the Exome Aggregation Consortium (ExAC), indicating they might not have a strong correlation with epilepsy or might need other factors for manifestation.

When these genetic variants were analysed for their biophysical properties, most showed only slight alterations or none at all. These variants aren't concentrated in areas critical for channel function but are spread throughout the channel sequence (Weiss and Zamponi 2020). Some mutations might impact the channel's alternative splicing, potentially affecting native T-type currents (Zhong et al. 2006). While many mutations indicate a channel gain-of-function, indicating a propensity for neurons to fire more, a few show a loss. Some mutations may influence the channel's presence on the cell surface (Khosravani et al. 2004; 2005; Vitko et al. 2005; Peloquin et al. 2006; Arias-Olguín et al. 2008). Simulations and in vitro tests have supported this idea, showing that these gain-of-function (GoF) mutations might increase neuronal activity similar to that seen in absence seizures (Vitko et al. 2005; Eckle et al. 2014).

However, many of the identified *CACNA1H* gene variants don't correlate with specific epilepsy types in family studies (Heron et al. 2007). A definitive mutation connecting $Ca_v3.2$ with genetic epilepsies was found in the genetic absence epilepsy rat from Strasburg (GAERS) (Marescaux et al. 1984). This GoF mutation, associated with increased seizures, enhances the $Ca_v3.2$ channel's recovery from inactivation and increases expression to the surface through a change in the interaction with calnexin (Proft et al. 2017; Powell et al. 2009). This variant's impact is seen particularly in a specific $Ca_v3.2$ splice variant containing exon 25 which is highly expressed in thalamic tissue (Powell et al. 2009). This could explain why GAERS rats display no other physiological dysfunctions except seizures, as much of the pathogenic effect is focused in the thalamus.

It remains ambiguous how much these *CACNA1H* variants contribute to human epilepsies. They might only be low-risk factors and might require other genetic or environmental factors to trigger the condition. As such one of the studies in this thesis will aim to explore the link between *CACNA1H* and a different epileptic syndrome, previously not associated with *CACNA1H*.

Pain: These channels are integral to the understanding of neuropathic and inflammatory pain. Any dysregulation or aberrant expression of $Ca_v3.2$, has been implicated in several chronic pain conditions (M. Nelson, Todorovic, and Perez-Reyes 2006; Jagodic et al. 2007; Souza et al. 2016; Garcia-Caballero et al. 2016; Duzhyy et al. 2015; Orestes et al. 2013; Marger et al. 2011; Jagodic et al. 2008). In animal models, T-type channel inhibitors yield analgesic effects (Todorovic and Jevtovic-Todorovic 2007). In particular, the downregulation of the $Ca_v3.2$ channel counters both neuropathic and inflammatory pain (Bourinet et al. 2005; S. Choi et al. 2007). Similarly, knockdown of $Ca_v3.2$ and $Ca_v3.3$ channels alleviates symptoms such as tactile allodynia and thermal hyperalgesia (Wen et al. 2006).

For instance, there's a heightened activity of $Ca_v3.2$ channels in primary afferent fibres (nerve fibres that transmit sensory signals to the central nervous system) in cases of diabetic neuropathy, nerve injury, irritable bowel syndrome, and peripheral inflammation (García-Caballero et al. 2014; Duzhyy et al. 2015; Jagodic et al. 2008; Marger et al. 2011). These conditions are often linked with chronic pain. The GoF of $Ca_v3.2$ channels in these situations is thought to be a driving factor behind the initiation and persistence of chronic pain.

However, it's important to note that these GoF effects in $Ca_v3.2$ channels are not due to mutations in the channel's genetic sequence. Instead, they stem from altered post-translational modifications, more specifically, changes in processes like deubiquitination and glycosylation (García-Caballero et al. 2014; Gadotti et al. 2015; Garcia-Caballero et al. 2016; Stemkowski et al. 2016; Orestes et al. 2013). The influence of glycosylation of $Ca_v3.2$ and its impact on diabetic neuropathic pain will be further explored in two studies in this thesis.

More recently, there was a documented case of a paediatric patient experiencing chronic pain who had two heterozygous missense mutations in the *CACNA1H* gene (Souza et al. 2016). When these mutations were functionally studied using a heterologous expression system, the findings were ambiguous. Specifically, the functional implications of these mutations on the $Ca_v3.2$ channel seemed to be contingent on the specific experimental conditions used (Souza et al. 2016). When co-expressed in tsA-201 cells, they discovered a loss-of-function (LoF) in the channel with significantly reduced $Ca_v3.2$ current density. However, when co-expressing these variants in neuronal-derived CAD cells in a different extracellular media, they had a non-significant GoF, instead increasing in current density. This poses challenges and implies that understanding the exact impacts of these mutations requires further exploration, perhaps under a variety of experimental conditions or in more physiologically relevant systems.

Autism Spectrum Disorder (ASD): Autism spectrum disorders (ASD) are neurodevelopmental disorders with communication difficulties, social interaction deficits, and unusual sensory-motor behaviours (Lord et al. 2018). While the genetic foundation of ASD is incredibly diverse with numerous risk genes identified, around 5% of those with ASD consistently exhibit a subset of high-risk mutations (Ramaswami and Geschwind 2018; de la Torre-Ubieta et al. 2016). Among these, several missense mutations have been identified in the *CACNA1H* gene in ASD patients (Splawski et al. 2006). These mutations altered the $Ca_v3.2$ channel's function, causing a significant positive shift in activation kinetics and a reduction in channel conductance. This suggests a LoF in the channel. Given the intersection of ASD and epilepsy in many individuals (Tuchman, Cuccaro, and Alessandri 2010), and T-type channels' role in neuronal development, it's speculated that mutations in *CACNA1H* might affect neuronal function during early brain development, potentially influencing the onset of autism (Lory, Bidaud, and Chemin 2006). The extent and nature of these changes seemed to correlate with the mutation's location within the channel protein, but there is still

much to understand about this complex disorder and its relationship with Cav3.2 (Splawski et al. 2006).

Neuromuscular disorder: Neuromuscular disorders refer to a broad set of conditions that are marked by both voluntary and involuntary muscle degeneration and weakness. Amyotrophic lateral sclerosis (ALS), commonly known as Lou Gehrig's disease, stands out as a the most severe and prevalent neuromuscular disorder (Chiò et al. 2013). It involves the gradual degeneration of cortical, brain stem, and spinal motor neurons, leading to muscle weakness and eventual paralysis (Taylor, Brown, and Cleveland 2016). Only about 5%–10% of ALS cases are familial (fALS) with a clear Mendelian inheritance pattern. The majority of ALS patients have no family history of the condition, making it 'sporadic' or 'isolated' (sALS). However, genetics still plays a role, as numerous genes and loci in sporadic ALS cases have been linked to increased disease risk or an effect on its onset or progression (Nguyen, Van Broeckhoven, and van der Zee 2018).

In recent studies, whole exome sequencing analysis pinpointed two compound heterozygous recessive missense mutations in the *CACNA1H* gene (Steinberg et al. 2015). When these mutations were functionally analysed, it was found that they resulted in a minor alteration of the $Ca_v3.2$ channel activity, indicative of a LoF of the channel. Computer simulations showed reduced neuronal activity in nerve cells containing these channel variants (Rzhepetskyy, Lazniewska, Blesneac, et al. 2016).

Though T-type channels have been identified in motor neurons, their exact role in these cells hasn't been thoroughly investigated (Chang and Martin 2016; Canto-Bustos et al. 2014; Z. Zhang and David 2016). Increased neuronal excitability, often characterised by increased sodium conductance and reduced axonal potassium currents, is a known feature of ALS (Pieri et al. 2009; J. J. Kuo et al. 2005; Jason J. Kuo et al. 2004; Vucic and Kiernan 2006; Bostock et al. 1995; Y.-M. Jiang et al. 2005). Given the influence of T-type channels on calcium-activated potassium channels, it's plausible that a decrease in T-type channel activity from ALS-associated mutations could affect potassium currents (Womack, Chevez, and Khodakhah 2004). Recent research has also highlighted the role of T-type channels in preserving the viability of neuronal progenitor cells (J.-W. Kim et al. 2018). This is especially significant when considering neurodegenerative diseases like ALS.

Furthermore, another recent study reported on an individual with severe infantile onset amyotrophy who had inherited two heterozygous *CACNA1H* mutations (Carter et al. 2019). Functional tests of the associated $Ca_v3.2$ variants indicated a loss-of-channel function, mainly evidenced by a reduced window current. This broadens the potential link between *CACNA1H* and motor neuron diseases. As such, this thesis functionally analysis several new *CACNA1H* mutations associated with ALS to expand on this relationship.

In the backdrop of all these revelations, there's a renewed interest in exploring T-type current modulators. While some blockers, like mibefradil, were known even before the molecular cloning of T-type channels, the recent surge in research emphasises the hunt for novel compounds. The hope is to find new molecules capable of fine-tuning T-type channels, which could be immensely valuable therapeutically.

1.1.2.5.2 CACNA1G Channelopathies

Cerebellar Ataxia: This condition affects the cerebellum and its connecting pathways, resulting in poor coordination (Bernard and Shevell 2008). While some forms are non-genetic and arise from acquired conditions or spontaneous degenerative processes, many hereditary cerebellar ataxias have been traced back to specific genes, particularly those coding for ion channels (Mancuso et al. 2014). The R1715H variant of *CACNA1G* has been documented in several families with autosomal dominant cerebellar ataxia (Coutelier et al. 2015; Morino et al. 2015; Kimura et al. 2017; Ngo et al. 2018). This mutation resides in a critical voltage-sensing region, and it brings about a LoF of the channel found both in electrophysiological studies and computer simulations. Another crucial piece of evidence is the LoF of T-type current found in patient-derived iPSC Purkinje cells carrying this R1715H variant (Morino et al. 2015).

Other GoF mutations in *CACNA1G* are linked to childhood-onset cerebellar atrophy (Chemin et al. 2018). These mutations, including A961T and M1531V, inhibit the channel's inactivation, thus causing a GoF. Furthermore, a specific mutation, M1574L, found in Chinese patients has been associated with spinocerebellar ataxia type 42, characterised not only by ataxia but also cerebellar atrophy and defects in the brainstem (Chemin et al. 2018).

This mutation has not been functionally characterised as of yet and without testing it is difficult to hypothesise the potential effects.

Essential Tremor: T-type calcium channel antagonists also show promise as potential treatments for essential tremor (Handforth et al. 2010; Sinton et al. 1989). The link between T-type channels, specifically $Ca_v3.1$, and essential tremor suggests that they might also be relevant in conditions like parkinsonian tremor (Matthews et al. 2023).

Epilepsy: The connection between *CACNA1G* and epilepsy has also been explored. In a study that observed a group of patients with idiopathic generalised epilepsies (IGEs), 13 *CACNA1G* variants were discovered, five of which caused amino acid substitutions (B. Singh et al. 2007). The biophysical properties of these mutations, however, were not significantly different from the WT channel. Intriguingly, *CACNA1G* has also been seen as a potential modifier for Dravet syndrome, a severe form of epilepsy, when defects are present in the sodium channel $Na_v1.2$ (Calhoun et al. 2016; 2017). This revelation hints at the possibility that mutations in *CACNA1G* might not always be directly pathogenic but could sometimes modify the manifestation of disorders influenced by other genes.

1.1.2.5.3 CACNA1I Channelopathies

Unlike *CACNA1G* and *CACNA1H*, which have known associations with a variety of neurological conditions, *CACNA1I's* links are still being uncovered, and its implications are not as well-established.

Schizophrenia: This is a complex psychiatric disorder with a genetic basis that encompasses both common and rare genetic variations (Henriksen, Nordgaard, and Jansson 2017). Recently, two de novo missense mutations in *CACNA1I* were found in individuals with schizophrenia (Gulsuner et al. 2013). These mutations are situated in the pore-forming region of the $Ca_v3.3$ channel, both displaying LoF effects. The mutation A1346H led to a significant reduction in the channel's expression, potentially as a result of an impact on the glycosylation of $Ca_v3.3$. The other mutation, T797M, did not exhibit any noticeable impact on the channel (Andrade et al. 2016). When the effects of the R1346H mutation were examined through computer simulations focused on thalamic reticular nucleus neurons, a decrease in neuronal

excitability, which is associated with this mutation, was observed (Andrade et al. 2016). However, considering the complexity of this disorder, it is difficult to elucidate the overall physiological effects in the development of schizophrenia.

The genetic landscape of neurological and psychiatric disorders is incredibly complex, and as research continues, it's becoming clear that even small variations in genes like *CACNA1I* can potentially have significant implications for brain function and, consequently, for behaviour and cognition. The absence of epilepsy associations for $Ca_v3.3$ compared to other T-type channels underscores the need for nuanced, gene-specific research (Juli Wang et al. 2006). Understanding these distinctions will be pivotal for the development of targeted therapeutic interventions for conditions like schizophrenia and epilepsy.

1.1.2.6 T-type channel drugs

As the role of T-type channels in diseases has continued to be documented, so has the number of drugs targeting these channels. For many years there has only been a few T-type channel blockers approved, but recently there has been a large influx in these drugs. This includes; ethosuximide, zonisamide, verapamil, trazodone, haloperidol, and several types of dihydropyridines (DHP)(Melgari et al. 2022). There are many more T-type channel blockers that have either been withdrawn from the market, failed clinical trials or are still in clinical trials (for a full review of these see (Melgari et al. 2022; Nam 2018).

Those that have been approved are used to treat a variety of different disorders through a variety of different mechanisms. Ethosuximide for example, is primarily used in the treatment of absence seizures. It is an antagonist of T-type channels predominantly in thalamic neurons, reducing the ability of thalamic neurons to generate abnormal rhythmic discharges (Coulter, Huguenard, and Prince 1989). Zonisamide is another antiepileptic drug, but the mechanism of action is not fully understood. However, studies find that it targets numerous pathways to reduce neuronal excitability. These include inhibition of T-type channels, particularly in thalamic neurons, but also voltage-gated sodium channels. Other effects include inhibition of carbonic anhydrase, modulation of neurotransmitter release, and free radical sCavenging (Biton 2007).

Verapamil is a medication primarily used in the management of high blood pressure, angina (chest pain), and certain types of irregular heartbeats (B. N. Singh, Ellrodt, and Peter 1978). It blocks both L-type and T-type channels with a higher affinity at more depolarised membrane potentials. However, this state-dependent block is more dramatic in L-type channels than T-type channels. Studies have found that verapamil preferentially blocks from the extracellular side of the channel, binding inside the pore (Bergson et al. 2011).

Trazodone is a medication primarily used in the treatment of major depressive disorder, but it also has several off-label uses, such as insomnia (Fagiolini et al. 2012; Jaffer et al. 2017). It belongs to the class of drugs known as serotonin antagonist and reuptake inhibitors (SARIs). Along with its various effects on other pathways, trazodone also inhibits T-type currents (Stahl 2009). Studies have found that this block occurs when the channel is at rest, with a weak voltage-dependence of inhibition (Kraus et al. 2007).

Haloperidol, a typical antipsychotic medication, is primarily used in the treatment of schizophrenia and acute psychotic states, including delirium. It is also used in controlling severe tics and vocal utterances in Tourette syndrome (Kudo and Ishizaki 1999). In addition to its antipsychotic properties, it has a role in treating severe behaviour problems in children, including combative and explosive behaviour (Werry and Aman 1975). These effects are thought to be primarily due to its activity on dopamine receptors as although it inhibits T-type currents, it is not very potent (Santi et al. 2002).

Dihydropyridines constitute a category of drugs mainly used for treating cardiovascular diseases (Epstein, Vogel, and Palmer 2007; Messerli et al. 2006). These drugs function by blocking the entry of calcium ions into the heart and vascular smooth muscle cells. This mechanism plays a crucial role in treating conditions like hypertension, angina pectoris, and certain types of arrhythmias (Messerli et al. 2006). They are selective in their action, focusing on L-type and T-type calcium channels (Schaller et al. 2018; Weiland and Oswald 1985; Wappl et al. 2001). Their ability to selectively inhibit calcium entry into vascular smooth muscle stems from their unique splicing variations and their increased affinity to bind with calcium channels when these channels are in an inactive state. Since vascular smooth muscle cells typically keep their membrane potential at relatively depolarized levels, a greater number of calcium channels are found in their inactive state (Perez-Lemus et al., n.d.)

2 Aims of the thesis

This comprehensive thesis delves into several critical aspects of T-type channels, highlighting their significance in medical science and pharmacology. These channels play a vital role in many physiological systems in the body from their neuronal activity to regulating heart rate and rhythm and their implication in cancer cell proliferation and embryonic development. This underscores the need for research into all facets of T-type channels to combat not only neurological but also various other disorders.

The thesis is thus structured around three main areas: understanding the regulation pathways of T-type channels, including their processing and expression on the plasma membrane; investigating mutations in T-type channels associated with various neurological disorders; and developing drugs targeting T-type calcium channels.

Part 1: Regulation of T-type channel expression
It is already well established that T-type channels go through various post-translational modifications which influence the expression and function of the channel at the cell membrane. Despite acceptance of N-glycosylation at N-X-S/T motifs, it is unknown if non-canonical motifs undergo N-glycosylation.

Another area of focus is the role of trafficking proteins, such as the Secretory carrier-associated membrane proteins (SCAMPs), in the regulation of ion channels. SCAMPs form a family of intrinsic membrane proteins expressed in the trans-Golgi network and recycling endosome membranes, where they help modulate vesicular trafficking and vesicle recycling processes (Castle and Castle 2005). SCAMP2, found across all tissues, including neuronal tissues (https://www.proteinatlas.org/ENSG00000140497-SCAMP2/tissue), has been linked to ion channel and transporter regulation (Müller, Wiborg, and Haase 2006; Diering, Church, and Numata 2009; Zaarour et al. 2011; Fjorback et al. 2011), but its specific role in T-type channel expression remains unexplored.

Part 2: T-type channelopathies associated with neurological disorders

ALS has been linked to the dysfunction of many genes, and new genetic mutations associated with ALS are still being discovered (Nguyen, Van Broeckhoven, and van der Zee 2018). Studies have also long known that excitability dysfunction in neurons is associated with ALS pathology (Zanette et al. 2002). As ion channels are intrinsic to the excitability of the neuron, and one of the only drugs available for treatment targets ion channels (Lamanauskas and Nistri 2008). It suggests that dysfunction in ion channels is linked to ALS pathology. However, as of yet, there has been only one other study identifying the functional effect of *CACNA1H* variants associated with ALS (Rzhepetskyy, Lazniewska, Blesneac, et al. 2016). Therefore, we will further explore this link between *CACNA1H* and ALS.

CACNA1H GoF variants have long been associated with various forms of epilepsy, including childhood absence epilepsy and other idiopathic generalised epilepsies (Zhong et al. 2006; Khosravani and Zamponi 2006; M. Nelson, Todorovic, and Perez-Reyes 2006; Eckle et al. 2014; Powell et al. 2009; Cain et al. 2018; J. Liang et al. 2007; Heron et al. 2007; 2004; Peloquin et al. 2006; Vitko et al. 2005; Yi Zhang et al. 2004; Y. Chen et al. 2003; Tsakiridou et al. 1995). Although the link between *CACNA1H* and various forms of epilepsy is evident, the implications of these mutations is still largely unknown. Furthermore, *CACNA1H* LoF variants have been associated with various developmental disorders such as autism (Splawski et al. 2006). Recently, a young patient with severe developmental and epileptic encephalopathy (DEE) was found to carry a *CACNA1H* variant. However, this variant has not been functionally characterised for its biophysical properties and therefore its pathological impact.

Recently, through whole exome sequencing analysis of familial trigeminal neuralgia patients, a study has identified 19 *CACNA1H* variants (Dong et al. 2020). As of yet only four of these have been biophysically characterised (Gambeta et al. 2022), so the potential pathogenicity of the rest is still unknown.

Part 3: Drugs targeting T-type channels

Finally, the thesis emphasises the urgency of developing new drugs targeting T-type calcium channels, given the increasing recognition of their connection to various disorders and the limited number of approved drugs in this category (Melgari et al. 2022). This highlights the essential nature of ongoing research and development in this field.

Specific aims were therefore:

Part 1: Regulation of T-type channel expression

- To determine the role of non-canonical N-glycosylation sites on the post-translational processing of $Ca_V3.2$ channels to the cell membrane.
- To determine the role of SCAMP2 on cell membrane expression of T-type channels

Part 2: T-type channelopathies associated with neurological disorders

- To ascertain the effect of diabetes on glycan-processing genes and their functional effect on *CACNA1H*.
- To ascertain the functional effect of various *CACNA1H* variants associated with ALS.
- To ascertain the functional effect of a *CACNA1H* variant associated with severe developmental and epileptic encephalopathy.
- To ascertain the functional effect of *CACNA1H* variants associated with familial trigeminal neuralgia.

Part 3: Drugs targeting T-type channels

- To identify the effects of a group of surfen derivatives on T-type channel activity.

3 Methods

Biophysical Characterization (Study 1-7): We conducted patch clamp recordings to study T-type currents in tsA-201 cells expressing $Ca_v3.2$ channel variants and associated regulatory proteins when applicable (study 1, 2, and 3). These recordings were performed 72 hours after transfection in a whole-cell configuration at room temperature (22-24 °C). This study utilized specific bath and pipette solutions for the experiment. They used an Axopatch 200B amplifier and software like pClamp 10 and Clampfit 10 for data acquisition and analysis. We correcting the linear leak component of the current using a subtraction protocol, and the data was digitized at 10 kHz and filtered at 2 kHz. These studies focused on analyzing the voltage dependence of activation, conductance, and steady-state inactivation of Cav3.2 channels in response to, various mutations (study 1, 4, 5, 6), or regulatory proteins (study 2 and 3). This involved measuring the peak T-type current amplitude in response to various protocols and fitting the data to modified Boltzmann equations. Additionally, the recovery from inactivation was assessed using a double-pulse protocol, and the results were fitted to a single-exponential function to determine the time constant for channel recovery from inactivation. For study 7 a stab protocol was used to measure the current induced at one voltage step and drugs were applied to see the resulting inhibition. This was carried out on $Ca_v1.2$, $Ca_v2.1$, $Ca_v2.2$, $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$ recombinant channels.

Biotinylation studies (study 1): This process was carried out using EZ-Link Sulfo-NHS-SS-Biotin. After quenching the reaction, the cells were lysed using a modified RIPA buffer. Two milligrams of these lysates were then incubated with Neutravidin beads to isolate the biotinylated proteins. Subsequent to the incubation, the beads were washed, and the proteins were eluted using 2× Laemmli sample buffer. The biotinylated proteins, along with the lysates, were then subjected to SDS-PAGE for separation. Following this, a Western blot analysis was conducted using specific antibodies: anti-Cav3.2, and anti-Na+/K+ ATPase, to detect and analyze the presence of these proteins.

Co-Immunoprecipitation (study 2): tsA-201 cells expressing Cav3.2-HA (hemagglutinin-tagged) in combination with SCAMP2-Myc were used. These cells were first solubilized in a lysis buffer supplemented with a protease inhibitor cocktail obtained from Sigma. After solubilization, the cell lysates were cleared by centrifugation. All these steps were performed at a temperature of 4°C to preserve protein integrity. Following centrifugation, the lysates

were incubated overnight at 4°C with a rat monoclonal anti-HA antibody. This step allows the antibody to bind to the HA-tagged Cav3.2 proteins. After the overnight incubation, the lysates were further incubated for two hours at 4°C with magnetic protein G beads. Once the incubation with the beads was complete, the beads were washed to remove non-specifically bound proteins. The beads, with the bound protein complexes, were then resuspended in Laemmli buffer. Finally, the samples were heated at 100°C.

Western Blot (study 2): immunoprecipitation samples and total cell lysates were separated using 10% SDS-PAGE and transferred onto a PVDF membrane. Detection involved incubating the membrane with specific primary antibodies: mouse monoclonal anti-Myc for SCAMP2-Myc, rat monoclonal anti-HA for Cav3.2-HA, and mouse monoclonal anti-actin for actin. Post-incubation, membranes were washed and treated with HRP-conjugated secondary antibodies. Immunoreactive bands were detected using enhanced chemiluminescence and analyzed with ImageJ software.

Transcriptomic analysis (study 3): Transcriptomic analysis was conducted on total RNA from the dorsal root ganglia (L4/L6) of wild-type and db/db mice. The study utilized the Glycosylation RT2 Profiler PCR Array (Qiagen) for analyzing glycan-modifying enzymes, following the manufacturer's guidelines. PCR and qRT-PCR were performed using a LightCycler® 480 (Roche) under specific conditions: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 20 seconds. Each test was repeated thrice to ensure accuracy, averaging the results to minimize discrepancies. The focus was on 84 key genes encoding enzymes processing glycans, including glycosyltransferases and glycosidases for various sugars like galactose, glucose, mannose, N-acetylgalactosamine, N-acetylglucosamine, fucose, and sialic acid.

Computational modeling (study 6): We simulated thalamic reticular neuron (nRT) firing using the NEURON simulation environment, based on a three-compartment model. Electrophysiological properties of both wild-type and TG-associated Cav3.2 variants were incorporated into the model using Hodgkin-Huxley equations. Recognizing the relative expression of Cav3.2 channels in nRT neurons (about 40% Cav3.2 and 60% Cav3.3) and the heterozygous nature of TN-associated Cav3.2 variants, only 20% of the T-type channel conductance in the model was modified with experimental values for WT and TN-associated

Cav3.2 variants. Simulations were conducted at a holding potential of -70 mV, monitoring the virtual soma's electrical membrane potential in response to 200ms hyperpolarizing and depolarizing current injections to assess rebound and tonic firing.

Cell toxicity assay (study 7): The study involved evaluating the cytotoxicity of compounds on various human cancer cell lines, including cervix cancer (HeLa), hepatocellular carcinoma (Hep G2), acute lymphoblastic leukemia (CCRF-CEM), and acute promyelocytic leukemia (HL-60), all sourced from ATCC. Each cell line was cultured in specific media: HeLa in DMEM high glucose, CCRF-CEM and HL-60 in RPMI-1640 (Dutch modification), and Hep G2 in αMEM, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM glutamine, at 37 °C in a 5% CO2 humidified atmosphere. The CellTiter-Glo® 2.0 Cell Viability Assay kit from Promega was used for measuring cytotoxicity. Cells were seeded into white 384-well plates and treated with compounds or DMSO (control) for 72 hours. Post-treatment, CellTiter-Glo® reagent was added, mixed, and allowed to stabilize, with luminescence readings indicating cell number and hence viability. IC50 values, indicating the concentration reducing viable cells by half, were calculated using nonlinear regression analysis with GraphPadPRISM® 7, based on a sigmoidal concentration-response curve.

Molecular docking (study 7): This study involved protein and ligand preparation and docking using the Schrödinger Docking Suite (2023-2 release). Ligands were prepared using LigPrep, considering ionization states at pH 7.0. The cryo-EM structure of Ca v 2.2 (PDB ID: 7mix) was prepared with the Protein Preparation Wizard, while the homology model of Ca v 3.2 was based on previous descriptions. Docking simulations were conducted using Glide in Standard Precision mode with enhanced sampling to generate up to 100 poses per site. Furthermore, Schrödinger Induced-Fit Docking allowed for flexible receptor docking, involving 20 docking runs with subsequent optimization of amino acid positions and conformations within 5 Å of the docked site. The ligand-receptor complexes were then ranked based on the energies of the induced-fit complexes.

Preclinical efficacy (study 7): The assessment of mechanical allodynia in rats involved measuring their paw withdrawal threshold in response to probing with a series of fine calibrated filaments (von Frey filaments, Stoelting, Wood Dale, IL). For the test, rats were placed in suspended plastic cages with a wire mesh floor. The von Frey filaments were applied perpendicularly to the plantar surface of the rat's paw. The "up-down" method, which

sequentially increases and decreases stimulus strength, was used to determine the paw withdrawal threshold. Data analysis was conducted using Dixon's nonparametric method. Mechanical allodynia in the rats was indicated by a decrease in the paw withdrawal threshold, meaning the rats withdrew their paws at lower filament pressures compared to normal.

4 Results

Comments to the results

According to the specific aims for this thesis, seven papers have been designed, six of which have been published with the last one going for review. In the first part, two studies were designed to assess the regulation and expression of T-type channels to the surface by 1) determining the potential role of non-canonical N-glycosylation sites within $Ca_v3.2$ and 2) regulatory properties of SCAMP2 on T-type channels. In the second part, three studies were carried out to identify T-type channelopathies associated with neurological disorders and conditions including 3) peripheral diabetic neuropathy, 4) ALS, 5) DEE, and 6) familial TN. The final part includes one study that 7) characterises the action of a group of compounds that target T-type channels.

Part 1: Regulation of T-type channel expression

The research presented in these studies offers significant insights into the mechanisms regulating the expression and function of T-type channels, specifically focusing on $Ca_v3.2$ channels and the role of non-canonical glycosylation motifs and a novel regulator, SCAMP2.

Study 1: Non-Canonical Glycosylation Motifs in $Ca_v3.2$

Key findings include:

Biophysical Characterization: Using patch-clamp electrophysiology, we found that the motifs at N345 and N1780 were crucial for the proper trafficking of $Ca_v3.2$ to the membrane. This was evident from the significant reduction in channel conductance in cells expressing these mutations, compared to those expressing the wild-type (WT) channel.

Biotinylation Studies: To discern whether the reduced current was due to channel dysfunction or decreased surface expression, biotinylation was employed. The total cellular expression of all channels was consistent, but the surface expression of the N345 and N1780 mutated channels was notably reduced. This reduction in surface expression correlated with the

decreased channel conductance, suggesting that these motifs are essential for the glycosylation and correct trafficking of $Ca_v3.2$ to the plasma membrane.

Study 2: SCAMP2 as a regulator of T-Type Channels

Key findings include:

Co-Immunoprecipitation: This confirmed a physical interaction between SCAMP2 and $Ca_v3.2$, forming a protein complex. Though we cannot confirm if there is a direct interaction or if there is an intermediate protein.

Electrophysiological Analysis: Patch-clamp recordings showed a 91% reduction in Cav3.2 channel conductance when co-expressed with SCAMP2 and a 35% and 98% reduction in $Ca_v3.1$ and $Ca_v3.3$ conductance, respectively. Further analysis through Alanine mutagenesis on SCAMP2's E peptide residues (C201A and W202A) reduced this inhibition effect, suggesting the E peptide's involvement in the regulation process.

Western Blot Analysis: This revealed a non-significant increase in total $Ca_v3.2$ expression, possibly due to a reduced rate of vesicular exocytosis, which in turn may prevent the channel from being targeted for degradation.

Intramembrane Charge Movement Measurement: Indicated a decrease in channel expression at the surface and a change in the coupling between voltage-sensor activation and pore opening, suggesting a role for SCAMP2 in gating regulation.

<u>Part 2:</u> T-type channelopathies associated with neurological disorders

Study 3: Glycan-processing genes in diabetic mice

Key findings include:

Transcriptomic Analysis: This study employed transcriptomic analysis to examine changes in gene expression in DRGs. It revealed that several genes encoding glycosyltransferases and sialic acid-modifying enzymes were upregulated in diabetic conditions.

Functional Analysis of Glycosylation Enzymes: When these enzymes were applied to recombinant $Ca_v3.2$ channels, there was an unexpected loss of channel function. Despite the observed upregulation of T-type channels in diabetic conditions, the individual action of these glycosylation enzymes on recombinant $Ca_v3.2$ did not align with this upregulation.

Study 4: *CACNA1H* variants linked to ALS patients

Key findings include:

Whole genome sequencing: This technique was used on a small cohort of ALS patients, revealing two heterozygous *CACNA1H* variants.

Patch Clamp Electrophysiology: c.454GTAC > G Variant (ΔI153): This newly discovered variant led to an inframe deletion of a highly conserved isoleucine residue in $Ca_v3.2$, causing a complete loss of channel function. Notably, this variant also exerted a dominant-negative effect on the wild-type channel when co-expressed, further impairing channel function. c.3629C > T Variant (P1210L): This variant caused a missense substitution (proline to leucine) and resulted in a mild reduction of the $Ca_v3.2$ channel activity and thus is unlikely to be pathogenic.

Study 5: *SCN8A* and *CACNA1H* variants associated with DEE

Key findings include:

Whole exome sequencing: This technique uncovered genetic variations in two channels that could be responsible for the condition, a de novo heterozygous variant in *SCN8A* (c.4873-4881 duplication) and an inherited heterozygous variant in *CACNA1H* (c.952G>A).

Patch Clamp Electrophysiology: *SCN8A* (c.4873-4881 duplication): A mild leftward shift in voltage dependence of activation, consistent with a gain-of-function. *CACNA1H* (c.952G>A): A mild rightward shift in the voltage dependence of activation, suggesting a loss-of-function.

Study 6: *CACNA1H* variants associated with TN

Key findings include:

Patch Clamp Electrophysiology: Seven variants showed a GoF. Six variants showed altered gating properties, exhibiting a hyperpolarizing shift in voltage dependence of activation and/or inactivation, leading to an enhanced window current through $Ca_v3.2$ channels. One variant enhanced recovery from inactivation which could potentially contribute to increased channel activity.

Computational Modelling: Using a computational model of reticular thalamic neurons, this study simulated the neuronal electrical membrane potential. The results suggest that TN-associated $Ca_v3.2$ GoF variants could enhance neuronal excitability, potentially contributing to the development of TN.

Part 3: Drugs targeting T-type channels

Study 7: Quinolone-based calcium channel blockers

Key findings include:

Patch Clamp Electrophysiology: Among the synthesised compounds, S13 effectively blocks several VGCC subtypes, including $Ca_v2.2$ and $Ca_v3.2$, which are particularly relevant to pain. Biophysical studies conducted on cultured DRG neurons confirmed S13's blocking activity on native LVA and HVA VGCCs, without affecting sodium and potassium channels.

