Title
Microanatomy of Voltage-Gated Ion Channels Targeted by Tarantula Venom Toxins

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Publication Date
2022

Peer reviewed|Thesis/dissertation

Microanatomy of Voltage-Gated Ion Channels
Targeted by Tarantula Venom Toxins

By

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THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Pharmacology and Toxicology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2022

Abstract

This thesis investigates ion channels modulated by tarantula peptide toxins. Through the research, two primary questions are investigated: (1) Can a fluorescent peptide tarantula toxin reveal activation of neuronal ion channels? and (2) Where in the body do tarantula peptide toxins bind ion channels? I address these questions through several different approaches using immunohistochemistry, tissue culture, and confocal and two-photon imaging. Resulting findings provide evidence that a toxin-based fluorescent probe viably demonstrates the location and activation of K_v2 channels in male and female rat brain neurons. Findings also demonstrate that the $Na_v1.7$ channel, a pain therapeutic target, is found throughout the cell bodies and central processes of female mouse nonpeptidergic nociceptors. This research provides a basis for further exploration that may lead to new types of ion channel activity imaging and ion channel targeting therapeutics.

Acknowledgements

I am grateful to the UC Davis community, who, from my mentors to my fellow students, to my academic advisors, have been my partners in creating this thesis. I'd like to thank my leaders in the Pharmacology and Toxicology graduate group, including my graduate advisor Kermit Carraway and my academic advisor Katherine Moffat. I'd also like to thank my graduate committee for their gracious guidance throughout my time at UC Davis, including my mentor Jon Sack, and committee members Theanne Griffith and Vladimir Yarov-Yarovoy. I am also indebted to the members of the Sack-Yarov-Yarovoy Lab including staff, my fellow graduate students, and undergraduates, without whom this work could not have been accomplished. I would also like to thank my wife Emily for her ongoing support and patience, and of course for the countless hours she spent walking the dog while I was imaging in the lab.

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Introduction

The design and implementation of novel probes that enable the imaging of neuronal electrical activity will yield crucial information on how to counteract the disruption to signaling and systems caused by neuronal diseases, disorders, and toxicants. It is possible to design targeted therapies to address the issues that arise from channel-based maladies, such as chronic pain.

In cell membranes, there are voltage-sensitive proteins that conduct ions across the lipid bilayer; this class of proteins is called voltage-gated ion channels. While each type of voltage-gated ion channel is only active under certain circumstances, their harmonious interaction results in complex wave forms transmitted along neurons. To decipher the individual contribution of a single ion channel, one must understand the channel's contribution to the organized action of a multitude of voltage-gated ion channels working in concert to execute neuronal signaling. To disentangle the characteristic performance of individual channels it is necessary to have specific recording methods that identify channels of interest during cell activity. These methods of targeted cell monitoring can be modified to not only identify specific channels, but also to inhibit the channelopathies that contribute to chronic maladies that impact humans.

There is a pharmaceutical opportunity to design a pain dampener that functions through channel inhibition. It is possible to look to our natural surroundings for inspiration for agents that bind selectively to channels. One such inhibitor from the natural world is guangxitoxin-1E (GxTX), part of the venom cocktail emitted by tarantulas from the Guangxi province in China[1]. GxTX binds selectively to the

potassium (K) delayed rectifier channel (K_v2) family, which allows fluorescent toxin labeling to produce a fluorescent probe showing the channel's activity in live tissue. The probe's conformational selectivity is due to the peptide toxin's higher affinity for the voltage sensor of the channel at rest over any other conformation, so channel activation results in increased unbinding. The voltage-induced disengagement can be observed as a reduction of fluorescence intensity at the cell membrane. The K_v2 channel is prevalent throughout the mammalian central nervous system[2]. These channels collect in distinct puncta[3] of neuronal endoplasmic reticulum-membrane junctions[4] which have been shown to have distinct physiological purposes[5], from trafficking proteins to the cell surface[6] to sequestering interacting membrane proteins[7] to membrane voltage control[5].

