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T-type calcium channels in neuropathic pain

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Pain is a quite frequent complaint accompanying numerous pathologies. Among these pathological cases numerous neuropathies are retrieved with identified etiologies (chemotherapies, diabetes, surgeries...) and also more diffuse syndromes such as fibromyalgia. More broadly, pain is one of the first consequences of the majority of inherited diseases. Despite its importance for the quality of life, current pain management is limited to drugs that are either old, or with a limited efficacy or that possess a bad benefit/risk ratio. As no new pharmacological concept has led to new analgesics in the last decades, the discovery of new medications is needed, and to this aim the identification of new druggable targets in pain transmission is a first step. Therefore studies of ion channels in pain pathways are extremely active. This is particularly true with ion channels in peripheral sensory neurons in Dorsal Root Ganglia (DRG) known now to express unique sets of these channels. Moreover, both spinal and supra spinal levels are clearly important in pain modulation. Among these ion channels, we and others revealed the important role of low voltage-gated calcium channels in cellular excitability in different steps of the pain pathways. These channels, by being activated nearby resting membrane potential have biophysical characteristics suited to facilitate action potential generation and rhythmicity. In this review we will review the current knowledge on the role of these channels in the perception and modulation of pain.

Calcium channel subtypes and molecular composition

While a number of ionic conductances contribute to neuronal firing, voltage-gated calcium channels are unique in being involved in both shaping the action potential and triggering downstream an array of physiological cytoplasmic processes. Calcium entry via voltage gated calcium channels triggers cytoplasmic processes including the activation of calcium dependent enzymes, gene transcription, and the release of neurotransmitter from presynaptic nerve terminals. Most neurons express multiple types of calcium channels which

have been classified into high and low voltage activated channels based on their voltage-dependences of activation [89]. High voltage-activated calcium channels include pharmacologically distinct N-, P/-, Q-, R- and L-types, all of which are heteromultimers that consist of a pore forming $\alpha 1$ subunit which defines the channel type, plus one of four different types of β subunits and one of four different $\alpha 2$ - δ subunits as largely described in the present book. The low voltage-activated channels (also known as T-types) appear to consist only of the $\alpha 1$ subunit since its sole expression in recombinant systems recapitulates the properties of native channels. Moreover, no evidence of essential auxiliary subunits has been provided to date. The present picture arising from the completion of sequencing of multiple mammalian genomes shows that ten different types of calcium channel $\alpha 1$ subunits are distributed into 3 families (Cav1, Cav2, and Cav3) based on their homology of sequence. They correspond to the native calcium channel functionally identified in excitable cells by electrophysiological means. These subunits share a similar transmembrane topology, being comprised of four homologous domains, each containing six putative transmembrane helices (S1 through S6) plus a re-entrant pore forming loop. The four domains are connected via large cytoplasmic linker regions which are molecular platforms for protein kinases, and form interaction sites for multiple regulatory proteins, such as G protein $\beta\gamma$ subunits or the calcium channel β subunits in the case of Cav1 and Cav2 $\alpha 1$ subunits.

Different calcium channel isoforms show distinct cellular and subcellular distributions, and fulfill specific functional roles. The Cav1 family is more involved in excitation contraction coupling, while the Cav2 family members by being expressed at presynaptic terminals are more involved in the control of neurotransmitter release from nerve terminals. These two $\alpha 1$ families may also partake in the activation of calcium dependent enzymes and gene transcription [141]. However, the exact roles and distributions of each channel subtypes are neuron subtype dependent, such that most types of calcium channels are expressed at various subcellular loci

and do in fact support a wider range of functions. These diverse functional roles ultimately pose a challenge when designing new calcium channel therapeutics with a low risk of side effects.