Cell Toxicity Assay: S13 had improved cell tolerance compared to surfen and other derivatives.

Molecular Docking Analysis: Predicted the direct binding of S13 to $Ca_v2.2$ and $Ca_v3.2$ channels.

Preclinical Efficacy: Intrathecal administration of S13 in a rat model of nerve ligation-induced mechanical allodynia showed substantial antinociceptive effects.

5 Discussion

The increase in diseases associated with T-type channels has increased the need to further research them. This starts at basic research elucidating their modulation and trafficking to the surface and then expands to the need to find out what the effect of channel variants and regulatory proteins have on the biophysical function of the channel. Finally, as many T-type channel blockers have been produced with only a handful of them being approved for use due to efficacy (Melgari et al. 2022), there is a need to develop novel drugs to combat the growing number of diseases associated with T-type channels.

As such seven studies have been carried out to research the three aspects involved in treating these diseases; 1) regulation of T-type channels, 2) T-type channelopathies, and 3) novel drug development.

Part 1: Regulation of T-type channel expression

Two studies were focused on the regulation of T-type channels. The first study revealed a previously unknown aspect of T-type channel regulation. While N-glycosylation at canonical sites is well-known, we identified additional regulatory processes at two non-canonical sites. This discovery is key for a more comprehensive understanding of how T-type channels are controlled. Our findings showed a reduced presence of these channels at the cell surface when these sites were mutated, highlighting their role in the channel's proper trafficking and expression. Our study didn't pinpoint the exact mechanisms behind this effect. Nevertheless, since we replaced the asparagines with glutamine to maintain similar secondary structures and charge distributions, we deduce that the change isn't due to the changes in the channel's biophysical properties. Instead, it appears to result from disrupting the glycosylation process. We also noted no difference in the overall cellular expression of the mutated channels, only a decrease at the surface identified through biotinylation. This suggests the mutations likely don't influence the channel's ubiquitination, as this would lead to decreased overall cellular expression due to marked degradation.

Glycosylation's role in T-type channels is notably crucial, impacting their function, location, and broader cellular effects (Lazniewska and Weiss 2017). Commonly occurring in the channels' extracellular loop, glycosylation can alter their gating, conductance, and drug responses. These modifications affect how the channels interact with their surroundings,

influencing their distribution, stability, and response to regulatory elements (Q. Wang, Groenendyk, and Michalak 2015; Weiss et al. 2013; Ferris, Kodali, and Kaufman 2014; Vagin, Kraut, and Sachs 2009). Therefore, identifying glycosylation sites is vital for understanding the fundamental regulatory mechanisms of T-type channels.

The second study brought to light a novel modulator of T-type channels, SCAMP2, which plays a critical role in various cellular processes, particularly in regulating membrane trafficking and signal transduction (Castle and Castle 2005). As a part of the SCAMP family, SCAMP2 is integral to post-Golgi recycling pathways, facilitating the efficient transport of proteins and lipids within cells. Its involvement in key physiological processes such as exocytosis, endocytosis, and membrane repair underscore its importance (Castle and Castle 2005).

SCAMP2's ability to interact with a diverse range of proteins and lipids enables it to function as a versatile regulator. It coordinates the trafficking of cargo molecules to specific destinations within the cell, a process vital for maintaining cellular homeostasis and responding to environmental changes. Disruptions in SCAMP2 function are linked to various diseases, including neurodegenerative disorders and cancer, highlighting its significance in both normal cellular functioning and disease pathology (C. Yue et al. 2021).

T-type channels have already been found to be modulated by a variety of interacting proteins including Stac1 (Rzhepetskyy, Lazniewska, Proft, et al. 2016), Rack-1 (Gandini et al. 2022), KLHL1 (Perissinotti et al. 2014), calnexin (Proft et al. 2017), spectrins (Garcia-Caballero et al. 2018), Caveolins (Markandeya et al. 2011), calmodulin (Ben-Johny and Yue 2014), syntaxin (Weiss et al. 2012), and USP5 (García-Caballero et al. 2014). Here we have identified an additional interacting protein to add to these and increase our understanding of the basic regulatory mechanism T-type channels are under.

Part 2: T-type channelopathies associated with neurological disorders

In the third study, we explored further the role of glycosylation in the trafficking of T-type channels, particularly in the context of diabetes. Glycosylation is known to be crucial for the proper trafficking of T-type channels to the cell surface, and dysfunctions in this process have been linked to various disorders (Jagodic et al. 2007; 2008; Andrade et al. 2016; Dogrul et al.

2003; Bourinet, Francois, and Laffray 2016; J. Yue et al. 2013; Watanabe et al. 2015; Scanzi et al. 2016; Latham et al. 2009).

Our study initially aimed to provide evidence for peripheral diabetic neuropathy (PDN) by analysing the transcriptomic profiles of glycan-processing genes in a mouse model of diabetes. We observed an increase in these genes, leading us to theorise an associated increase in $Ca_v3.2$ expression. This hypothesis was based on existing knowledge that elevated $Ca_v3.2$ expression in primary afferent nociceptive fibres is linked to the development of peripheral painful neuropathy in various conditions (Jagodic et al. 2007).

However, contrary to this, when we tested this in a recombinant expression system using tsA-201 cells, we observed a significant reduction in $Ca_v3.2$ surface expression. In fact, three out of the five glycan-processing genes we studied caused an almost complete loss of $Ca_v3.2$ surface expression.

We speculate that these contradictory results may stem from the limitations of the tsA-201 cell expression system in replicating the complexities of primary neurons, such as DRGs. While this system is a common choice for such studies due to practical constraints, it cannot always accurately mimic native systems, especially as the cell line does not come from neuronal origins. Due to this there may be a difference in cell expression profiles, changing cellular dynamics in comparison to neurons (Y.-C. Lin et al. 2014).

To reach a definitive conclusion about the interaction of these glycan-processing genes with $Ca_v3.2$ expression in diabetes and its impact on PDN, further investigation in native conditions is necessary. For instance, this could involve experiments in DRGs from normal animals. By increasing the expression of these glycan-processing genes, we could measure the changes in the biophysical properties of $Ca_v3.2$ channels compared to WT DRGs. This could be achieved through various methods, such as gene therapy with viral vectors (Lundstrom 2018), CRISPR/Cas9 (Hsu, Lander, and Zhang 2014) to enhance gene expression, or RNA interference (RNAi) targeting repressor genes to indirectly boost the expression of these glycan-processing genes (D. H. Kim and Rossi 2008).

In the fourth study, we investigated the role of rare genetic variants in sporadic Amyotrophic Lateral Sclerosis (sALS), focusing on two heterozygous *CACNA1H* variants identified in

ALS patients through whole genome sequencing. Our functional analysis revealed varying degrees of loss-of-function in $Ca_v3.2$ channel variants.

The P1210L missense mutation, located in a variable region of $Ca_v3.2$, was initially not predicted to be deleterious. This is supported by the fact that this variant is found abundantly in the general population. However, our electrophysiological analysis showed a mild reduction in cell surface expression and T-type conductance of the P1210L channel variant. Even then, we would still predict it has no pathogenic activity. To confirm this, we would need to introduce the mutation into a knock-in rodent model to elucidate the phenotypic effects, as has been previously carried out for ALS associated genes (Kao et al. 2020; S.-L. Huang et al. 2020). We can also express them in more native conditions such as motor neurons, which have already been found to express $Ca_v3.2$ (Chang and Martin 2016; Canto-Bustos et al. 2014; Z. Zhang and David 2016). We could then analyse any potential biophysical changes that may occur in the firing properties of these neurons to elucidate if these changes correspond with dysfunction of excitability found in ALS motor neurons (Jason J. Kuo et al. 2004; Filipchuk et al. 2021).

In contrast, the ΔI153 variant, previously unreported and predicted to be deleterious, showed a complete loss of functional expression. Electrophysiological analysis and charge movement recordings indicated its absence from the cell surface, and biochemical analysis suggested extensive degradation of the channel protein. Notably, the ΔI153 variant exerted a dominant-negative effect on the WT channel when co-expressed, likely due to $Ca_v3.2$ subunit dimerization, which could impede the trafficking of the WT channel to the cell surface. This effect might extend to other ion channels, given $Ca_v3.2$'s known interactions with various calcium- and voltage-activated potassium and sodium channel subunits (Garcia-Caballero, Gandini, et al. 2019; Yi Zhang et al. 2002; Gackière et al. 2013; Wolfart and Roeper 2002).

It is difficult to predict the effect of this loss-of-function in a patient due to the ubiquitous expression of $Ca_v3.2$ throughout the body (J.-H. Lee et al. 1999). However, due to the nature of ALS we can hypothesise the effect based on the neurons known to be affected. For example, evidence for a decrease in synaptic transmission in thalamic neurons has been noted in ALS patients (Sharma et al. 2011), and it is well known that $Ca_v3.2$ are expressed in reticular thalamic neurons, where they contribute to NMDA receptor-mediated synaptic transmission (Talley et al. 1999; G. Wang et al. 2015). The loss-of-function seen could then

explain this reduced synaptic transmission. Additionally, the presence of T-type channels has been documented in motor neurons and their role in cellular excitability and motor functions suggest that a loss-of-function in $Ca_v3.2$ could lead to a hypoexcitable state, causing neuronal degeneration (Chang and Martin 2016; Canto-Bustos et al. 2014; Z. Zhang and David 2016). This could explain the hypoexcitability identified in some models of ALS (Filipchuk et al. 2021). It would be interesting to continue studying the effect of this loss-of-function mutation in more native conditions, and to screen other ALS patients for *CACNA1H* mutations to provide stronger evidence in the link between ALS and *CACNA1H*. It would also be intriguing to test the biophysical properties of motor neurons from ALS patients to elucidate the role of $Ca_v3.2$ in neuronal degeneration. However, as this is unethical to extract motor neurons from patients other methods would have to suffice. We then should turn to expressing these $Ca_v3.2$ variants in mice models and in human iPSC cells to come to a better understanding of the potential biophysical changes.

In the fifth study, we reported the case of a child with severe developmental and epileptic encephalopathy (DEE), who was found to have a de novo mutation in *SCN8A* and an inherited rare variant in *CACNA1H*. Pathogenic variants in *SCN8A*, particularly de novo missense variants in the conserved transmembrane domains of Nav1.6, have been previously described in DEE patients and the GoF we found aligns with this data (Veeramah et al. 2012; Meisler et al. 2016; Jiaping Wang et al. 2017). In contrast, *CACNA1H* has not been previously associated with DEEs. It is interesting to see that we found a LoF in *CACNA1H* activity as this is usually associated with autism spectrum disorders, amyotrophic lateral sclerosis, and congenital amyotrophy (Splawski et al. 2006; Rzhepetskyy, Lazniewska, Blesneac, et al. 2016; Carter et al. 2019). Whereas GoF variants in CACNA1H have been linked to conditions like absence epilepsy and primary aldosteronism (Daniil et al. 2016; Khosravani et al. 2005). We would then have expected a GoF in CACNA1H. Although this variant was inherited from the father and he had no symptoms, it suggests that this alone would have no significant effect on disease development. From our results we could hypothesise that when expressed together, they could negate each other's effect on the biophysical properties in neurons. Therefore, to ascertain the impact these two variants have on the pathogenicity of DEE, we would need to express both in more native conditions, such as in iPSC derived neurons.

In the sixth study, we investigated the functional characteristics of 13 $Ca_V3.2$ missense variants identified in patients with trigeminal neuralgia (TN). Interestingly, 6 of the 13 $Ca_V3.2$ variants (S187L, A802V, E819K, P1120L, P2280H, and E2291K) exhibited a hyperpolarized shift in the voltage dependence of activation, indicative of a GoF effect. Additionally, A802V and Q1049H variants showed an increased recovery from inactivation, also displaying a GoF effect. It is interesting to see that despite the spread of these variants across the *CACNA1H* channel, they displayed similar biophysical modifications. Moreover, these six variants caused a hyperpolarizing shift in the voltage dependence of the window current, potentially leading to increased passive calcium influx near the resting membrane potential. This could further enhance neuronal activity, as the window current's voltage range is crucial for neuronal electrical activities and calcium oscillations (S. R. Williams et al. 1997).

When these 6 variants were introduced into a computational model of nRT neurons, they reduced the threshold for rebound burst firing, suggesting an overall GoF effect. This finding aligns with previous studies demonstrating that increased T-type channel activity can lower the threshold for rebound burst firing in various neuron types (Woodward et al. 2019). Although our modelling was based on nRT neurons, it may reflect potential effects of TN-associated $Ca_V3.2$ variants on the trigeminal pathway. This is supported by several factors, including the potential for T-type dependent rebound burst firing in trigeminal ganglion neurons (Guido, Günhan-Agar, and Erzurumlu 1998; Zhu, Wei, and Wang 2022), the presence of low-threshold calcium conductance in brainstem trigeminal nuclei neurons (Guido, Günhan-Agar, and Erzurumlu 1998), and the role of T-type channels in thalamocortical rhythmic activities implicated in trigeminal pain (Soonwook Choi et al. 2016). To discover the role that these variants have in the development of TN, we would need to express these channels in trigeminal neurons, possibly through CRISPR/Cas9 on WT neurons (Hsu, Lander, and Zhang 2014). We would also need to find out why these GoF $Ca_V3.2$ variants did not cause idiopathic generalised epilepsy or primary aldosteronism, which have been previously seen with GoF variants (Daniil et al. 2016; J. Liang et al. 2007; Jianmin Liang et al. 2006; Khosravani et al. 2004). This would need to be carried out by expressing these variants in different subsets of neurons, such as thalamic neurons to see if they’re effect is somewhat neuron specific.

In conclusion, our functional analysis of 13 $Ca_v3.2$ variants in TN patients revealed a GoF in 7 variants, potentially contributing to trigeminal pathway sensitization. While these effects are similar to those reported for TN-associated variants in other channels, it's important to note that one variant showed a loss-of-function (LoF), indicating that GoF phenotypes may not be universal in TN. Interestingly, all idiopathic TN patients with continuous pain harboured GoF $Ca_v3.2$ variants, while those with congenital TN did not show channel alterations. Further studies are needed to fully understand $Ca_v3.2$'s role in trigeminal sensory processing.

Part 3: Drugs targeting T-type channels

Previous research has highlighted the analgesic potential of mixed VGCC blockers. For instance, TROX-1, which inhibits $Ca_v2.1$, $Ca_v2.2$, and $Ca_v2.3$ channels, has shown efficacy in reversing inflammation-induced hyperalgesia and nerve-injury-induced allodynia (Abbadie et al. 2010; R. Patel et al. 2015). Similarly, A-1264087, a blocker of $Ca_v2.1$, $Ca_v2.2$, and Ca_v3 channels, has been effective in reducing mechanical hyperalgesia induced by spinal nerve ligation (SNL) in animal models (Xu et al. 2014). Additionally, physalin F, a natural product, acts as a dual blocker of $Ca_v2.2$ and $Ca_v2.3$ channels and has provided relief from mechanical hyperalgesia in rodent models of neuropathic pain (Shan et al. 2019).

In the seventh study, we therefore introduce S13, a novel quinolone-based compound, as a promising addition to the arsenal of broad spectrum VGCC blockers. S13 effectively inhibits voltage-activated calcium currents in nociceptive DRGs, while not affecting sodium and potassium currents. This specificity is ideal, as it suggests a targeted action on calcium channels involved in pain signalling (Bourinet et al. 2014).

S13 has shown significant analgesic potential in a preclinical rat model of SNL-induced neuropathic pain. It successfully alleviated mechanical allodynia in both male and female subjects, indicating its effectiveness across genders. These results are encouraging and highlight the therapeutic potential of broad-spectrum calcium channel blockers in the treatment of neuropathic pain (Rivas-Ramirez et al. 2017; Radwani et al. 2016; Dobremez et al. 2005; de Amorim Ferreira and Ferreira 2023).

The findings from this study also suggest that the quinolone backbone structure of S13 could be a valuable foundation for developing new analgesic compounds. The efficacy of S13 in

preclinical models warrants further investigation into quinolone derivatives, potentially leading to the development of novel analgesics for clinical use. This approach could open new avenues for the treatment of neuropathic pain, a condition that is often challenging to manage with current therapies (Gordon and Dahl 2004; Johnson, Collett, and Castro-Lopes 2013).

6 Conclusions

This thesis was focused on three aspects of T-type channels. It includes basic research on the modulation of T-type channel expression with two studies. This is followed by four papers on channelopathies of T-type channels. Finally, focusing on the development of novel compounds for targeting disorders associated with T-type channel dysfunction. What we found was the following:

- We discovered two key non-canonical glycosylation sites required for correct expression of $Ca_v3.2$ to the surface. We also found that two other potential non-canonical glycosylation sites were unnecessary.
- SCAMP2 is a key regulatory protein in the trafficking of T-type channels to the surface, and overexpression of SCAMP2 in a recombinant system displays a varied reduction of channel expression to the surface, dependent on channel subtype.
- Diabetes impacts the glycosylation of $Ca_v3.2$, but we could not find definitive evidence of how this takes place. This was due to the unexpected differences between recombinant expression of glycan-enzymes and phenotypic data in diabetic mice. Further studies in more native conditions would need to be carried out to elucidate the impact of diabetes on glycosylation.
- We identified a LoF in *CACNA1H* variants associated with ALS. This increases the evidence for characterising *CACNA1H* as a susceptible gene in the development of ALS.
- We discovered a GoF in *SCN8A*, consistent with previous studies. We also identified a LoF in *CACNA1H* associated with DEE. A gene that has not been previously linked to DEE. It is unknown what the significance of this variant is to the development of DEE, especially considering LoF in *CACNA1H* is usually associated with developmental disorders such as autism.
- We discovered seven *CACNA1H* variants associated with TN had a GoF. This is indicative of an increase in excitability, which coincides with an increase in pain as previously documented. Interestingly, there was a correlation between TN patients with idiopathic TN with concomitant continuous pain and GoF

CACNA1H variants. Whereas there was no dysfunction of the channel in patients with congenital TN and concomitant pain.

- Finally, we identified and characterised a group of broad-spectrum VGCC blockers and their use as pain therapeutics. Suggesting the importance of using broad-spectrum VGCC blockers over selective VGCC blockers in treating some disorders.

7 Summary

T-type channels are integral in many physiological responses evident by their abundant expression in the cardiovascular, neuroendocrine, and nervous system. Due to their unique biophysical properties compared to other VGCC subtypes they are essential in hormone release and shaping neuronal excitability. As such this thesis aims to further our understanding of this unique set of VGCCs.

First, we aimed to understand the regulation of T-type channel expression through two studies. The first study identified non-canonical sites for N-glycosylation on T-type channels, affecting their trafficking and surface expression. This study suggests these glycosylation sites play a crucial role in channel regulation, but the exact mechanisms remain unclear and requires additional in native conditions. The second study introduced SCAMP2 as a novel modulator of T-type channels, essential for various cellular processes including membrane trafficking and signal transduction. SCAMP2's dysfunction is linked to diseases like neurodegenerative disorders and cancer further increasing the requirement of basic research to understand the link with pathophysiology.

Secondly, we explored various T-type channelopathies associated with neurological disorders. To do this we conducted four studies on T-type channels and their role in four different disorders. The third study therefore investigated the role of glycosylation in T-type channels in the context of diabetes, finding contradictory results regarding $Ca_v3.2$ expression in a mouse model and a recombinant expression system. As such this would need to be carried out in primary neurons such as DRGs, which are integral in processing peripheral pain, to elucidate the role of these glycan-enzymes in increasing T-type current and peripheral diabetic neuralgia. The fourth study analysed two *CACNA1H* variants in ALS patients, revealing varying degrees of loss-of-function in $Ca_v3.2$ channel variants. This increases the link between *CACNA1H* and ALS pathology and warrants further investigation in motor neurons, which are known to be functionally affected in ALS patients. The fifth study reported on a child with DEE, having a de novo mutation in *SCN8A* and an inherited rare variant in *CACNA1H*, showing contrasting effects of gain-of-function and loss-of-function, respectively. This adds to the known complexity in channel function and interactions, and could add *CACNA1H* to the DEE disorder. The sixth study examined $Ca_v3.2$ missense variants in patients with trigeminal neuralgia, finding mostly gain-of-function

effects that could contribute to trigeminal pathway sensitization. Interestingly, this study found a noteworthy correlation in patients with idiopathic TN who also experienced continuous pain. Specifically, these patients showed a presence of GoF variants in the *CACNA1H* gene. However, in cases of congenital TN accompanied by pain, there was no observed dysfunction in these channels. This distinction suggests different underlying mechanisms in idiopathic vs. congenital TN, particularly regarding the role and behaviour of T-type channels.

Finally, in the seventh study we aimed to develop novel VGCC blockers in the search for finding more efficacious drugs to alleviate pain and potentially other conditions displaying a GoF in these channels. This study also underscores the potential of broad-spectrum VGCC blockers in treating neuropathic pain.

Overall, this thesis emphasizes the importance of understanding T-type channels' regulation, the impact of channelopathies on neurological disorders, and the development of novel drugs targeting these channels to combat associated diseases.

8 References

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9 List of publications

Part 1: Regulation of T-type channel expression

1. **Functional identification of potential non-canonical N-glycosylation sites within $Ca_v3.2$ T-type calcium channels**
   Ficelova, Vendula, Ivana A. Souza, Leos Cmarko, Maria A. Gandini, Robin N. Stringer, Gerald W. Zamponi, and Norbert Weiss. 2020. 'Functional Identification of Potential Non-Canonical N-Glycosylation Sites within Cav3.2 T-Type Calcium Channels'. *Molecular Brain* 13 (1): 149. https://doi.org/10.1186/s13041-020-00697-z.
   Impact factor: 4.399

2. **Secretory carrier-associated membrane protein 2 (SCAMP2) regulates cell surface expression of T-type calcium channels**
   Cmarko, Leos, Robin N. Stringer, Bohumila Jurkovicova-Tarabova, Tomas Vacik, Lubica Lacinova, and Norbert Weiss. 2022. 'Secretory Carrier-Associated Membrane Protein 2 (SCAMP2) Regulates Cell Surface Expression of T-Type Calcium Channels'. *Molecular Brain* 15 (1): 1. https://doi.org/10.1186/s13041-021-00891-7.
   Impact factor: 4.399

Part 2: T-type channelopathies associated with neurological disorders

3. **Transcriptomic analysis of glycan-processing genes in the dorsal root ganglia of diabetic mice and functional characterization on $Ca_v3.2$ channels**
   Stringer, Robin N., Joanna Lazniewska, and Norbert Weiss. 2020. 'Transcriptomic Analysis of Glycan-Processing Genes in the Dorsal Root Ganglia of Diabetic Mice and Functional Characterization on $Ca_v3.2$ Channels'. *Channels* 14 (1): 132–40. https://doi.org/10.1080/19336950.2020.1745406.
   Impact factor: 3.493

4. **A rare *CACNA1H* variant associated with amyotrophic lateral sclerosis causes complete loss of $Ca_v3.2$ T-type channel activity**

Stringer, Robin N., Bohumila Jurkovicova-Tarabova, Sun Huang, Omid Haji-Ghassemi, Romane Idoux, Anna Liashenko, Ivana A. Souza, et al. 2020. 'A Rare CACNA1H Variant Associated with Amyotrophic Lateral Sclerosis Causes Complete Loss of Cav3.2 T-Type Channel Activity'. *Molecular Brain* 13 (1): 33. https://doi.org/10.1186/s13041-020-00577-6.
Impact factor: 4.399

5. **De novo *SCN8A* and inherited rare *CACNA1H* variants associated with severe developmental and epileptic encephalopathy**
   Stringer, Robin N., Bohumila Jurkovicova-Tarabova, Ivana A. Souza, Judy Ibrahim, Tomas Vacik, Waseem Mahmoud Fathalla, Jozef Hertecant, Gerald W. Zamponi, Lubica Lacinova, and Norbert Weiss. 2021. 'De Novo SCN8A and Inherited Rare CACNA1H Variants Associated with Severe Developmental and Epileptic Encephalopathy'. *Molecular Brain* 14 (1): 126. https://doi.org/10.1186/s13041-021-00838-y.
   Impact factor: 4.399

6. **Electrophysiological and computational analysis of $Ca_v3.2$ channel variants associated with familial trigeminal neuralgia**
   Mustafá, Emilio R., Eder Gambeta, Robin N. Stringer, Ivana A. Souza, Gerald W. Zamponi, and Norbert Weiss. 2022. 'Electrophysiological and Computational Analysis of Cav3.2 Channel Variants Associated with Familial Trigeminal Neuralgia'. *Molecular Brain* 15 (1): 91. https://doi.org/10.1186/s13041-022-00978-9.
   Impact factor: 4.399

Part 3: Drugs targeting T-type channels

7. **Synthesis and pharmacological evaluation of quinolone-based calcium channel blockers with analgesic properties (Unpublished)**
   Leoš Cmarko, Mikhail Klychnikov, Kimberly Gomez, Robin N. Stringer, Samantha Perez-Miller, Miroslav Hájek, Michel De Waard, Rajesh Khanna, Ullrich Jahn, Norbert Weiss

10 Appendix

Paper 1

Functional identification of potential non-canonical N-glycosylation sites within $Ca_V3.2$ T-type calcium channels

Ficelova, Vendula, Ivana A. Souza, Leos Cmarko, Maria A. Gandini, Robin N. Stringer, Gerald W. Zamponi, and Norbert Weiss. 2020. 'Functional Identification of Potential Non-Canonical N-Glycosylation Sites within Cav3.2 T-Type Calcium Channels'. *Molecular Brain* 13 (1): 149. https://doi.org/10.1186/s13041-020-00697-z.

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Functional identification of potential non-canonical N-glycosylation sites within $Ca_v3.2$ T-type calcium channels

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Abstract

Low-voltage-activated T-type calcium channels are important contributors to nervous system function. Post-translational modification of these channels has emerged as an important mechanism to control channel activity. Previous studies have documented the importance of asparagine (N)-linked glycosylation and identified several asparagine residues within the canonical consensus sequence N-*X*-S/T that is essential for the expression and function of $Ca_v3.2$ channels. Here, we explored the functional role of non-canonical N-glycosylation motifs in the conformation N-*X*-C based on site directed mutagenesis. Using a combination of electrophysiological recordings and surface biotinylation assays, we show that asparagines N345 and N1780 located in the motifs NVC and NPC, respectively, are essential for the expression of the human $Ca_v3.2$ channel in the plasma membrane. Therefore, these newly identified asparagine residues within non-canonical motifs add to those previously reported in canonical sites and suggest that N-glycosylation of $Ca_v3.2$ may also occur at non-canonical motifs to control expression of the channel in the plasma membrane. It is also the first study to report the functional importance of non-canonical N-glycosylation motifs in an ion channel.

Keywords: Asparagine-linked glycosylation, N-glycosylation, Non-canonical glycosylation, Calcium channel, T-type channel, $ca_v3.2$ Channel, Trafficking

Low-voltage-activated T-type calcium channels are widely expressed throughout the nervous system where they generate low-threshold calcium spikes that contribute to neuronal electrical excitability [1]. Over recent years, post-translational modification of the channel protein including phosphorylation [2–4], ubiquitination [5], and glycosylation [6] has emerged as an important level of control over the expression and function of the channel in the plasma membrane, and alteration of these regulations is known to contribute to the development of several neurological disorders. Therefore, the identification of channel loci undergoing post-translational modification is essential not only to enhance our fundamental understanding of the channel, but also to gain insights into how alteration of these regulations may compromise channel function in pathological conditions.

We and others have previously documented the importance of asparagine (N)-linked glycosylation in the expression of the $Ca_v3.2$ T-type channels and identified several asparagines essential for the expression of the channel in the plasma membrane [7, 8]. These asparagine residues are located within the sequence N-*X*-S/T commonly referred to as the canonical N-glycosylation motif where the asparagine is located at the N-terminal to any amino acid (except proline) followed by either a serine (S)

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or threonine (T). However, while N-glycosylation at N-*X*-S/T motifs is an established dogma, there is evidence for the occurrence of N-glycosylation at non-canonical motifs falling into the conformation N-*X*-C (cysteine) [9]. In the present study, we aimed to further explore the glycosylation loci of $Ca_v3.2$ channels and assess whether asparagines located within such non-canonical motifs contribute to the expression of the channel. The human $Ca_v3.2$ channel contains four potential non-canonical motifs defined by asparagines N258, N335 and N345 located within the first pore-forming loop (P-loop), and asparagine N1780 within the forth P-loop of the channel (Fig. 1a) and in-silico analysis using NetNGlyc 1.0 server (https://www.cbs.dtu.dk/services/NetNGlyc/) predicted that these sites could be potentially glycosylated (Fig. 1b). To assess the functional importance of these residues in the expression of $Ca_v3.2$ channels, we used site directed mutagenesis to disrupt these motifs. We replaced asparagine residues with glutamine (Q) and such recombinant mutated channels were expressed in tsA-201 cells for functional characterization by patch clamp electrophysiology. Representative current traces for cells expressing the wild-type (WT) channel and the various mutated variants (N258Q, N335Q, N345Q, and N1780Q) are shown in Fig. 1c. While all channel variants produced a characteristic low-threshold voltage-activated T-type current, currents recorded from cells expressing the N345Q and N1780Q variants were strongly reduced compared to cells expressing the WT channel (Fig. 1d). The maximal whole cell slope conductance was reduced by 50% ($p=0.0050$) in N345Q (480 ± 68 pS/pF, $n=17$) and by 56% ($p=0.0021$) in N1780Q-expressing cells (423 ± 90 pS/pF, $n=11$) compared to cells expressing the WT channel (970 ± 110 pS/pF, $n=27$). This decrease of the maximal conductance was associated with a mild but significant shift of the voltage-dependence of activation without any additional alteration of the voltage-dependence of inactivation and recovery from inactivation (Additional file 1: Figure S1). Furthermore, mutations at asparagines N345 and N1780 did not alter the ability of nickel to block T-type currents (Additional file 1: Figure S2). Next, we aimed to determine whether the impaired T-type conductance in cells expressing the N345Q and N1780Q channels was caused by an alteration of the channel activity or due to reduced expression of the channels in the plasma membrane. To do so, we used cell surface biotinylation followed by immunodetection of the channels. Representative immunoblots of total and surface biotinylated channels are shown in Fig. 1f and g, respectively. No immunoreactivity was detected in non-biotinylated cells expressing WT $Ca_v3.2$ channels demonstrating the absence of contamination from other cellular fractions and also the absence of non-specific interaction of the channel with NeutrAvidin beads. While the total channel expression was statistically similar across all channel variants (Fig. 1f), the expression of the N345Q and N1780Q channels in the plasma membrane was reduced by 50% ($p=0.0031$) and 56% ($p=0.0301$) respectively, a decrease that is closely correlated with the reduction of the whole cell conductance.

Glycosylation of T-type channels has emerged as an important post-translational modification to control the expression and functioning of the channel in the plasma membrane, and it was suggested that alteration of the glycoproteome of $Ca_v3.2$ may contribute to the development of peripheral pain associated with diabetes [8, 10]. While several studies have previously reported the importance of canonical glycosylation sites in the expression and function of $Ca_v3.2$ channels [7, 8, 11, 12], the potential role for non-canonical motifs has never been explored. Here, we identified two asparagine residues, N345 and N1780 located within non-consensus glycosylation motifs in the conformation N-*X*-C that contribute to the expression of the channel in the plasma membrane. Although the exact underlying mechanisms by which these two asparagines influence the expression of the channel at the cell surface was not explored in this study, it is likely that they either enhance the trafficking of the channel to the cell surface, or stabilize the channel protein in the plasma membrane by slowing down its internalization as it was previously reported for other glycosylation loci [11]. Because of the large molecular weight of the full-length channel and the existence of several canonical glycosylation sites, it is challenging to demonstrate by Western blot analysis that mutagenesis of the non-canonical sites leads to small molecular weight shifts that are consistent with fewer sugar groups. We can therefore not exclude the possibility that alteration of $Ca_v3.2$ expression upon mutagenesis of asparagines N345 and N1780 may have resulted from an alteration of the channel itself rather than from disruption of its glycosylation. However, this mutagenesis approach is commonly used to functionally assess the functional importance of glycosylation motifs, and glutamine as a replacement of asparagine was chosen because of its similarity, which is therefore expected to preserve the local charge distribution and secondary structure of the channel. Moreover, we cannot totally exclude that mutagenesis of asparagines N345 and N1780 may have interfered with the ubiquitination of the channel, although this process occurs at a different locus (III-IV linker) and therefore is not expected to be directly impacted by the mutations [5]. Our observation that the total expression of mutated channels remained unaltered would also argue against an effect on the ubiquitination pathway.