The fluorescent toxin technique can be expanded to identify other types of channels. One channel of interest is the voltage-gated sodium (Na) channel $Na_v1.7$ because of its involvement in nociceptive signaling[8], as studies have shown KO of $Na_v1.7$ produces analgesia without abolishing peripheral nociceptors[9], indicating the channel acts during pain signal transduction, and it's absence doesn't remove nociceptors at the periphery. Pinpointing the $Na_v1.7$ channel location could help disentangle their contribution to pain signaling, and where specifically they should be targeted by therapeutics.

The experience of pain is initiated by a noxious stimulus creating an electrical signal that is conducted to the brain along nerve fibers from the periphery to the central nervous system. The unmyelinated axons of sensory neurons are called C nerve fibers, which can be identified with specific markers. Isolating key components of pain signaling allows for production of direct targeting inhibitors. The sodium channel $Na_v1.7$

has been identified as a contributor to pain[8]. Loss-of-function mutations in humans lead to anosmia and an insensitivity to pain, but there is not a loss of peripheral sensation[10]. In this research, I focused on $Na_v1.7$ channels because of availability of multiple modulators that selectively target the channel that could become potential drug candidates for pain modulation. The $Na_v1.7$ channel plays a key role in setting the threshold for firing action potentials and tuning the gain in pain signaling neurons[11]. Loss-of-function mutations in human $Na_v1.7$ ($hNa_v1.7$) have been identified in families with congenital insensitivity to pain[11] whereas gain-of-function mutations in $hNa_v1.7$ lead to inherited pain disorders[12]. While there are multiple types of afferent variants to transmit sensory signals from sensation, proprioception, and pain it is important to isolate just the nociceptive signaling neurons. One method of identifying neurons involved in pain transmission has been to establish if the cell expresses the Mrgprd receptor[13].

In this research, I developed and optimized an imaging method to visually record conformational changes of ion channels in living tissue in response to voltage activation. I used immunohistochemistry to identify nociceptive neurons in the dorsal root ganglion (DRG) and used confocal imaging to record colocalization of $Na_v1.7$ channels with nociceptor identifiers.

Section 1 – Contribution to *EVAP: A two-photon imaging tool to study conformational changes in endogenous Kv2 channels in live tissues*

Through conceptualization, experimental design, and writing, I contributed to the published scientific journal article *EVAP: A two-photon imaging tool to study conformational changes in endogenous Kv2 channels in live tissues*[14]. We used a

fluorescently tagged toxin (GxTX-594)[15] to image the activation of K_v2 channels in live tissue. Since GxTX-594 binds with higher affinity to voltage sensor domain of the K_v2 channel in its resting conformation, activation of the channels causes the probe to detach. This conformation-induced unbinding can be recorded by imaging the fluorescent changes at the cell surface, which were measured with two-photon microscopy.

Methods

Two-photon excitation slice imaging

Image stacks (512x512 pixels, 1 mm Z-steps, 0.035 μm/pixel) were acquired using a custom two-photon excitation microscope (LUMPLFLN 60XW/IR2 objective, 60/1.0 NA; Olympus) with two pulsed Ti:sapphire lasers (Mai Tai; Spectra Physics) tuned to 810 nm (for GxTX-594 imaging) and 930 nm (for GFP imaging) and controlled with ScanImage software[16]. After identifying a neuron expressing $K_v2.1$-GFP, perfusion was stopped, and GxTX-594 was added to the static bath solution to a final concentration of 100 nM. After a five-minute incubation, perfusion was restarted, leading to washout of GxTX-594 from the slice bath. Red and green photons (565dcxr, BG-22 glass, HQ607/45; Chroma Technology) emitted from the sample were collected with two sets of photomultiplier tubes (R3896; Hamamatsu).

Hippocampal slice preparation and transfection

Organotypic hippocampal brain slices were prepared by the Zito lab from postnatal day 5-7 rats as previously described[17]. DIV15–30 neurons were transfected 2–6 d before imaging via biolistic gene transfer (160 psi, Helios gene gun; Bio-Rad) as

described in a detailed video protocol[18]. 10 µg of plasmid was coated to 6–8 mg of 1.6-µm gold beads.

