REVIEW ARTICLE
Redox and trace metal regulation of ion channels in the pain pathway

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Given the clinical significance of pain disorders and the relative ineffectiveness of current therapeutics, it is important to identify alternative means of modulating nociception. The most obvious pharmacological targets are the ion channels that facilitate nervous transmission from pain sensors in the periphery to the processing regions within the brain and spinal cord. In order to design effective pharmacological tools for this purpose, however, it is first necessary to understand how these channels are regulated. A growing area of research involves the investigation of the role that trace metals and endogenous redox agents play in modulating the activity of a diverse group of ion channels within the pain pathway.

In the present review, the most recent literature concerning trace metal and redox regulation of T-type calcium channels, NMDA (N-methyl-D-aspartate) receptors, GABA$_A$ (γ-aminobutyric acid A) receptors and TRP (transient receptor potential) channels are described to gain a comprehensive understanding of the current state of the field as well as to provide a basis for future thought and experimentation.

Key words: ion channels, nociception, pain, pharmacology, redox agents, trace metals.

INTRODUCTION

The process of nociception relies on the proper function of several types of ion channels working together to transfer information encoding the presence of potentially dangerous stimuli. Abnormal channel activity can contribute to the development and maintenance of painful neuropathies, which pose a large and persistent clinical problem [1]. Several studies, both *in vitro* and *in vivo*, have shown that T-channels (T-type calcium channels), NMDA (N-methyl-D-aspartate) receptors, GABA$_A$ (γ-aminobutyric acid A) receptors and TRP (transient receptor potential) channels play significant roles in both normal and pathological nociception, and can potentially be exploited as valuable pharmacological targets [2–5]. However, in order to provide effective therapeutic options, it is necessary to gain a better understanding of how these ion channels are regulated. Although these channels play distinct roles, several studies have shown that modulation via redox agents and trace metals is a common regulatory feature. The results of these studies are summarized in Table 1.

T-TYPE CALCIUM CHANNELS

LVA (low-voltage-activated) or T-type calcium channels play a distinct role in modulating excitability in a variety of nervous tissues [6,7]. Of particular interest is the Ca$_V$3.2 isoform, which is highly expressed in nociceptive neurons of the DRG (dorsal root ganglia) and dorsal horn of the spinal cord [8,9]. Electrophysiological studies have established that Ca$_V$3.2 currents are augmented upon application of reducing agents such as DTT and L-cysteine, and diminished upon treatment with the oxidizing agent DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)] [10,11]. Furthermore, additional studies have suggested that prevalent endogenous gasotransmitters, such as hydrogen sulfide (H$_2$S) and carbon monoxide (CO), play a distinct role in T-channel regulation through redox-related mechanisms [12–14]. Since DTT and L-cysteine are both reducing agents with non-specific chelating abilities, channel modification can occur via the reduction of cysteine residues or by chelation of trace metals from high-affinity binding sites, as several previous studies have shown [15–20]. To determine the specific mechanism of action, cysteine residues were modified by agents such as NEM (N-ethylmaleimide) or by pulses of UV light to disrupt hypothetical disulfide bonds. Neither of these treatments affected DTT- or L-cysteine-induced current increases, suggesting that Ca$_V$3.2 current augmentation is not mediated by reduction of cysteine residues, but is instead likely to be altered via trace metal chelation. In addition, the effects of L-cysteine on T-currents were mimicked with metal chelators such as DTPA (diethylenetriaminepenta-acetic acid) and TPEN [N,N,N,N-tetrakis(2-pyridylmethyl)ethylenediamine], providing further evidence suggesting that the Ca$_V$3.2 channel isoform is altered via metal chelation [11].

Interestingly, the Ca$_V$3.2 channel isoform is much more potently inhibited by trace metals than the Ca$_V$3.1 and Ca$_V$3.3 isoforms [21,22]. Thus the locus of inhibition was determined by identifying non-conserved amino acids between the Ca$_V$3.2 and Ca$_V$3.1/Ca$_V$3.3 channels, specifically looking for residues that are capable of binding with trace metals, such as glutamate, aspartate, cysteine and histidine. Subsequent site-directed mutagenesis studies demonstrated that substitution of a glutamine for an extracellular histidine residue located at position 191 (H191Q)

Table 1   Summary of ion channel current responses to redox and trace metal regulation

Signs $+$ and $-$ indicate increased and decreased current respectively after treatment with designated agent. Representative studies are cited for each channel type.