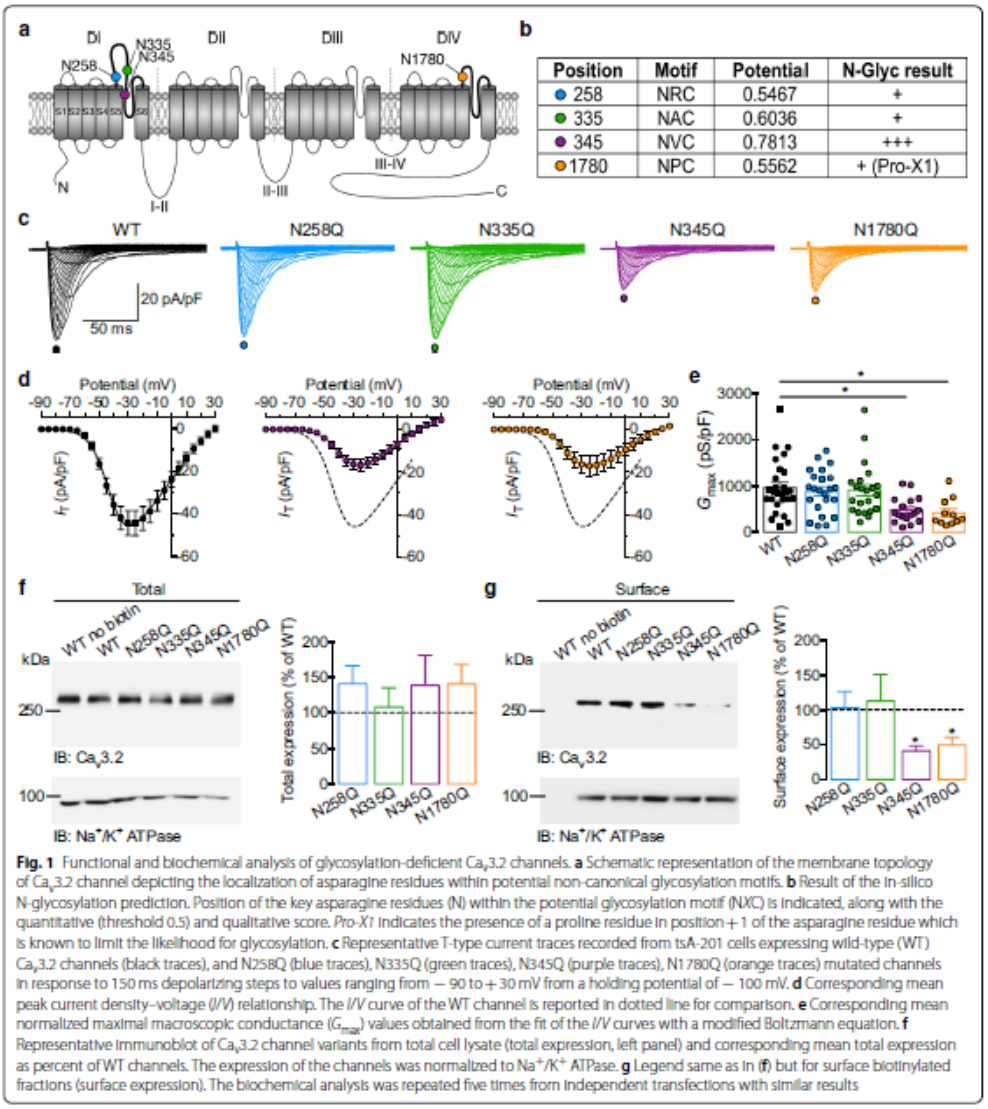

Fig. 1 Functional and biochemical analysis of glycosylation-deficient $Ca_v3.2$ channels. **a** Schematic representation of the membrane topology of $Ca_v3.2$ channel depicting the localization of asparagine residues within potential non-canonical glycosylation motifs. **b** Result of the in-silico N-glycosylation prediction. Position of the key asparagine residues (N) within the potential glycosylation motif (NXC) is indicated, along with the quantitative (threshold 0.5) and qualitative score. *Pro-X1* indicates the presence of a proline residue in position + 1 of the asparagine residue which is known to limit the likelihood for glycosylation. **c** Representative T-type current traces recorded from tsA-201 cells expressing wild-type (WT) $Ca_v3.2$ channels (black traces), and N258Q (blue traces), N335Q (green traces), N345Q (purple traces), N1780Q (orange traces) mutated channels in response to 150 ms depolarizing steps to values ranging from − 90 to + 30 mV from a holding potential of − 100 mV. **d** Corresponding mean peak current density–voltage (*I/V*) relationship. The *I/V* curve of the WT channel is reported in dotted line for comparison. **e** Corresponding mean normalized maximal macroscopic conductance (G_{max}) values obtained from the fit of the *I/V* curves with a modified Boltzmann equation. **f** Representative immunoblot of $Ca_v3.2$ channel variants from total cell lysate (total expression, left panel) and corresponding mean total expression as percent of WT channels. The expression of the channels was normalized to Na^+/K^+ ATPase. **g** Legend same as in (**f**) but for surface biotinylated fractions (surface expression). The biochemical analysis was repeated five times from independent transfections with similar results

Altogether, this study supports the notion that $Ca_v3.2$ channels may undergo N-glycosylation at non-canonical motifs and identified two sites defined by asparagines N345 and N1780 important for expression of the channel at the cell surface. To our knowledge, this study is the first to document the functional role of non-canonical N-glycosylation motifs within an ion channel.

Supplementary information

Supplementary Information accompanies this paper at https://doi.org/10.1186/s13041-020-00697-z.

Additional file 1. **Figure S1** Electrophysiological properties of $Ca_v3.2$ channel variants. **a** Mean normalized voltage-dependence of activation for wild-type (WT) $Ca_v3.2$ channels (black circles), and N258Q (blue circles), N335Q (green circles), N345Q (purple circles), N1780Q (orange circles). **b** Corresponding mean half-activation potential values obtained from the fit of the activation curves with a modified Boltzmann equation. **c-d** Legend same as for (a-b) but for the voltage-dependence of steady state inactivation. **e** Mean normalized recovery from inactivation kinetics. **f** Corresponding mean time constant values of recovery from inactivation obtained from the fit of the recovery curves with a single-exponential function. **Figure S2** Effect of nickel on $Ca_v3.2$ channel variants. **a** Representative T-type current traces recorded from tsA-201 cells expressing wild-type (WT, black traces), N345Q (purple traces) and N1780Q (orange traces) $Ca_v3.2$ variants recorded in response to 150 ms depolarizing steps to -20 mV from a holding potential of -100 mV before (Ctrl) and after application of 50 μM nickel (Ni^+). **b** Corresponding mean peak current inhibition.

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Not applicable.

Authors' contributions
VF, LC, and RNS performed electrophysiological recordings and analyzed the data. MAG performed biotinylation experiments. IAS generated plasmid cDNAs encoding for $Ca_v3.2$ mutants. NW designed and supervised the study. GWZ and NW wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate
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Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

Paper 2

Secretory carrier-associated membrane protein 2 (SCAMP2) regulates cell surface expression of T-type calcium channels

Cmarko, Leos, Robin N. Stringer, Bohumila Jurkovicova-Tarabova, Tomas Vacik, Lubica Lacinova, and Norbert Weiss. 2022. 'Secretory Carrier-Associated Membrane Protein 2 (SCAMP2) Regulates Cell Surface Expression of T-Type Calcium Channels'. *Molecular Brain* 15 (1): 1. https://doi.org/10.1186/s13041-021-00891-7.

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Secretory carrier-associated membrane protein 2 (SCAMP2) regulates cell surface expression of T-type calcium channels

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Abstract

Low-voltage-activated T-type Ca^{2+} channels are key regulators of neuronal excitability both in the central and peripheral nervous systems. Therefore, their recruitment at the plasma membrane is critical in determining firing activity patterns of nerve cells. In this study, we report the importance of secretory carrier-associated membrane proteins (SCAMPs) in the trafficking regulation of T-type channels. We identified SCAMP2 as a novel $Ca_v3.2$-interacting protein. In addition, we show that co-expression of SCAMP2 in mammalian cells expressing recombinant $Ca_v3.2$ channels caused an almost complete drop of the whole cell T-type current, an effect partly reversed by single amino acid mutations within the conserved cytoplasmic E peptide of SCAMP2. SCAMP2-induced downregulation of T-type currents was also observed in cells expressing $Ca_v3.1$ and $Ca_v3.3$ channel isoforms. Finally, we show that SCAMP2-mediated knockdown of the T-type conductance is caused by the lack of $Ca_v3.2$ expression at the cell surface as evidenced by the concomitant loss of intramembrane charge movement without decrease of total $Ca_v3.2$ protein level. Taken together, our results indicate that SCAMP2 plays an important role in the trafficking of $Ca_v3.2$ channels at the plasma membrane.

Keywords: Ion channels, Calcium channels, T-type channels, $Ca_v3.2$ channels, Secretory carrier-associated membrane protein 2, SCAMP2, Trafficking

Through their ability to pass calcium ions (Ca^{2+}) near the resting membrane potential, low-voltage-activated T-type channels have an important physiological role in shaping firing activity patterns of nerve cells, both in the central and peripheral nervous system. The implication of T-type channels in the control of neuronal excitability is partly defined by the density of channels embedded in the plasma membrane. Therefore, a number of molecular mechanisms and signaling pathways come into play to underly precise control of cell surface expression of T-type channels [1] and defects whether genetic or acquired can lead to severe neuronal conditions [2, 3].

Secretory carrier-associated membrane proteins (SCAMPs) form a family of integral membrane proteins essentially expressed in the trans-Golgi network and recycling endosome membranes where they regulate vesicular trafficking and vesicle recycling processes [4]. Of the five known mammalian SCAMPs, SCAMP2 shows a ubiquitous expression pattern including in neuronal tissues where SCAMP2 transcripts are observed for instance in the cerebellum, thalamus, hippocampus, and spinal cord (https://www.proteinatlas.org/ENSG00000140497-SCAMP2/tissue). SCAMP2 consists of four transmembrane helices with cytoplasmic amino- and carboxy-termini and a so-called E peptide located between

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transmembrane helices 2 and 3 essential for mediating SCAMP2 function [5]. This E domain is highly conserved among SCAMP isoforms and represents an essential molecular determinant for SCAMP2-mediated inhibition of exocytosis [6]. Only a few reports have documented the role of SCAMP2 in the regulation of ion channels and transporters [7–10]. In the present study, we aimed to assess the functional role of SCAMP2 in the regulation of T-type channels.

To address this issue, we assessed whether $Ca_v3.2$ channels and SCAMP2 associate at the protein level. Co-immuniprecipitation from tsA-201 cells expressing recombinant HA-tagged $Ca_v3.2$ and Myc-tagged SCAMP2 using an anti-HA-antibody precipitated SCAMP2-Myc with $Ca_v3.2$-HA revealing the existence of a $Ca_v3.2$/SCAMP2 protein complex (Fig. 1a). We note that co-immunoprecipitation experiments from total cell lysates do not address whether this interaction is direct or not and it is a possibility that formation of $Ca_v3.2$/SCAMP2 protein complex may also involve another intermediate protein. Next, we aimed to analyze the functional effect of SCAMP2 on $Ca_v3.2$ channels. Patch-clamp recordings from tsA-201 cells expressing $Ca_v3.2$ showed that co-expression of SCAMP2 produces an almost complete drop of the whole-cell T-type current (Fig. 1b and c). For instance, the maximal macroscopic conductance (G_{max}) was reduced by 91% ($p<0.0001$) in cells co-expressing SCAMP2 (61 ± 18 pS/pF, $n=18$) compared to cells expressing $Ca_v3.2$ alone (692 ± 62 pS/pF, $n=25$) (Fig. 1d). Alanine mutagenesis of the E peptide of SCAMP2 at cysteine 201 (C201A) and tryptophan 202 (W202A) reduced this effect to 64% ($p=0.0269$) and 39% ($p<0.0001$) inhibition, respectively, indicating that SCAMP2-induced knockdown of $Ca_v3.2$ currents is at least partly mediated by the E peptide (Fig. 1b–d). These data also indicate that the reduction in $Ca_v3.2$ current density in the presence of SCAMP2 is not merely due to the co-expression of just any protein given that the W202A mutant construct has no big effect. With regard to the effect of SCAMP2 on the other T-type channel isoforms, co-expression of SCAMP2 in cells expressing recombinant $Ca_v3.1$ and $Ca_v3.3$ reduced G_{max} by 35% ($p<0.0001$) and 98% ($p<0.0001$) respectively (Fig. 1e and f and Additional file 1: Fig. S1) indicative of a differential susceptibility to SCAMP2-dependent modulation ($Ca_v3.3 \approx Ca_v3.2 > Ca_v3.1$). Next, we aimed to assess the underlying mechanism by which SCAMP2 induced knockdown of the T-type conductance. The alteration of the T-type conductance in the presence of SCAMP2 could originate from an overall decreased level of $Ca_v3.2$ proteins or from a reduced expression of the channel in the plasma membrane. Western blot analysis from total cell lysates showed that $Ca_v3.2$ protein levels were not decreased by the presence of SCAMP2. Instead, we observed a non-significant trend toward higher expression levels which may have arisen from a lower rate of vesicular exocytosis therefore preventing the channel from being targeted to the proteasomal degradation machinery (Fig. 1g and h). In contrast, recording of intramembrane charge movements (Q) that provide an accurate assessment of the number of channels embedded in the plasma membrane revealed an 85% decrease ($p<0.0001$) of Q_{max} in cells expressing SCAMP2 (from 6.1 ± 0.7 fC/pF, $n=16$ to 0.9 ± 0.2 fC/pF, $n=17$) (Fig. 1i and j) indicating a decreased channel expression at the cell surface. Moreover, while the kinetics of intramembrane charge movements remained unaltered (Fig. 1k), the G_{max}/Q_{max} dependency in the presence of SCAMP2 was reduced by 52% ($p<0.0001$) (from 0.169 ± 0.007 pS/fC, $n=16$ to 0.080 ± 0.014 pS/fC, $n=11$) suggesting an additional alteration of the coupling between the activation of the voltage-sensor and the pore opening of the channel (Fig. 1l). This observation is consistent with a previous report showing that besides to be concentrated primarily in intracellular membranes, SCAMP2 is also found in the plasma membrane [11] and therefore could potentially modulate the gating of the channel in addition to its insertion in the membrane. We note that the reduction

(See figure on next page.)

Fig. 1 SCAMP2 regulates T-type channel expression. **a** Co-immunoprecipitation of Myc-tagged SCAMP2 (SCAMP2-Myc) from tsA-201 cells co-transfected with HA-tagged $Ca_v3.2$ channel ($Ca_v3.2$-HA). The upper panel shows the result of the co-immunoprecipitation of SCAMP2-Myc with $Ca_v3.2$-HA using an anti-HA antibody. The lower panels show the immunoblot of Cav3.2-HA and SCAMP2-Myc from total cell lysates using an anti-HA and anti-Myc antibody, respectively. *HC*, heavy chain antibody; *LC*, light chain antibody. This experiment was performed four times from independent transfections and $Ca_v3.2$/SCAMP2 interaction was consistently observed. **b** Representative T-type current traces from tsA-201 cells expressing $Ca_v3.2$ alone (black traces) and in combination with wild-type SCAMP2 (blue traces), as well as with C201A (purple traces) and W202A (orange traces) SCAMP2 mutants in response to 150 ms depolarizing steps varied from − 90 mV to + 30 mV from a holding potential of − 100 mV. **c** Corresponding mean current/voltage (*I/V*) relationships. **d** Corresponding mean maximal macroscopic conductance values (G_{max}) obtained from the fit of the *I/V* curves with the modified Boltzmann Eq. (1). **e–f** Mean G_{max} values for tsA-201 cells expressing $Ca_v3.1$ and $Ca_v3.3$ channels, respectively. **g.** Immunoblot of $Ca_v3.2$-HA expressed in tsA-201 cells in the absence (−) and presence (+) of SCAMP2-Myc. The immunoblot shows the results of three independent sets of transfections. **h** Corresponding mean expression levels of $Ca_v3.2$-HA normalized to actin. **i** Representative intramembrane charge movement traces recorded at the ionic reversal potential from cells expressing $Ca_v3.2$ alone (black trace) and in the presence of SCAMP2 (blue trace). The doted lines depict the time course of the intramembrane charge mouvement integral. **J** Corresponding mean maximal intramembrane charge movement values (Q_{max}). **k** Corresponding mean 10–90% rise time values calculated from the integral time course shown in **i**. **l** Corresponding mean G_{max}/Q_{max} values

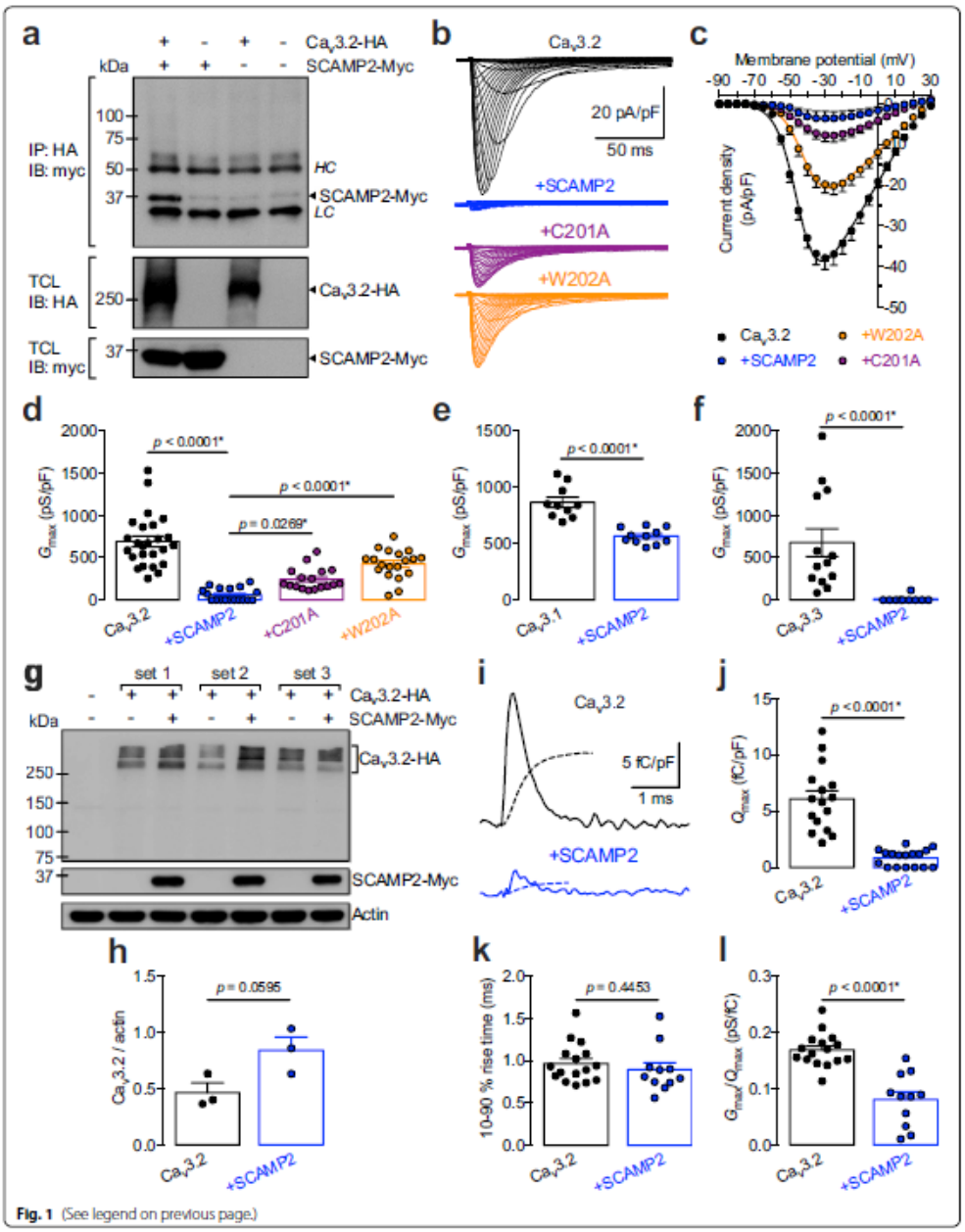

Fig. 1 (See legend on previous page.)

of Q_{max} combined with the reduction of G_{max}/Q_{max} of the small fraction of channels that still reached the plasma membrane in the presence of SCAMP2 is very similar to the reduction of the maximal T-type conductance we previously observed (91%, Fig. 1d).

Several $Ca_v3.2$ interacting proteins including KLHL1 [12], USP5 [13], Stac1 [14], calnexin [15], and Rack-1 [16] have been reported to modulate the sorting and trafficking of the channel to the plasma membrane. In this study, we reported SCAMP2 as a novel $Ca_v3.2$-interacting partner and potent repressor of the expression of the channel at the cell surface. Further investigations will be necessary to fully explore the importance of this regulation in native conditions. Importantly, altered expression of SCAMP2 has been reported in several types of cancer [17]. Given the importance of $Ca_v3.2$ channels in the development of peripheral painful neuropathies [18], it will be interesting to assess to what extent SCAMP2-mediated regulation of $Ca_v3.2$ could possibly contribute to cancer-related neuropathic pain.

Abbreviations

G_{max}: Maximal macroscopic conductance; KLHL1: Kelch-like 1; Q_{max}: Maximal intra membrane charge movement; Rack-1: Receptor for activated C kinase 1; SCAMP2: Secretory carrier membrane protein 2; Stac1: Stac adaptor protein 1; USP5: Ubiquitin-specific proteinase 5.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13041-021-00891-7.

Additional file 1. Fig. S1. Functional effect of SCAMP2 on $Ca_v3.1$ and $Ca_v3.3$ channels. **a** Representative T-type current traces from tsA-201 cells expressing $Ca_v3.1$ alone (black traces) and in combination with SCAMP2 (blue traces) in response to 150 ms depolarizing steps varied from -90 mV to +30 mV from a holding potential of -100 mV. **b** Corresponding mean current/voltage (*I/V*) relationships. **c** Corresponding mean maximal macroscopic conductance values (*Gmax*) obtained from the fit of the *I/V* curves with the modified Boltzmann Eq. (1). **d**–**e** Same legend as for **a**–**c** but for cells expressing $Ca_v3.3$ channel.

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Authors' contributions

LC, RNS and BJT performed experiments and analyzed the data. TV generated SCAMP2 C201A mutant cDNA. LL supervised recordings and analysis of intramembrane charge movement. NW designed and supervised the study and wrote the manuscript. All authors critically revised the manuscript and contributed significantly to this work. All authors read and approved the final manuscript.

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All data generated or analyzed during this study are included in this published article and its additional information files.

Declarations

Ethics approval and consent to participate

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Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

Paper 3

Transcriptomic analysis of glycan-processing genes in the dorsal root ganglia of diabetic mice and functional characterization on $Ca_v3.2$ channels

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BRIEF REPORT

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Transcriptomic analysis of glycan-processing genes in the dorsal root ganglia of diabetic mice and functional characterization on $Ca_v3.2$ channels

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ABSTRACT

$Ca_v3.2$ T-type calcium channels play an essential role in the transmission of peripheral nociception in the dorsal root ganglia (DRG) and alteration of $Ca_v3.2$ expression is associated with the development of peripheral painful diabetic neuropathy (PDN). Several studies have previously documented the role of glycosylation in the expression and functioning of $Ca_v3.2$ and suggested that altered glycosylation of the channel may contribute to the aberrant expression of the channel in diabetic conditions. In this study, we aimed to analyze the expression of glycan-processing genes in DRG neurons from a leptin-deficient genetic mouse model of diabetes (*db*/*db*). Transcriptomic analysis revealed that several glycan-processing genes encoding for glycosyltransferases and sialic acid-modifying enzymes were upregulated in diabetic conditions. Functional analysis of these enzymes on recombinant $Ca_v3.2$ revealed an unexpected loss-of-function of the channel. Collectively, our data indicate that diabetes is associated with an alteration of the glycosylation machinery in DRG neurons. However, individual action of these enzymes when tested on recombinant $Ca_v3.2$ cannot explain the observed upregulation of T-type channels under diabetic conditions.

Abbreviations: Galnt16: Polypeptide N-acetylgalactosaminyltransferase 16; B3gnt8: UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 8; B4galt1: Beta-1,4-galactosyltransferase 1; St6gal1: Beta-galactoside alpha-2,6-sialyltransferase 1; Neu3: Sialidase-3

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Introduction

It is well established that increased expression of the low-voltage-activated $Ca_v3.2$ T-type calcium channel within neurons of the dorsal root ganglia contribute to the sensitization of nociceptive sensory fibers in response to hyperglycemia associated with diabetes, leading to painful symptoms of peripheral diabetic neuropathy [1–3]. This notion is further exemplified by the observation that pharmacological blockade of T-type channels alleviates diabetes-induced hyperalgesia in a leptin-deficient genetic mouse model of diabetes (*ob*/*ob*) [4]. Furthermore, it has been reported that removal of terminal sialic acid moieties from complex glycan structures can normalize T-type currents in DRG neurons isolated from *ob*/*ob* mice, and reverse neuropathic pain *in vivo* [5], suggesting that glycosylation of $Ca_v3.2$ could possibly represent an underlying mechanisms contributing to the enhanced expression of the channel during diabetes.

Protein glycosylation is a posttranslational modification that refers to the co-valent addition of a sugar molecule oligosaccharide (glycan) to specific residues within the target protein. It is an essential chemical process that contributes to the proper maturation, sorting, and functioning of proteins including ion channels [6,7], and several studies have documented the importance of glycosylation for the expression of $Ca_v3.2$ channels [8,9]. However, the underlying cellular mechanisms by which $Ca_v3.2$ channel may undergo aberrant glycosylation during diabetes have remained unknown.

In this study, we aimed to specifically analyzed the transcriptomic profile of glycan-modifying enzymes in DRG neurons from diabetic *db*/*db* mice and assess the effect of these enzymes on the expression of recombinant $Ca_v3.2$ channels.

Materials and methods

Animals

8 weeks old male *db*/*db* mice and their control hibernates were purchased from Janvier Labs and were

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kept under standard conditions for 3 weeks to allow sufficient adaptation. The mean glycemia values measured using a glucocard X-meter ARKAY from blood samples drawn from the tail were 8.2 ± 0.6 mmol/L for wild-type animals ($n = 6$) and 29.9 ± 0.7 mmol/L for *db*/*db* animals ($n = 7$).

Transcriptomic analysis

Transcriptomic analysis of glycan-modifying enzymes was performed on total RNA harvested from the dorsal root ganglia (lumbar L4/L6) of wild-type and *db*/*db* mice using the Glycosylation RT2 Profiler PCR Array (Qiagen) according to the manufacturer's instructions. The PCR array and qRT-PCR were performed on a LightCycler® 480 (Roche) with the following PCR conditions: 95°C for 5 min, 40 cycles at 95°C for 15 sec, 60°C for 15 sec, and 72°C for 20 sec. Each test was run three times and the mean values were taken to eradicate any discrepancies. 84 key genes encoding glycan-processing enzymes were analyzed and included glycosyltransferase and glycosidase for several important sugars (galactose, glucose, mannose, N-acetylgalactosamine, N-acetylglucosamine, fucose and sialic acid).

Plasmid cDNA constructs

The cDNA construct encoding for the human $Ca_v3.2$ wild-type in pcDNA3.1 was previously described [10]. The plamid cDNAs encoding for the human glycan-modifying enzymes Galnt16, B3gnt8, B4galt1, St6gal1, and Neu3 in pCMV3 were purchased from Sino Biological.

Cell culture and heterologous expression

Human embryonic kidney tsA-201 cells were grown in high glucose DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all media were purchased from Invitrogen) and maintained under standard conditions. Cells were transfected using the calcium/phosphate method using 2.5 ± g of $Ca_v3.2$ plasmid and 2.5 ± g of plasmid encoding for the glycan-modifying enzymes. For transfections using the channel alone, 2.5 ± g of empty pcDNA3 vector was added to the mixture to maintained the total amount of cDNA.

Electrophysiology

Patch clamp recording of T-type currents in tsA-201 cells expressing $Ca_v3.2$ channels was performed 72 h after transfection in the whole-cell configuration at room temperature (2224°C) as previously described [11]. The external solution contained (in millimolar): 5 $BaCl_2$, 5 KCl, 1 $MgCl_2$, 128 NaCl, 10 TEA-Cl, 10 D-glucose, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.2 with NaOH). Patch pipettes were filled with an internal solution containing (in millimolar): 110 CsCl, 3 Mg-ATP, 0.5 Na-GTP, 2.5 $MgCl_2$, 5 D-glucose, 10 EGTA, and 10 HEPES (pH 7.4 with CsOH), and had a resistance of 2–4 MΩ. Recordings were performed using an Axopatch 200B amplifier (Axon Instruments) and acquisition and analysis were performed using pClamp 10 and Clampfit 10 software, respectively (Axon Instruments). The linear leak component of the current was corrected online and current traces were digitized at 10 kHz and filtered at 2 kHz. The voltage dependence of activation of $Ca_v3.2$ channels was determined by measuring the peak T-type current amplitude in response to 150 ms depolarizing steps to various potentials applied every 10 s from a holding membrane potential of −100 mV. The current-voltage relationship (I/V) curve was fitted with the following modified Boltzmann Equation (1):

$$I(V) = Gmax\frac{(V - V\text{rev})}{1 + \exp\frac{(V0.5 - V)}{k}} \quad (1)$$

with $I(V)$ being the peak current amplitude at the command potential V, G_{max} the maximum conductance, V_{rev} the reversal potential, $V_{0.5}$ the half-activation potential, and k the slope factor. The voltage dependence of the whole-cell Ba^{2+} conductance was fitted with the following modified Boltzmann Equation (2):

$$G(V) = \frac{Gmax}{1 + \exp\frac{((V0.5 - V)}{k}} \quad (2)$$

with $G(V)$ being the Ba^{2+} conductance at the command potential V.

The voltage dependence of the steady-state inactivation of $Ca_v3.2$ channels was ascertained by measuring the peak T-type current amplitude in response to a 150 ms depolarizing step to −20 mV

applied after a 5 s-long conditioning prepulse ranging from −120 mV to −30 mV. The current amplitude obtained during each test pulse was normalized to the maximal current amplitude and plotted as a function of the prepulse potential. The voltage dependence of the steady-state inactivation was fitted with the following two-state Boltzmann function (3):

$$I(V) = \frac{Imax}{1 + \exp\frac{(V - V0.5)}{k}} \quad (3)$$

with I_{max} as the maximal peak current amplitude and $V_{0.5}$ as half-inactivation voltage.

The recovery from inactivation was determined using a double-pulse protocol from a holding potential of −100 mV. The cell membrane was depolarized for 2 s at 0 mV (inactivating prepulse) to ensure complete inactivation of the channel, and then to −20 mV for 150 ms (test pulse) after an increasing time period (interpulse) ranging between 0.1 ms and 2 s at −100 mV. The peak current from the test pulse was plotted as a ratio of the maximum prepulse current versus interpulse interval. The data were fitted with the following single-exponential function (4):

$$\frac{I}{Imax} = A \times \left(1 - exp\frac{-t}{\tau}\right) \quad (4)$$

where τ denotes the time constant of channel recovery from inactivation.

Statistical analysis

Data values are presented as mean ± S.E.M. for n measurements. Statistical analysis was performed using GraphPad Prism 7. Statistical significance was determined using a one-way ANOVA test and datasets were considered significantly different for $p \leq 0.05$.

Results

Expression of glycan-processing enzymes in the dorsal root ganglia of diabetic mice

In order to assess the expression of glycan-processing enzymes in diabetic conditions, we performed a differential transcriptomic analysis on the dorsal root ganglia isolated from a transgenic mouse model of diabetes (*db*/*db*) versus wild-type animals (Figure 1). 19 out of 84 enzymes analyzed were found significantly upregulated ($p < 0.05$) in diabetic conditions (Figure 2am). The majority of these enzymes (53%) belonged to the family of glycosyltransferases (Galnt1, Galnt4, Galnt12, Galnt16, B3gnt8, Gcnt1, Mgat4 c, Uggt2, B3glct, and B4galt1) that catalyze the transfer of saccharide moieties from an activated nucleotide sugar to a nucleophilic glycosyl acceptor molecule. In addition, 16% belonged to the family of mannosidases (Man1a, Man2a1, and Man2b1) that hydrolyze mannose moieties; 16% to the family of fucosidases/fucosyltransferases (Fuca1, Fut8, and Pofut2); 5% to the family of galactosides/glucosidases/hexosaminidases (Ganab); and 10% to the family of sialidases/sialyltransferases (St6gal1, and Neu3) involved in the processing of sialic acid moieties from complex glycan structures (Figure 2m). In contrast, we did not observe any enzymes that were significantly downregulated.