Results

To determine whether expression of K_v2 proteins embedded in tissue can be imaged with GxTX-594 and provide evidence that an endogenous channel activity probe is possible, we overexpressed $K_v2.1$-GFP in rat brain slices and examined CA1 pyramidal neurons of the hippocampus. We chose CA1 neurons for several reasons: They express K_v2 channels at a density typical of central neurons, the physiology of these neurons has been intensively studied, and their electrical properties are relatively homogeneous[19]. Organotypic hippocampal slice cultures prepared from postnatal day 5–7 rats were sparsely transfected with $K_v2.1$-GFP, resulting in a subset of neurons displaying green fluorescence. When imaged 2–4 days after transfection, GFP fluorescence was observed in the plasma membrane surrounding neuronal cell bodies and proximal dendrites (Supplemental Figure 2, A and B).

Six days or more after transfection, $K_v2.1$-GFP fluorescence organized into clusters on the surface of the cell soma and proximal processes (Figure 1, A), a pattern consistent with a prior report of endogenous $K_v2.1$ in CA1 neurons[19]. After identifying a neuron expressing $K_v2.1$-GFP, solution flow into the imaging chamber was stopped, and GxTX-594 was added to the static bath solution to a final concentration of 100 nM. After 5 minutes of incubation, solution flow was restarted, leading to washout of excess GxTX-594 from the imaging chamber. After washout, GxTX-594 fluorescence remained colocalized with $K_v2.1$-GFP (Figure 1), indicating that GxTX-594 can permeate through

dense neural tissue and bind to K_v2 proteins on neuronal surfaces. Pearson correlation coefficients confirmed the colocalization of GxTX-594 with $K_v2.1$-GFP in multiple slices (Figure 1, C). In most images of $K_v2.1$-GFP–expressing neurons, GxTX-594 also labeled puncta on neighboring neurons that did not express $K_v2.1$-GFP but at intensities that were roughly an order of magnitude dimmer (Figure 1, B, white arrow). The clustered GxTX-594 fluorescence patterns on the cell body and proximal processes of CA1 neurons were strikingly similar to reported patterns of anti-K_v2 immunofluorescence patterns and are consistent with GxTX-594 labeling of endogenous K_v2 proteins in CA1 neurons.

While I cannot exclude the possibility that CA1 neurons have a subset of K_v2 proteins on their surface that is not labeled by GxTX-594, I saw no indication of $K_v2.1$-GFP on neuronal surfaces that are not labeled by GxTX-594. While we observed GxTX-594 fluorescence that morphologically resembles endogenous K_v2 protein localization, I also found that GxTX-594 occasionally labels structures not consistent with K_v2 proteins (Supplemental Figure S2, B, bottom panel, arrows). This non-K_v2 labeling was most prevalent at the surface of the hippocampal slices and progressively decreased as the imaging plane was moved deeper into the tissue (data not shown). Our interpretation of this phenomenon is that GxTX-594 can accumulate in the dead tissue and debris that is present at the surface of a hippocampal section after it is cut. For this reason, I analyzed only GxTX-594 fluorescence with subcellular localizations consistent with K_v2 channels.