	Reduction	Oxidation	Chelation	Trace metal
T-channel [10,11,24]	+	−	+	−
NMDA receptor [16–18]	+	−	+	−
GABA$_A$ receptor [32–35]	+	−	+	−
TRPV1/TRPA1/TRPM8 channel [40–44]	+ (TRPV1)	+ (TRPV1)	− (TRPA1)	+ (TRPA1)
				− (TRPM8)

completely abolished $Ca_V 3.2$ channel sensitivity to metal chelators, indicating that His[191] plays a critical role in $Ca_V 3.2$ channel regulation by divalent trace metals, such as zinc, copper and nickel [11]. The prevalence of zinc in physiological systems made it an early target of investigation, and it was eventually determined to be the most commonly found trace metal bound to His[191] [11]. Further mutagenesis studies have refined the structural features that confer zinc sensitivity on the $Ca_V 3.2$ channel to show that additional residues near His[191], specifically Asp[189] and Gly[190], contribute to a Asp-Gly-His motif which facilitates the binding of zinc, resulting in tonic inhibition through stabilization of the closed conformation of the channel [23].

In vivo studies have shown that injection of either DTT or L-cysteine has a profound effect on nociception, resulting in the development of thermal and mechanical hyperalgesia [24]. The possibility that these effects are mediated through $Ca_V 3.2$ channel potentiation was explored using genetic knockout animals [11,25]. In one such study, wild-type mice were injected with L-cysteine, resulting in significant thermal hyperalgesia, whereas no effect was observed in $Ca_V 3.2$-knockout animals [11]. These results corroborate the electrophysiological data discussed previously and add to the growing amount of evidence suggesting that hyperactive $Ca_V 3.2$ channels contribute to abnormal nociception [2]. Additional *in vivo* studies utilizing site-specific knockin animals are needed to investigate the physiological importance of the His[191] regulatory site, as well as its role in pain disorders, which would provide a better understanding of the function of endogenous chelators of the $Ca_V 3.2$ channel in nociceptive transmission.

N-METHYL-D-ASPARTATE RECEPTORS

The ligand-gated ionotropic NMDA receptor is expressed in nociceptive neurons of the dorsal horn of the spinal cord, where it receives and facilitates transmission of excitatory signals from peripheral glutamatergic neurons [26]. Similar to the $Ca_V 3.2$ channel, NMDA currents are augmented by DTT and inhibited by DTNB and trace metals, most notably zinc [27]. Site-directed mutagenesis studies have shown that several extracellular histidine residues within the NR2A subunit comprise in part the high-affinity metal-binding site [17,18]. A more recent study has shown that substitution of serine for the histidine residue located in the NR2A subunit at position 128 (H128S) nearly abolishes recombinant NMDA receptor sensitivity to inhibition by zinc, establishing the importance of the His[128] site in zinc binding [28]. In addition, multiple cysteine residues outside the binding site have been implicated as being important for both redox and trace metal modulation [16]. The authors of this study suggest that, instead of contributing to the direct binding and stabilization of trace metals, the redox state of these cysteine residues contributes to the channel's overall affinity for trace metals, thereby providing an additional means of channel regulation [16].

The NMDA receptor plays an important role in nociception, and channel hyperactivity has been shown to contribute to the development of central sensitization of pain responses [3,29]. Several studies have utilized pharmacological antagonists of the NMDA receptor, such as AP-5 (DL-2-amino-5-phosphonovaleric acid), ketamine, MK-801 and dextromethorphan, to investigate its role in abnormal nociceptive transmission. An appreciable amount of evidence suggests that antagonizing the NMDA receptor results in reduced hyperalgesia and normalization of pain sensation in several experimental models of pain [3].