Functional effect of glycan-processing enzymes on the expression of recombinant $Ca_v3.2$ channels

Next, we aimed to assess the functional impact of up-regulated glycan-modifying enzymes on recombinant $Ca_v3.2$ channels expressed in tsA-201 cells. Six enzymes responsible for the processing of the glycan structure at different stages were assessed: Galnt16 (*N*-acetylgalactosaminyltransferase) responsible for catalyzing the initial addition of N-acetylgalactosamine to a serine or threonine residue on early protein precursors [12]; B3gnt8 (*N*-acetylglucosaminyltransferase) responsible for the elongation of the polylactosamine chains on tetraantennary *N*-glycans [13]; B4galt1 (Galactosyltransferase) which catalyzes the addition of galactose moieties to N-acetylglucosamine of complex *N*-glycans in the Golgi apparatus [14]; St6gal1 (Sialyltransferase) responsible for catalyzing the final transfer of sialic acid moieties from CMP-sialic acid to galactose acceptor substrates [15]; and Neu3 (sialidase) expressed in the plasma membrane and responsible for removing sialic acid moieties from glycoproteins and glycolipids, acting in the opposite way of St6gal1 [16]. Representative T-type current traces recorded

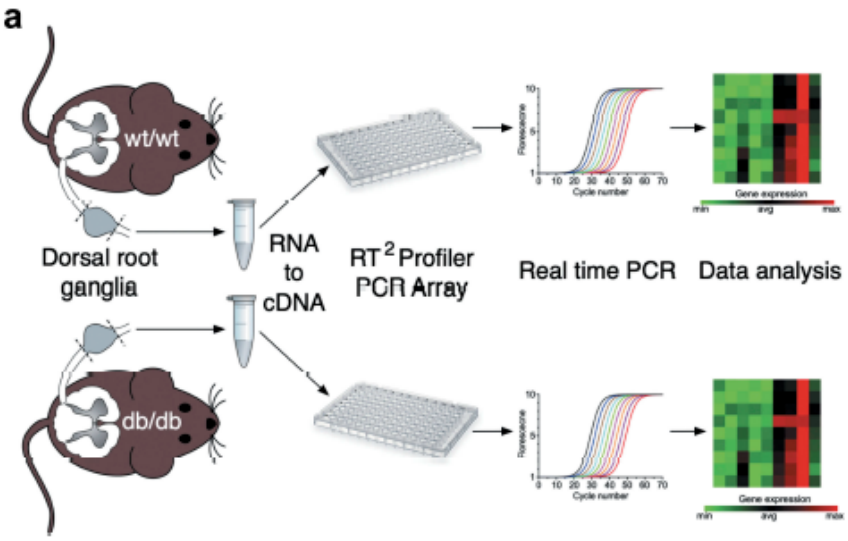

Figure 1. Schematic representation of the transcriptomic analysis process. (a) Total RNA harvested from the dorsal root ganglia (lumbar L4/L6) of wild-type and *db/db* mice and subjected to the Glycosylation RT^2 Profiler PCR Array to analyze the expression level of 84 genes encoding for glycan-processing enzymes.

from cells co-expressing $Ca_v3.2$ with glycosyltransferases (Galnt16, B3gnt8, or B4galt1) and sialic acid-modifying enzymes (St6gal1 or Neu3) are shown in Figure 3a in response to 150 ms depolarizing steps ranging between −90 mV and 30 mV from a holding potential of −100 mV. Unexpectedly, co-expression of glycosyltransferases with $Ca_v3.2$ nearly abolished T-type currents. For instance, the maximal T-type conductance (G_{max}) in cells expressing $Ca_v3.2$ with Galnt16, B3gnt8, and B4galt1 was reduced by 98% (p = 0.0001) (20 ± 20 pS/pF, n = 14), 92% (p = 0.0003) (67 ± 28 pS/pF, n = 5), and 80% (p = 0.0002) (165 ± 21 pS/pF, n = 7), respectively, compared to cells expressing $Ca_v3.2$ alone (821 ± 68 pS/pF, n = 37) (Figure 3b,c). We also observed a significant decrease of G_{max} in cells co-expressing the sialyltransferase St6gal1 by 52% (p = 0.0028) (395 ± 74 pS/pF, n = 13) (Figure 3ac and Table 1). In contrast, we did not observe a significant alteration (p = 0.7542) of G_{max} in cells co-expressing the sialidase Neu3 (921 ± 104 pS/pF, n = 24) (Figure 3ac and Table 1). Altogether, these data indicate that some of the glycan-processing enzymes tested here can have a potent influence on the expression of $Ca_v3.2$ that is consistent with a loss-of-channel function.

Electrophysiological properties of $Ca_v3.2$ channels in the presence of sialic acid-processing enzymes

Previous studies have shown that the terminal sialic acid moiety attached to complex glycan structures can affect the gating of voltage-gated ion channels [17]. Therefore, we further assessed the voltage-dependence of activation and inactivation of $Ca_v3.2$ channels in the presence of the sialyltransferase St6gal1 and sialidase Neu3. The mean half-activation potential in cells expressing St6gal1 was shifted by 4.9 mV (p = 0.0001) toward depolarized potentials (−38.7 ± 0.7 mV, n = 13) compared to cells expressing the channel alone (−43.6 ± 0.6 mV, n = 37) (Figure 4a,b and Table 1). In contrast, co-expression of Neu3 had no significant effect of the voltage-dependence of activation of $Ca_v3.2$. Furthermore, neither St6gal1 nor Neu3 altered the voltage-dependence of inactivation (Figure 4c,d and Table 1) or the recovery from inactivation of $Ca_v3.2$ channels (Figure 4e,f

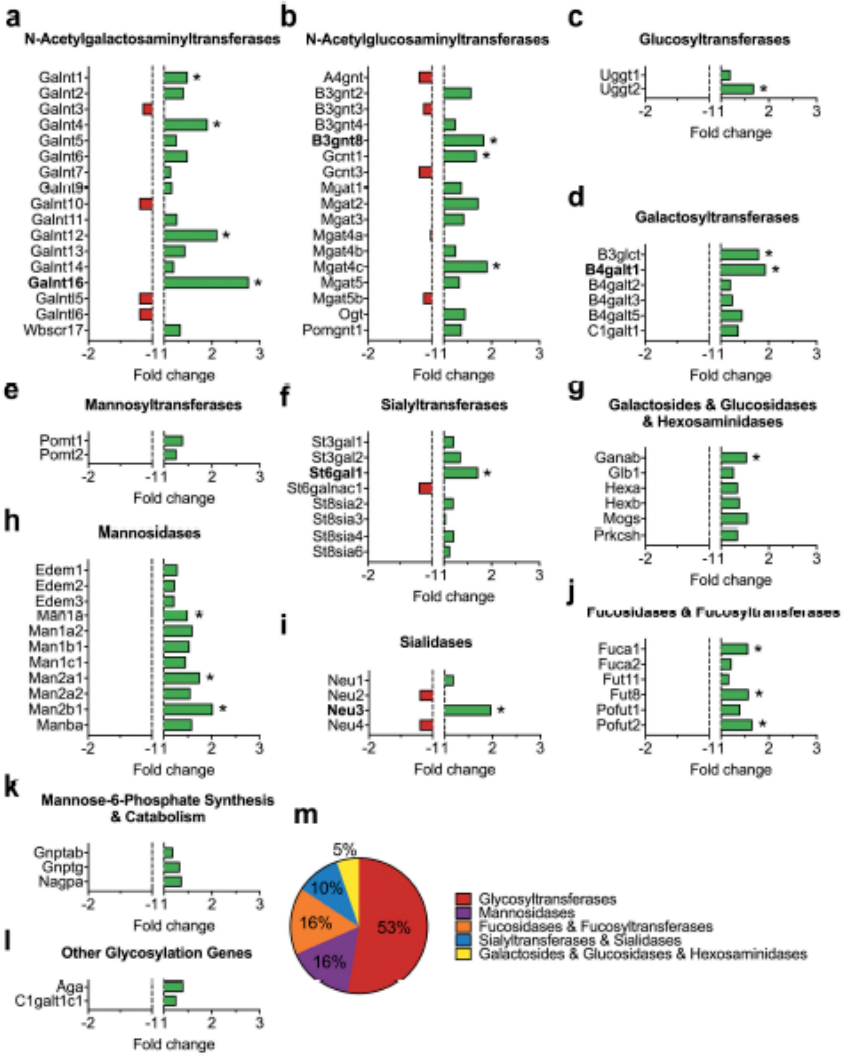

Figure 2. Summary of the transcriptomic profiling of glycan-modifying genes in the dorsal root ganglia of *db/db* mice. Data are presented as fold change compared to wild-type animals for (a) N-acetylgalactosaminyltransferases, (b) N-acetylglucosaminyltransferases, (c) Glucosyltransferases, (d) Galactosyltransferases, (e) Mannosyltransferases, (f) Sialyltransferases, (g) Galactosides/Glucosidases/Hexosaminidases, (h) Mannosidases, (i) Sialidases, (j) Fucosidases/Fucosyltransferases, (k) Mannose-6-Phosphate synthesis/catabolism, and (l) other glycosylation genes. Enzymes indicated in bold were functionally characterized on $Ca_v3.2$ channels. (m) Summary of up-regulated genes.

and Table 1). Altogether, these data indicate that increased sialylation activity tends to negatively modulate recombinant $Ca_v3.2$ channels when expressed in tsA-201 cells.

Discussion

Increased expression of $Ca_v3.2$ in primary afferent nociceptive fibers is causally linked to the

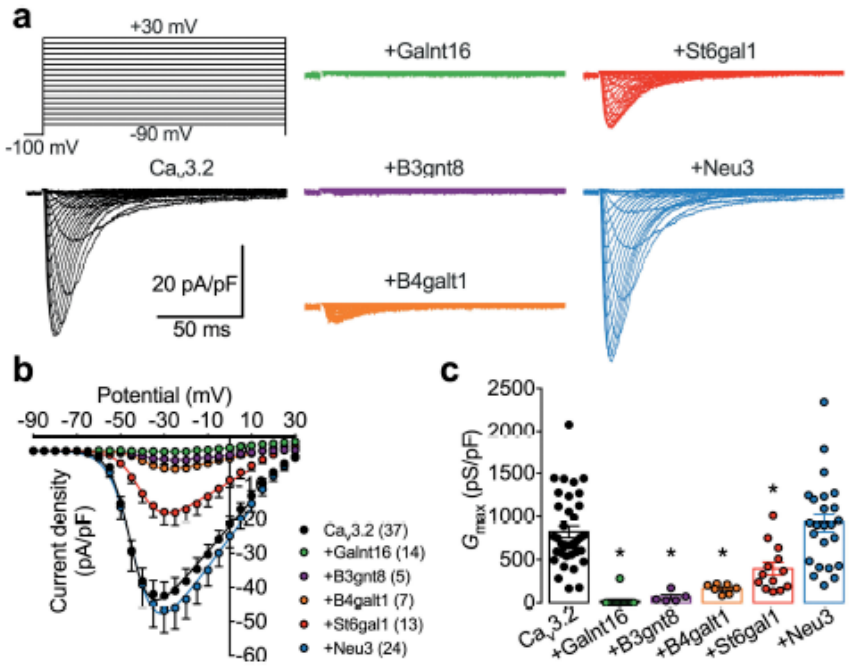

Figure 3. Influence of glycan-modifying enzymes on the functional expression of Ca$_v$3.2 channels. (a) Representative T-type current traces recorded from cells expressing Ca$_v$3.2 alone (black traces) and in combination with Galnt16 (green traces), B3gnt8 (purple traces), B4galt1 (orange traces), St6gal1 (red traces), Neu3 (blue traces) glycan-modifying enzymes in response to 150 ms depolarizing steps ranging between −90 mV and 30 mV from a holding potential of −100 mV. (b) Corresponding mean peak current density-voltage (*I/V*) relationship. (c) Corresponding mean maximal macroscopic conductance (G_{max}) values obtained from the fit of the *I/V* curves with the modified Boltzmann equation (1).

Table 1. Electrophysiological properties of human Ca$_v$3.2 channels expressed in tsA-201 cells in the presence of sialic acid-processing enzymes.

	Activation					Inactivation			RFI	
Channel	$V_{0.5}$ (mV)	*k*	(n)	G_{max} (pS/pF)	(n)	$V_{0.5}$ (mV)	*k*	(n)	τ (ms)	(n)
Ca$_v$3.2	−43.6 ± 0.6	4.5 ± 0.2	37	820 ± 68	37	−68.3 ± 0.9	2.9 ± 0.7	13	447 ± 34	11
+St6gal1	−38.7 ± 0.7*	5.5 ± 0.3	13	395 ± 74*	13	−70.7 ± 1.6	3.2 ± 0.3	8	550 ± 48	8
+Neu3	−41.8 ± 0.9	5.3 ± 0.2*	24	921 ± 104	24	−66.6 ± 1.3	3.7 ± 0.2	16	422 ± 39	7

development of peripheral painful neuropathy associated with nerve injury [18–20], antineoplastic drugs [21–23], inflammation [24,25], and diabetes [1,3]. Several studies have unraveled some of the mechanisms underlying the pathological expression of Ca$_v$3.2 and alteration of the posttranslational regulation of the channel including ubiquitinylation [26], SUMOylation [27] and phosphorylation [28,29]. Defects in these processes have emerged as some of the primary reasons leading to enhanced expression of the channel. Furthermore, altered glycosylation of Ca$_v$3.2 was proposed to contribute to the sensitization of nociceptive fibers in response to hyperglycemia associated with diabetes [5]. In this study, we show using a differential transcriptomic approach that several glycan-modifying enzymes are upregulated in DRG neurons from *db/db* mice compared to wild-type animals. These results are consistent with previous studies reporting an alteration of glycan-processing enzymes in the kidney of diabetic mice [30]. Several of these enzymes contribute to the processing of important sugars including glucose,

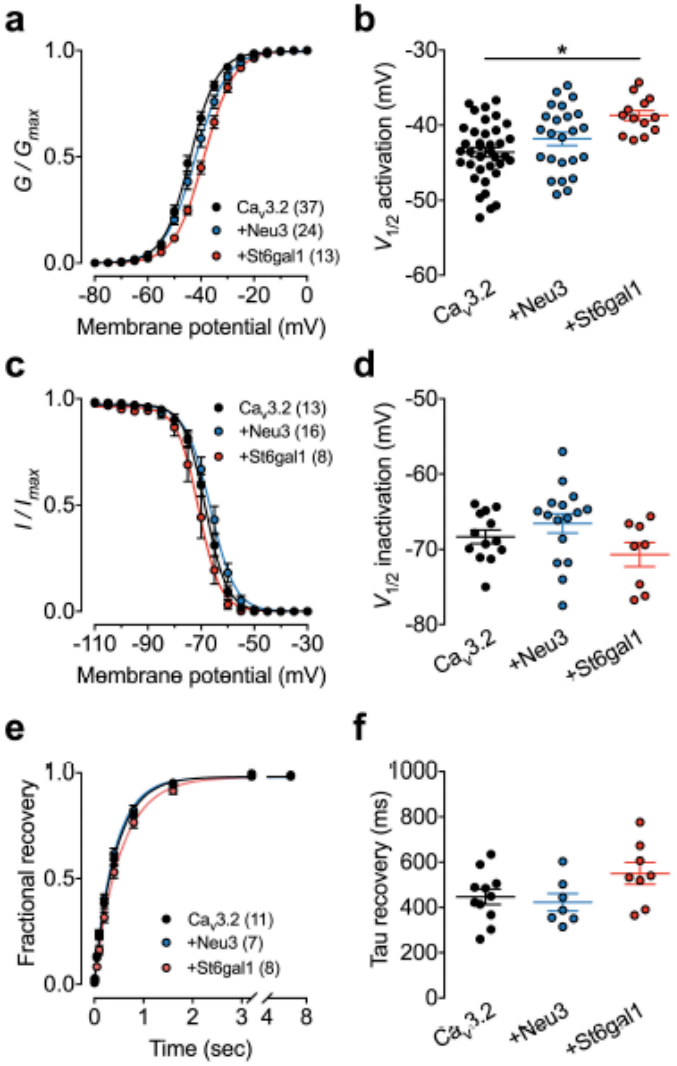

Figure 4. Influence of sialic acid-processing enzymes on the electrophysiological properties of $Ca_v3.2$ channels. (a) Mean normalized voltage-dependence of T-type current activation for cells expressing $Ca_v3.2$ alone (black circles) and in combination with Neu3 (blue circles) or St6gal1 (red circles). (b) Corresponding mean half-activation potential values obtained from the fit of the activation curves with the modified Boltzmann Equation (2). (c,sd) Legend same as for (a,b) but for the voltage-dependence of steady state inactivation. Half-inactivation potential values was obtained from the fit of the inactivation curves with the two-state Boltzmann function (3). (e) Mean normalized recovery from inactivation kinetics. (f) Corresponding mean time constant values of recovery from inactivation obtained from the fit of the recovery curves with the single-exponential function (4).

galactose, mannose, and fucose, and therefore alteration of their expression level could potentially alter the processing and maturation of the glycan structures. Furthermore, we found that several enzymes involved in the processing of the terminal sialic acid moieties found in complex glycan structures were upregulated in diabetic conditions. This aspect is particularly relevant in the context of PDN since

enzymatic removal of sialic acid moieties with neuraminidase was reported to normalize T-type currents in DRG neurons isolated from diabatic mice and to alleviate PDN *in vivo* [5]. Furthermore, sialylation was reported to contribute to the hyperexcitability of DRG neurons following peripheral nerve injury [31]. However, our functional analysis on recombinant Cav3.2 channels did not provide evidence in support of a role for these enzymes in the upregulation of $Ca_v3.2$ when co-expressed individually with the channel. For instance, co-expression of glycosyltransferases Galnt16, B3gnt8, and B4galt1 with $Ca_v3.2$ produced an almost complete loss of functional expression of the channel. However, several studies have previously shown that glycosyltransferases can form heterodimers that contribute to their subcellular expression, enzymatic activity, efficient biosynthesis of glycan chains, trafficking through intracellular vesicles, and substrate specificities [32]. For instance, binding of B3gnt8 appears to cause a conformational change in the catalytic site of B3gnt2 and increases its enzymatic activity [33]. Therefore, we cannot exclude that overexpression of individual enzymes with $Ca_v3.2$ in tsA-201 cells as performed in our study may not fully capture the more complex situation in DRG neurons where the expression several genes encoding for glycan-modifying enzymes is altered at the same time and there could be synergetic effects among the various players. Furthermore, tsA-201 cells were grown in high glucose medium which represents another variable that could have influenced the phenotypic effect of these enzymes on $Ca_v3.2$ channels. In contrast to glycosyltransferases, co-expression of the sialyltransferase St6gal1 produced a relatively mild decreased expression of the channel with a depolarized shift of the voltage-dependence of activation, indicating that sialylation contributes to the functioning of $Ca_v3.2$. However, co-expression of the neuraminidase Neu3 that removes sialic acid moieties did not altered expression of the channel, nor its gating properties. These results are consistent with previous studies showing that application of neuraminidase on tsA-201 cells expressing $Ca_v3.2$ channels did not alter channel function [8], which could suggest a low basal level of sialylation in these cells.

Altogether, this study identified several glycan-modifying genes whose expression is altered in DRG neurons under diabetic condition. However, we did not find evidence for a role of these enzyme in the upregulation of $Ca_v3.2$ channels. At this stage, we cannot exclude that expression of glycan-modifying enzymes in DRG neurons may have produced a different phenotypic effect on $Ca_v3.2$ and this aspect would deserve further investigations in native conditions.

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Disclosure statement

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Paper 4

A rare *CACNA1H* variant associated with amyotrophic lateral sclerosis causes complete loss of $Ca_V3.2$ T-type channel activity

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Molecular Brain

RESEARCH Open Access

A rare *CACNA1H* variant associated with amyotrophic lateral sclerosis causes complete loss of $Ca_v3.2$ T-type channel activity

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Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by the progressive loss of cortical, brain stem and spinal motor neurons that leads to muscle weakness and death. A previous study implicated *CACNA1H* encoding for $Ca_v3.2$ calcium channels as a susceptibility gene in ALS. In the present study, two heterozygous *CACNA1H* variants were identified by whole genome sequencing in a small cohort of ALS patients. These variants were functionally characterized using patch clamp electrophysiology, biochemistry assays, and molecular modeling. A previously unreported c.454GTAC > G variant produced an inframe deletion of a highly conserved isoleucine residue in $Ca_v3.2$ (p.ΔI153) and caused a complete loss-of-function of the channel, with an additional dominant-negative effect on the wild-type channel when expressed in *trans*. In contrast, the c.3629C > T variant caused a missense substitution of a proline with a leucine (p.P1210L) and produced a comparatively mild alteration of $Ca_v3.2$ channel activity. The newly identified ΔI153 variant is the first to be reported to cause a complete loss of $Ca_v3.2$ channel function. These findings add to the notion that loss-of-function of $Ca_v3.2$ channels associated with rare *CACNA1H* variants may be risk factors in the complex etiology of ALS.

Keywords: ALS, Amyotrophic lateral sclerosis, Motor neuron disease, *CACNA1H*, Mutation, Calcium channel, $Ca_v3.2$ channel, T-type channel, Biophysics

Introduction

Amyotrophic lateral sclerosis (ALS), also known as motor neuron disease or Lou Gehrig's disease, is a heterogeneous neuromuscular disease characterized by the degeneration of cortical, brain stem and spinal motor neurons that leads to muscle weakness and paralysis. Disease onset averages between 40 and 70 years of age [1], and the annual incidence worldwide is estimated to be between one to three per 100,000 people [2]. ALS is best regarded as a complex genetic disorder with a Mendelian pattern of inheritance in approximately 5–10% of patients (familial ALS, fALS), but most patients have no discernable family history of the disease which is then referred to being "sporadic" or "isolated" in nature (sALS) [3]. However, the observation that established fALS genes are also implicated in sALS makes the distinction between fALS and sALS more abstruse [4]. For instance, mutations in the most common ALS genes (*SOD1*, *FUS*, *TARDBP*, *C9orf72*, *VCP*, and *PFN1*) account for up to 70% of fALS patients and about 10% of

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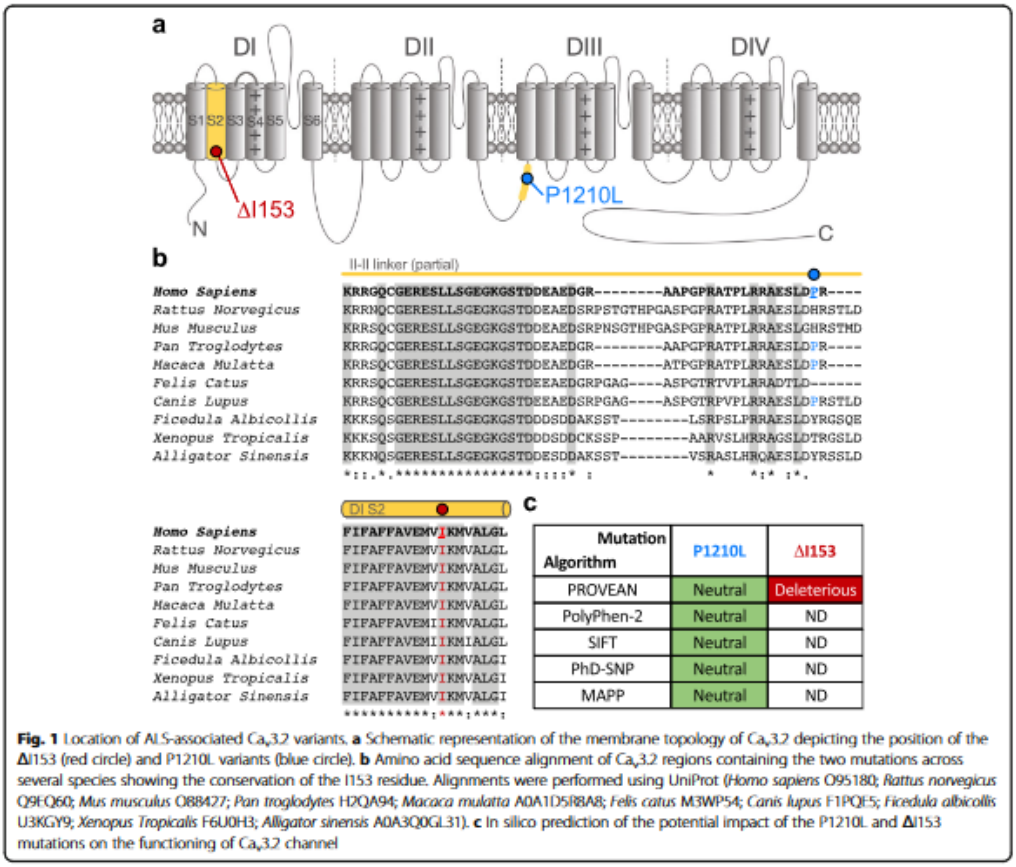
sALS [5]. In addition, several genes and loci in apparent sALS patients have been proposed to be associated with an increased risk of ALS, or to modify the onset or progression of the disease, which highlights the importance of genetic risk factors [6]. Among these genes, the most prominent are *ATXN2* [7], *UNC13A* [8], *ANG* [9], and *SMN1* [10]. Recently, whole exome sequence analysis of case-unaffected-parents trios identified two compound heterozygous recessive missense mutations in the gene *CACNA1H* [11, 12].

In the present study, we report two additional *CACNA1H* variants (c.3629C > T, p.P1210L and c.454GTAC > G, p.ΔI153) identified using whole genome sequencing of a cohort of 34 sALS patients, with sequencing undertaken at the Genome Institute, Washington University, St Louise USA. The method of whole genome analysis was the same as that reported in a separate study [11]. Whole genome analyses reveal no pathogenetic single nucleotide or structural differences between monozygotic twins discordant for amyotrophic lateral sclerosis [13]. No unaffected parent DNA was subjected to whole genome sequencing, so it was not possible to determine if the variants were recessive or de novo in nature [11]. Functional analysis of these two variants revealed a complete loss of $Ca_v3.2$ channel function associated with the ΔI153 variant and a dominant-negative effect of this variant on the wild-type channel when expressed in *trans*.

Results

Whole genome sequencing identifies heterozygous CACNA1H mutations in ALS patients

In a previous study, using case-unaffected parents trio exome analyses, we reported an ALS patient with two heterozygous *CACNA1H* missense mutations causing a partial loss-of-function of $Ca_v3.2$ channel, suggesting



Mutation / Algorithm	P1210L	ΔI153
PROVEAN	Neutral	Deleterious
PolyPhen-2	Neutral	ND
SIFT	Neutral	ND
PhD-SNP	Neutral	ND
MAPP	Neutral	ND

Fig. 1 Location of ALS-associated $Ca_v3.2$ variants. **a** Schematic representation of the membrane topology of $Ca_v3.2$ depicting the position of the ΔI153 (red circle) and P1210L variants (blue circle). **b** Amino acid sequence alignment of $Ca_v3.2$ regions containing the two mutations across several species showing the conservation of the I153 residue. Alignments were performed using UniProt (*Homo sapiens* O95180; *Rattus norvegicus* Q9EQ60; *Mus musculus* O88477; *Pan troglodytes* H2QA94; *Macaca mulatta* A0A1D5R8A8; *Felis catus* M3WP54; *Canis lupus* F1PQE5; *Ficedula albicollis* U3KGY9; *Xenopus Tropicalis* F6U0H3; *Alligator sinensis* A0A3Q0GL31). **c** In silico prediction of the potential impact of the P1210L and ΔI153 mutations on the functioning of $Ca_v3.2$ channel

that rare *CACNA1H* variants may represent a risk factor for ALS [11, 12]. In the present study, using whole genome sequencing of a small cohort of ALS patients, we identified two additional heterozygous variants in *CACNA1H*. The first variant (c.3629C > T, p.P1210L) was identified in a man with ALS onset aged 55 years who died aged 62 years. He had no family history of ALS, though his father had Alzheimer's disease and his mother bipolar disorder. The P1210L variant is located in a non-conserved region of the intracellular linker connecting transmembrane domains II and III (II-III loop) of $Ca_v3.2$ (Fig. 1a and b). This variant has previously been reported in 188 out of 240,876 individuals in the gnomAD database (https://gnomad.broadinstitute.org/), including 144 of 193,008 alleles only from individuals who were not ascertained for having a neurological condition in a neurological case/control study. Furthermore, in silico analysis predicted the amino acid change to be neutral (Fig. 1c), suggesting that this variant is likely to not have a major pathological role. The second variant (c.454GTAC > G, p.ΔI153) was identified in a man with ALS onset aged 53 years who died aged 54 years. Although he had no family history of ALS, his mother developed insulin-dependent diabetes mellitus and narcolepsy, and his father presented with early onset dementia, a condition known to precede motor impairment in some people with ALS [14]. This mutation produces an inframe deletion of the isoleucine 153 located in the second transmembrane helix of $Ca_v3.2$, a region highly conserved across $Ca_v3.2$ channel orthologs (Fig. 1a and b). The ΔI153 variant has only been reported in 1 out of 198,036 individuals in the gnomAD database and this deletion was predicted to be deleterious on the channel (Fig. 1c). Hexanucleotide repeat number in C9orf72, the most common genetic cause of ALS, was normal in both patients.

The ΔI153 mutation causes a complete loss of $Ca_v3.2$ function

In the first series of experiments we assessed the functional expression of $Ca_v3.2$ P1210L and ΔI153 channel variants expressed in tsA-201 cells by whole-cell patch clamp electrophysiology. Cells expressing the P1210L channel variant displayed a characteristic low-threshold voltage-activated T-type current (Fig. 2a and b) that only differed from cells expressing the wild-type (WT) channel by a 32% reduction ($p = 0.0125$, Mann-Whitney test) of the maximal conductance (G_{max}) (from 571.3 ± 58.4 pS/pF, $n = 42$ to 387.7 ± 33.9 pS/pF, $n = 41$) (Fig. 2c). The main electrophysiological properties, including voltage-dependence of activation and inactivation (Fig. 2d), and recovery from inactivation (Fig. 2e), remained unaffected. In cells expressing the ΔI153 channel variant, we did not record any T-type conductance (Fig. 2a-c). It is noteworthy that experimental conditions known to favor the expression of misfolded proteins, such as treatment of cells with the proteasome inhibitor MG132 or decrease of cell incubation temperature to 30 °C, were used but failed to restore a T-type conductance. Additionally, co-expression of the ΔI153 channel variant with Stac1 or with a calnexin-derived peptide that has previously been reported to potentiate the expression of $Ca_v3.2$ in the plasma membrane [15, 16] also failed to restore T-type currents (data not shown). The lack of functional expression of the ΔI153 channel variant could have been inherent in our experimental conditions using recombinant channels, so we aimed to further assess the phenotypic effect of the ΔI153 mutation on native $Ca_v3.2$ channels in a neuronal environment. Therefore, we used a CRISPR/Cas9 approach to introduce the ΔI153 mutation in native $Ca_v3.2$ channels in cultured dorsal root ganglion (DRG) neurons. DRG neurons were used as a model system since these neurons are known to display a T-type conductance that is almost exclusively carried by $Ca_v3.2$ channel subtype [17]. Consistent with our observation with recombinant $Ca_v3.2$ channels, T-type currents recorded from $Ca_v3.2$ ΔI153 DRG neurons 3 days after gene editing were reduced by 73% (Mann-Whitney $p < 0.0001$) compared to wild type neurons (from 15.4 ± 2.5 pA/pF, $n = 12$ to 4.1 ± 0.8 pA/pF, $n = 12$) (Fig. 2f and g).

Collectively, these data revealed a mild loss of channel function associated with the P1210L variant, and the deleterious effect of the ΔI153 mutation leading to a complete loss of $Ca_v3.2$ activity.