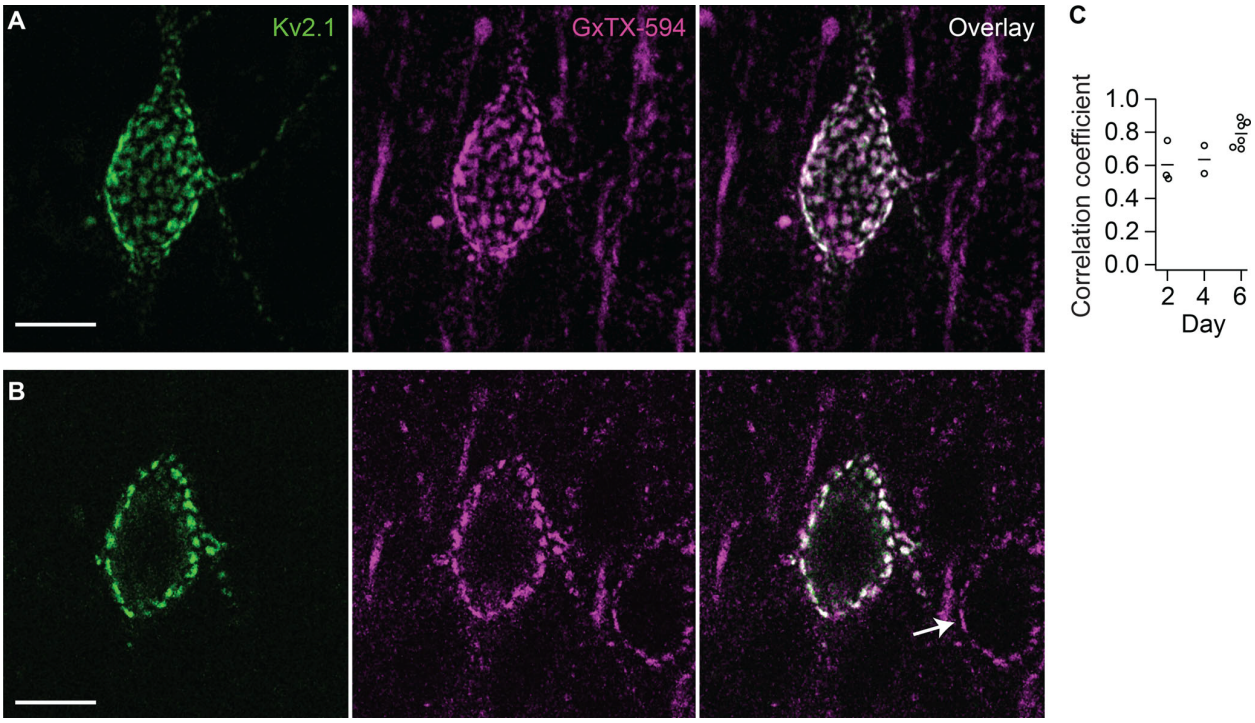

Figure 1. GxTX-594 labels CA1 hippocampal pyramidal neurons transfected with Kv2.1-GFP

(A) Two-photon excitation images of fluorescence from the soma and proximal dendrites of a rat CA1 hippocampal pyramidal neuron in a brain slice six days after transfection with K_v2.1-GFP (left), labeled with 100 nM GxTX-594 (middle), and the overlay (right). The image represents a Z-projection of 20 optical sections. Scale bar, 10 µm. (B) A single optical section of the two-photon excitation image shown in A. GxTX-594 labels both K_v2.1-GFP puncta from a transfected cell and apparent endogenous K_v2 proteins from an untransfected cell in the same cultured slice (right, arrow). Scale bar, 10 µm.

(C) Pearson correlation coefficients from CA1 hippocampal neurons two, four, and six days after transfection with K_v2.1-GFP. Each circle represents a different neuron. Bars are arithmetic means. Coefficients were calculated by Robert Stewart.

Discussion

The molecular targeting, conformation selectivity, and spatial precision of fluorescence from GxTX-594 show the location within tissue where the conformational status of K_v2 voltage sensors becomes altered. However, the utility of GxTX-594 as an EVAP is limited by several factors, including emission intensity, variability between experiments, and inhibition of K_v2 proteins. While this EVAP may have drawbacks (such

as speed), it is the only established imaging method for measuring voltage-sensitive conformational changes of a specific, endogenous protein. GxTX binds to the K_v2 voltage sensor when the sensor is in the resting state[15]. Knowing this, one can expect that reversible GxTX-594 labeling will bind with the highest affinity specifically to the fully resting conformation of the K_v2 voltage sensor[20]. The images of GxTX-594 fluorescence in Figure 1 reveal this conformation's occurrence with subcellular spatial resolution. From these labeled channels, it is possible to infer the behavior of the unlabeled channels. This enables the subcellular locations where K_v2 voltage sensing occurs to be seen for the first time.

