

# Regulation of the $\text{Ca}_v3.2$ calcium channels in health and disease

## Regulácia $\text{Ca}_v3.2$ vápnikových kanálov v zdraví a chorobe

Original Paper

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**Abstract** Family of T-type or low-voltage activated calcium channels consists of three members:  $\text{Ca}_v3.1$ ,  $\text{Ca}_v3.2$ , and  $\text{Ca}_v3.3$ .  $\text{Ca}_v3.2$  channel has almost identical biophysical properties as the  $\text{Ca}_v3.1$  channel, but is distinguished by a specific tissue expression profile and a prominent role in several pathologies, including neuropathic pain, epilepsy, and dysregulation of cardiac rhythm. Further, it may be involved in phenotype of autism spectrum disorders, and amyotrophic lateral sclerosis. It represents a promising target for future pharmacotherapies.

**Slovak abstract** Skupina nízkonapätových vápnikových kanálov nazývaných aj kanály T-typu má troch členov:  $\text{Ca}_v3.1$ ,  $\text{Ca}_v3.2$ , a  $\text{Ca}_v3.3$ .  $\text{Ca}_v3.2$  kanál má temer identické biofyzikálne vlastnosti ako  $\text{Ca}_v3.1$  kanál, ale líši sa od neho tým, že je exprimovaný v iných tkanivách a má dôležitú úlohu pri viacerých závažných ochoreniach ako sú neuropatická bolesť, epilepsia a poruchy regulácie srdcového rytmu. Preto tento kanál môže byť cieľom nových liečiv.

**Keywords** T-type calcium channel –  $\text{Ca}_v3.2$  – regulation – trafficking – glycosylation

**Kľúčové slová:** vápnikové kanály T-typu – regulácia  $\text{Ca}_v3.2$  kanála – transport do membrány – glykozylácia

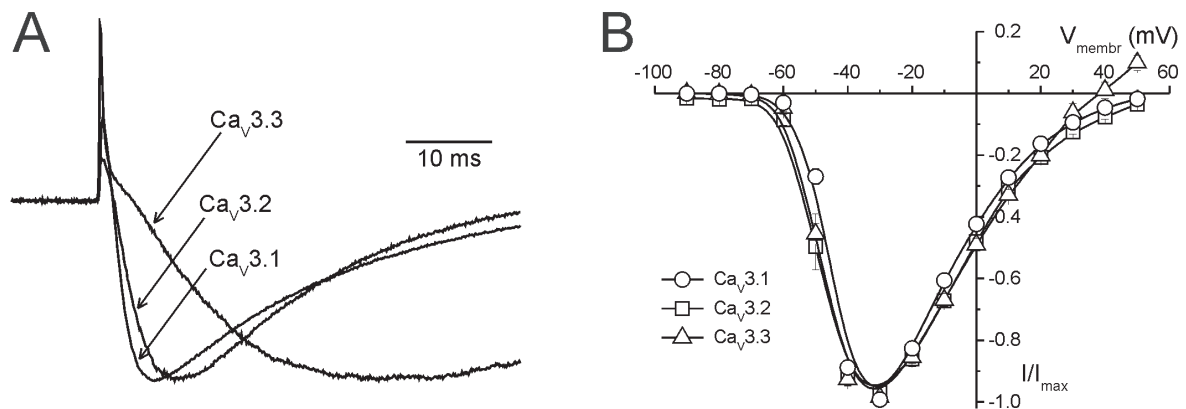
## INTRODUCTION

T-type calcium channels, also known as low-voltage-activated calcium channels, or  $\text{Ca}_v3$  channels, are characterised by a relatively negative voltage activation threshold around -60 mV. This feature enables them to operate near the resting membrane potential of most electrically excitable cells and contribute to the initiation of an action potential. T-type calcium channels play a fundamental role in shaping neuronal excitability, as they generate the so-called low threshold calcium action potential in neurons lacking sodium channels and support burst firing (Perez-Reyes, 2003). In the heart, they contribute to the pacemaker function of sinoatrial nodal cells. Three subtypes of  $\text{Ca}_v3$  channels are currently known:  $\text{Ca}_v3.1$ ,  $\text{Ca}_v3.2$  and  $\text{Ca}_v3.3$ , with  $\alpha_1$  subunits encoded by CACNA1G, CACNA1H, and CACNA1I genes (Lacinova et al., 2000) 2000.  $\text{Ca}_v3.1$  and  $\text{Ca}_v3.2$  channels share the basic biophysical properties, for example, kinetics of activation and inactivation (Figure 1 A),