The ΔI153 mutation disrupts $Ca_v3.2$ biogenesis

The alteration of T-type currents in ALS-associated $Ca_v3.2$ variants could originate from an overall decreased expression of channel proteins, reduced channel density in the plasma membrane, altered gating of the channel, or from a combination of several of these. Therefore, we first assessed the expression levels of P1210L and ΔI153 channel variants in tsA-201 cells by western blot (Fig. 3a). Immunoblot analysis from total cell lysates showed that the P1210L channel variant was present at a similar level as the WT channel (Fig. 3b). In contrast, the expression level of the ΔI153 channel variant was reduced by 78% (Mann-Whitney $p = 0.0286$), suggesting that this variant may undergo extensive degradation (Fig. 3b). Next, we aimed to assess the expression of Cav3.2 channel variants at the cell surface. Therefore, we analyzed charge movements (Q) that refer to the movement of the channel voltage-sensor in the plasma membrane in response to electrical membrane depolarizations. Total charges (Q_{max}) were assessed at the reversal potential of the ionic current, where we can consider Q_{rev} to be equal to Q_{max}, providing an accurate assessment of the total number of channels in the plasma membrane (Fig. 3c). In cells expressing the P1210L

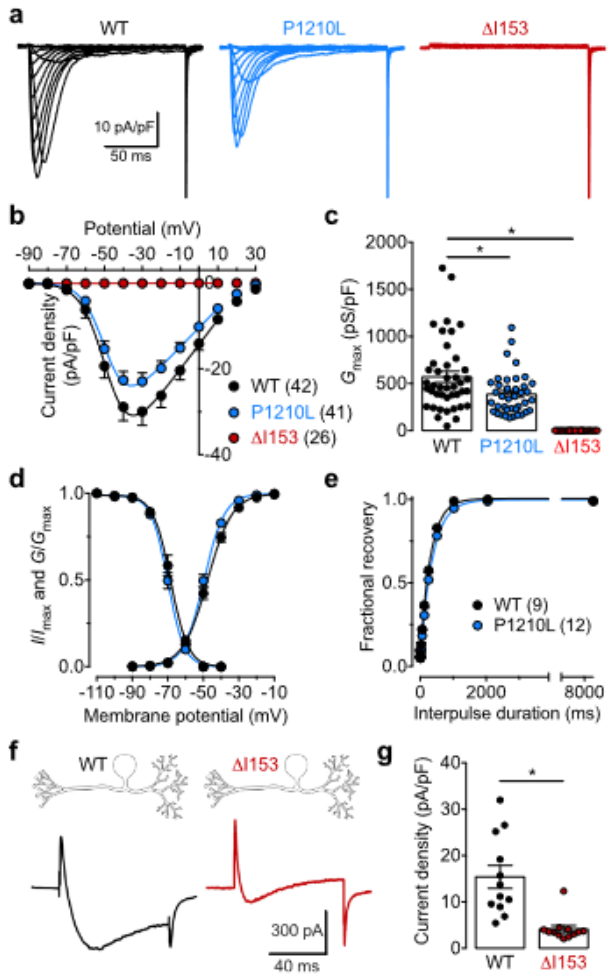


Fig. 2 Electrophysiological characterization of $Ca_v3.2$ P1210L and ΔI153 variants. **a** Representative T-type current traces recorded in response to 150 ms depolarizing steps to values ranging between − 90 mV and + 30 mV from a holding potential of − 100 mV for wild-type (WT, black traces), P1210L (blue traces), and ΔI153 (red traces) channel variants expressed in tsA-201 cells. **b** Corresponding mean current-voltage relationship (*I/V*) for WT (black circles), P1210L (blue circles), and ΔI153 (red circles) channels. **c** Corresponding mean maximal macroscopic conductance (G_{max}) obtained from the fit of the I/V curves with the modified Boltzmann eq. (1). **d** Mean normalized voltage-dependence of activation and inactivation for WT (black circles) and P1210L channels (blue circles). **e** Mean normalized recovery from inactivation kinetics. **f** Representative T-type current traces recorded from WT (black trace) and ΔI153 DRG neurons (red trace) 3 days after editing of *Cacna1h* by CRISPR/Cas9 in response to 80 ms depolarizing steps to − 25 mV from a holding potential of − 90 mV. **g** Corresponding mean peak T-type current density at − 25 mV in WT and ΔI153 mutant DRG neurons

variant, we observed a 27% reduction of Q_{max} (t-test $p = 0.0467$) compared to cells expressing the WT channel (from 12.0 ± 1.3 fC/pF, $n = 19$ to 8.7 ± 0.8 fC/pF, $n = 18$) (Fig. 3d). This reduction of Q_{max} is similar to the reduction of the maximal T-type conductance we previously observed (32%, Fig. 2c), suggesting that the decrease of the T-type conductance in cells expressing the P1210L channel variant is likely caused by a reduced expression of the channel in the plasma membrane. This notion is further supported by the observation that neither the G_{max}/Q_{max} dependency (Fig. 3e), nor the kinetics of charge movements (Fig. 3f), were modified, indicating

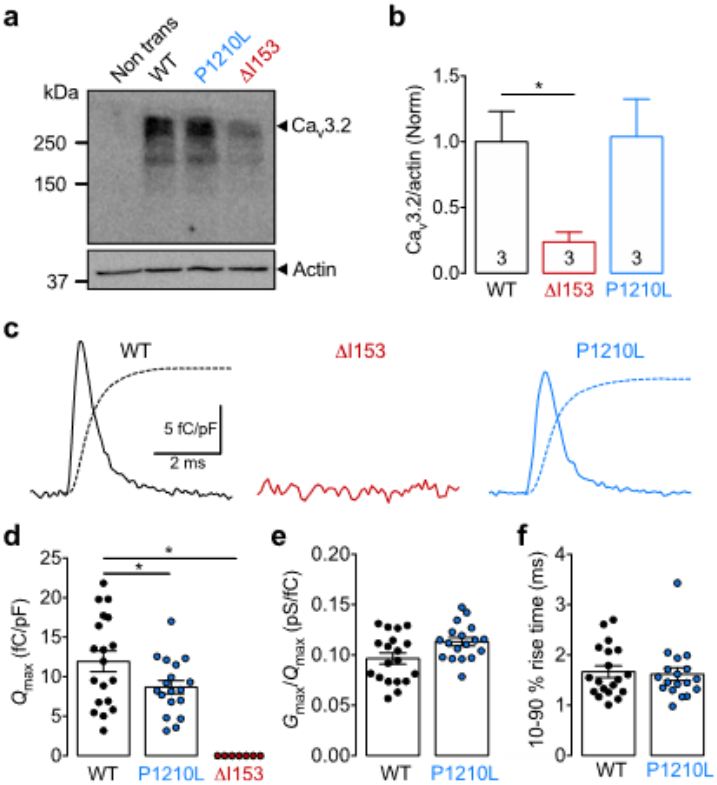


Fig. 3 Expression of $Ca_v3.2$ P1210L and ΔI153 variants. **a** Representative immunoblot of $Ca_v3.2$ from tsA-201 cells expressing wild-type (WT), P1210L, and ΔI153 channel variants. **b** Corresponding mean expression levels of P1210L and ΔI153 variants relative to WT channels. **c** Representative charge movement traces recorded at the ionic reversal potential from cells expressing wild-type (WT, black trace), P1210L (blue trace), and ΔI153 (red trace) channel variants. The dotted line depicts the time course of the integral for each trace. **d** Corresponding mean Q_{max} values calculated for each investigated cell. **e** Corresponding mean G_{max}/Q_{max} ratios. **f** Corresponding mean 10–90% rise times calculated from the integral time course shown in panel **c**

that the gating properties of the P1210L channel variant remained unaltered. In contrast, we did not detect any charge movement in cells expressing the ΔI153 channel variant (Fig. 3c and d), suggesting that despite being biochemically expressed, this variant is not present in the plasma membrane.

Altogether, these data are consistent with a mildly decreased surface expression of the P1210L variant without additional alterations. Importantly, these data demonstrate the profound deleterious effect of the ΔI153 mutation on the biogenesis and surface trafficking of $Ca_v3.2$ channels.

Dominant-negative effect of the ΔI153 channel variant

Given the heterozygosity of the ΔI153 mutation and the defective trafficking of the ΔI153 channel variant, we aimed to test whether this variant could have a dominant-negative effect on WT channels. Therefore, we co-expressed the WT and ΔI153 channels in tsA-201 cells in a 1:1 ratio (equal amount of cDNAs) and compared T-type currents with cells expressing the WT channel in combination with a cation-impermeant but trafficking-competent channel (PM). Recording of T-type currents in cells expressing a combination of WT:ΔI153 channels (Fig. 4a) revealed a 35% reduction (Mann-Whitney p = 0.0080) of the maximal T-type conductance compared to cells expressing a combination of WT:PM channels (from 569 ± 73 pS/pF, n = 38 to 372 ± 27 pS/pF, n = 58) (Fig. 4b and c), indicating that the ΔI153 variant produced a dominant-negative effect on the WT channel when expressed in *trans*. In contrast, the voltage-dependence of

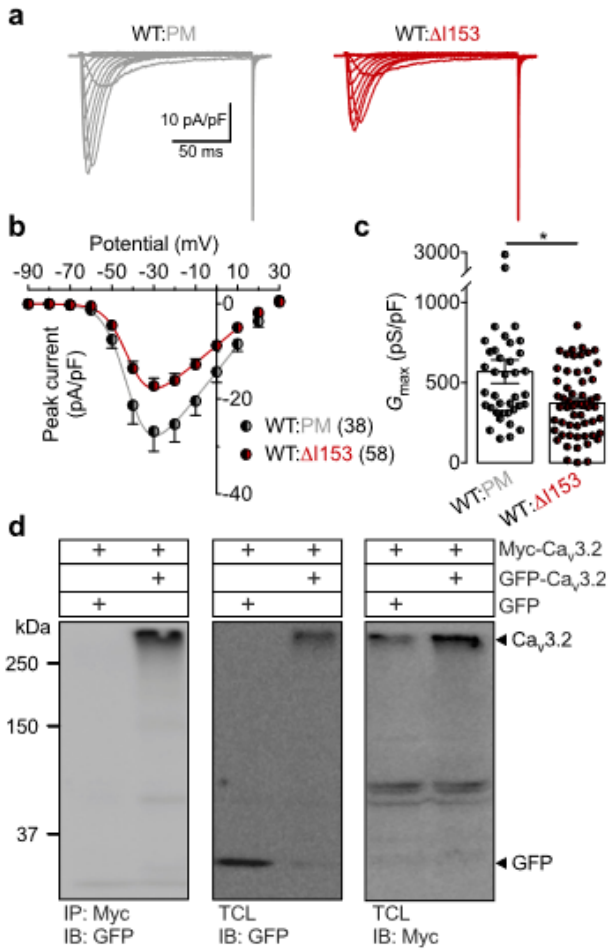


Fig. 4 Electrophysiological characterization of Cav3.2 WT and ΔI153 expressed in *trans*. **a** Representative T-type current traces recorded from tsA-201 cells expressing WT channels in combination with either the ΔI153 variant (WT:ΔI153, red traces) or the cation-impermeant but trafficking-competent $Ca_v3.2$ pore mutant (WT:PM; grey traces) in a ratio 1:1. **b** Corresponding mean current-voltage relationship (*I/V*) for WT:ΔI153 (black|red circles), and WT:PM (black|grey circles) conditions. **c** Corresponding mean maximal macroscopic conductance (G_{max}). **d** Co-immunoprecipitation of $Ca_v3.2$ from tsA-201 cells co-transfected with a Myc-tagged and GFP-tagged $Ca_v3.2$. The left panel shows the result of the co-immunoprecipitation of Myc-$Ca_v3.2$ with GFP-$Ca_v3.2$ using an anti-Myc antibody. The middle and right panels show the immunoblot of GFP-$Ca_v3.2$ and Myc-$Ca_v3.2$ using and anti-GFP and anti-Myc antibody, respectively

activation and inactivation remained unaltered. Given the comparatively mild phenotype produced by the P1210L mutation, the P1210L variant was not tested in combination with the WT channel. Finally, to test whether this dominant-negative effect could be mediated by an interaction between $Ca_v3.2$ subunits, we performed co-immunoprecipitations from tsA-201 cells co-expressing Myc-tagged and GFP-tagged $Ca_v3.2$ to discriminate between the two channels. We observed that the GFP-tagged $Ca_v3.2$ was immunoprecipitated with the Myc-tagged $Ca_v3.2$ using a specific anti-Myc antibody, revealing the ability of $Ca_v3.2$ channels to dimerize (Fig. 4d).

Collectively, these data revealed the dominant-negative effect of the ΔI153 variant on the WT channel, a phenomenon likely to be mediated by the interaction between $Ca_v3.2$ subunits.

Discussion

While several common genes are implicated in familial ALS, the occurrence of rare genetic variants in patients with no family history of the disease has emerged as a potential contributing factor in sporadic ALS [11]. In this study, we report two heterozygous *CACNA1H* variants identified by whole genome sequencing of a small cohort of ALS patients. Functional analysis revealed mild to severe alterations of $Ca_v3.2$ variants that were consistent with a loss-of-function of the channels.

The P1210L missense mutation was located in a variable region of $Ca_v3.2$ and was not predicted to be deleterious. Our electrophysiological analysis showed a moderate reduction of the expression of the P1210L channel variant at the cell surface and an associated reduction in the T-type conductance. We cannot entirely rule out the possibility that the phenotypic expression of the P1210L variant could have differed when introduced into a different $Ca_v3.2$ splice variant [18], or when functionally assessed under different experimental conditions [19], but our experimental data together with the relatively high occurrence of this variant in the general population strongly suggest that it is indeed unlikely to be pathogenic. In contrast, the ΔI153 variant had never been reported and was predicted to be deleterious. Electrophysiological analysis revealed a complete loss of functional expression of the ΔI153 variant, and recording of charge movements suggested that this variant was absent from the cell surface. Furthermore, our biochemical analysis revealed a dramatic decrease of the expression level of the channel protein, suggesting that this variant may have undergone extensive degradation. Of particular importance was the dominant-negative effect produced by the ΔI153 variant on the WT channel when the two channels were expressed in *trans*. This effect was likely to be mediated by the ability of $Ca_v3.2$ subunits to dimerize, which could have prevented the proper trafficking of the WT channel to the cell surface in the presence of the impaired ΔI153 variant. In this regard, it is worth considering that this dominant-negative effect may also have an effect on other ion channels. Indeed, $Ca_v3.2$ channels are known to biochemically interact with several calcium- and voltage-activated potassium and sodium channel subunits [20–23] whose surface trafficking and activity could be affected by the $Ca_v3.2$ ΔI153 variant.

The molecular mechanisms underlying the deleterious effect of the ΔI153 variant can be appreciated by examining the 3-dimensional environment of I153, and the possible impact of its deletion in the homology model of $Ca_v3.2$ we have developed, using the 3.3 Å CryoEM structure of $Ca_v3.1$ [24]. In this model, I153 is located within the transmembrane S2 alpha helix of domain I (Fig. 5a), where it is surrounded by hydrophobic residues near the membrane-cytosol interface (Fig. 5b). The nearby hydrophobic residues are highly conserved between L- and T-type channels and I153 shows a clear involvement in the helical packing (Fig. 5b). Therefore, deletion of I153 that results in a net loss of hydrophobicity within the transmembrane segment is likely to alter helix packing in domain I which would result in the misfolding of the channel. Additionally, deletion of I153 would also affect downstream residues in the helix due to a change in the helical register, thus further affecting the helical packing in the voltage-sensing domain.

From a clinical point of view, the loss-of-channel function associated with the ΔI153 variant could have several pathological implications. First, $Ca_v3.2$ is present in several central neurons, including reticular thalamic neurons [25], where they contribute to NMDA receptor-mediated synaptic transmission [26]. Given that gain-of-function mutations associated with childhood absence epilepsy were shown to enhance synaptic activities [26], the reciprocal theory would suggest that loss-of-channel function could, in contrast, decrease synaptic transmission. Along these lines, neuroimaging studies have revealed decreased thalamic activity in ALS [27–32], and a recent MRI study reported alterations of thalamic connectivities that mirrored the progressive motor functional decline in ALS [33]. Second, although the functional expression of $Ca_v3.2$ in mammalian motor neurons remains elusive, several studies suggest that T-type channels may have a functional role. For instance, $Ca_v3.1$ channels are present in turtle spinal motor neurons where they contribute to cellular excitability [34]. In addition, a low-threshold voltage-activated calcium conductance was reported at nodes of Ranvier in mouse spinal motor neurons, suggesting the presence of T-type channels [35]. Third, a T-type channel ortholog is present in motor neurons of the nematode *C. elegans* [36] where it contributes to motor-related functions [37, 38]. Finally, a recent study documented the role of T-type channels in the maintenance of neuronal progenitor cells [39]. A loss-of-function of $Ca_v3.2$ could compromise the architecture of nerve cells and precipitate neuronal degeneration.

In conclusion, this newly identified ΔI153 variant is the first to be reported to cause a complete loss of $Ca_v3.2$ channel function [40]. Although its pathogenic role in the context of ALS remains to be established, these findings add to the notion that rare *CACNA1H* variants represent a risk factor for ALS. Furthermore, several T-type channels blockers are currently being used for the treatment of epilepsy [41]. The question then arises as to whether long term use of these molecules may present a risk to the development of ALS. This notion should be given particular attention, especially considering that several other T-type channel blockers are currently evaluated in clinical trials for the management of epilepsy and chronic pain symptoms.

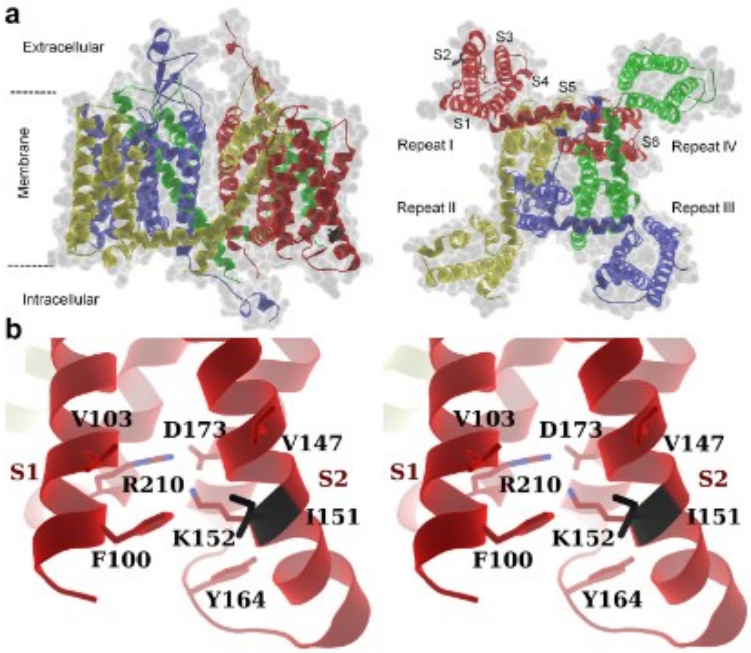


Fig. 5 Homology model of human $Ca_v3.2$. **a** Cartoon representation of secondary structural elements of human $Ca_v3.2$ (Uniprot O95180) homology model (residues 97–1974) based on $Ca_v3.1$ (PDB: 6KZO), showing side (left panel) and bottom (right panel) views of the channel. The four domains of $Ca_v3.2$ are colored in red, yellow, blue and green. The S1-S6 helices are indicated in red for domain I. Some of the flexible loops connecting the transmembrane helices are not shown, or could not be modeled, due to poor model accuracy or lack of structural information, respectively. The isoleucine 153 (Ile153) is shown in black. **b** Stereo diagram of Ile153 and nearby hydrophobic residues showing its involvement in the helical packing

Methods

Plasmids cDNA constructs and site-directed mutagenesis

The $Ca_v3.2$ P1210L and ΔI153 channel variants were created by introducing the respective mutations into the human wild-type HA-tagged $Ca_v3.2$ in pcDNA3.1 [42] by PCR-based site-directed mutagenesis using Q5® Site-Directed Mutagenesis Kit (New England Biolabs) and the following mutagenic primers: delI153: 5′-TCAAGA TGGTGGCCTTGG-3′ (forward) and 5′-CCATCT CCACCGCAAAAAAG-3′ (reverse); P1210L: 5′-GCCGCCCTCCtGCCTACCAAGTGC-3′ (forward) and 5′-CGGCCGCAGGGGCCGTGG-3′ (reverse). The cation-impermeant $Ca_v3.2$ channel was generated by replacing the glutamic acid 378 in domain I with a lysine (E378K) by site-directed mutagenesis. Final constructs were verified by sequencing of the coding sequence of the plasmid cDNAs.

Cell culture and heterologous expression

Human embryonic kidney tsA-201 cells were grown in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all media purchased from Invitrogen) and maintained under standard conditions at 37 °C in a humidified atmosphere containing 5% CO_2. Heterologous expression of $Ca_v3.2$ channels was performed by transfecting cells with 5 μg plasmid cDNAs encoding for $Ca_v3.2$ channel variants using the calcium/phosphate method. For experiments aiming at investigating the dominant negative effect of the ΔI153 variant, cells were co-transfected with 2.5 μg plasmid cDNA encoding for WT channels with either 2.5 μg plasmid cDNA encoding for the ΔI153 channel variant or 2.5 μg plasmid cDNA encoding for a non-conducting but trafficking-competent $Ca_v3.2$ (PM).

Patch clamp electrophysiology

Patch clamp recordings of T-type currents in tsA-201 cells expressing $Ca_v3.2$ channel variants were performed 72 h after transfection in the whole-cell configuration at room temperature (22-24 °C) as previously described [43]. The bath solution contained (in millimolar): 5 $BaCl_2$, 5 KCl, 1 $MgCl_2$, 128 NaCl, 10 TEA-Cl, 10 D-glucose, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.2 with NaOH). Patch pipettes

were filled with a solution containing (in millimolar): 110 CsCl, 3 Mg-ATP, 0.5 Na-GTP, 2.5 $MgCl_2$, 5 D-glucose, 10 EGTA, and 10 HEPES (pH 7.4 with CsOH), and had a resistance of 2–4 MΩ. Recordings were performed using an Axopatch 200B amplifier (Axon Instruments) and acquisition and analysis were performed using pClamp 10 and Clampfit 10 software, respectively (Axon Instruments). The linear leak component of the current was corrected online and current traces were digitized at 10 kHz and filtered at 2 kHz. The voltage dependence of activation of $Ca_v3.2$ channels was determined by measuring the peak T-type current amplitude in response to 150 ms depolarizing steps to various potentials applied every 10 s from a holding membrane potential of − 100 mV. The current-voltage relationship (I/V) curve was fitted with the following modified Boltzmann eq. (1):

$$I(V) = Gmax \frac{(V - Vrev)}{1 + \exp\frac{(V0.5 - V)}{k}} \quad (1)$$

with $I(V)$ being the peak current amplitude at the command potential V, G_{max} the maximum conductance, V_{rev} the reversal potential, $V_{0.5}$ the half-activation potential, and k the slope factor. The voltage dependence of the whole-cell Ba^{2+} conductance was calculated using the following modified Boltzmann eq. (2):

$$G(V) = \frac{Gmax}{1 + \exp\frac{(V0.5 - V)}{k}} \quad (2)$$

with $G(V)$ being the Ba^{2+} conductance at the command potential V.

The voltage dependence of the steady-state inactivation of $Ca_v3.2$ channels was determined by measuring the peak T-type current amplitude in response to a 150 ms depolarizing step to − 20 mV applied after a 5 s-long conditioning prepulse ranging from − 120 mV to − 30 mV. The current amplitude obtained during each test pulse was normalized to the maximal current amplitude and plotted as a function of the prepulse potential. The voltage dependence of the steady-state inactivation was fitted with the following two-state Boltzmann function (3):

$$I(V) = \frac{Imax}{1 + \exp\frac{(V - V0.5)}{k}} \quad (3)$$

with I_{max} corresponding to the maximal peak current amplitude and $V_{0.5}$ to the half-inactivation voltage.

The recovery from inactivation was assessed using a double-pulse protocol from a holding potential of − 100 mV. The cell membrane was depolarized for 2 s at 0 mV (inactivating prepulse) to ensure complete inactivation of the channel, and then to − 20 mV for 150 ms (test pulse) after an increasing time period (interpulse) ranging between 0.1 ms and 2 s at − 100 mV. The peak current from the test pulse was plotted as a ratio of the maximum prepulse current versus interpulse interval. The data were fitted with the following single-exponential function (4):

$$\frac{I}{Imax} = A \times \left(1 - exp\frac{-t}{\tau}\right) \quad (4)$$

where τ is the time constant for channel recovery from inactivation.

Measurement of charge movements

Recording of charge movements was performed 72 h after transfection as previously described [44, 45]. The bath solution contained (in millimolar): CsCl 95; TEACl 40, $BaCl_2$ 5; $MgCl_2$ 1; HEPES 10; glucose 10; pH 7.4 (adjusted with CsOH). Patch pipettes had a resistance ranging from 1.8 MΩ to 2.2 MΩ when filled with a solution containing (in millimolar): CH_3SO_3Cs 130; Na-ATP 5; TEACl 10; HEPES 10; EGTA 10; $MgCl_2$ 5; pH 7.4 (adjusted with CsOH). Osmolarity of the intracellular solution was approximately 300 mOsmol/L. Osmolarity of the extracellular solution was adjusted by adding sucrose so that the final value was about 2–3 mOsmol/L lower than the osmolarity of the corresponding intracellular solution. Recordings were performed using HEKA EPC10 amplifier (HEKA Electronics). Acquisition and analysis were performed using Patchmaster v90.2 and Fitmaster v2x73.1 and Origin Pro 2015 software, respectively. Only cells with an input resistance less than 5 MΩ were considered. The input resistance and capacity transients were compensated by up to 70% with in-built circuits of the EPC 10 amplifier. Remaining artifacts were subtracted using a -P/8 procedure. ON-gating currents were recorded in response to a series of 5 depolarizing pulses at the reversal potential of the ionic current assessed for each cell, and total gating charge Q_{ON} was calculated as the integral of area below the averaged current traces.

CRISPR/Cas9 genome editing in DRG neurons

Male rats (6-week-old) were purchased from Charles River and DRG neurons were harvested as described previously [46]. The next day, neurons were transfected with Crispr-Cas9 plasmids (Cas9-sgRNA plasmid and donor plasmid purchased from GeneCopoeia) using Lipofectamine 2000 from Invitrogen (Cat. 11,668–019). The sequence of Crispr RNA was CGTGGAGATG

GTGATCAAGA. The donor plasmid contained the homologous arms of the genomic DNA without I153. Whole-cell voltage-clamp recordings of T-type currents were performed 3 days post transfection. The external solution contained (in mM): 40 TEACl, 65 CsCl, 20 $BaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 D-glucose, pH 7.4. The internal solution contained (in mM): 140 CsCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 5 EGTA, 10 HEPES, 2 Na-ATP, 0.3 Na-GTP, pH 7.3. We used GFP fluorescence to specifically identify neurons that were transfected with the CRISPR plasmids. The overall percentage of GFP positive neurons in a dish was relatively low, and hence we cannot use bulk genomic sequencing for verification. However, given the large functional effect on current densities, we are confident that the use of GFP fluorescence is an appropriate means of identifying neurons that were targeted with these plasmids. We specifically targeted medium diameter neurons for our analysis. The mean capacitance of the neurons that we recorded from was 24.79 ± 4.40 pF for control neurons versus 21.89 ± 1.31 pF for CRISPR-edited neurons.

SDS-PAGE and immunoblot analysis

Immunoblot of HA-tagged $Ca_v3.2$ channel was performed as previously described [16]. Briefly, total cell lysate from tsA-201 cells expressing HA-$Ca_v3.2$ channels was separated on a 5–20% gradient SDS-PAGE gel and transferred onto PVDF membrane (Millipore). Detection of HA-$Ca_v3.2$ was performed using a primary rat monoclonal anti-HA antibody (1:1000, Roche) and secondary HRP-conjugated antibody (1:10,000, Jackson ImmunoResearch). Immunoreactive products were detected by enhanced chemiluminescence and analyzed using ImageJ software.

For co-immunoprecipitation, cell lysates containing GFP-tagged and Myc-tagged $Ca_v3.2$ were incubated for 3 h with a biotinylated mouse monoclonal anti-Myc antibody (Santa Cruz Biotechnology), and then for 45 min with streptavidin beads (Invitrogen) at 4 °C, and washed with PBS/Tween-20 buffer. Beads were resuspended in Laemmli buffer and immunoprecipitation samples were separated on SDS-PAGE gel.

Generation of human $Ca_v3.2$ homology model

The homology model of the human $Ca_v3.2$ channel was prepared using the $Ca_v3.1$ structure as a template (PDB: 6KZO) in conjunction with Swiss-Model server (https://swissmodel.expasy.org/) [47]. Figures were prepared using Pymol (v2.2 Schrödinger, LLC.).

Statistics

Data values are presented as mean ± SEM for *n* measurements. Statistical analysis was performed using GraphPad Prism 7. For datasets passing the D'Agostino & Pearson omnibus normality test, statistical significance was determined using either Student's t-test or a Mann-Whitney test. Datasets were considered significantly different for $p \leq 0.05$.

Abbreviations

ALS: Amyotrophic lateral sclerosis; DRG: Dorsal root ganglia; GFP: Green fluorescent protein; G_{max}: Maximal macroscopic conductance; MRI: Magnetic resonance imaging; PM: Pore mutant; Q_{max}: Maximal charge movements; Q_{rev}: Charge movements at the reversal calcium potential; WT: Wild-type

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Authors' contributions

N.W., L.L., F.V.P., G.W.Z., and R.P. designed and conceptualized the study. R.N.S., R.I., B.I.T., O.J.H., S.J.t., I.A.S., A.L., Y.R., and N.W. collected data, performed analysis and interpreted the results. N.W. and R.P. wrote the manuscript. All authors critically revised the manuscript and contributed significantly to this work. The authors read and approved the final manuscript.

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Availability of data and materials

The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The whole genome sequencing of white blood cell DNA that gave rise to the finding of the genetic variants further characterised in the present study was undertaken by RP in a joint University of Sydney (Australia) and the Genome Institute Washington University (St Louis, USA) project using DNA samples from the Australian Motor Neuron Disease DNA Bank, with approval from the Sydney South West Area Health Service Human Research Ethics Committee. Informed written consent was obtained from each individual for their DNA to be used for research purposes.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Paper 5

De novo *SCN8A* and inherited rare *CACNA1H* variants associated with severe developmental and epileptic encephalopathy

Stringer, Robin N., Bohumila Jurkovicova-Tarabova, Ivana A. Souza, Judy Ibrahim, Tomas Vacik, Waseem Mahmoud Fathalla, Jozef Hertecant, Gerald W. Zamponi, Lubica Lacinova, and Norbert Weiss. 2021. 'De Novo SCN8A and Inherited Rare CACNA1H Variants Associated with Severe Developmental and Epileptic Encephalopathy'. *Molecular Brain* 14 (1): 126. https://doi.org/10.1186/s13041-021-00838-y.

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Molecular Brain

MICRO REPORT Open Access

De novo *SCN8A* and inherited rare *CACNA1H* variants associated with severe developmental and epileptic encephalopathy

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Abstract

Developmental and epileptic encephalopathies (DEEs) are a group of severe epilepsies that are characterized by seizures and developmental delay. DEEs are primarily attributed to genetic causes and an increasing number of cases have been correlated with variants in ion channel genes. In this study, we report a child with an early severe DEE. Whole exome sequencing showed a de novo heterozygous variant (c.4873–4881 duplication) in the *SCN8A* gene and an inherited heterozygous variant (c.952G > A) in the *CACNA1H* gene encoding for $Na_v1.6$ voltage-gated sodium and $Ca_v3.2$ voltage-gated calcium channels, respectively. In vitro functional analysis of human $Na_v1.6$ and $Ca_v3.2$ channel variants revealed mild but significant alterations of their gating properties that were in general consistent with a gain- and loss-of-channel function, respectively. Although additional studies will be required to confirm the actual pathogenic involvement of *SCN8A* and *CACNA1H*, these findings add to the notion that rare ion channel variants may contribute to the etiology of DEEs.

Keywords: Ion channels, Channelopathy, Calcium channel, *CACNA1H*, $Ca_v3.2$ channel, Sodium channel, *SCN8A*, $Na_v1.6$ channel, Epilepsy, Encephalopathy

Main text

Developmental and epileptic encephalopathies (DEEs) are a group of severe epilepsies that are characterized by seizures often drug-resistant, and developmental delay leading to varying degrees of intellectual, psychiatric, behavioral, and motor disabilities [1]. DEEs are primarily attributed to genetic causes and while recessive and X-linked variants have been found, the majority of patients show de novo pathogenic variants [2]. Recently, an increasing number of DEE cases have been correlated with variants in ion channel genes [3].

In the present study, we report a girl with an early severe DEE. She was born by emergency caesarean section at 37 weeks due to placenta previa and was the first child of non-consanguineous parents. Immediately after birth, she presented with trembling despite normal blood sugar levels. In the early postnatal period, she developed myoclonic jerks in all limbs, diagnosed as infantile spasms but did not respond to steroids. By the age of 2 months, she started having generalized tonic–clonic seizures and recurrent status epilepticus that poorly responded to antiepileptic medication including clobazam, levetiracetam, phenobarbital and topiramate. Seizures were characterized by right eye deviation and generalized tonic posturing. She also presented with

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(See figure on next page.)

Fig. 1 Electrophysiological properties of $Na_v1.6$ and $Ca_v3.2$ channel variants associated with developmental and epileptic encephalopathy. **a** Family pedigree chart. Filled and open symbols indicate affected and unaffected individuals, respectively. **b** Location of the $Na_v1.6$ G1625_I1627 duplication (red circle) and $Ca_v3.2$ G318S missense variants (blue circle) within the secondary membrane topology of the channels. **c** Representative sodium current traces recorded from cells expressing wild-type $Na_v1.6$ ($Na_v1.6^{wt}$, black traces) and $Na_v1.6$ duplication variant ($Na_v1.6^{dup}$, red traces) in combination with Na_vb_2. **d** Corresponding mean current–voltage (*I/V*) relationship. **e** Corresponding mean maximal macroscopic conductance (G_{max}) values obtained from the fit of the *I/V* curves with the modified Boltzmann Eq. (1). **f** Corresponding mean normalized voltage-dependence of activation. The voltage-dependence of activation for $Na_v1.6^{wt}$ in the absence of Na_vb_2 is shown for comparison (dotted line). *Inset* shows corresponding mean half-activation potential values obtained from the fit of the activation curve with the modified Boltzmann Eq. (2). **g** Mean normalized voltage-dependence of steady-state inactivation for $Na_v1.6^{wt}$ and Nav1.6^{dup}. *Inset* shows corresponding mean half-inactivation potential values obtained from the fit of the inactivation curves with the two-state Boltzmann function (3). **h** Mean normalized recovery from inactivation kinetics. *Inset* shows corresponding mean time constant τ values of recovery from inactivation obtained by fitting recovery curves with a single-exponential function (4). **i–n** Legend same as in (c–h) but for cells expressing wild type $Ca_v3.2$ ($Ca_v3.2^{wt}$, black) and $Ca_v3.2$ G318S ($Ca_v3.2^{G>S}$, blue) channel variants

additional complications including scoliosis, bilateral hip dislocation and recurrent pneumonia, and by the age of 3 she developed myoclonus, spastic quadriplegia with generalized hypertonia and hyperreflexia with clonus. Secondary skeletal abnormalities were also observed including flattening of the head and chest, severe kyphoscoliosis and flexion contractures. An MRI brain scan showed generalized brain atrophy with marked insular atrophy and bright white matter on flair. Blood tests were in general normal and only creatine phosphokinase levels were increased, probably as secondary consequence of seizures. The patient died at the age of 4. Whole exome sequencing (EGL Genetics) showed a de novo heterologous duplication (c.4873_4881dup) in *SCN8A* (Fig. 1a) causing the duplication of amino acid G1625_I1627 (p.G1625_I1627dup) within the highly conserved transmembrane IVS4 segment (voltage sensor) of the voltage-gated sodium channel $Na_v1.6$ (Fig. 1b). This variant has never been reported in the Genome Aggregation Database (gnomAD) and was predicted to be deleterious (PROVEAN algorithm). In addition, a rare heterozygous missense variant (c.952G > A) in *CACNA1H* (Fig. 1a) was inherited from the father who was asymptomatic. This variant that caused the substitution of a glycine at position 318 by a serine (p.G318S) within the first pore-forming loop of the voltage-gated calcium channel $Ca_v3.2$ (Fig. 1b) has never been reported and was not predicted to be deleterious. To assess the impact of these mutations, the G1625_I1627 duplication and G318S missense variant were introduced into the human $Na_v1.6$ (UniProt Q9UQD0-1) and $Ca_v3.2$ (UniProt O95180-1) channels, respectively, and recombinant channels were expressed in HEK cells for electrophysiological analysis. The sodium conductance recorded from cells expressing the duplication variant ($Na_v1.6^{dup}$) in combination with the human Na_vb_2 ancillary subunit (Uniprot O60939) was similar to the one measured from cells expressing the wild-type channel ($Na_v1.6^{wt}$) (Fig. 1c–e and Additional file 1: Table S1). However, the mean half activation potential of $Na_v1.6^{dup}$ was shifted toward more hyperpolarized potentials by − 5.4 mV ($p = 0.0005$) (Fig. 1f and Additional file 1: Table S1) to values similar to $Na_v1.6^{wt}$ expressed without the Na_vb_2 subunit (Additional file 1: Fig. S1 and Table S1). In contrast, we did not observe any gating alteration of $Na_v1.6^{dup}$ in the absence of Na_vb_2. While the current literature on the effect of Na_vb on the regulation of $Na_v1.6$ is rather sparse and conflicting [4, 5], these results suggest that phenotypic expression of *SCN8A* duplication variant may depend on the molecular composition of $Na_v1.6$, possibly by disrupting Na_vb-dependent regulation of the channel. Other gating properties including steady-state inactivation and recovery from inactivation were not affected (Fig. 1g, h and Additional file 1: Table S1). In addition, recording of T-type currents from cells expressing the $Ca_v3.2$ G318S variant ($Ca_v3.2^{G>S}$) did not reveal any alteration of the T-type conductance compared to cells expression the wild-type channel ($Ca_v3.2^{wt}$) (Fig. 1i–k and Additional file 1: Table S1). However, the mean half activation potential of the $Ca_v3.2^{G>S}$ variant was shifted toward more positive potentials by + 4.3 mV ($p = 0.0048$) (Fig. 1l and Additional file 1: Table S1) without any additional alteration of the other gating properties (Fig. 1m, n and Additional file 1: Table S1).