In an effort to ameliorate abnormal nociception, researchers have investigated the effects of redox agents such as DTT and DTNB on NMDA receptor-mediated pain sensation *in vivo*. A simple, yet important, study revealed that intrathecal injection of mice with DTT resulted in increased response to a variety of pain assays after pharmacological activation of the receptor using NMDA, whereas pre-treatment with DTNB prevented these effects [30]. These behavioural responses mirror the electrophysiological data discussed above, thereby strengthening the claim that NMDA receptor potentiation by reducing agents results in abnormal and enhanced pain transmission, which can be prevented and ameliorated by the use of oxidizing agents [30]. In addition to traditional redox modulation, the role of zinc in NMDA receptor-mediated nociceptive transmission was investigated using NR2A-H128S knockin mice, which lack the high-affinity zinc-binding site discussed above [28]. In wild-type animals, both subcutaneous and intrathecal injection of zinc resulted in profound analgesia after induction of chronic inflammatory and neuropathic pain using either CFA (complete Freund's adjuvant) or SNL (sciatic nerve ligation) respectively. In contrast, NR2A-H128S knockin mice were unaffected by treatment. Taken together, these studies emphasize the important role that redox agents and zinc play in NMDA receptor modulation of pain responses *in vivo* [28].

γ-AMINOBUTYRIC ACID RECEPTORS

It is well known that modulation of GABA$_A$ receptors in the pain pathway at the level of the dorsal horn and DRG can influence nociception. For example, GABAergic interneurons found within the dorsal horn of the spinal cord provide a source of inhibition to primary nociceptive afferents and their post-synaptic nociceptive targets [31]. Several studies have shown that both recombinant GABA$_A$ currents in heterologous systems and native GABA$_A$ currents in nociceptive DRG neurons are augmented by treatment with DTT and decreased by the oxidizing agent DTNB [11,32]. In addition, treatment with the high-affinity zinc chelator TPEN potentiates GABA$_A$ currents, indicating that zinc chelation plays a role in the regulation of the GABA$_A$ receptor [32]. Although all GABA$_A$ receptor compositions exhibit some degree of sensitivity to zinc, the degree of sensitivity has been shown to be dependent on receptor subunit composition [33,34]. The $\alpha\beta$ composition is
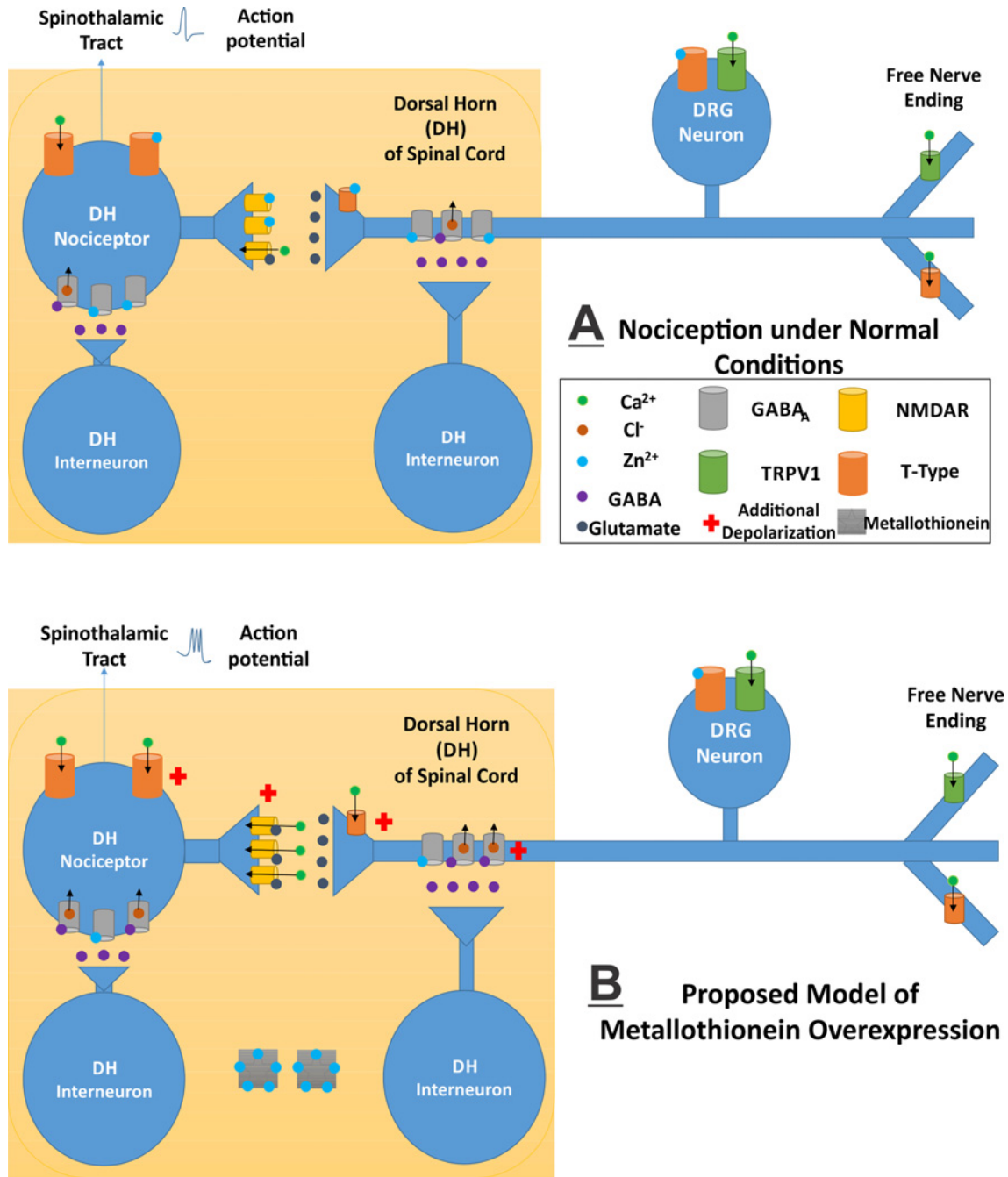
Figure 1   Proposed mechanism of metallothionein regulation of extracellular zinc in nociception