voltage dependence of activation and inactivation (Figure 1 B), and kinetics of recovery from an inactivation (Lacinova, 2005).  $\text{Ca}_v3.3$  channels have distinguished biophysical properties (Chemin et al., 2002) having one decimal order slower activation and inactivation kinetics (Figure 1 A), but virtually identical voltage dependence of activation (Figure 1 B) compared to the  $\text{Ca}_v3.1$  and  $\text{Ca}_v3.2$  channels. These channels are expressed almost exclusively in the brain (Monteil et al., 2000).  $\text{Ca}_v3.1$  and  $\text{Ca}_v3.2$  channels are ubiquitously expressed; nevertheless, their expression patterns only partly overlap in the mammalian brain (Lacinova, 2004; Aguado et al., 2016) and throughout the mammalian body at large. In addition to neuronal tissue,  $\text{Ca}_v3.2$  was identified in the heart (Cribbs et al., 1998), skeletal muscle (Berthier et al., 2002), pancreas (Braun et al., 2008), kidney (Hayashi et al., 2007), and also in female (Ohkubo et al., 2005), and male reproductive tissues (Darszon et al., 2006).

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**Figure 1.** Basic biophysical profile of T-type calcium channels. *a.* Examples of current traces activated by a rectangular depolarising pulse from a potential of -100 mV to a -20 mV. Traces corresponding to individual channel subtypes (Ca<sub>v</sub>3.1, Ca<sub>v</sub>3.2, and Ca<sub>v</sub>3.3) are marked. *b.* Current-voltage relations for all three subtypes (Ca<sub>v</sub>3.1, Ca<sub>v</sub>3.2, and Ca<sub>v</sub>3.3) of T-type calcium channel, as marked.

The altered function of T-type calcium channels was implied in several pathological states. Ca<sub>v</sub>3.2 channels seem to be of a particular importance as they were implied in several heritable diseases.

### CA<sub>v</sub>3.2 CHANNELS IN NEURONS

Typical resting membrane potential of most neurons corresponds to a voltage range at which T-type calcium channels are partly inactivated. Minor hyperpolarisation recruits additional T-type calcium channels, enhances T-type calcium current and enables the generation of low-threshold action potentials. Low-threshold action potentials form the backbone for neuronal burst firing (Huguenard, 1998). Ability of Ca<sub>v</sub>3 channels to initiate an action potential firing may contribute to epileptiform activity. Indeed, mutations in Ca<sub>v</sub>3.2 channel were associated with the childhood absence epilepsy (Chen et al., 2003) and with other forms of idiopathic generalised epilepsy (Heron et al., 2004; Heron et al., 2007). Identified mutations were either gain-of-function or did not alter electrophysiological properties of the channel. Mechanism by which these mutations increase neuronal excitability, and subsequently increase seizure susceptibility, was investigated (Eckle et al., 2014). These authors suggested that increased current density caused by an epilepsy mutation C456S may be at least partly due to the increased surface expression of Ca<sub>v</sub>3.2 channels. Further, this mutation enhanced glutamatergic transmission by the enhancement of local calcium influx at synapses (Wang et al., 2015). It is interesting that in spite of that, Ca<sub>v</sub>3.1 channel has the same electrophysiological profile as Ca<sub>v</sub>3.2 channel, Ca<sub>v</sub>3.1<sup>-/-</sup> mice are resistant to baclofen-induced epilepsy (Kim et al., 2001), and overexpression of Ca<sub>v</sub>3.1 channel induced absence epilepsy in mice (Ernst et al., 2009), no consistent linkage of this channel to epilepsy was found until now.

Autism spectrum disorders (ASD) represent complex neurodevelopmental conditions. Causes of ASD remain a