In summary, we reported the case of a child with severe DEE in whom a de novo mutation in *SCN8A* and an inherited rare *CACNA1H* variant were found. Pathogenic variants in *SCN8A* have originally been described in patients with DEE [6–9]. Most are de novo missense variants clustered in the highly conserved transmembrane domains of $Na_v1.6$ and are in general consistent with a gain-of-function pathogenic mechanism predicted to increase neuronal excitability and seizure susceptibility [6, 10, 11]. Our observation that the *SCN8A* duplication variant produced a hyperpolarizing shift of the voltage-dependence of activation of

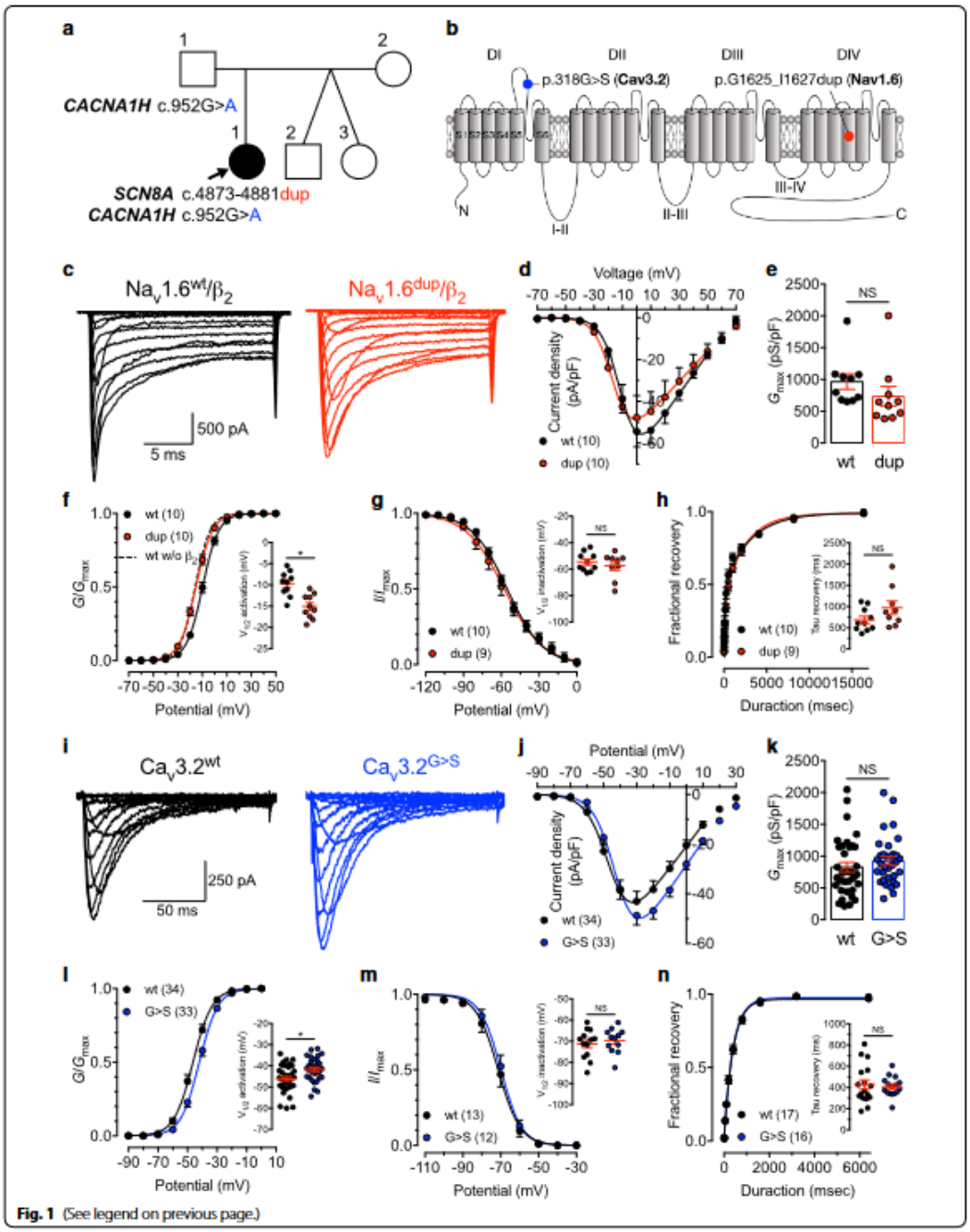

Fig. 1 (See legend on previous page.)

$Na_v1.6$ is also consistent with a gain-of-function (GoF) of the channel. Although future studies will be required to further assess the importance of the molecular composition of the channel in the phenotypic expression of *SCN8A* variants, the results presented here strengthen the notion that GoF *SCN8A* mutations may represent a general pathogenic mechanism in DEEs. In contrast, *CACNA1H* has never been associated with DEEs. Instead, GoF *CACNA1H* variants have been linked to absence epilepsy and primary aldosteronism [12] while loss-of-function (LoF) variants have been reported in autism spectrum disorders [13], amyotrophic lateral sclerosis [14, 15], and congenital amyotrophy [16]. It is not clear to which extent the LoF *CACNA1H* variant we identified in our patient may have contributed to the disease. Given that the father from whom the child inherited this variant was asymptomatic, this variant may not have had a major contribution to the development of the disease on its own. However, it is a possibility that it may have precipitated its development by interacting with other genes. This notion is supported by previous studies showing that *CACNA1G* ($Ca_v3.1$) and *CACNA1A* ($Ca_v2.1$) are genetic modifiers of epilepsy associated with Dravet syndrome [17–19]. While additional studies using primary neurons will be required to uncover the detailed underlying pathogenic mechanisms of $Na_v1.6$ and $Ca_v3.2$ variants, the current findings add to the notion that rare ion channel variants may contribute to the etiology of DEEs.

Abbreviations
DEEs: Developmental and epileptic encephalopathies; GoF: Gain-of-function; LoF: Loss-of-function.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13041-021-00838-y.

Additional file 1: Fig. S1. Electrophysiological properties of Nav1.6 variant expressed in the absence of Navb2. a Representative sodium current traces recorded from cells expressing wild type Nav1.6 (Nav1.6wt, black traces) and Nav1.6 duplication variant (Nav1.6dup, red traces). b Corresponding mean current–voltage (I/V) relationship. c Corresponding mean maximal macroscopic conductance (Gmax) values obtained from the fit of the I/V curves with the modified Boltzmann Eq. (1). d Corresponding mean normalized voltage dependence of activation. Inset shows corresponding mean half-activation potential values obtained from the fit of the activation curve with the modified Boltzmann Eq. (2). e Mean normalized voltage-dependence of steady-state inactivation for Nav1.6wt and Nav1.6dup. Inset shows corresponding mean half-inactivation potential values obtained from the fit of the inactivation curves with the two-state Boltzmann function (3). f Mean normalized recovery from inactivation kinetics. Inset shows corresponding mean time constant t values of recovery from inactivation obtained by fitting recovery curves with a single-exponential function (4). **Table S1.** Electrophysiological properties of human Nav1.6 and Cav3.2 variants expressed in tsA-201 cells. *p < 0.05.

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Authors' contributions
R.N.S. and B.J.T. performed electrophysiological recordings and analyzed the data. I.V.A. and T.V. generated $Ca_v3.2$ and $Na_v1.6$ variant cDNAs, respectively. J.A.I., W.M.F., and J.J.H. performed medical examination. N.W., G.W.Z., and I.L. designed the study. N.W. supervised the study and wrote the manuscript. All authors critically revised the manuscript and contributed significantly to this work. All authors read and approved the final manuscript.

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All data generated or analyzed during this study are included in this published article and its additional information files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Written consent to publish information related to the patient was obtained from the father.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

Paper 6

Electrophysiological and computational analysis of $Ca_V3.2$ channel variants associated with familial trigeminal neuralgia

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Molecular Brain

SHORT REPORT Open Access

Electrophysiological and computational analysis of $Ca_v3.2$ channel variants associated with familial trigeminal neuralgia

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Abstract

Trigeminal neuralgia (TN) is a rare form of chronic neuropathic pain characterized by spontaneous or elicited paroxysms of electric shock-like or stabbing pain in a region of the face. While most cases occur in a sporadic manner and are accompanied by intracranial vascular compression of the trigeminal nerve root, alteration of ion channels has emerged as a potential exacerbating factor. Recently, whole exome sequencing analysis of familial TN patients identified 19 rare variants in the gene *CACNA1H* encoding for $Ca_v3.2$ T-type calcium channels. An initial analysis of 4 of these variants pointed to a pathogenic role. In this study, we assessed the electrophysiological properties of 13 additional TN-associated $Ca_v3.2$ variants expressed in tsA-201 cells. Our data indicate that 6 out of the 13 variants analyzed display alteration of their gating properties as evidenced by a hyperpolarizing shift of their voltage dependence of activation and/or inactivation resulting in an enhanced window current supported by $Ca_v3.2$ channels. An additional variant enhanced the recovery from inactivation. Simulation of neuronal electrical membrane potential using a computational model of reticular thalamic neuron suggests that TN-associated $Ca_v3.2$ variants could enhance neuronal excitability. Altogether, the present study adds to the notion that ion channel polymorphisms could contribute to the etiology of some cases of TN and further support a role for $Ca_v3.2$ channels.

Keywords: Trigeminal neuralgia, Ion channel, Calcium channel, *CACNA1H*, $Ca_v3.2$ channel, Channelopathy

Introduction

Trigeminal neuralgia (TN) also referred as "tic douloureux" is a rare form of chronic neuropathic pain syndrome originating from the trigeminal nerve that supplies sensation to the face. TN is characterized by recurrent and chronic paroxysms of electric shock-like or stabbing pain in the orofacial region (for reviews see [1, 2]). The pain usually lasts from a few seconds to a few minutes and may be so intense that it triggers involuntary wincing, hence the term tic. Most cases of TN are sporadic but familial forms exist and are likely to be underestimated [3]. In both situations, the etiology of TN remains largely unknown and neurovascular compression of the trigeminal root nerve represents the primary theory for the underlying cause of the disease. However, the observation that many TN patients do not show any sign of neurovascular compression, and conversely that individuals with compression do not necessarily develop symptoms, suggested the existence of additional factors. Hence, an alteration of neuronal excitability resulting from abnormal functioning of ion channels has emerged

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as a potential underlying mechanism [4–7] and consistent with this notion, the sodium channel blockers carbamazepine and oxcarbazepine represent the first line therapy in TN [8]. Moreover, alterations of the expression of several ion channels including sodium, calcium, and potassium channels have been reported in TN patients [9] as well as in preclinical rodent models [10–17]. In addition, rare polymorphisms in ion channel genes were identified in TN patients [18–20] suggesting the existence of predisposing genetic factors and gain-of-function mutations (GoF) were reported for $Na_v1.6$ [18], $Ca_v2.1$ [21], TRPM7 [22, 23], and TRPM8 channels [24].

Recently, TN-associated polymorphisms in the gene *CACNA1H* encoding $Ca_v3.2$ calcium channels were reported [25]. $Ca_v3.2$ channels belong to the subfamily of low-voltage-activated T-type channels and are widely expressed throughout the nervous system where they play an essential role in the control of neuronal excitability [26]. Importantly, $Ca_v3.2$ is expressed in all structures of the trigeminal pathway including trigeminal ganglion sensory neurons [27, 28], the spinal trigeminal nucleus (SpV) [17] as well as several thalamic nuclei such as the ventroposterior nucleus (VPM) [29] that receives projections from the SpV. Hence, $Ca_v3.2$ channels may be of direct relevance for the transmission of trigeminal sensory information and a role for $Ca_v3.2$ in TN-like syndrome was reported in a preclinical rodent model [17].

In this study, we aimed to provide a comprehension analysis of TN-associated *CACNA1H* variants with regard to their impact on the functioning of $Ca_v3.2$ channels. Of the 19 variants reported [25], four had already been assessed for their impact on the biophysical properties of $Ca_v3.2$ channels and revealed a variant-dependent effect such that G563R and P566T produced a GoF of the channel, E286K caused a mild loss-of-function (LoF), and H526Y did not cause any alteration [17, 30]. We now report the functional characterization of 13 additional variants. Seven of these variants are located within cytoplasmic regions of $Ca_v3.2$ including the N-terminal region (P30L), the loop connecting domains II and III (Q1049H and P1120L), the loop connecting domains III and IV (P1605H), the linker connecting transmembrane segments S2-S3 of domain IV (R1674), and the C-terminal region (P2280H and E2291K). Four additional variants are mapped within important structural determinants of the channel including the transmembrane segment S1 of domain II (I799V and A802V), the end of the S4 voltage-sensor of domain IV (R1736C), and the fourth pore-forming loop (D1779Y). The two remaining variants are localized within the extracellular linkers connecting transmembrane segments S3-S4 of domain I (S187L) and S1-S2 of domain II (E819K) (Fig. 1). Electrophysiological analysis of recombinant TN-associated $Ca_v3.2$ variants in tsA-201 cells revealed a significant alteration in the gating properties of 7 out of the 13 variants analyzed. In addition, introduction of these variants in a computational model of reticular thalamic neuron (nRT) enhanced rebound burst firing of action potentials. Taken together, these data suggest that altered gating of TN-associated $Ca_v3.2$ variants may enhance neuronal excitability which could potentially contribute to the etiology of TN.

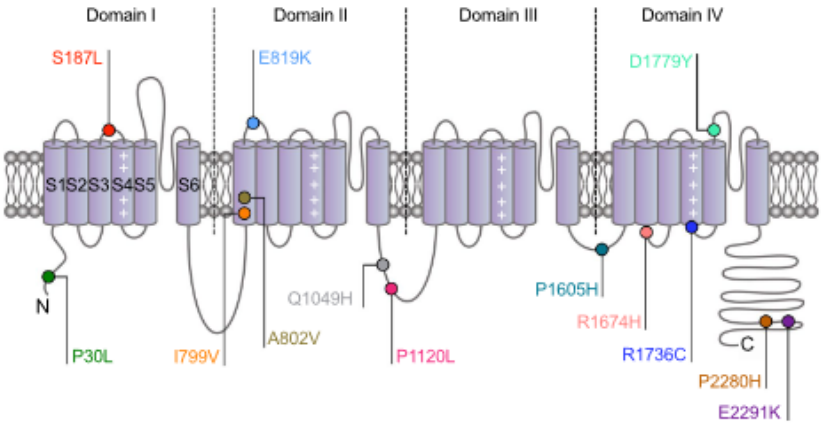

Fig. 1 Schematic representation of the membrane topology of $Ca_v3.2$ depicting the amino acid position of TN-associated variants characterized in this study

Materials and methods

Plasmid cDNA constructs and site-directed mutagenesis

The $Ca_v3.2$ variants were generated by site directed mutagenesis performed by GenScript using the wild-type human $Ca_v3.2$ (containing exon 26) in pcDNA3.1 (kindly provided by Dr. Terrance Snutch) as template. The fidelity of all constructs was confirmed by full-length sequencing of the coding region.

Cell culture and heterologous expression

Human embryonic kidney tsA-201 cells were grown in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all media purchased from Invitrogen) and maintained under standard conditions at 37 °C in a humidified atmosphere containing 5% CO_2. Heterologous expression was performed by transfecting cells with 5 µg of plasmid cDNAs encoding for $Ca_v3.2$ variants and empty pEGFP vector as transfection marker using the calcium/phosphate method.

Patch clamp electrophysiology

Patch clamp recordings of T-type currents in tsA-201 cells expressing $Ca_v3.2$ variants were performed 72 h after transfection in the whole-cell configuration at room temperature (22–24 °C) in a bath solution containing (in millimolar): 10 $BaCl_2$, 125 CsCl, 1 $MgCl_2$, 10 D-glucose, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4 with CsOH). Patch pipettes were filled with a solution containing (in millimolar): 110 CsCl, 3 Mg-ATP, 0.5 Na-GTP, 2.5 $MgCl_2$, 5 D-glucose, 10 EGTA, and 10 HEPES (pH 7.4 with CsOH), and had a resistance of 2–4MΩ. The calculated liquid junction potential was about − 2.6 mV and therefore was corrected from the recordings. Recordings were performed using an Axopatch 200B amplifier (Axon Instruments) and acquisition and analysis were performed using pClamp 10 and Clampfit 10 softwares, respectively (Axon Instruments). The linear leak component of the current was corrected using a P/4 subtraction protocol and current traces were digitized at 10 kHz and filtered at 2 kHz.

The voltage dependence of activation of $Ca_v3.2$ channels was determined by measuring the peak of the T-type current in response to 140 ms depolarizing steps from − 80 mV to + 20 mV in 5 mV increments preceded by a 200 ms hyperpolarizing prepulse to − 110 mV from a holding membrane potential of − 100 mV. The current–voltage relationship (I/V) curve was fitted with the following modified Boltzmann Eq. (1):

$$I(V) = Gmax\frac{(V - V\mathrm{rev})}{1 + \exp\frac{(V0.5 - V)}{k}} \quad (1)$$

with $I(V)$ being the peak current amplitude at the command potential V, G_{max} the maximum conductance, V_{rev} the reversal potential, $V_{0.5}$ the half-activation potential, and k the slope factor. The voltage dependence of the whole-cell T-type channel conductance was calculated using the following modified Boltzmann Eq. (2):

$$G(V) = \frac{Gmax}{1 + \exp\frac{(V0.5 - V)}{k}} \quad (2)$$

with $G(V)$ being the T-type channel conductance at the command potential V.

The voltage dependence of the steady-state inactivation of $Ca_v3.2$ channels was determined by measuring the peak T-type current amplitude in response to a 50 ms depolarizing step to -30 mV applied after a 1 s-long conditioning prepulse ranging from -110 mV to -15 mV in 5 mV increments. The current amplitude obtained during each test pulse was normalized to the maximal current amplitude and plotted as a function of the prepulse potential. The voltage dependence of the steady-state inactivation was fitted with the following two-state Boltzmann function (3):

$$I(V) = \frac{Imax}{1 + \exp\frac{(V - V0.5)}{k}} \quad (3)$$

with I_{max} corresponding to the maximal peak current amplitude and $V_{0.5}$ to the half-inactivation voltage.

The recovery from inactivation was assessed using a double-pulse protocol preceded by a 50 ms-long hyperpolarizing prepulse to -110 mV from a holding potential of -100 mV. The cell membrane was depolarized for 2 s at -20 mV (inactivating prepulse) to ensure complete inactivation of the channel, and then to -20 mV for 150 ms (test pulse) after an increasing time period (interpulse) ranging between 1 ms and 8 s at -110 mV. The peak current from the test pulse was plotted as a ratio of the maximum prepulse current versus interpulse interval. The data were fitted with the following single-exponential function (4):

$$\frac{I}{Imax} = A \times (1 - exp\frac{-t}{\tau}) \quad (4)$$

where τ is the time constant for channel recovery from inactivation.

Computational modeling

Simulation of thalamic reticular neuron (nRT) firing was performed using the NEURON simulation environment (https://senselab.med.yale.edu/ModelDB/) [31] in the three-compartment model previously described [32].

The electrophysiological properties of wild-type and TG-associated $Ca_v3.2$ variants obtained experimentally were modeled using Hodgkin-Huxley equations as previously described [33] and introduced into the model. To take into account the relative expression of Cav3.2 channels in nRT neurons (about 40% of $Ca_v3.2$ and 60% of $Ca_v3.3$ [34]) and the heterozygous nature of TN-associated $Ca_v3.2$ variants, only 20% of the T-type channel conductance described in the original model was altered with experimental values obtained for WT and TN-associated $Ca_v3.2$ variants. The simulation was performed at a holding potential set to -70 mV and the electrical membrane potential of the virtual soma was monitored in response to a 200ms-long hyperpolarizing and depolarizing current injection in order to assess rebound and tonic firing, respectively.

Statistical analysis

Average data are presented as mean ± S.E.M. for n measurements. Statistical analysis was performed using GraphPad Prism 8. A Kolmogorov–Smirnov normality test was performed and statistical significance was assessed using Kruskal–Wallis test with Dunn's post-test. Datasets were considered significantly different for $p \leq 0.05$.

Results

Expression of TN-associated $Ca_v3.2$ variants

To assess the functional impact of TN-associated *CACNA1H* variants, tsA-201 cells were transiently transfected with plasmids encoding human $Ca_v3.2$ wild-type (WT) and TN-associated variants for electrophysiological analysis. Whole-cell patch clamp recordings in tsA-201 cells expressing wild-type (WT) and TN-associated $Ca_v3.2$ variants revealed that all variants were functionally expressed and generated a characteristic low-voltage-activated T-type current similar to WT channels (Fig. 2a–n, left panels). The maximal whole-cell macroscopic T-type channel conductance (G_{max}) obtained from the fit of the current–voltage relationships (Fig. 2a–n, right panels) revealed no significant difference between cells expressing $Ca_v3.2$ variants compared to cells expressing the WT channel except for the R1674H variant were G_{max} was reduced by 56% ($p = 0.0185$) (Fig. 2o and Table 1).

TN-associated CACNA1H variants alter the gating properties of $Ca_v3.2$ channels

Next, we aimed to assess the gating properties of TN-associated $Ca_v3.2$ variants. First, we analyzed the voltage dependence of activation of the channels. In 6 (S187L, A802V, E819K, P1120L, P2280H, and E2291K) out of the 13 variants analyzed, the mean half-activation potential of the T-type current was significantly shifted toward more hyperpolarized potentials by − 8.0 mV (E2291K, $p = 0.0268$) up to − 15.0 mV (P2280H, $p < 0.0001$) relative to WT channels which is consistent with a GoF of the channels (Fig. 3a–o and Table 1). In addition, a significant decrease of the activation slope factor (k) was observed for S187L, A802V, E819K, and P2280H variants suggesting an increased coupling between the channel voltage-sensor and the pore opening again consistent with a GoF which may be particularly relevant for voltage changes close to the resting membrane potential where first openings of the channel occur (Table 1). To gain additional insights into the electrophysiological properties of TN-associated $Ca_v3.2$ variants, we then assessed their voltage dependence of inactivation. A statistically significant hyperpolarizing shift of the voltage dependence of inactivation by -12.6 mV ($p = 0.0015$) relative to WT channels was observed for the P2280H variant and a similar trend albeit not statistically significant was observed for S187L (− 8.6 mV, $p = 0.8716$) and A802V variants (− 9.2 mV, $p = 0.3699$) whereas the remaining variants remained unaltered (Fig. 4a–o and Table 2). The alteration of the voltage dependence of inactivation is consistent with a LoF of the channel variants although the extent to which it may affect channel activity will largely depend on the resting membrane potential of cells, with a more pronounced effect in cells with a comparatively depolarized resting potential. In contrast, the kinetics of recovery from inactivation were accelerated by 2.7-fold for A802V ($p = 0.0001$) and by 3.5-fold for Q1049H variants ($p = 0.0005$) compared to WT channels, and a similar trend (albeit not statistically significant) was observed for several other variants indicative of a GoF (Fig. 5a–o and Table 2).

$Ca_v3.2$-dependent window current is altered by TN-associated CACNA1H variants

Because several TN-associated $Ca_v3.2$ variants showed alterations in the voltage dependence of activation and/or inactivation, we aimed to assess the impact on the T-type window current by visualizing the overlapping area between the activation and inactivation curves (Fig. 6a–g). In all TN-associated $Ca_v3.2$ variants for which the voltage dependence of activation and/or inactivation was altered, the window current was displaced toward more hyperpolarized potentials with the peak-voltage shifted by − 5 mV (P1120L) up-to

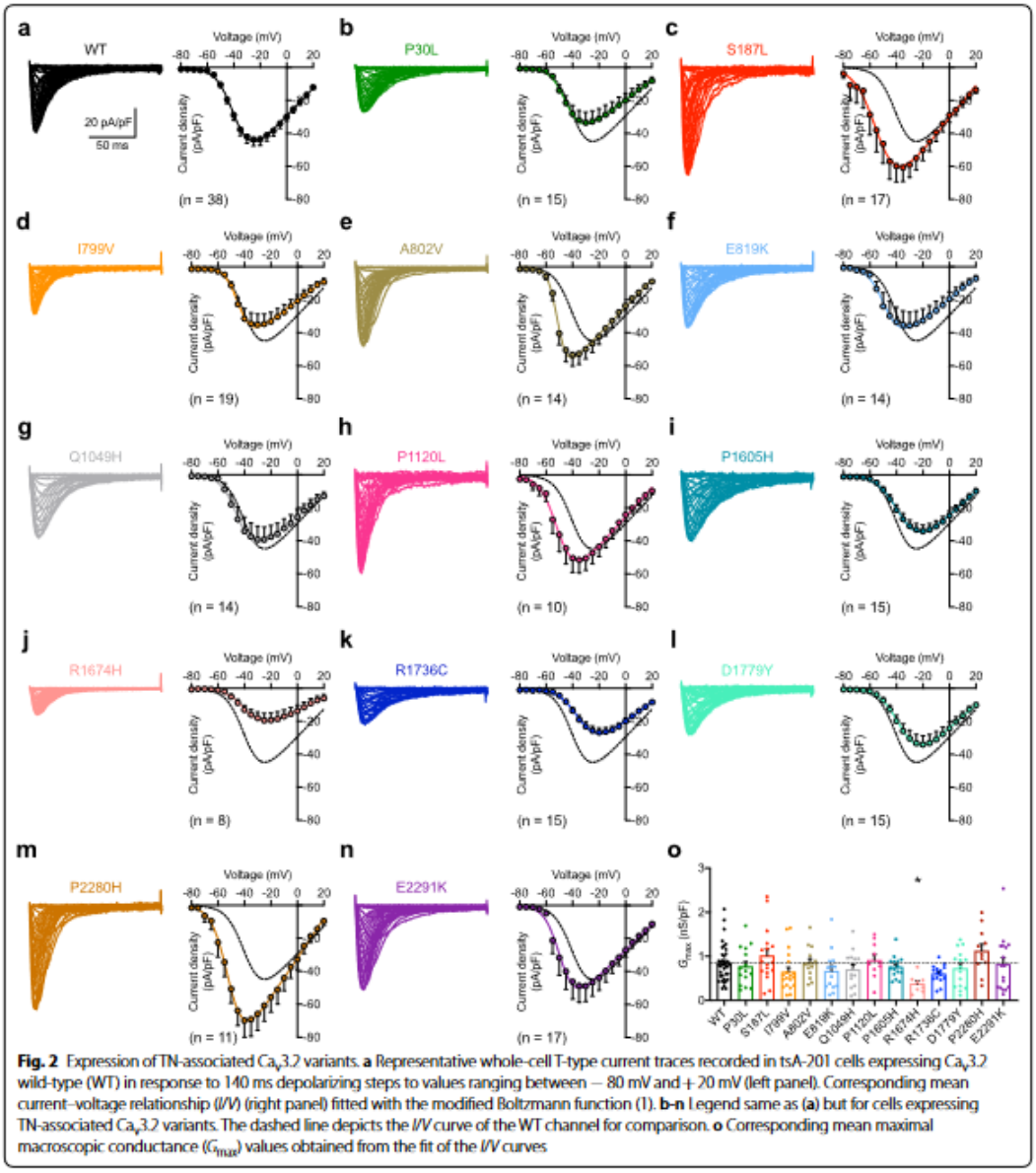

Fig. 2 Expression of TN-associated $Ca_v3.2$ variants. **a** Representative whole-cell T-type current traces recorded in tsA-201 cells expressing $Ca_v3.2$ wild-type (WT) in response to 140 ms depolarizing steps to values ranging between − 80 mV and + 20 mV (left panel). Corresponding mean current–voltage relationship (*I/V*) (right panel) fitted with the modified Boltzmann function (1). **b-n** Legend same as (**a**) but for cells expressing TN-associated $Ca_v3.2$ variants. The dashed line depicts the *I/V* curve of the WT channel for comparison. **o** Corresponding mean maximal macroscopic conductance (G_{max}) values obtained from the fit of the *I/V* curves

− 12 mV (P2280H) (Fig. 6h). This effect was accompanied by an increased magnitude of the window current (except for the P2280H variant) ranging from 14% (E2291K) up-to 165% increase (P1120L) (Fig. 6i).

TN-associated $Ca_v3.2$ variants increase neuronal firing in a computational model of thalamic neurons

Given that $Ca_v3.2$ channels are highly expressed in thalamic neurons where they play an essential role in

Table 1 Steady-state activation properties of TN-associated human $Ca_v3.2$ variants expressed in tsA-201 cells

	Activation						
$Ca_v3.2$	G_{max} (nS/pF)	*p*	$V_{0.5}$ (mV)	*p*	*k* (mV)	*p*	(n)
WT	0.84 ± 0.07		− 38.17 ± 0.80		6.38 ± 0.35		38
P30L	0.77 ± 0.11	> 0.9999	− 44.18 ± 1.24	0.1251	4.70 ± 0.51	0.2024	15
S187L	1.01 ± 0.15	> 0.9999	− 51.63 ± 2.56	< 0.0001	3.47 ± 0.50	0.0006	17
I799V	0.65 ± 0.10	0.6889	− 41.78 ± 1.58	0.5519	5.62 ± 0.69	> 0.9999	19
A802V	0.85 ± 0.09	> 0.9999	− 48.82 ± 1.14	< 0.0001	3.56 ± 0.45	0.0015	14
E819K	0.66 ± 0.12	> 0.9999	− 48.19 ± 2.97	0.0208	4.31 ± 0.53	0.0491	14
Q1049H	0.70 ± 0.11	> 0.9999	− 41.88 ± 2.42	> 0.9999	4.99 ± 0.67	0.7660	14
P1120L	0.91 ± 0.13	> 0.9999	− 49.55 ± 3.17	0.0106	4.23 ± 0.56	0.1175	10
P1605H	0.76 ± 0.06	> 0.9999	− 34.53 ± 1.60	> 0.9999	8.17 ± 0.34	0.2336	15
R1674H	0.37 ± 0.07	0.0185	− 38.23 ± 2.30	> 0.9999	6.49 ± 0.99	> 0.9999	8
R1736C	0.60 ± 0.05	0.8669	− 32.42 ± 1.38	0.4164	7.39 ± 0.29	> 0.9999	15
D1779Y	0.72 ± 0.11	> 0.9999	− 33.86 ± 1.12	> 0.9999	8.08 ± 0.27	0.2457	15
P2280H	1.12 ± 0.16	> 0.9999	− 53.12 ± 2.31	< 0.0001	3.87 ± 0.37	0.0159	11
E2291K	0.81 ± 0.15	> 0.9999	− 46.13 ± 2.01	0.0268	4.91 ± 0.69	0.1754	17

regulating neuronal excitability [35] and considering that the thalamus is a key relay station in the trigeminal sensory pathway [36], we aimed to simulate the functional consequence of TN-associated $Ca_v3.2$ variants on neuronal electrical activities using a computational model of reticular thalamic neuron (nRT). The simulation was performed with $Ca_v3.2$ variants for which an alteration of the voltage dependence of activation and/or inactivation was observed and the original model was altered in order to account for the relative contribution of $Ca_v3.2$ channels to the overall native T-type conductance and also to account for the heterozygous nature of TN-associated $Ca_v3.2$ variants (see *Methods*). Simulation of the neuronal membrane potential showed that hyperpolarizing current injections triggered rebound burst firing with WT as well as with TN-associated $Ca_v3.2$ variants (Fig. 7a–g). However, the minimum current necessary to trigger rebound firing (rheobase) was significantly less for TN-associated $Ca_v3.2$ variants (except A802V) compared to WT channels (Fig. 7h). Moreover, the firing frequency was increased (Fig. 7i). In contrast, when the firing was triggered with depolarizing current injections there was no major effect between WT and TN-associated $Ca_v3.2$ variants (Fig. 7j–r).

Discussion

Polymorphisms in the *CACNA1H* gene have been reported in a number of human disorders [37] and GoF mutations in $Ca_v3.2$ are linked to primary aldosteronism (PA) [38–40] and idiopathic generalized epilepsy (IGE) [41]. In contrast, LoF mutations were documented in autism spectrum disorders [42], neuromuscular disorders [43–45], and developmental and epileptic encephalopathy [46].