At the molecular and biochemical level, T-type calcium channels are formed by a single Cav α 1 subunit – a ~250 KDa protein that is comprised, as for the HVA calcium channel subunits of four membrane domains that are connected by cytoplasmic regions and whose N- and C-termini are also cytoplasmic [16]. Each membrane domain contains six membrane spanning helices (S1 through S6) that include a voltage sensor region plus a re-entrant p-loop motif that lines the pore of the channel and controls ion selectivity. The mammalian genome encodes three distinct T-type calcium channel α 1 subunits, termed Cav3.1, Cav3.2 and Cav3.3 [24; 68; 99; 100] and which show distinct brain tissue distributions [80; 121]. In addition to the multiplicity of genes, all of the known Cav α 1 subunits undergo alternate splicing, in some cases giving rise to channels with dramatically different functional behavior [1; 12; 72; 114; 122; 123]. Each of the Cav3.x subunits are subject to alternate splicing [17; 26; 84; 102; 110] with alteration of functional properties. The consequence of splicing for Cav3.x subcellular or tissue distribution is largely unknown so far.

Primary afferent neurons as well as spinal and supraspinal neurons of the pain circuitry express multiple types of voltage gated calcium channels, including the three Cav3 isoforms coding for T-type channels. Below, we will highlight the roles of Cav3.1, Cav3.2 and Cav3.3 in pain transmission, and their usefulness as targets for analgesics.

T-type calcium channels and pain signaling

By virtue of their hyperpolarized voltage-activation range and window current, T-type (Cav3) calcium channels are ideally suited to regulate neuronal excitability, as evident from their role in the development of spike and wave discharges in the epileptic brain (for review, see [59; 145]). They share this peculiarity with a number of other “threshold” ion channels all

important for initiating and boosting cell excitability. These includes HCN channels, some potassium channel isoforms such as KCNQs, or the famous Nav1.7 sodium channel largely studied in the context of pain pathophysiology owing its gain or loss of function in congenital pain diseases that respectively increase or dampen nociception. In this electrogenic effect of T-type channels compared to the different other threshold channels, one could note that their range of activation is even lower than Nav1.7 for example suggesting a role of preamplifier that favors sodium spike generation. In addition to their direct electrogenic role of calcium influx mediated regulation of a numbers of other conductances such as potassium channels[77; 129], T-type calcium channels also support secretion from neuroendocrine cells [40; 91] and are capable of associating with the synaptic vesicle release machinery [138]. T-type channels have been firstly well described functionally in primary sensory neurons [9; 15; 35; 37]. Following this initial discovery, DRG neurons subtypes have been show to differentially express T-type calcium channels with medium sized neurons being the cells with the highest expressers followed with small putative nociceptors [109]. In addition kinetics differences suggested the presence of two distinct T-type channels in these neurons [23; 101]. With the molecular identification of the Cav3.x gene family and the subsequent in situ hybridization analysis, it was clearly shown that Cav3.2 is highly expressed in small and medium sized DRGs as well as Cav3.3 to a lower extent in small neurons [115; 121] (Allen Brain Atlas: www.brain-map.org). The popularisation of large scale transcriptomic profiling combined to individual cell analysis or cell sorting of genetically marked DRG classes recently confirmed that Cav3.2 and Cav3.3 are selectively expressed in defined subclasses of primary sensory neurons [103; 131]. Along these lines, T-type calcium channels have been implicated in synaptic release in the dorsal horn of the spinal cord [51; 126]. Cav3.2 calcium channels are expressed in various subpopulations of primary afferent neurons [11; 125] altogether suggesting a role of these channels in pain processing. Consistent with this idea, systemic or intrathecal delivery of T-type calcium channel blockers

such as ethosuximide and mibefradil mediates analgesia in rodents [27; 36]. On the flip side, T-type calcium channel activity is increased in afferent pain fibers in a number of chronic pain conditions, such as after different types of traumatic nerve injury [52; 140; 144], metabolic nerve alteration in diabetic neuropathy [14; 53], or toxic neuropathies induced by chemotherapies [36; 57; 92]. At least in the case of diabetic neuropathy, blocking T-type channel activity restores a normal pain phenotype [65; 81; 90]. Of particular note, Cav3.2 activity is modulated by glycosylation that is altered in diabetic situation [94; 137] offering a molecular substrate to pharmacologically control Cav3.2 cell surface expression in a therapeutic perspective. Reinforcing the notion of pronociceptive effect of Cav3.2, the T-type channel agonist ST-101 efficient on Cav3.2 was shown to increase DRG cell excitability [29], although the selectivity of this remains to be fully demonstrated. Redox modulation is another means that effectively affect Cav3.2 function and that could be a major component of inflammatory or neuropathic pain via the action of L cysteine or the gasotransmitter hydrogen sulfide [74; 127], although pronociceptive effect of H₂S are debated [32]. As a consequence, anti-oxidants could be analgesic by targeting Cav3.2 [69; 87; 88]. Mechanically, pathological increase of Cav3.2 currents in neurons has been show to results from multiple mechanisms from initial increased transcription [132] involving Egr1 a transcription factor activated by electrical activity particularly in the context pain [75], to increased channel trafficking to the membrane [76]. Recently, the molecular basis of this increased Cav3.2 presence at DRG cell membrane in pain syndromes was identified as the result of a deubiquitination mechanism selectively orchestrated by the USP5 deubiquitinase enzyme forming a molecular complex with the channel [42]. These findings offers an opportunity to discover novel analgesics designed to shorten Cav3.2 channel residence time at the cell membrane by disrupting their interaction with USP5. Dr Zamponi and coworkers validated this elegant approach in both inflammatory and neuropathic pain models [41].