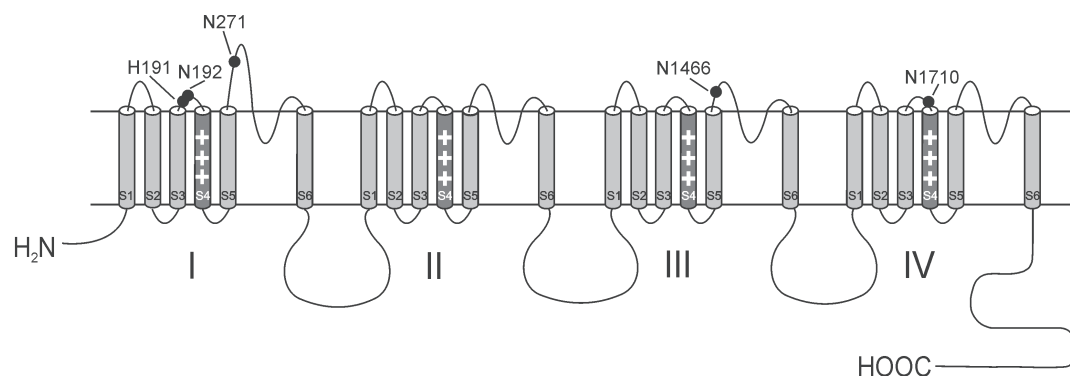
mystery, however, heritable components were demonstrated (Zoghbi, 2003). Interestingly, mutations in voltage-dependent calcium channels associated with ASD were reported. Initially, the gain-of-function mutation of Ca<sub>v</sub>1.2 L-type calcium channel was described by Splawski and coauthors (Splawski et al., 2004; Splawski et al., 2005). Soon, a group led by the same authors reported four loss-of-function point mutations in Ca<sub>v</sub>3.2 channel, associated with ASD (Splawski et al., 2006). How an altered conductance of Ca<sub>v</sub>3.2 channels arising from these mutations may lead to ASD phenotype remains unknown.

Two rare recessive variants of the CACNA1H gene associated with sporadic amyotrophic lateral sclerosis (ALS) were recently discovered (Steinberg et al., 2015). When these missense mutations (V1689M and A1705T) were introduced into recombinant channels and expressed, they produced moderate but significant changes in the channel properties consistent with loss-of-function of the mutated channel (Rzhetsky et al., 2016). Mathematical modelling suggested that these changes may result in decreased excitability of thalamic neurons (Rzhetsky et al., 2016).

Key role of neuronally expressed Ca<sub>v</sub>3.2 channels in nociception was established (Todorovic and Jevtovic-Todorovic, 2013). Downregulation of these channels in rat dorsal root ganglion neurons resulted in major antinociceptive, anti-hyperalgesic, and anti-allodynic effects (Bourinet et al., 2005). Inhibition of Ca<sub>v</sub>3.2 channels contributed to antinociceptive effect of epipregnanolone (Ayoola et al., 2014), KYS-05090S (M'Dahoma et al., 2016), and to the peripheral anti-nociceptive effect of substance P (Huang et al., 2016). Ca<sub>v</sub>3.2 channel emerged as a prospective target of future analgesics.

### CA<sub>v</sub>3.2 CHANNELS IN THE CARDIOVASCULAR SYSTEM

Ca<sub>v</sub>3.2 channel gene CACNA1H was originally cloned from a human heart by a screening of cDNA library with the Ca<sub>v</sub>3.1 gene (Cribbs et al., 1998). These channels are expressed in



**Figure 2. Scheme of T-type  $\alpha 1$  subunit.**  $\alpha 1$  subunit of T-type calcium channel consists of four homological domains (I – IV). Each domain consists of six transmembrane segments S1–S6. S4 segment contains 5–6 positively charged amino acids and is responsible for voltage sensing. Segments S5–S6 create conducting pore of the channel. Location of histidine 191 (H191) and four extracellularly located asparagines (N192, N271, N1466, and N1710) are marked by filled circles.

ventricular myocytes, where they mediate chronotropic action of corticosteroids (Maturana et al., 2009) and aldosterone (Lalevee et al., 2005). In sinoatrial nodal cells,  $\text{Ca}_v3.2$  channels contribute to cardiac pacemaking due to their ability to be activated during initial slow depolarising phase of sinoatrial action potential, which enables them to contribute to this depolarisation process (Husse and Franz, 2016). Their expression in sinoatrial cells is increased in streptozotocin-induced diabetic rats (Ferdous et al., 2016) and thus, they may contribute to cardiac-related comorbidity in diabetic patients. Recently, gain-of-function mutation CACNA1H(M1549V) of the  $\text{Ca}_v3.2$  channel was identified, which results in an early onset hypertension caused by increased aldosterone production (Scholl et al., 2015).