In this study, we report the functional characterization of 13 $Ca_v3.2$ missense variants identified in TN patients. Patch clamp recordings of T-type currents in tsA-201 cells expressing recombinant TN-associated $Ca_v3.2$ variants showed that all variants were functional with no significant alteration in their maximal macroscopic conductance except for the R1674H variant for which the conductance was reduced. This effect was not further investigated but may have been caused by a decreased trafficking of the channel to the plasma membrane and/or decreased stability. In contrast, of the 13 $Ca_v3.2$ variants analyzed, 6 variants (S187L, A802V, E819K, P1120L, P2280H, and E2291K) displayed alterations in their gating properties evidenced by a recurrent hyperpolarized shift of the voltage dependence of activation consistent with a GoF of the channels. An additional acceleration of the recovery from inactivation was also observed for A802V and Q1049H. Although these variants showed similar alterations in their gating properties, they did not segregate into a particular region of $Ca_v3.2$. Nonetheless, some of the channel molecular determinants containing TN-associated variants are known to contribute to the gating of $Ca_v3.2$. For instance, the II-III loop containing variant P1120L and the C-terminus containing variants P2280H and E2291K were previously reported to affect the voltage dependence of T-type channels [47–49]. Moreover, the GoF effect of TN-associated variants in the C-terminus of $Ca_v3.2$ is reminiscent of what was reported

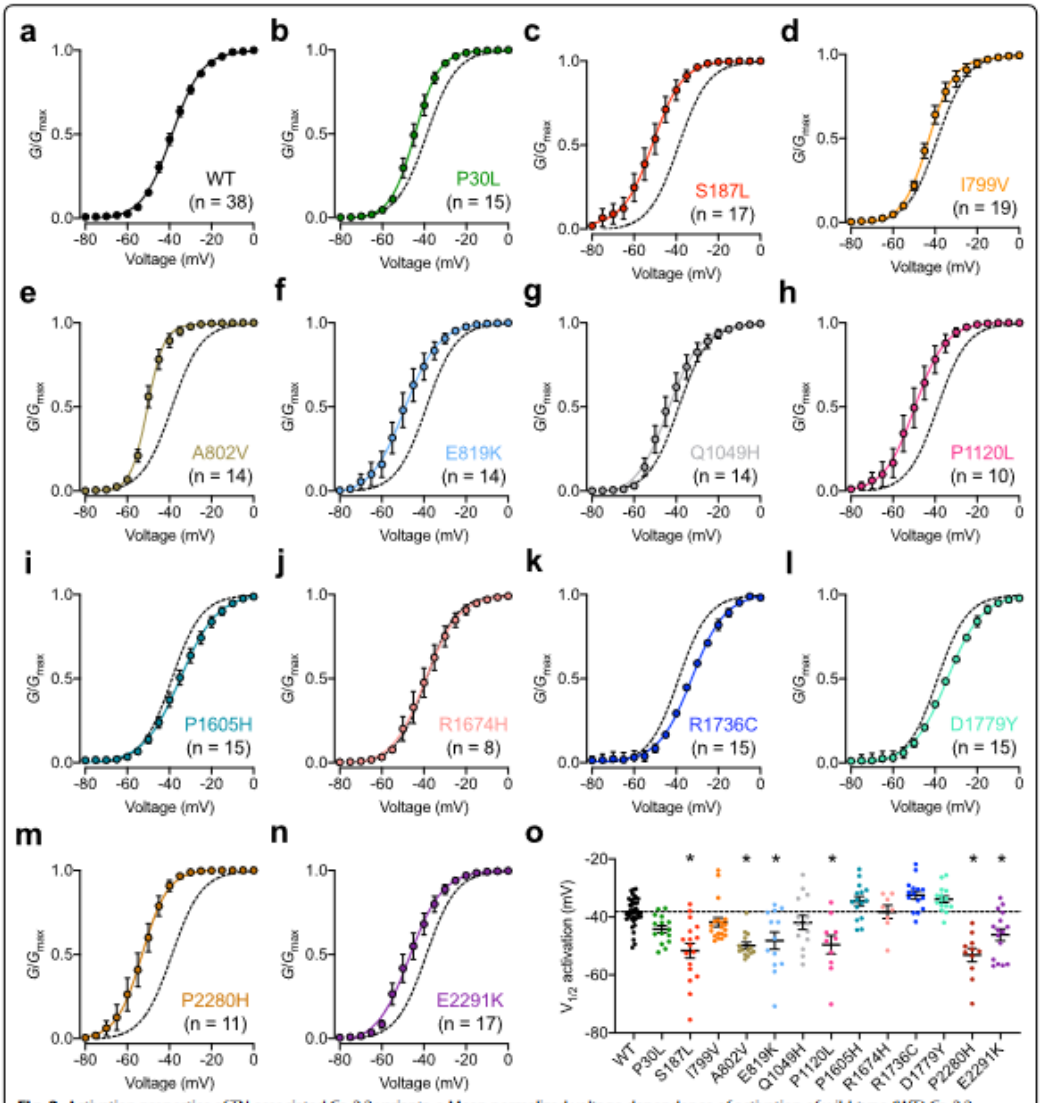

Fig. 3 Activation properties of TN associated $Ca_v3.2$ variants. **a** Mean normalized voltage dependence of activation of wild type (WT) $Ca_v3.2$ channels fitted with the modified Boltzmann function (2). **b**–**n** Legend same as (**a**) but for TN-associated $Ca_v3.2$ variants. The dashed line depicts the activation curve of the WT channel for comparison. **o** Corresponding mean half-activation potential ($V_{1/2}$ activation) values obtained from the fit of the conductance curve

for several variants associated with (IGE) and PA [40, 50]. Importantly, when introduced into a computational model of nRT neuron, the 6 variants reduced the threshold for rebound burst firing implying an overall GoF effect. This is consistent with previous findings in various types of neurons showing that upregulation of T-type channel activity underlies reduced threshold for rebound burst firing [51–55]. While our modeling was

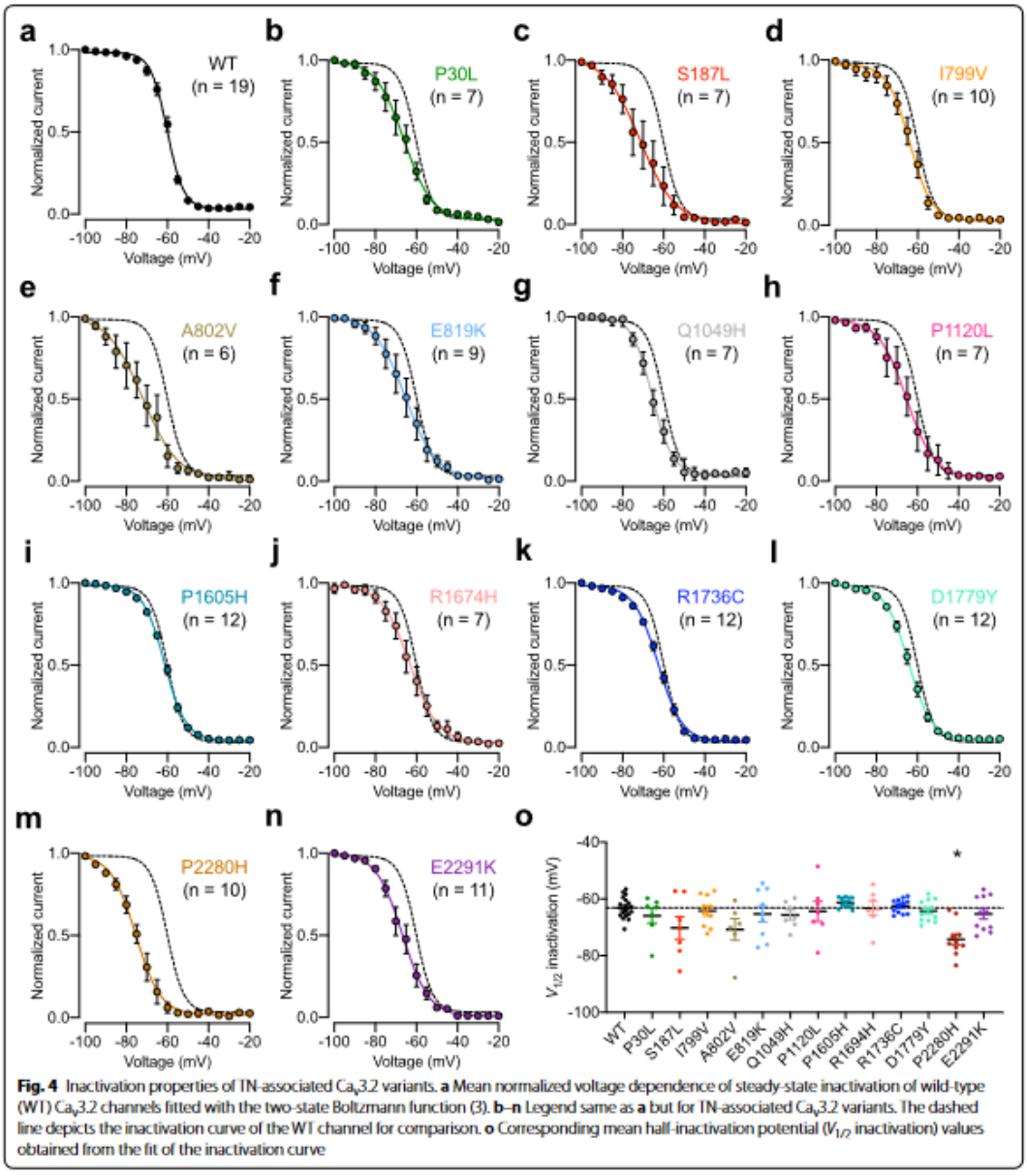

Fig. 4 Inactivation properties of TN-associated $Ca_v3.2$ variants. **a** Mean normalized voltage dependence of steady-state inactivation of wild-type (WT) $Ca_v3.2$ channels fitted with the two-state Boltzmann function (3). **b**–**n** Legend same as **a** but for TN-associated $Ca_v3.2$ variants. The dashed line depicts the inactivation curve of the WT channel for comparison. **o** Corresponding mean half-inactivation potential ($V_{1/2}$ inactivation) values obtained from the fit of the inactivation curve

performed in a computational model of nRT neurons, it may anticipate some of the possible effects of TN-associated $Ca_v3.2$ variants on the functioning of the trigeminal pathway for several reasons. First, although T-type dependent rebound burst firing has yet to be shown in trigeminal ganglion (TG) sensory neurons, it has been documented in dorsal root ganglion sensory neurons [56] and it is a possibility that it does also occur

Table 2 Steady-state inactivation and recovery from inactivation properties of TN-associated human $Ca_v3.2$ variants expressed in tsA-201 cells

	Inactivation					Recovery from inactivation		
$Ca_v3.2$	$V_{0.5}$ (mV)	*p*	*k* (mV)	*p*	(n)	τ (ms)	*p*	(n)
WT	−61.61 ± 1.75		−4.48 ± 0.22		19	644 ± 49		15
P30L	−65.95 ± 2.67	> 0.9999	−4.75 ± 0.44	> 0.9999	7	705 ± 200	> 0.9999	5
S187L	−70.21 ± 3.99	0.8716	−4.10 ± 0.34	> 0.9999	7	400 ± 39	0.6749	6
I799V	−64.22 ± 1.71	> 0.9999	−4.27 ± 0.51	> 0.9999	10	382 ± 76	0.1542	8
A802V	−70.79 ± 3.80	0.3699	−4.16 ± 0.39	> 0.9999	6	241 ± 28	0.0001	10
E819K	−65.31 ± 2.86	> 0.9999	−5.18 ± 0.55	> 0.9999	9	335 ± 38	0.1564	5
Q1049H	−65.68 ± 1.74	> 0.9999	−4.44 ± 0.28	> 0.9999	7	185 ± 24	0.0005	5
P1120L	−64.38 ± 3.53	> 0.9999	−3.64 ± 0.57	> 0.9999	7	489 ± 197	0.4634	6
P1605H	−61.32 ± 0.51	> 0.9999	−5.10 ± 0.19	> 0.9999	12	470 ± 46	> 0.9999	10
R1674H	−63.29 ± 2.49	> 0.9999	−6.05 ± 1.04	> 0.9999	7	397 ± 103	0.3918	6
R1736C	−62.48 ± 0.73	> 0.9999	−5.50 ± 0.11	0.0191	12	665 ± 68	> 0.9999	7
D1779Y	−64.23 ± 1.07	> 0.9999	−5.03 ± 0.11	> 0.9999	12	662 ± 58	> 0.9999	8
P2280H	−74.22 ± 1.89	0.0015	−4.56 ± 0.37	> 0.9999	10	334 ± 42	0.0724	7
E2291K	−65.27 ± 1.83	> 0.9999	−4.23 ± 0.19	> 0.9999	11	429 ± 80	0.3967	8

in TG neurons. Second, a low-threshold calcium conductance (presumably mediated by T-type channels) leading to calcium spikes and rebound burst firing has been reported in neurons of the brain stem trigeminal nuclei [57]. Third, the trigeminal pathway gates through the thalamus in particular via the VPM where T-type channels contribute to rebound burst firing [58]. And finally, alteration of thalamocortical rhythmic activities mediated by T-type channels has been implicated in the development of trigeminal pain [14]. Hence, all of these aspects suggest that alteration of rebound burst firing caused by TN-associated $Ca_v3.2$ variants could potentially contribute to the sensitization of the trigeminal pathway. In addition, alteration of the channel gating properties resulted in a hyperpolarizing displacement of the voltage dependence of the window current which implies an increased passive influx of calcium around the resting membrane potential of cells. Considering that the voltage range of the window current is an important determinant of neuronal electrical activities and calcium oscillations [59], this may further contribute to enhance neuronal activity. These data are consistent with a previous report showing that re-expression of a GoF TN-associated $Ca_v3.2$ variant in cultured TG neuron increased neuronal excitability [17]. The question then remains as to why TN patients harboring GoF $Ca_v3.2$ variants did not show signs of IGE or PA. It is a possibility that the gating alterations caused by these variants and affecting only the rebound burst firing in the absence of general alteration of the tonic firing is not enough to cause additional disease phenotypes.

In conclusion, our functional analysis of 13 $Ca_v3.2$ variants identified in TN patients revealed an overall GoF of the channel for 7 of these variants that could potentially contribute to the sensitization of the trigeminal pathway. Although these gating effects are reminiscent of what was previously reported for TN-associated variants in $Na_v1.6$ [18], $Ca_v2.1$ [21], TRPM7 [22, 23], and TRPM8 channels [24], it is important to consider that our functional analysis in a heterologous expression system provides only a snapshot of the phenotype of a mutation. Hence, additional analysis of these variants in native conditions will be necessary to further validate these findings. Moreover, it is a possibility that the variants for which we did not observe any gating alteration will show different phenotypes in a more complex physiological environment. Finally, although most of the gating alterations were in general consistent with a GoF of the channel, it is important to consider that one variant was associated with a LoF suggesting that GoF phenotypes in ion channels may not represent a universal feature in TN. For instance, the expression level of *SCN9A* ($Na_v1.7$) and *SCN10A* ($Na_v1.8$) is reduced in gingival tissue of TN patients [9], as well as in preclinical models of TN [11] implying a LoF phenotype, although this mechanism may occur as a protective mechanism to normalize neuronal excitability. Nonetheless, it is striking to note that all patients exhibiting idiopathic TN with concomitant continuous pain (iTN-2) harbored GoF $Ca_v3.2$ variants. In contrast, all $Ca_v3.2$ variants identified in patients with congenital TN and concomitant pain (cTN-2) did not cause any alteration of the channel (Table 3). While

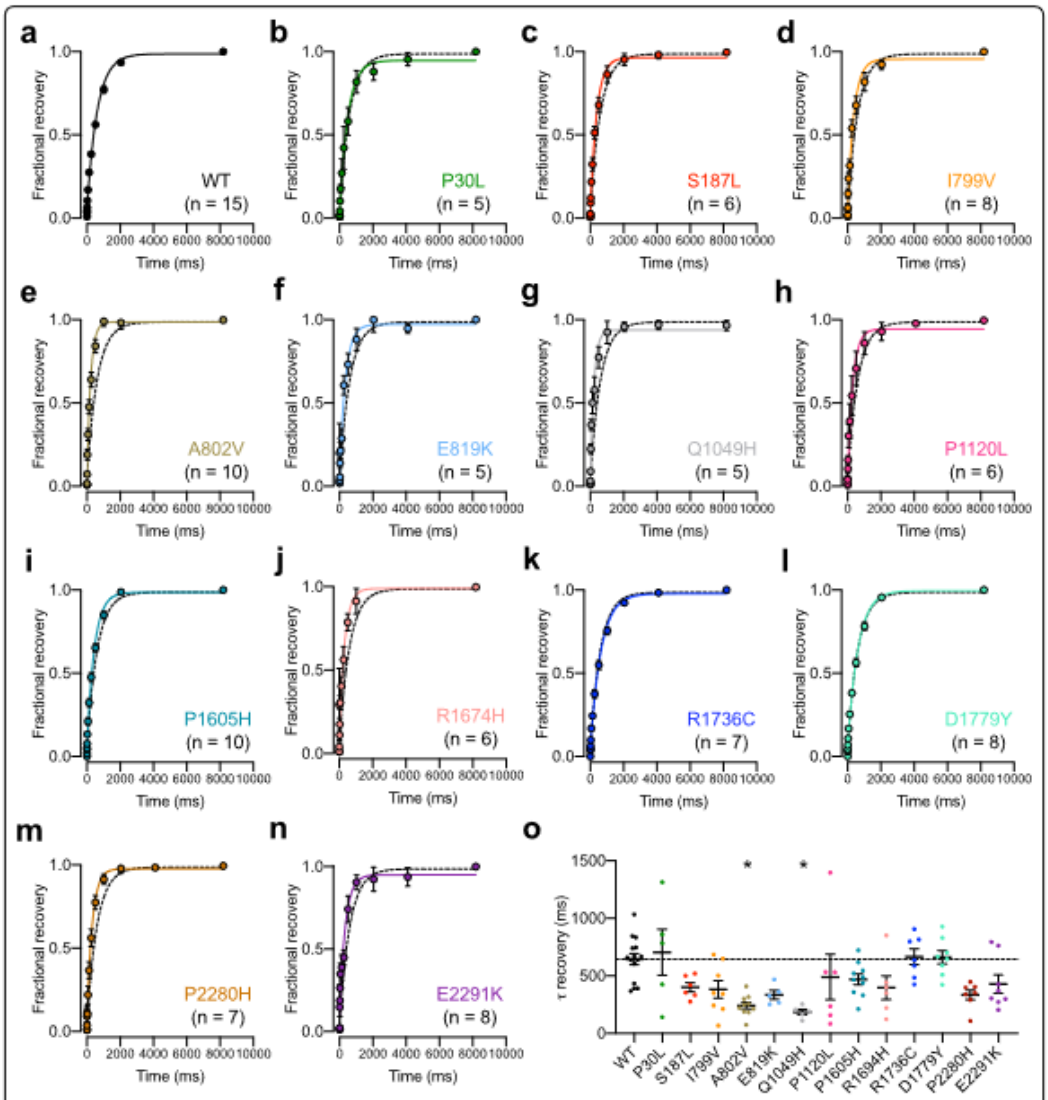

Fig. 5 Recovery from inactivation properties of TN-associated $Ca_v3.2$ variants. **a** Mean normalized recovery from inactivation kinetic of wild type (WT) $Ca_v3.2$ channels fitted with the single-exponential function (4). **b**–**n** Legend same as (**a**) but for TN-associated $Ca_v3.2$ variants. The dashed line depicts the recovery from inactivation of the WT channel for comparison. **o** Corresponding mean time constant τ values of recovery obtained from the fit of the recovery from inactivation curve

additional studies are necessary to assess the exact role of $Ca_v3.2$ in the processing of trigeminal sensory information, our data add to the notion that rare *CACNA1H* variants may contribute to the etiology of TN. In that respect, the T-type channel blocker valproic acid was shown to be effective to mitigate pain in some TN patients [60] suggesting that other antiepileptic T-type channel blockers ethosuximide, zonizamide, and nimodipine [26] should also be considered especially in patients resistant to first line therapies.

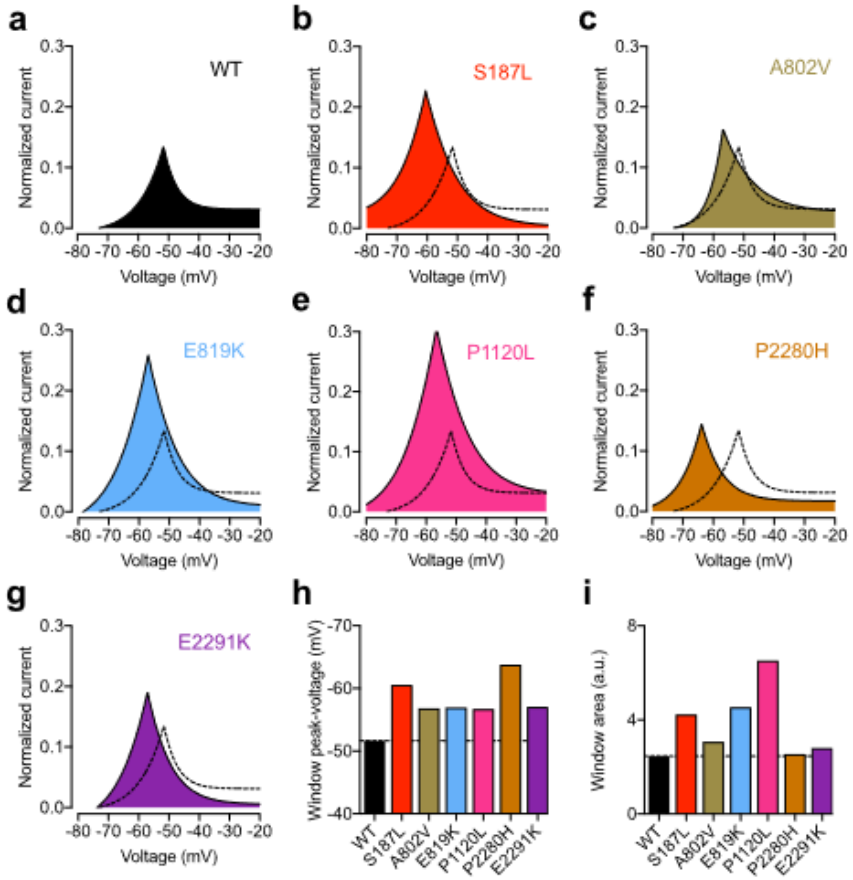

Fig. 6 Window current of TN-associated $Ca_v3.2$ variants. **a** Window current of wild-type (WT) $Ca_v3.2$ illustrated by the overlap of the mean activation and inactivation curves. **b**–**g** Legend same as (**a**) but for TN-associated $Ca_v3.2$ variants. The dashed line depicts the window current of the WT channel for comparison. **h** Corresponding peak-voltage values of the window current: WT (— 51.6 mV), S187L (— 60.5 mV), A802V (— 56.8 mV), E819K (— 56.9 mV), P1120L (— 56.7 mV), P2280H (— 63.8 mV), E2291K (— 57.0 mV). **i** Corresponding magnitude values of the window current measured as the area under the curve: WT (2.5 a.u.), S187L (4.2 a.u.), A802V (3.1 a.u.), E819K (4.5 a.u.), P1120L (6.5 a.u.), P2280H (2.5 a.u.), E2291K (2.8 a.u.)

(See figure on next page.)

Fig. 7 Computer simulation of nRT neuron firing. **a** Representative electrical membrane potential of the virtual soma containing wild-type (WT) $Ca_v3.2$ channels in response to a 700 ms-long hyperpolarizing current injection of — 0.65 nA (left panel) and corresponding number of spikes during the rebound as a function of the current injected (right panel). **b**–**g** Legend same as **a** but for TN-associated $Ca_v3.2$ variants. The dashed line depicts the number of spikes for WT channels for comparison. **h** Minimum current injection (rheobase) necessary to trigger rebound action potentials: WT (— 0.585 nA), S187L (— 0.568 nA), A802V (— 0.606 nA), E819K (— 0.561 nA), P1120L (— 0.528 nA), E2280H (— 0.471 nA), and E2291K (— 0.559 nA). **i** Rebound firing frequency at -0.65 nA current injection: WT (15 Hz), S187L (35 Hz), A802V (10 Hz), E819K (45 Hz), P1120L (55 Hz), E2280H (55 Hz), and E2291K (20 Hz). **J**–**p** Legend same as **a**–**g** but for depolarizing current injections. Representative membrane potentials are shown in response to 0.2 nA current injection. **q** Minimum current injection (rheobase) necessary to trigger tonic action potentials: WT (0.0257 nA), S187L (0.0258 nA), A802V (0.0260 nA), E819K (0.0255 nA), P1120L (0.0251 nA), E2280H (0.0254 nA), and E2291K (0.0255 nA). **r** Action potential frequency at 0.2 nA current injection: WT (170 Hz), S187L (180 Hz), A802V (170 Hz), E819K (180 Hz), P1120L (185 Hz), E2280H (175 Hz), and E2291K (175 Hz)

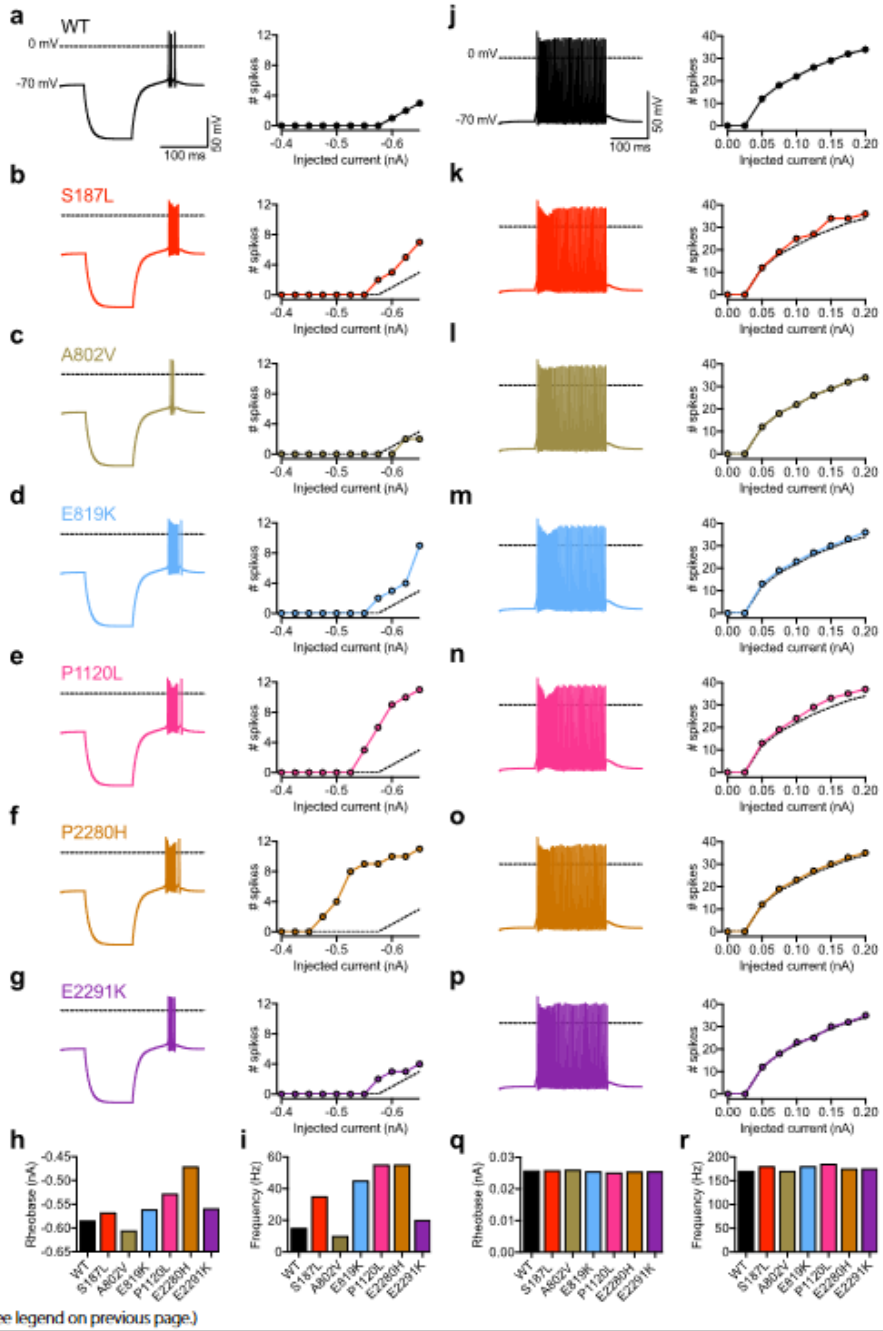

Fig. 7 (See legend on previous page.)

Table 3 Summary of gating effects of TN-associated $Ca_v3.2$ variants in relation to the clinical phenotype of patients

Idiopathic TN**	$Ca_v3.2$ variant	Classical TN**	$Ca_v3.2$ variant
iTN-1	**E286K***	cTN-1	*E229K*
iTN-1	*G563R**		
iTN-1	**R1674H**	cTN-2	***P30L***
iTN-1	***D1779Y***	cTN-2	***H526Y****
		cTN-2	***I799V***
iTN-2	*P566T**	cTN-2	***P1605H***
iTN-2	*E819K*	cTN-2	***R1736C***
iTN-2	*Q1049H*	cTN-2	***P30L***
iTN-2	*P1120L*		
iTN-2	*P2280H*		

Italic: GoF

Bold: LoF

Bolditalic: Neutral

iTN idiopathic trigeminal neuralgia, *cTN* classical trigeminal neuralgia; (– 1), purely paroxysmal; (– 2) with concomitant continuous pain. *According to [17]. **According to [25]. Two GoF variants (S187L and A802V) are not included in this table since their clinical phenotype was not fully defined (atypical facial pain and TN without further information, respectively)

Abbreviations

cTN-1: Classical trigeminal neuralgia purely paroxysmal; cTN-2: Classical trigeminal neuralgia with concomitant continuous pain; GoF: Gain-of-function; IGE: Idiopathic generalized epilepsy; iTN-1: Idiopathic trigeminal neuralgia purely paroxysmal; iTN-2: Idiopathic trigeminal neuralgia with concomitant continuous pain; LoF: Loss-of-function; nRT: Reticular thalamic neuron; PA: Primary aldosteronism; SpV: Spinal trigeminal nucleus; TG: Trigeminal ganglion; TN: Trigeminal neuralgia; VPM: Ventroposterior nucleus; WT: Wild-type.

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Author contributions

ERM, EG, RNS, and IAS performed experiments and analyzed the data. ERM performed the computational simulation. GWZ and NW designed and supervised the study and wrote the manuscript. All authors critically revised the manuscript and contributed significantly to this work. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors have no competing interests to declare.

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Publisher's Note

Paper 7

Synthesis and pharmacological evaluation of quinolone-based calcium channel blockers with analgesic properties (Unpublished)

Leoš Cmarko, Mikhail Klychnikov, Kimberly Gomez, Robin N. Stringer, Samantha Perez-Miller, Miroslav Hájek, Michel De Waard, Rajesh Khanna, Ullrich Jahn, Norbert Weiss

Synthesis and pharmacological evaluation of quinoline-based calcium channel blockers with analgesic properties

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ABSTRACT

In the neurons of the dorsal root ganglia (DRG), various voltage-gated calcium channels (VGCCs) play a role in the processing of peripheral nociceptive information, serving as validated targets for pain therapeutics. Despite significant efforts to develop selective pharmacological blockers of VGCC subtypes for pain management, progress in clinical translation has been slow. Alternatively, polypharmacological blockers targeting multiple VGCC subtypes may offer additional advantages over highly selective inhibitors due to their synergistic activity. Previously, we identified surfen (*bis(2-methyl-4-amino-quinolin-6-yl)urea) as a broad-spectrum VGCC blocker with analgesic properties. In this study, a series of 15 quinoline-based surfen analogs were rationally synthesized and evaluated for their pharmacological activity on VGCCs. Our results demonstrate that compound* ***S13*** *exhibits improved cell tolerance compared to the reference compound surfen, while maintaining blocking activities on several recombinant VGCCs subtypes, including the primary pain-relevant* $Ca_v2.2$ *(N-type) and* $Ca_v3.2$ *(T-type) channels. Molecular docking analyses predicted direct binding of* ***S13*** *to* $Ca_v2.2$ *and* $Ca_v3.2$*, suggesting potential interactions with ion-conducting pathways. Additional electrophysiology analyses of acutely dissociated DRG neurons in culture confirmed the blocking activity of* ***S13*** *on both native low-voltage-activated (LVA) and high-voltage-activated (HVA) VGCCs, while sparing sodium and potassium channels. Notably, we show that intrathecal administration of* ***S13*** *in a preclinical rat model of nerve ligation-induced mechanical allodynia produced substantial antinociceptive effects. Altogether, these findings underscore the potential of broad-spectrum VGCC blockers for pain therapy, and establish the quinoline-based backbone structure of* ***S13*** *as the basis for the development of novel analgesics.*

KEYWORDS

Calcium channels, Voltage-gated calcium channels, Quinoline, Molecular docking, Pain, Neuropathy

INTRODUCTION

Voltage-gated calcium channels (VGCCs) play an essential role in processing peripheral nociceptive information within the neurons of the dorsal root ganglia (DRG) [10]. Among the diverse VGCCs expressed in DRG neurons [14, 47], two stand out as primary contributors to pain signaling in primary afferent nociceptive nerve fibers: the low-voltage-activated (LVA) $Ca_v3.2$ (T-type) and the high-voltage-activated (HVA) $Ca_v2.2$ (N-type) channels. For example, $Ca_v3.2$ channels, found in the soma and along the axon [32], drive neuronal excitability [26]. Moreover, presynaptic $Ca_v3.2$ channels directly participate in nociceptive transmission between primary afferent fibers and second-order neurons of the lamina I and II in the dorsal horn of the spinal cord [20]. Likewise, $Ca_v2.2$ channels, predominantly located in presynaptic nerve terminals, facilitate the release of key pronociceptive neurotransmitters such as glutamate, substance P, and calcitonin gene-related peptide (CGRP) [13, 24, 33, 39]. Beyond $Ca_v3.2$ and $Ca_v2.2$ channels, there is also evidence pointing to the involvement other VGCC members, especially the HVA $Ca_v1.2$ [7, 29, 31] and $Ca_v2.3$ channels [6] to the processing of peripheral nociception. Consequently, selective inhibition of VGCC subtypes with small organic molecules or peptides has been recognized for its potential in mediating analgesia in a wide range of preclinical rodent pain models [27, 48]. Significant effort has been directed towards the development of selective VGCC blockers for pain therapy. However, progress in clinical development of new drugs for chronic pain treatment has been slow. Despite promising preclinical drug candidates, no selective small-molecule channel blockers have received clinical approval thus far [40]. The limitations of established rodent pain models in predicting drug responses may be one factor, but it is also evident that these molecules often fail due to their adverse side effects.