In vivo, we and others evidenced that direct silencing of Cav3.2 calcium channels (but not other T-type calcium channel isoforms) in DRGs via antisense oligonucleotides or siRNA reduces mechanical nociception, and tactile allodynia arising from traumatic or metabolic nerve injury [11; 81; 120]. This fits globally with observations showing that Cav3.2 channels regulate mechanosensitivity of A δ -LTMR fibers [31; 136], but the notion that the main impact on somatosensation of Cav3.2 is due to their presence in A δ -LTMRs is certainly excessive. Moreover this class of fiber, although present in rodents, has not been identified functionally in humans in contrary to other LTMR subtypes (A β and C) [67; 79]. To address this issue, we engineered a unique knockin/flox mouse strain where Cav3.2 is replaced by a functional Cav3.2-surface-eclipticGFP fusion that also has a floxed exon[39]. Using this approach we first demonstrated that Cav3.2 is indeed a selective marker of two major low-threshold mechanoreceptors, A δ - and C-LTMRs, innervating the most abundant hair follicles in the skin. Moreover by generating of a C-LTMR specific Cav3.2 conditional knock out we uncovered that Cav3.2 withing these C-LTMRs regulates light-touch perception, noxious mechanical cold and chemical sensations, and is essential to build-up debilitating allodynic symptoms of neuropathic pain, a mechanism thought and reported in the literature to be entirely A-LTMRs-specific. Collectively these findings globally help to further understand the fundamental role for Cav3.2 in touch/pain pathophysiology, validating their critic pharmacological relevance to relieve mechanical and cold allodynia. These data confirm recent description of a number of C-LTMR specific genes by deep RNA Seq sequencing following DRG subtype FACS sorting or individual single cell DRG isolation showing that Cav3.2 is indeed a marker of these neurons [103; 131]. These findings on somatic nociception can be extended to painful situations linked to visceral origin. In a rodent model of colonic hypersensitivity mimicking the Irritable Bowel Syndrome [10], *in vivo* knockdown of Cav3.2 channels reverses pain hypersensitivity in response to colorectal distension [76]. These findings are further supported by analysis of other

visceral chronic pain models [74; 78; 83; 124]. Altogether, these data indicate that T-type channel membrane expression is dynamically regulated and increased under conditions of chronic pain, and that counteracting this aberrant upregulation may constitute an effective means of mediating analgesia. It is interesting to note that mice lacking Cav3.2 in the constitutive knock out [18] show hyposensitivity to basal nociception, to formalin induced pain in both phases, and a more limited effect on neuropathic pain [21] suggesting possible compensatory phenomenon. The availability of floxed Cav3.2 model opens interesting perspective on inducible localized KO that will limit these compensations.