### REGULATION OF $\text{Ca}_v3.2$ CHANNELS BY GASOTRANSMITTERS

Gasotransmitters, such as nitric oxide, carbon monoxide and hydrogen sulphide may act as calcium channel modulators. In recent years, (patho)physiological importance of  $\text{H}_2\text{S}$  is being acknowledged (Li et al., 2011). Acutely applied micromolar  $\text{H}_2\text{S}$  inhibited the current through the recombinant  $\text{Ca}_v3.2$  channel, but not through  $\text{Ca}_v3.1$  or  $\text{Ca}_v3.3$  channels (Elies et al., 2014). It was shown that  $\text{H}_2\text{S}$  interacts with the  $\text{Ca}_v3.2$  channel at an amino acid histidine in a position 191 (H191, Figure 2), which is absent in  $\text{Ca}_v3.1$  and  $\text{Ca}_v3.3$  channels (Elies et al., 2015). The same amino acid residue is responsible for channel inhibition by  $\text{Zn}^{2+}$  (Nelson et al., 2007), by  $\text{Ni}^{2+}$  (Kang et al., 2006), and for redox regulation of the channel. Elies and coauthors suggested that  $\text{H}_2\text{S}$  may enhance channel sensitivity to  $\text{Zn}^{2+}$  ions (Elies et al., 2016). However, chronic exposure to millimolar  $\text{H}_2\text{S}$  enhanced T-type calcium current in NG108-15 cells and evoked hyperalgesia in rats (Kawabata et al., 2007). Acute augmentation of the current through recombinant  $\text{Ca}_v3.2$ , but not  $\text{Ca}_v3.1$  channel was also achieved by millimolar  $\text{H}_2\text{S}$  (Elies et al., 2014). Mechanism of this dual effect remains unknown.

Carbon monoxide inhibited all three recombinant  $\text{Ca}_v3$  channels and reached half-maximal inhibition in concentration around  $3 \mu\text{M}$  (Boycott et al., 2013). The effect of nitric monoxide on T-type calcium channels was not investigated.

### REGULATION OF $\text{Ca}_v3.2$ CHANNELS BY OTHER ENDOGENOUS MOLECULES

T-type channels are also sensitive to a number of endogenous agents. For instance, arachidonic acid potently shifts the steady-state inactivation of  $\text{Ca}_v3.2$  channels by  $-25$  mV, eliminating the window current (Zhang et al., 2000). Considering that arachidonic acid is used in a number of anabolic bodybuilding supplements, the alteration of immune response may represent a side effect of these products. Endogenous cannabinoid anandamide inhibits all three  $\text{Ca}_v3$  channels with order of efficiency  $\text{Ca}_v3.2 > \text{Ca}_v3.3 > \text{Ca}_v3.1$  (Chemin et al., 2001). Inhibition of  $\text{Ca}_v3.2$  channels by endocannabinoid N-arachidonoyl dopamine (Ross et al., 2009), by endogenous lipoamino acids (Barbara et al., 2009), and by N-arachidonoyl serotonin (Gilmore et al., 2012) may contribute to their anti-nociceptive effect attributed to the interaction with TRPV1 channel.

### REGULATION OF $\text{Ca}_v3.2$ CHANNELS BY GLUCOSE

Glycosylation represents one of the most prevalent mechanisms of post-translational modification of proteins (Moremen et al., 2012). It is reversible and proteins can undergo dynamic glycosylation and deglycosylation. Asparagine (N)-linked glycosylation of various voltage- and ligand-gated channels (Ufret-Vincenty et al., 2001; Watanabe et al., 2003; Cohen, 2006) including  $\text{Ca}_v3.2$  channel (Weiss et al., 2013) was documented. Consensus N-linked glycosylation site has an amino acid sequence N-X-S/T. Four corresponding asparagines are located in the extracellular loops of the  $\text{Ca}_v3.2$  channel: N192, N271, N1466 and N1710 (Figure 2). Inhibition of N-linked glycosylation by tunicamycin reduced an expression