(**A**) Simplified model of nociception under normal conditions. Free nerve endings transduce painful stimulus into a neural signal, which propagates centrally and eventually synapses on a nociceptive neuron within the dorsal horn (DH) of the spinal cord. The information encoded in the signal is carried via the spinothalamic tract to the processing areas of the brain. A portion of T-channels, NMDA receptors and GABA$_A$ receptors are tonically inhibited by the presence of extracellular zinc. (**B**) Proposed model of nociception under conditions of MT overexpression. Pain signal is carried towards the dorsal horn, where it is modulated and augmented by hyperactive T-channels, NMDA receptors and paradoxically depolarizing GABA$_A$ receptors on the presynaptic nerve terminals after subsequent relief of tonic inhibition from zinc via sequestration by excess MT.

much more sensitive to zinc inhibition than the $\alpha\beta\gamma$ composition, indicating that the γ-subunit prevents zinc binding and inhibition. The molecular sites of zinc inhibition were determined in a comprehensive site-directed mutagenesis study, which resulted in the identification of five critical amino acid residues within the α and β subunits [35]. Notably, replacement of all five amino acid residues with alanine (subunit α1: E137A and H141A; subunit β3:

E182A, H267A and E270A) led to complete insensitivity to zinc inhibition.

Numerous studies have shown the importance of the GABA$_A$ receptor in modulating nociception at the level of the DRG and dorsal horn of the spinal cord [4,36]. For example, GABA$_A$ agonists, such as muscimol and THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol hydrochloride), have been

shown to alleviate both thermal and mechanical hyperalgesia in rat models of painful neuropathy after peripheral nerve injury, whereas application of GABA$_A$ antagonists such as bicuculline and picrotoxin led to more pronounced hyperalgesia [37]. Hence it is reasonable to propose that redox modulation of these channels in the pain pathway can powerfully influence nociception in acute and pathological conditions associated with neuropathic pain. Future studies are needed to address this issue.