Compared to the DRGs, at the spinal level little is known on the impact of T-type channels but nickel sensitive low voltage gated evoked and spontaneous neurotransmitter release has been described between primary afferent neurons and outer laminae spinal projection or interneurons [2]. Recent evidences using the Cav3.2 KO mice confirmed these results [42; 51]. Moreover the implication of Cav3.2 in the presynaptic neurotransmitter release machinery is an emerging concept [48; 138; 139]. In this context, our morphological data with the Cav3.2-GFP mouse argue for a specific expression of Cav3.2 in inner lamina II receiving low threshold mechanoreceptive fibers namely the C-LTMRs and the A- δ LTMRs[39]. Moreover, electron microscopy analysis of the glomeruli structure of the synapses at this level reveals the presence of Cav3.2 at both sides of the synapses [39]. Postsynaptically, spinal projection and/or interneurons express functional T-type channels [49; 64; 134] with a selective expression in a subclass of lamina II interneurons whose function remains to be established [134]. Considering that the vast majority of spinal neurons are interneurons, the wiring underlying the spinal networks relaying sensory signaling is clearly not fully understood. In this context, a lot of explorations remain to be performed. Are T-type channels expressed in excitatory or inhibitory inter-neurons, in projection neurons? Specific genetic inactivation

within the spinal cord sparing the primary afferent neurons is still waiting for behavioral analysis.

Anatomically, several ascending spinal pathways are heading towards the brain. These pathways serve not only thalamic targets but also a number of key brainstem nuclei, such as the parabrachial nucleus and periaqueductal grey, each of which also project higher in the brain. Moreover, pain-related functional changes across brain regions consistently reported activation of the thalamus, somatosensory cortex, Anterior Cingulate Cortex (ACC), prefrontal cortex (PFC), insula, amygdala, forming a so-called pain matrix. The excitability of the thalamocortical loop is based on the reciprocal connections between thalamus and cortex, TC neurons project to the cortex and receive a dense cortical excitatory feedback. Within this loop, a peculiar nucleus, the Nucleus Reticularis Thalami (NRT), exclusively composed of GABAergic neurons expressing a high density of Cav3.2 channels is considered as the “clock” of the system[25] due to its anatomical localization. Indeed NRT neurons receive excitatory inputs from both thalamocortical collaterals and cortical afferences, and provide inhibitory input to the TC neurons. Thalamic ventral posterior nucleus receives spino thalamic afferent tracts and projects to the primary somatosensory cortex implicated in sensory discrimination of noxious stimuli. Additional thalamic nuclei[56] project to other cerebral areas governing the emotional aspects of pain, including the insula and the AAC[56]. Of note the insular cortex has been shown to be the essential integrator of the inputs coming from the Cav3.2 expressing C-LTMR fibers[39; 93]. In addition, nociceptive information reaches the thalamus through parallel pathways via the lateral hypothalamus or the amygdala [13; 112], two areas also implicated in the emotional aspects of pain.

Mechanistically, neuroplasticity secondary to neuropathies decreases neuronal firing threshold, increases spontaneous firing and enhances firing evoked by repetitive stimulation[105; 142]. However to what extent T-type calcium channels are involved in these changes of excitability is unknown, despite their abundance in nearly all the regions of the pain network [121]. In the thalamus it is well known that both TC and NRT neurons display two modes of firing, the tonic and burst modes[105]. Indeed, the most remarkable feature characterizing the transition of the thalamocortical activity from wakefulness to sleep is the change in the firing patterns of thalamic neurons that is tightly linked to the biophysical properties of the T-type calcium channels. During sleep the prolonged hyperpolarization of the thalamic neurons massively de-inactivate the T-type channels and promote low threshold calcium spikes (LTS) generation and the occurrence of rhythmic high frequency bursting activities[119]. The cortical consequences of this T-type channel dependent bursting mode is reflected on the electroencephalogram (EEG) which is dominated during sleep by high amplitude, low frequency oscillatory activities (<15 Hz) and, by contrast, by low amplitude, high frequency oscillatory activities in the gamma band (30–50 Hz) during waking states. However, it is of great importance to note that clinical studies reported the presence of abnormal slow TC oscillations in the theta frequency band (4-8Hz) in awake neuropathic patients[55; 73; 135]. Moreover, thalamic bursting can lead to synaptic potentiation in the ACC, with important consequences regarding cortical integration of the nociceptive input and memory formation related to painful events and comorbidities such as anxiety[63; 117]. While plasticity of these synapse seems to rely on multiple mechanisms, it is worth to mention a recent brief report indicating the potential role of T-type channels in these neurons with an increased expression of Cav3.2 linked to neuropathic state[113]. As additional layer of regulation, interconnectivity within the cortical areas amplify chronic pain as described for the connection from the somatosensory S1 to the AAC[34]. Inversely, inhibition of ACC neurons with targeted

inhibitory opsins recently showed the analgesic potential of impeding the excitability of this region[46]. This is largely due to the fact that the enhanced cingulate cortical output information augmented in chronic pain influence multiple subcortical targets such as the periaqueductal grey (PAG) and mesolimbic nuclei further building up the multidimensional aspects of pain including the motivational processing of the hedonic value of pain and pain relief [86].