of the Ca<sub>v</sub>3.2 channel in cell membrane (Weiss et al., 2013) and reduced the current density. Increase of extracellular glucose concentration from 5 mM (physiological level) to 25 mM (corresponding to clinically observed hyperglycaemia) potentiated the trafficking of channel protein to the plasma membrane (Lazniewska et al., 2016) and increased the current density (Weiss et al., 2013). An uneven role of glycosylation of individual asparagines was demonstrated by a substitution of each asparagine by glutamine. Mutations N271Q and N1710Q disrupted the channel expression and no detectable current was observed in cells transfected with these constructs (Weiss et al., 2013; Ondacova et al., 2016). Total protein expression of N192Q and N1466Q channels was unaffected but their trafficking to the surface membrane was decreased (Weiss et al., 2013). Glycosylation could regulate the current amplitude solely by altering the number of functional channel expressed in the plasma membrane. Alternatively, changes in channel gating could contribute to decreased current amplitude. To test for involved mechanisms, the charge movement in wild type and mutant channels was investigated by Ondacova and coauthors. The charge movement arises from upwards movement of S4 segments in all four channel domains (Figure 2) and is proportional to the number of functional channels in a cell membrane. Ratio maximal current/maximal charge movement is proportional to the channel opening probability (Aglar et al., 2005). Individual mutations (N192Q or N1466Q) did not alter this ratio significantly. However, when both asparagines were replaced by glutamines, the ratio maximal current/maximal charge movement decreased significantly, suggesting that proper glycosylation of the Ca<sub>v</sub>3.2 channel may also upregulate the channel's opening probability (Ondacova et al., 2016). Considering that the elevated glucose caused the enhanced activity of Ca<sub>v</sub>3.2 channels, which plays an important role in the peripheral nociception, this mechanism may be involved in the genesis of diabetic neuropathy. Therefore, these channels are emerging targets for its therapy (Orestes et al., 2013).

### REGULATION OF THE CA<sub>v</sub>3.2 CHANNELS BY PHOSPHORYLATION

Ca<sub>v</sub>3.2 channels are regulated by multiple phosphorylation mechanisms. Using high resolution mass spectroscopy, 34 putative phosphorylation sites, represented by serine and threonine residues, were identified in the intracellular loops of the rat Ca<sub>v</sub>3.2 channel and 43 such sites were identified in the human Ca<sub>v</sub>3.2 channel (Blesneac et al., 2015). Dephosphorylation of the channel by non-specific phosphatase AP accelerated its activation and inactivation kinetics, and shifted the voltage dependencies of channel activation and inactivation in hyperpolarising direction

(Blesneac et al., 2015). Several of these phosphorylation sites were described previously. S1198 in the cytoplasmic loop, connecting the channel domains II and III, is phosphorylated by CaMKII (Welsby et al., 2003; Yao et al., 2006). This amino acid residue is unique to the CACNA1H sequence (Perez-Reyes, 2003). Its phosphorylation potentiates calcium entry through the Ca<sub>v</sub>3.2 channels by shifting the half-maximal activation voltage towards the more negative membrane potentials and by increasing voltage sensitivity of the channel (Welsby et al., 2003). Phosphorylation of S1107 located in the same intracellular loop by protein kinase A is necessary for channel inhibition by Gβγ dimers (Hu et al., 2009). This signalling pathway is specific for the Ca<sub>v</sub>3.2 channel and may be a mechanism mediating dopamine inhibition of this channel (Hu et al., 2009).

Vast majority of experiments are conducted at a room temperature. Chemin and coauthors demonstrated that protein kinases A and C, but not G, can enhance the current through all three Ca<sub>v</sub>3 channel subtypes at a physiological temperature (30–37 °C), while no effect was observed at room temperature (20–27 °C) (Chemin et al., 2007). This observation should be taken into account when we think about regulation of T-type calcium current in a mammalian organism.

### REGULATION OF THE CA<sub>v</sub>3.2 CHANNELS BY UBIQUITINATION

Ubiquitination is a common enzymatic post-translational modification of proteins occurring in eukaryotic cells. Ca<sub>v</sub>3.2 channels are under control by ubiquitinating and deubiquitinating enzymes as well (Zamponi et al., 2015). Deubiquitination of the Ca<sub>v</sub>3.2 channel by deubiquitinase USP5 results in an increased T-type calcium current that is responsible for inflammatory and neuropathic pain (Gadotti et al., 2015). Disrupting USP5/Ca<sub>v</sub>3.2 channel interactions prevented such pain and represents a concept for a new class of analgesics (Garcia-Caballero et al., 2014; Gadotti et al., 2015; Garcia-Caballero et al., 2016).

### CONCLUSION

Ca<sub>v</sub>3.2 channels play a unique role in nociception, in heart automaticity, in several types of epilepsy and convulsion. They may be involved in diabetic neuropathy, autism spectrum disorders, or amyotrophic lateral sclerosis. These features make them valuable pharmacological targets.

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