TRANSIENT RECEPTOR POTENTIAL ION CHANNELS

The role of individual members of the TRP ion channel family in the pain pathway has been studied extensively [5]. Of particular interest is the vanilloid receptor subtype 1 (TRPV1) channel, which is expressed in nociceptive neurons of the DRG and plays a fundamental role in transducing thermal stimuli [38,39]. Although TRPV1 channel activity is influenced by many different physical conditions and chemical agents, such as membrane voltage, pH, capsaicin and phosphorylation via second messenger pathways, it has also been shown to be sensitive to reducing agents, such as DTT [38,40]. In this study, the authors showed that application of DTT resulted in a dramatic increase in current magnitude elicited by noxious heat in both rat DRG neurons and HEK (human embryonic kidney)-293 cells expressing the TRPV1 channel [40]. More recently, the same research group identified three potential extracellular cysteine residues (Cys^{616}, Cys^{621} and Cys^{634}) as potential candidates for DTT modulation. The site of action was determined through site-directed mutagenesis, which showed that substituting glycine for Cys^{621} (C621G) nearly abolished DTT sensitivity, suggesting that the Cys^{621} site plays a critical role in TRPV1 redox modulation [41]. Interestingly, the same group reported that membrane-permeant oxidizing agents (e.g. diamide), but not the membrane-impermeant agent DTNB, also enhance heat-induced activation of TRPV1 channels. This suggests that distinct unidentified site(s) located within the cytoplasmic domain mediate the observed increase in recombinant TRPV1 currents via oxidation [41].

In addition to modulation by traditional redox agents, the TRP family of ion channels includes a few members which are sensitive to trace metals [42–44]. Specifically, a fairly recent study has identified a subset of sensory neurons within mouse DRGs that were sensitive to both zinc and mustard oil, an established TRPA1 (transient receptor potential ankyrin 1) channel agonist [42,45]. TRPA1 channel sensitivity to zinc and cadmium was confirmed using *in vitro* electrophysiological recordings from transiently expressing TRPA1 HEK-293 cells [42]. Additional studies corroborated these findings through the use of the copper/zinc chelator and ionophore clioquinol, which acts by increasing the intracellular concentrations of these trace metals, and furthermore demonstrated that the TRPA1 channel is activated by the divalent copper cation (Cu^{2+}), but not directly by iron (Fe^{2+}) [43]. Subsequent site-directed mutagenesis studies revealed the identity of several key amino acid residues which appear to confer the TRPA1 channel's sensitivity on zinc [42]. These intracellular sites include two cysteine residues located at positions 641 and 1021, and one histidine residue located at position 983. Substitution with non-metal binding serine or alanine residues for these residues substantially reduced zinc sensitivity.

The role of TRPA1 channel-expressing sensory neurons in the pain pathway was investigated using genetic knockout mice (*Trpa1*$^{-/-}$) [42]. When compared with wild-type controls, these animals exhibited a marked decrease in stereotypical pain behaviour when injected with a noxious dose of zinc acetate, which is known to cause pain and irritation in the affected areas, suggesting that zinc-induced pain is in part mediated through the TRPA1 channel [42,46].

Along with the TRPA1 channel, the cold- and menthol-activated TRPM8 (transient receptor potential melastatin 8) channel, found in peripheral thermoreceptors, exhibits diminished recombinant currents in HEK-293 cells after treatment with Zn^{2+} [44]. However, in spite of this pharmacological attribute, the authors demonstrated that TRPM8-expressing neurons of the DRG can be activated by Zn^{2+}, presumably caused by alterations in TASK-3 potassium (K$^+$) leak channel activity. This study not only demonstrates the importance of obtaining a comprehensive knowledge of ion channel expression and function in order to understand neuronal activity on a macroscopic level, but also underscores the complex and varied role that trace metals play in the modulation of sensory transmission.

CLINICAL STUDIES SUPPORTING THE ROLE OF TRACE METALS IN NOCICEPTION

In addition to the basic science studies presented, multiple clinical studies have aimed to uncover the relationship between serum trace metal concentrations and the incidence of pain. More specifically, multiple studies have found an inverse correlation between the pain associated with a variety of diseases, such as fibromyalgia and myofascial pain syndrome, and the concentration of serum zinc [47,48]. In each of these studies, the severity of the disease was characterized using a variety of clinical features, such as the number of tender points and extent of fatigue or pain pressure thresholds, in individuals suffering from fibromyalgia and myofascial pain syndrome respectively. These clinical features were then correlated with serum levels of prevalent trace metals in addition to other serum factors. Both studies found serum zinc levels to be decreased in diseased individuals when compared with normal healthy controls. Although merely correlative, these conclusions mirror the findings provided by numerous basic science studies, and warrant further and more extensive clinical investigation.