Alteration of thalamic bursting, underlain by T-type currents, is therefore mechanistically a key element of pain network malfunctions as reported in patients[70; 71; 82] in association to theta rhythmicity[55; 107]. Animal studies corroborates these findings with enhanced bursting associated to spontaneous slow rhythmic oscillations [43; 50], reduced coherence between electrocorticogram and thalamic local field potential, and increased theta oscillations power[66] in neuropathic models. Similarly, bursting in ACC cortex of neuropathic rats has been linked to overexpressed Cav3.2 and modulated by T-type channel blocker [113]. As a whole, these data point to a crucial role played by supra spinal T-type channels in both the transfer of nociceptive information and the development of abnormal brain activities.

A more direct way to estimate the involvement of T channels in pain response is the use of genetically modified mice for the different Cav3 isoforms. However, experiments performed so far have involved general KO mice for the Cav3.1 or Cav3.2 channels and mostly non-specific T channel pharmacology (Mibefradil, Ethosuximide). The Cav3.3 KO model has not been explored for pain behavior yet. Hyperalgesia was first reported in response to visceral stimulation in the Cav3.1 null mice [60] exhibiting a total absence of T-type currents in somatosensory TC neurons [61; 128]. In contrast the global Cav3.2 KO exhibits an analgesic phenotype regarding different models of pain [3; 19; 21; 53]. Moreover our recent work shows that the analgesic effects of *per os* administered newly developed T-type channel agonist (affecting all 3 Cav3 isoforms) are abolished in the null mice showing that the blockade of T-

type channels overall mediates analgesia[38]. Direct proof of central pronociceptive role of Cav3.x channels will require either local pharmacological approaches using specific T channel antagonist [20; 28; 38] or conditional Cav3.x KO mice. This conclusion stands equally to unravel the impact of Cav3.x in the other regions of the pain matrix in the ascending as well as in the descending pathways. Although little is known on the impact of Cav3.x channels in descending pain modulation, it should be noted that in the bulbospinal area, namely in inhibitory periaqueductal gray gabaergic neurons, the lack of Cav3.1 KO mice perturb morphine analgesia [95]. These findings could indicate that PAG located Cav3.1 may limit persistent pain, but the use of TTA-A2, a Cav3.x pan antagonist do not result in similar effects. Further work need to be done to clarify this issue. Of note in that respect, we reported recently that intra cerebro ventricular (ICV) injection of TTA-A2 result in an analgesic effect that depends on Cav3.2 expression [33]. Moreover this study demonstrates that blockade of supraspinal Cav3.2 is a key step of the mechanism underlying the analgesic effect of paracetamol, the most widely use remedy to treat mild pain [33]. Interestingly, this study shows that the end up inhibition of Cav3.2 subsequent to the action of AM404, the active paracetamol metabolite, is due to the activation of centrally expressed TRPV1 channels that in turn leads to an inhibition of Cav3.2. This regulation has been similarly proposed in rat primary afferent neurons [22].

For all the aspects presented the precise spatial and temporal resolution of Cav3.x expression in the pain neuronal pathways both at the cellular and subcellular levels are crucial. Up to recently the state of the art on this aspect was inexistent. However, recently two independent groups reported that T-type calcium channels are concentrated in the Axon Initial Segment (AIS), where they contribute to local subthreshold membrane depolarization and thereby influence action potential initiation. These data were based on conclusion from functional studies on isolated neurons using cell attached patch clamp recordings as well as calcium and sodium imaging. Furthermore, these studies shows that this localization is a