An alternative approach may hinge on broad-spectrum calcium channel blockers with relatively lower affinity. Such molecules offer dual advantages: 1) their lower affinity may be less harmful to tissues beyond the pain pathway, which generally display a more limited diversity of VGCCs, and 2) their analgesic effect arises from the synergistic blocking of multiple VGCC subtypes.

Our previous findings indicate that surfen (*bis(2-methyl-4-amino-quinolin-6-yl)urea) possesses analgesic properties in mouse models of acute and chronic inflammatory pain* [30]. *These analgesic effects were primarily attributed to the ability of surfen to inhibit VGCCs, irrespective of the specific channel isoform. As part of our ongoing endeavors to develop broad-spectrum calcium channel blockers for pain therapy, we rationally designed and synthesized a series of surfen analogs to generate structure-activity relationships, and assessed*

their pharmacological activity on heterologous and native VGCCs. Among these 16 compounds, ***S13*** *emerged as noteworthy, showing enhanced tolerance in cell toxicity assays compared to surfen, all the while retaining its ability to inhibit VGCCs. Molecular docking analyses provide further support for presumptive direct binding of* ***S13*** *to the channels. Importantly, in vivo evaluation of* ***S13*** *revealed potent analgesic effects in a preclinical rat pain model of neuropathic pain.*

RESULTS AND DISCUSSION

Chemical synthesis of quinoline-based compounds. The synthesis of the quinoline derivatives **S1** was performed according to the literature [35] by condensation of 5-nitro-2-aminobenzonitrile and acetone in the presence of tin tetrachloride and subsequent reduction of the nitro group to amino with Al/Ni alloy under basic conditions (**Figure 1A-B**). Quinoline **S1** and commercially available 4-desamino derivatives **S2** were subsequently subjected to selective N-acylation reaction at N6. Ureas **S5-S11** were synthesized by reaction with isocyanates (X=O), potassium cyanate (X = O, R^2 = H) or isothiocyanates (X = S). N-Quinolin-6-yl amides **S12** and **S14-S16** and carbamate **S13** were prepared by N-acylation with acid chlorides or phenyl chloroformate, respectively (see Supplemental information).

Screening of quinoline-based compounds on recombinant $Ca_v3.2$ channels. To evaluate the pharmacological activities of quinoline-based compounds, we conducted a primary screen on recombinant $Ca_v3.2$ channels expressed in tsA-201 cells using patch-clamp electrophysiology. The compounds were applied acutely at 30 μM and the steady-state inhibition was recorded (**Figure 2A**). Clear trends emerged from the results. The N'-aminoquinolinyl unit in surfen **S4** can be easily substituted by the sterically similar, yet significantly more hydrophobic, β-naphthyl unit in **S6** or the sterically less demanding N'-phenyl unit in urea **S5** without losing blocking activity. The carbonyl oxygen atom in **S4-S6** appeared to play a crucial role for the pharmacological action, as the thiourea **S7** displayed significantly less blocking activity. However, substituting the distal nitrogen atom in N'-phenylurea **S5** with a CH_2 group in phenylacetamide **S12** or an oxygen atom to O-phenyl carbamate **S13** resulted only in a slight decrease in channel blocking activity, while **S13** showed a more favorable biological profile (*vide infra*). Urea **S9**, featuring the sterically significantly different α-naphthyl group compared to surfen **S4**, **S5**, or **S6,** showed almost no blocking activity, demonstrating the importance of steric features at the N'-aryl group. However, substituting the N'-aryl unit in ureas **S4-S6** with aliphatic amide groups as in **S8** and **S15**, benzamide units as in **S14**, or having no N'-substituent at all as in **S10**, largely abolished the pharmacological activity on VGCCs. Finally, the importance of the free amino group at the 4-position of the quinoline ring is highlighted by the loss of blocking activity if it is absent, as observed in **S11**, or if acylated, as in **S16** (**Figure 2B**).

S13 is a broad-spectrum calcium channel blocker. Among the four surfen derivatives that produced greater than 50% inhibition of $Ca_v3.2$ channels (**Figure 3A**), **S13** showed significantly less cytotoxicity against various human cell lines in vitro. Notably, the half-maximal (IC_{50}) cytotoxic concentration of **S13** was approximately 5 to 7 times higher than that of our reference compound **S4** (surfen) (**Figure 3B**). Consequently, **S13** was selected as the lead compound for further analysis. To assess the pharmacological profile of **S13** across the voltage-gated calcium channel family, we conducted patch-clamp recordings in tsA-201 cells expressing recombinant channels to assess **S13** against $Ca_v1.2$ (L-type), $Ca_v2.1$ (P/Q-type), and $Ca_v2.2$ (N-type), as well as $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$ (T-type) channels. Acute application of **S13** (30 μM) resulted in pronounced inhibition of all channels, with the exception of $Ca_v2.1$, which appeared to be comparatively less sensitive (**Figure 4A**). The time course for **S13**-mediated inhibition of LVA channels was nearly instantaneous, whereas it occurred progressively over 3 to 4 minutes for HVA channels. While we cannot totally dismiss the possibility of two markedly different mechanisms of inhibition between LVA and HVA channels, one possibility for this difference could be due to **S13** binding to the channels with distinct accessibility (see Molecular docking section below for in-depth discussion). Notably, **S13** blocked the two prominent channels involved in the processing of peripheral nociception, $Ca_v3.2$ and $Ca_v2.2$ channels, by 67% and 58%, respectively (**Figure 4B**). The relative IC_{50} values were approximately 17 μM (Hill coefficient 1.8) for $Ca_v3.2$ channels (**Figure 4C**) and 25 μM (Hill coefficient 2.5) for $Ca_v2.2$ channels (**Figure 4D**).

Next, we conducted a further assessment of the effects of **S13** on the gating properties of recombinant $Ca_v3.2$ and $Ca_v2.2$ channels. T-type currents recorded in $Ca_v3.2$-expressing cells treated with 30 μM **S13** for 2 minutes were significantly reduced across a wide range of membrane potentials compared to cells treated with the vehicle (**Figure 5A**), and the maximal macroscopic T-type conductance (G_{max}) was reduced by 68% ($p<0.0001$) (**Figure 5B**). Furthermore, **S13** caused an additional depolarized shift of the voltage dependence of activation by 10.0 mV ($p<0.0001$) (**Figure 5C**), along with a depolarized shift of the voltage dependence of inactivation by 7.5 mV ($p<0.0001$) (**Figure 5D**). Likewise, **S13** caused inhibition of N-type currents in cells expressing $Ca_v2.2$ channels (**Figure 5E**) and decreased the maximal N-type conductance by 44% ($p=0.0004$) (**Figure 5F**). The voltage dependence of activation remained unaltered (**Figure 5G**), and only the voltage dependence of inactivation

was slightly shifted, by 3.5 mV (p=0.0038), in cells treated with **S13** (**Figure 5H**). Altogether, these data indicate that **S13** is effective in blocking several VGCC members including those involved in the processing of peripheral nociception.

Molecular docking of S13 to $Ca_v2.2$ and $Ca_v3.2$ channels. We used computational docking to predict binding sites for **S13**, focusing on the two primary pain-relevant channels, $Ca_v2.2$ and $Ca_v3.2$ which were most strongly inhibited by **S13**. Because a structure of $Ca_v3.2$ has not yet been published, we used our previously generated homology model [9]. Based on electrophysiology data showing minimal ($Ca_v2.2$) or moderate ($Ca_v3.2$) effects on channel gating (**Figure 5**), we docked **S13** to the extracellular vestibule and the pore (including open fenestrations) in each structure using Glide with enhanced sampling (see Methods). The top scoring sites were within the central pore for $Ca_v2.2$ (**Fig 6A**) and in the open fenestration between domains IV and I for $Ca_v3.2$ (**Fig 6B**). Each of these sites were then redocked using the Schrödinger Induced-Fit (flexible-receptor) protocol, resulting in improved docking scores for both targets due to optimization of favorable contacts (**Fig 6A-D**). In both receptors, the best **S13** pose is predicted to contact residues in the domain I S6 helix, partially obscuring the ion-conducting pathway, albeit with **S13** in different orientations (**Fig 6C-D**). This site differs from ligand binding sites observed in $Ca_v2.2$ and $Ca_v3.1$ or $Ca_v3.3$ structures to date. Cryo-EM structures of $Ca_v2.2$ showed that PD173212 and "blocker 1" bound within the open DIII-DIV fenestration and partially within the pore [8]. One overlap does occur for predicted **S13** and bound PD173212 which are both within hydrophobic contact distance of the Phe345 side-chain (**Fig 6E**). In the cases of $Ca_v3.1$ with bound Z944 [51] and $Ca_v3.3$ with mibefradil, otilonium bromide, or pimozide [15], these ligands bind to the central pore and also enter into fenestration DII-DIII to varying degrees (**Fig 6F**). In the $Ca_v3.2$ model, **S13** docks in the pore and the DIV-DI fenestration (**Fig 6F**). In this pose, **S13** is in the vicinity of two conserved contacts made by mibefradil and pimozide at Asn412 in DI-S6 (Asn388 in $Ca_v3.1$, Asn391 in $Ca_V3.3$) and at Leu1851 DIV-S6 (Leu1891 in $Ca_v3.1$, Leu1791 in $Ca_v3.3$). Interestingly, Cryo-EM structures have uncovered changes in pore-lining S6 helices with ligand binding, such as α/π transitions [46, 50, 51], straightening of kinks [50], and axial rotation [51]. S6 transitions have also been observed in other p-loop channels, including the highly structurally related Na_vs [18, 44, 49], and may impact opening and closing of the intracellular gate [4, 43]. Thus, interaction with the S6 helix suggests that **S13** could impair calcium current via direct channel block as well as through restriction of conformational transitions of the DI-S6 helix. Further, the predicted fenestration site for **S13**

for $Ca_v3.2$ is also of interest as the fenestrations, first proposed 45 years ago by Hille [16], have been found to be utilized to anchor various channel inhibitors and drugs within the pore of both Ca_v [8, 15, 46, 51] and Na_v channels [19, 21, 23, 49], demonstrating the importance of these sites for drug targeting. Recent studies in Na_v channels suggest that opening and closing of these hydrophobic drug access pathways may also be linked to α/π transitions and kinking in the S6 helices [5, 42] highlighting additional layers of complexity to inhibition of voltage-gated channels and raising a third possible mechanism for channel block by **S13**.

Finally, we venture to speculate that the slower onset of inhibition by **S13** observed for $Ca_v2.2$ channels could be due to slower entry into the pore or effects of VGCC ancillary subunits $Ca_v\beta$ and $Ca_v\alpha_2\delta$. We did not investigate docking to the latter based on the ability of **S13** to inhibit T-type channels, which are typically impervious to regulation by Ca_v ancillary subunits [22]. Finally, we also note several caveats of our *in-silico* studies. In addition to the obvious predictive nature of docking and uncertainties in homology modeling, we note that the Ca_v structures are missing about 40% of the amino acids (mainly the intracellular loops) precluding investigation into possible ligand binding sites in these regions. Thus, although our docking models are intriguing, the predicted binding sites remain to be experimentally validated.

S13 blocks voltage-activated calcium currents in DRG neurons but not sodium and potassium currents. Next, our aim was to confirm that **S13** not only inhibits recombinant calcium channels but is also effective on native channels in cultured mouse DRG neurons. We assessed the effects of **S13** on voltage-activated calcium currents in medium-sized DRG neurons, corresponding to thinly myelinated nociceptive Aδ fibers known to express both LVA and HVA calcium channels [2]. In line with our observations on recombinant VGCCs, acute application of 30 μM **S13** resulted in a significant reduction of both LVA and HVA calcium currents (**Figure 7A**). The maximal steady-state inhibition was 62% and 64% for LVA and HVA currents, respectively (**Figure 7C**). Our findings, showing that **S13** effectively inhibits LVA calcium currents, confirm its action on native $Ca_v3.2$ channels since $Ca_v3.2$ is the primary T-type channel isoform responsible for carrying LVA currents in DRG neurons [2]. On the other hand, the HVA calcium conductance in rodent DRG neurons comprises a combination of N-type (39%), P/Q-type (20%), L-type (22%), and R-type (19%) currents [14]. Therefore, our observation that **S13** blocked 64% of the total HVA current suggests that **S13** is also effective

on multiple native HVA channels, as none of them individually accounts for more than 40% of the total HVA current conductance. In contrast, **S13** had very minimal effects on total voltage-activated sodium and potassium currents (**Figure 7B**), causing only 8% and 9% inhibition, respectively (**Figure 7C**). Nonetheless, we note that **S13** induced a mild yet consistent slowdown of the inactivation kinetics of the sodium current. Altogether, these findings confirm that **S13** primarily functions as a broad-spectrum VGCC blocker, effectively reducing the calcium conductance in nociceptive DRG neurons.

S13 shows antinociceptive effects in a preclinical pain model of SNL-induced mechanical hyperalgesia. The data above revealed that pronociceptive VGCCs are the primary target of **S13**. Therefore, we sought to determine whether treatment with **S13** would demonstrate efficacy in a preclinical rat model of neuropathic pain induced by spinal nerve ligation (SNL). SNL induces partial denervation of the sensory zone of the spinal nerve, resulting in mechanical allodynia as evidenced by a pronounced decreased of the paw withdrawal thresholds (PWT) within 10 days post-surgery (**Figure 8A**). In this allodynic state, intrathecal treatment of male animals with **S13** (10 μg/5μL) resulted in a significant increase in PWT within 1 h after treatment (**Figure 8A**). The reversal of allodynia persisted for approximately 3 h post-treatment, as evidenced by the increase of the PWT integral compared to vehicle-treated animals (**Figure 8B**). We then conducted a similar experiment with female rats to determine whether there were any sex-based differences in the analgesic effects observed for **S13**. As shown in **Figure 8C-D**, **S13** was also effective at reversing mechanical hyperalgesia in female rats, indicating that its analgesic actions in rats are independent of sex. Collectively, these data demonstrate the antinociceptive potential of **S13** in experimentally induced neuropathic pain.

CONCLUSIONS

Previous studies have documented the analgesic effects of mixed VGCC blockers. For example, A-1264087, which acts as a mixed blocker of $Ca_v2.1$, $Ca_v2.2$, and Ca_v3 channels, has been shown to mitigate SNL-induced mechanical hyperalgesia [45, 52]. Similarly, TROX-1 exhibits inhibition of $Ca_v2.1$, $Ca_v2.2$, and $Ca_v2.3$ channels with comparable efficacy [41] and has been demonstrated to reverse inflammatory-induced hyperalgesia and nerve-injury-induced allodynia [1, 28]. In addition to these synthetic compounds, the natural product physalin F serves as a dual blocker of $Ca_v2.2$ and $Ca_v2.3$ channels, offering relief from mechanical hyperalgesia in rodent models of neuropathic pain [36]. Moreover, indirect modulation of $Ca_v2.2$ channels with a peptidomimetic was shown to be analgesic in models of neuropathic and inflammatory pain [13]. In this study, we introduce **S13**, a promising quinoline-based compound that exhibits activity against several pronociceptive VGCCs. **S13** effectively inhibits voltage-activated calcium currents in nociceptive DRG neurons while sparing sodium and potassium currents. Notably, **S13** demonstrates substantial potential as an analgesic agent in a preclinical rat model of SNL-induced neuropathic pain, successfully alleviating mechanical allodynia in both male and female animals. These findings further underscore the therapeutic promise of broad-spectrum calcium channel blockers in mitigating neuropathic pain and suggest that the quinoline backbone structure may serve as a valuable platform for the development of novel derivatives with analgesic properties for further preclinical investigation.

MATERIAL AND METHODS

Synthesis of quinoline-based compounds. See Supplemental information.

Compound preparation. All compounds were dissolved in DMSO at a stock concentration of 30 mM, aliquoted, and stored at -20ºC. For patch clamp recordings, compounds were diluted directly into the bath solution at the desired concentration, ensuring that the final DMSO concentration never exceeded 1/1000.

Heterologous expression of voltage-gated calcium channels. Human embryonic kidney tsA-201 cells were grown in DMEM high glucose medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (all media purchased from Invitrogen) and maintained under standard conditions at 37ºC in a humidified atmosphere containing 5% CO_2. Cells were transfected using the calcium/phosphate method with plasmid cDNAs encoding for the human $Ca_v1.2$ and $Ca_v2.1$ (along with $Ca_v\beta$ and $Ca_v\alpha2\delta$-1 ancillary subunits), $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$ channels. Patch-clamp recordings were performed 72h after transfection. The CHO cell line stably expressing the rat $Ca_v2.2$ channel is a generous gift from Dr. Klugbauer (University of Freiburg) and was previously described [25].

Isolation of DRG neurons. Lumbar L4-L6 dorsal root ganglia (DRGs) from adult C57 male and female mice were harvested has previously described [38] and dissociated enzymatically with 1 mg/mL collagenase (Sigma-Aldrich) in DMEM medium for 1 h at 37ºC, followed by mechanical trituration. Cells were seeded onto 12 mm glass coverslips coated with Poly-L-Lysine (Sigma-Aldrich) in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin (Thermo-Fisher). The cells were maintained at 37ºC in 5% CO_2 for 24 h before patch-clamp recordings.

Whole-cell patch clamp recordings (manual). Whole-cell patch clamp recordings were performed at room temperature (18-22 °C) using an Axopatch 200B amplifier in voltage-clamp mode and acquisition was performed using pClamp 10 (Axon Instruments). The linear leak component of the current was corrected using a P/4 subtraction protocol and current traces were digitized at 10 kHz and filtered at 2 kHz. Recording of recombinant VGCCs was performed in a bath solution containing (in millimolar): 5 $BaCl_2$, 5 KCl, 1 $MgCl_2$, 128 NaCl, 10 TEA-Cl, 10

D-glucose, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.2 with NaOH). Patch pipettes were filled with a solution containing (in millimolar): 110 CsCl, 3 Mg-ATP, 0.5 Na-GTP, 2.5 $MgCl_2$, 5 D-glucose, 10 EGTA, and 10 HEPES (pH 7.4 with CsOH), and had a resistance of 2–4 MΩ. For recording of VGCCs in DRG neurons, the extracellular solution contained (in mM): 2 $CaCl_2$, 160 TEA-Cl, 10 Glucose and 10 HEPES (pH 7.4). The intracellular solution contained (in mM): 134 CsCl, 10 EGTA, 10 HEPES, 4 Mg-ATP and 0.1 Leupeptin (pH 7.2). For recording of voltage gated sodium channels in DRG neurons, the extracellular solution contained (in mM): 128 NaCl, 2 $CaCl_2$, 5 KCl, 1 $MgCl_2$, 10 TEA-Cl, 10 Glucose and 10 HEPES (pH 7.4). The intracellular solution contained (in mM): 110 CsF, 2.5 $MgCl_2$, 5 Glucose, 10 EGTA, 10 HEPES, 3 Mg-ATP, 0.5 Na_2-GTP (pH 7.4). For recording of voltage-gated potassium channels in DRG neurons, the extracellular solution contained (in mM): 138 NaCl, 2 $CaCl_2$, 5 KCl, 1 $MgCl_2$, 10 Glucose and 10 HEPES (pH 7.4). The intracellular solution contained (in mM): 140 KCl, 4 NaCl, 1 $MgCl_2$, 10 EGTA, 10 HEPES and 2 Na_2-GTP (pH 7.4).

Whole-cell patch clamp recordings (automated). Some of the recordings of recombinant $Ca_v3.2$ and $Ca_v2.2$ channels (presented in Figure 5) were conducted using the SyncroPatch 384PE (Nanion) at room temperature (18-22ºC). Pulse generation and data acquisition were carried out with PatchControl384 v1.9.7 software (Nanion) and the Biomek v1.0 interface (Beckman Coulter). The extracellular solution contained (in mM): 10 $CaCl_2$, 140 NaCl, 4 KCl, 1 $MgCl_2$, 5 Glucose and 10 HEPES (pH 7.4). The intracellular solution contained (in mM): 10 CsCl, 110 CsF, 10 NaCl, 10 EGTA, and 10 HEPES (pH 7.2).

Electrophysiological analysis. The voltage dependence of activation of recombinant $Ca_v3.2$ and $Ca_v2.2$ channels was determined by measuring the peak of the currents in response to depolarizing steps from a holding potential of -120 mV ($Ca_v3.2$) and -100 mV ($Ca_v2.2$). The current-voltage relationship (I/V) curve was fitted with the following modified Boltzmann equation (1):

$$(1) \qquad I(V) = Gmax \frac{(V - V\mathrm{rev})}{1 + \exp\frac{(V0.5 - V)}{k}}$$

with $I(V)$ being the peak current amplitude at the command potential V, G_{rev} the maximum conductance, V_{rev} the reversal potential, $V_{0.5}$ the half-activation potential, and k the slope factor. The voltage dependence of the whole-cell calcium conductance was calculated using the following modified Boltzmann equation (2):

$$(2) \qquad G(V) = \frac{Gmax}{1 + \exp\frac{(V0.5 - V)}{k}}$$

with $G(V)$ being the conductance at the command potential V. The voltage dependence of the steady-state inactivation was determined by measuring the peak of the current amplitude in response to a 200 ms depolarizing step to +10 mV (Cav3.2) and +40 mV ($Ca_v2.2$) applied after a 1 s-long conditioning prepulse ranging from -120 mV to +80 mV. The current amplitude obtained during each test pulse was normalized to the maximal current amplitude and plotted as a function of the prepulse potential. The voltage dependence of the steady-state inactivation was fitted with the following two-state Boltzmann function (3):

$$(3) \qquad I(V) = \frac{Imax}{1 + \exp\frac{(V - V0.5)}{k}}$$

with I_{max} corresponding to the maximal peak current amplitude and $V_{0.5}$ to the half-inactivation voltage.

Cytotoxicity assay. Cervix cancer (HeLa), hepatocellular carcinoma (Hep G2), acute lymphoblastic leukemia (CCRF-CEM), and acute promyelocytic leukemia (HL-60) human cell lines were purchased from ATCC (LGC Standards Sp. z o.o., Poland). HeLa cells were cultured in DMEM high glucose medium, CCRF-CEM and HL-60 cells in RPMI-1640 medium (Dutch modification), and Hep G2 cells in αMEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 2 mM glutamine at 37°C in a humidified atmosphere containing 5% CO_2. All media and supplements were purchased from Sigma-Aldrich. The CellTiter-Glo® 2.0 Cell Viability Assay kit (Promega) was used to measure the cytotoxicity of tested compounds. Following the seeding of cells (20 µL) into white 384-well plates (Thermo Fisher Scientific Nunc™), cells were grown for 24 h before the addition of compounds or

DMSO (used as a vehicle control) into each well. Post 72 h treatment, CellTiter-Glo® reagent (20 μL) was added to each well, and the content was mixed on an orbital shaker in the dark for 2 min at 400 rpm. Subsequently, the luminescent signal was allowed to stabilize for 10 min at room temperature. Luminescence readings were taken using a microplate reader (Cytation 3, BioTek, USA). In this assay, the luminescence directly correlates with the cell number. Cytotoxicity is expressed using IC_{50} values, representing the concentration of a tested compound that reduces the number of viable cells by half. The data were normalized, and IC_{50} values were calculated using nonlinear regression analysis, assuming a sigmoidal concentration-response curve with a variable Hill slope (GraphPadPRISM® 7 software).

Molecular docking analysis. Protein and ligand preparation and docking were conducted using the Schrödinger Docking Suite (Schrödinger Release 2023-2: Schrödinger, LLC, New York, NY, 2023). Ligands were prepared using LigPrep with possible ionization states at pH 7.0 [34]. The cryo-EM structure of $Ca_v2.2$ (PDB ID: 7mix [12]) was prepared using the Protein Preparation Wizard [34]. The homology model of $Ca_v3.2$ was described previously [9]. Docking was conducted using Glide in Standard Precision (SP) mode with enhanced sampling (4x) to obtain up to 100 poses per site [11]. Schrödinger Induced-Fit Docking (IFD) was used for flexible receptor docking [37]. In this approach, 20 docking runs are each followed by optimization of the amino acid positions and conformations within 5 Å of the newly docked site, and the resulting ligand-receptor complexes are ranked by the energies of the resulting induced-fit complexes.

Animals. All experiments and procedures were performed in accordance with the guidelines recommended by the National Institutes of Health, the International Association for the Study of Pain, and the National Center for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) guidelines. Pathogen-free adult male and female Sprague-Dawley rats (100-150 g; Charles River Laboratories, Wilmington, MA) were used for behavioral experiments. Animals were housed in the New York University's Kriser Dental Center Animal Facility in light/dark cycle (12-h light: 12-h dark cycle; lights on at 07:00 h) and temperature (23 ± 3°C) controlled rooms, with standard rodent chow and water *ad libitum*. The Institutional Animal Care and Use Committees of the College of Dentistry at New York University approved all experiments.

L5/L6 spinal nerve ligation. Male and female rats (~150 g) were deeply anesthetized with isoflurane (4% for induction and 2% for maintenance). The lower half of the animal's back was shaved. After surgical preparation, the left L5 and L6 spinal nerves were exposed by removing the paraspinal muscles and ligated with a 5-0 silk suture in a region distal to the DRG [17]. After hemostasis was confirmed, muscle and fascia were closed in layers using 5-0 absorbable suture, and the skin was closed with wound clips. Animals were allowed to recover for 10 days.

Intrathecal administration. Ten days after spinal nerve ligation, **S13** was injected intrathecally (10 μg/5 μL) between L4/L5 intervertebral level into isoflurane anesthetized rats (4% for induction and 2% for maintaining) and behavior was measured every hour for 6 hours.

Measurement of mechanical allodynia. Mechanical allodynia was assessed by measuring rats' paw withdrawal threshold in response to probing with a series of fine calibrated filaments (von Frey, Stoelting, Wood Dale, IL). Rats were placed in suspended plastic cages with a wire mesh floor, and each von Frey filament was applied perpendicularly to the plantar surface of the paw. The "up-down" method (sequential increase and decrease of the stimulus strength) was used to determine the withdrawal threshold Dixon's nonparametric method was used for data analysis, as described by Chaplan et al. [3]. Data were expressed as the paw withdrawal threshold. Mechanical allodynia was manifested as a decrease in paw withdrawal threshold.

Data analysis and statistics. Data values are presented as mean ± S.E.M for n measurements. Statistical significance was evaluated by one-way or two-way ANOVA followed by a Turkey's test with GraphPad Prism 7 and datasets were considered significantly different for $p<0.05$.

AUTHOR CONTRIBUTIONS

M.K. synthesized the compounds. L.C. and R.N.S. performed electrophysiological recordings and analyzed the data. K.G. performed the behavioral analysis. S.P.M. performed the molecular docking analysis. M.H. performed the cytotoxicity analysis. N.W., U.J., R.K., and M.D.W. supervised the study. N.W. wrote the first draft of the manuscript. All authors edited and approved the final manuscript.

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FIGURE LEGENDS

Figure 1. Synthesis of quinoline-based compounds. (A) Schematic diagram of the various quinoline-based compounds synthesized and tested for their effects on voltage-gated calcium channels. (B) Chemical characteristics of synthesized compounds.

Figure 2. Screening of quinoline-based compounds on recombinant $Ca_v3.2$ channels. (A) Mean percentage of current inhibition produced by acute application of 30 μM quinoline-based compounds (*n* = 4-6 per compound). T-type currents were elicited by a step depolarization to -20 mV from a holding potential of -100 mV. (B) Summary of the structure-activity relationship (SAR). The structure of surfen (**S4**) is shown as reference compound.

Figure 3. In vitro cytotoxicity of quinoline-based compounds. (A) Structures of the four most potent compounds (**S5**, **S6**, **S12**, and **S13**) for their blocking activity on recombinant $Ca_v3.2$ channels. The structure of **S4** (surfen, reference compound) is shown for comparison. (B) Corresponding mean half-maximal cytotoxic concentration (IC_{50}) on several human cancer cells and primary fibroblasts (*n* = 3 per compound). CCRF-CEM, human lymphoblastic leukemia; HeLa, human cervical carcinoma; HepG2, human liver cancer; HL-60, human promyelocytic leukemia.

Figure 4. Pharmacological evaluation of **S13** across recombinant voltage-gated calcium channels. (A) Representative time course of current inhibition along with whole-cell current traces recorded from cells expressing LVA ($Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$) and HVA ($Ca_v1.2$, $Ca_v2.1$, and $Ca_v2.2$) channels in response to a step depolarization to -20 mV (LVA channels) and +10 mV (LVA channels) from a holding potential of -100 mV, before (black traces) and after (blue traces) acute application of **S13** (30 μM). (B) Corresponding mean percentage of maximal current inhibition (*n* = 4-6 per channel). (C-D) Corresponding dose-response curves of **S13** for $Ca_v3.2$ and $Ca_v2.2$ channels (*n* = 16-25 for each concentration).

Figure 5. Effects of **S13** on the gating properties of recombinant $Ca_v3.2$ and $Ca_v2.2$ channels. (A) Mean current-voltage (*I*/*V*) relationships of recombinant $Ca_v3.2$ channels recorded by automated patch-clamp in cells pre-treated for 2 min with vehicle (DMSO) and 30 μM **S13**

(blue symbols). The continuous lines represent the fit of the *I*/*V* curves with the modified Boltzmann Eq. (1). (B) Corresponding mean maximal macroscopic conductance values (G_{max}) obtained from the fit of the *I*/*V* curves. (C) Corresponding mean normalized voltage dependence of activation of $Ca_v3.2$ channels fitted (continuous lines) with the modified Boltzmann Eq. (2). The inset shows the mean half-activation potential values obtained from the fit of the conductance curves. (D) Mean normalized voltage dependence of steady-state inactivation of $Ca_v3.2$ channels fitted (continuous lines) with the two-state Boltzmann Eq. (3). The inset shows the mean half-inactivation potential values obtained from the fit of the inactivation curves. (E-H) Legend same as (A-D) but for recombinant $Ca_v2.2$ channels. Statistical significance was evaluated by one-way ANOVA in comparison to vehicle-treated cells.

Figure 6. Molecular docking of **S13** on $Ca_v2.2$ and $Ca_v3.2$ channels. (A-B) $Ca_v2.2$ structure (PDB ID: 7mix) [12] and $Ca_v3.2$ homology model [9] with top docking poses from Glide enhanced sampling (black lines) and from induced-fit (black sticks). The docking scores are given in kcal/mol, bold for induced-fit results. (C-D) Close-up views of best induced-fit poses for each structure (view rotated by 90° from top panels), with potential contacts within 4 Å shown as dashed lines. Domains colored as indicated, key helices labeled for domain I in lower panels. (E) Close-up view from the extracellular side showing best induced-fit pose for $Ca_v2.2$ overlaid with structures of $Ca_v2.2$ with bound PD173212 (PDB ID: 7vfv) and "blocker 1" (PDB ID: 7vfw) [8]. (F) Close-up view as in (E) showing best induced-fit pose for $Ca_v3.2$ overlaid with structures of $Ca_v3.1$ with bound Z944 (PDB ID: 6kzp) [51] and $Ca_v3.3$ with bound mibefradil, otilonium bromide, or pimozide (PDB IDs: 7wlj, 7wlk, 7wli, respectively) [15].

Figure 7. Effects of **S13** on native voltage-gated calcium (Ca_v), sodium (Na_v), and potassium (K_v) channels in DRG neurons. (A) Representative calcium current traces recorded in medium-sized DRG neurons in response to a step depolarization to -40 mV (LVA) channels, left panel) and +10 mV (HVA channels, right panel) from a holding potential of -100 mV, before (black traces) and after (blue traces) acute application of **S13** (30 μM). (B) Legend same as (A) but for sodium (left panel) and potassium (right panel) currents elicited by a step depolarization to -10 mV and +50 mV, respectively. (C) Corresponding mean percentage of current inhibitions.

Figure 8. Analgesic effect of **S13** on nerve injury-induced mechanical hyperalgesia. (A) Mean paw withdrawal threshold (PWT) in adult male rats before (BL, base line), and after 10 days following spinal nerve ligation (Pre-drug). A single intrathecal injection of **S13** (10 μg/5 μL, blue circles) produced a significant reduction of mechanical allodynia, which was not observed in control animals injected with saline (open circles). (B) Corresponding mean integral of the PWT measured over 6h following drug treatment. (C-D) Legend same as (A-B) but for female rats. Statistical significance was evaluated by two-way ANOVA followed by Turkey's post hoc test in comparison to vehicle-treated animals (panels A and C), and by Mann-Whitney test (panels B and D).