THE ROLE OF METALLOTHIONEINS IN THE REGULATION OF EXTRACELLULAR TRACE METAL CONCENTRATION AND POSSIBLE EFFECT ON NOCICEPTION

It is clear that trace metals, most notably zinc, play a critical role in maintaining proper nociception by modulating the activity of several important ion channels. Therefore, in order to gain a thorough understanding of how these channels are regulated, it is necessary to investigate how the cell's zinc environment is maintained.

Several biological molecules are involved in zinc binding and storage. For example, numerous studies have shown that MTs (metallothioneins), a family of low-molecular-mass cysteine-rich proteins, play a critical role in maintaining heavy-metal homoeostasis and prevention of oxidative stress [49–51]. Although MTs are considered to function primarily within the cell, appreciable amounts have been found in the blood stream at concentrations as high as 2.4 ng/ml in rats ($\sim$3.6$\times$10^{-10} M), which can become even higher under conditions of stress [52,53]. Thus it is possible that MTs play a physiologically significant role in the extracellular space [54].

A recent study has shown that MTI and MTII expression are elevated in animal models of inflammatory and neuropathic pain [55]. Specifically, injection with CFA into the rat hind

paw elicited a bilateral increase in MTI and MTII expression in vascular endothelial cells within the dorsal and ventral horns of the spinal cord, whereas chronic constrictive injury elicited a similar increase in the ipsilateral dorsal and ventral horn. Pre-injection of MTI siRNA resulted in near normalization of mechanical and thermal sensation. Taken together, these data support the authors' hypothesis that up-regulation of MTs within the spinal cord contributes to the development of pathological pain symptoms such as allodynia (innocuous stimulus becomes painful) and hyperalgesia (exaggerated perception of already painful stimuli) in these animals. These data are also consistent with the idea that zinc ions tonically inhibit the activity of neurons within the pain pathways of the spinal cord.

This study provides strong evidence indicting MTI and MTII as mediators of inflammatory and neuropathic pain. The authors suggest several possible means by which this could occur. Notably, they postulate that increased MT expression and secretion could lead to a decrease in zinc concentration in the spinal cord. To take the authors' hypothesis one step further, secretion of MTI and MTII into the extracellular space could decrease the concentration of free zinc in the dorsal horn of the spinal cord, thereby relieving $Ca_v3.2$ channels and NMDA receptors of inhibition, leading to increased nociceptor excitability and sensitivity to glutamatergic input from the periphery. In addition, owing to the high intracellular chloride (Cl^-) concentration maintained by primary sensory afferents via the NKCC1 and KCC2 transporters, the Cl^- equilibrium potential nears -30 mV [36]. Consequently, activation of the $GABA_A$ receptor in primary sensory afferents often increases membrane potential, through a process known as PAD (primary afferent depolarization), as Cl^- flows out of the neuron and down its electrochemical gradient [36,56]. Depolarizing the membrane can lead to premature inactivation of action-potential-generating voltage-gated sodium channels as well as voltage-gated calcium channels responsible for triggering synaptic release [36]. Under normal conditions, this form of presynaptic inhibition decreases the amount of glutamate released at the synaptic cleft and reduces the EPSP (excitatory postsynaptic potential) elicited within the target nociceptive neuron of the dorsal horn [4,36,56]. However, it is possible that widespread and excessive relief of $GABA_A$ receptors paired with sufficient stimulation can lead to an augmented nociceptive response, possibly leading to visible signs of hyperalgesia and allodynia [36,56,57]. Figure 1 depicts our proposed model of pain processing under conditions of MT overexpression, specifically showing how each ion channel could be affected by a systemic decrease in extracellular Zn^{2+} concentration.

Given zinc's diversity of action in the nociceptive pathway, it is possible that a small change in free extracellular zinc concentration could have a large effect on nociceptive transmission. Although no direct proof of $Ca_v3.2$ channel, NMDA receptor or $GABA_A$ receptor regulation via MTs has been shown to date, evidence supporting their involvement in the occurrence of pain disorders warrants further investigation into this potential mechanism.

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