dynamically regulated process enabling a fine tuning of intrinsic plasticity of neuronal excitability [4; 5; 44]. This tuning of T-type channel localization is moreover regulated by membrane receptors such as the dopamine receptors also expressed in regions enriched in Cav3.2 [6; 118; 121]. The plastic alterations of T-type channels distribution are likely involved during chronic pain states. For this aspect the Cav3.2-GFP knockin mouse will be of great utility. While plasticity of channel distribution in pathological pain remains to be addressed, this tool helped to uncover that physiologically Cav3.2 proteins are expressed in the receptive fields of LTMR fibers nearby the hair follicles in the putative triggering zone of action potential firing (thereby contributing to the extreme sensitivity of these fibers to low mechanical forces), as well as along the axons of these neurons with a specific presence at the nodes of Ranvier of A δ -LTMRs and in clusters along the C-LTMR fibers where it speed up conduction velocity [39]. This latter effect was retrieved recently in hippocampal and cerebellar neurons that respectively express Cav3.2 and Cav3.1 [45; 77]. Thus, the impact of T-type channel on axonal excitability is at its beginning but this is likely to drive increasing attention.

T-type channel pharmacology: toward a new class of analgesics?

Most of the work done so far validate the Cav3.2 isoform as a target for mediating analgesia, especially by acting in the periphery. It may be possible to exploit state dependence of drug action as a means to further preferentially inhibit T-type calcium channels in highly active pain fibers. Indeed, new generation of blockers appeared in the recent years with different scaffolds [7; 62; 96; 104; 116; 130; 143; 146]. In particular TTA-P2 and TTA-A2, the latter which interact preferentially with inactivated T-type calcium channels, both mediate analgesia in rodent models of pain [20; 38]. Z123212, a mixed blocker of voltage dependent sodium channels and T-type calcium channels mediates analgesia by selectively targeting the slow inactivated state of these channels [47]. In this context it is interesting to note that the

local anesthetic binding domain of voltage gated sodium channels is partially conserved in T-type calcium channels [8]. Given that there is now a crystal structure of a bacterial voltage gated sodium channels [97; 98] this knowledge may help guide further development of T-type channel blocking drugs. Finally, Z944, another state dependent T-type channel inhibitor is currently in phase I clinical trials for pain. Several pharmaceutical companies have T-type channels on their list of targets and near future will show if clinical drugs emerge. The use of tissue specific Cav3.X conditional KO will be in the future a nice perspective to decipher the important structures mediating the analgesic effects of T-type channels blockers. Translational value of preclinical research in the field of pain is always debated regarding the number of molecules that failed in their clinical development. Going from mouse to men targeting T-type channel may be a successful story regarding recent published data. Dr David Mahns and coworkers recently used the TTA-A2 in humans with local injection in the skin. They demonstrated that T-type channel blockade efficiently attenuated mechanical and cold allodynia by affecting C-LTMR (alias C-tactile in human) a striking parallel to our finding on mice [85; 106]. Although this contrast with the conclusions of a clinical trial on diabetic neuropathic patients with the ABT-639 molecule, a lower affinity new selective T-type channel blocker[54] that showed a disappointing lack of efficacy, although higher dose might have been needed to fully judge the drug efficacy [111; 147].

Concluding remarks

A number of questions concerning the role of T-type channels in pain remain unresolved. First, it is unclear precisely how T-type channels contribute to pain signaling. Possibilities include: 1) a lowering of the firing threshold for afferent pain fibers, 2) a direct contribution to neurotransmitter release at primary afferent synapses 3) a direct function of T-

type channels as mechanosensors, 4) an activation of pathways such as ERK which in turn is linked to increased pain [19] and 5) perhaps via interactions with other types of ion channels such as voltage- and calcium-activated potassium channels as described for different types of CNS neurons. Second, the mechanism by which T-type calcium channel activity is enhanced in chronic pain conditions remains to be determined such as direct modulations, post-translational modifications, and altered membrane turnover. This could potentially include the Cav α 2 δ subunit which has been shown to increase T-type channel amplitude in expression systems [30]. Finally, a number of inherited mutations have been described for the Cav3.2 coding gene in rats [102] and more importantly in humans [58; 108; 118; 133]. Although they are linked to some forms of epilepsies, autism or metabolic disorders, it remains to be explored if these patients suffer from altered pain perception.

In summary, among the calcium channel family the T-type calcium channels and in particular the Cav3.2 isoform appear to have a critical role in the excitability of pain neuronal circuits, and as a result are vigorously pursued as therapeutic targets.

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