Section 2 – Subcellular localization of $Na_v1.7$ in mouse nociceptors

To design peptides to attenuate nociceptive signaling, it was necessary to identify where the target sodium channel was expressed, and if it could be identified in the pain signaling neurons within the spinal cord and the DRG. As the protein MrgprD has been shown to be expressed in a subset neurons that transmit pain signaling[21,22], this protein was used to identify these mouse nociceptor sensory neurons. In this project, isolated spinal column slices from mice genetically modified to express GFP in place of MrgprD ($MrgprD^{GFP}$) were used for immunohistochemical staining against $Na_v1.7$ to indicate if the channels could be targeted outside the spinal cord.

Methods

Mice were anesthetized with isoflurane until deep breathing was observed. When there was no response from toe pinch the mice were decapitated. The spinal cords

were removed and excess tissue excised. Spines were cut in half in the middle of the vertebrae after the last rib. Spine segments were drop fixed in freshly made ice cold 4% paraformaldehyde for one hour. Time from decapitation to fix was approximately 3-4 minutes. Spines were washed for ten minutes repeated three times in phosphate buffer. Spines were placed in 30% sucrose for 24 hours to cryoprotect, then cut and frozen in OCT.

Samples were sectioned into 30-micron slices with a cryostat, collected on slides, and outlined with an Aqua Hold 2 hydrophobic pen. Slices were washed with a phosphate buffered solution with 4% milk and 0.2% Triton X-100 in 0.1 M phosphate buffer (blocking solution).

Blocking solution: 4% milk and 0.2% Triton X-100 in 0.1 M PB

4X PB (0.4 M phosphate buffer) Stock Solution (1 L)

- 45.69 g Na HPO_4 (MW 141.96) for 320 mM phosphate
- 10.49 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99) for 76 mM phosphate
- Allow reagents to dissolve into 80-90% final volume of dd$H_2$0
- Adjust pH to 7.4 and bring to final volume of 1 L

Samples were washed for one hour in 0.1mg/mL IgG F(ab) polyclonal IgG antibody (cat# is ab6668 from Abcam or 115-007-003 from Jackson Immuno Research lot#145216) diluted in blocking solution at room temperature (RT) while gently rocked. Slides were incubated with 0.1mg/mL primary antibody chicken anti-GFP AB from Dr. Theanne Griffith (Abcam cat # ab13970), and 0.1mg/mL mouse Anti-Na_v1.7

(Neuromab AB_2877500)[23] in blocking buffer for one hour at RT. Slides were washed three times for five minutes each in blocking solution. Samples were incubated in 0.5 µg/mL fluorescently-conjugated secondary antibodies (series specific Alexa Fluor 488 Thermo Fisher Scientific catalog # A-21121, and Alexa Fluor 647 Thermo Fischer Scientific catalog #A32728) diluted in blocking buffer. Slides were further washed three times for five minutes in blocking buffer, then mounted to coverslip with application of anti-fade medium (ProLong Gold Antifade Mountant, Thermo Fisher Scientific catalog # P36930) with a syringe according to manufacturer's instructions. Samples were stored at -20˚C.

Using an inverted confocal system (LSM 880 410900-247-075; Zeiss) run by ZEN Black 2.1 software, 6 by 2 confocal images were taken and stitched together to cover the entire spinal cord and two DRG on the slice. A Plan-Apochromat 20x/0.8 objective (440640-9903-000; Zeiss) was used to capture the images. The 488 nm band of the argon laser powered at 15% and the Neon Helium 647 nm laser at 35% were used to image the GFP and tagged $Na_v1.7$ respectively, resulting in an image of 2125x2125 microns with 2.89 pixels per µm.

Results

This experiment identified mouse nociceptor neurons by the GFP expression from the MrgprD locus[24] $MrgprD^{GFP}$, MGI:3521853. Transcript levels of ion channels relevant to action potential propagation, including Na_vs, are generally similar between NP1 and other nociceptor subtypes profiled in mouse and macaque[22,25] . Nonmyelinated

axons have largely homogeneous membrane structures[26], and this data (Figure 2) confirms that $Na_v1.7$ localizes to the cell bodies and axonal processes of NP1 neurons.

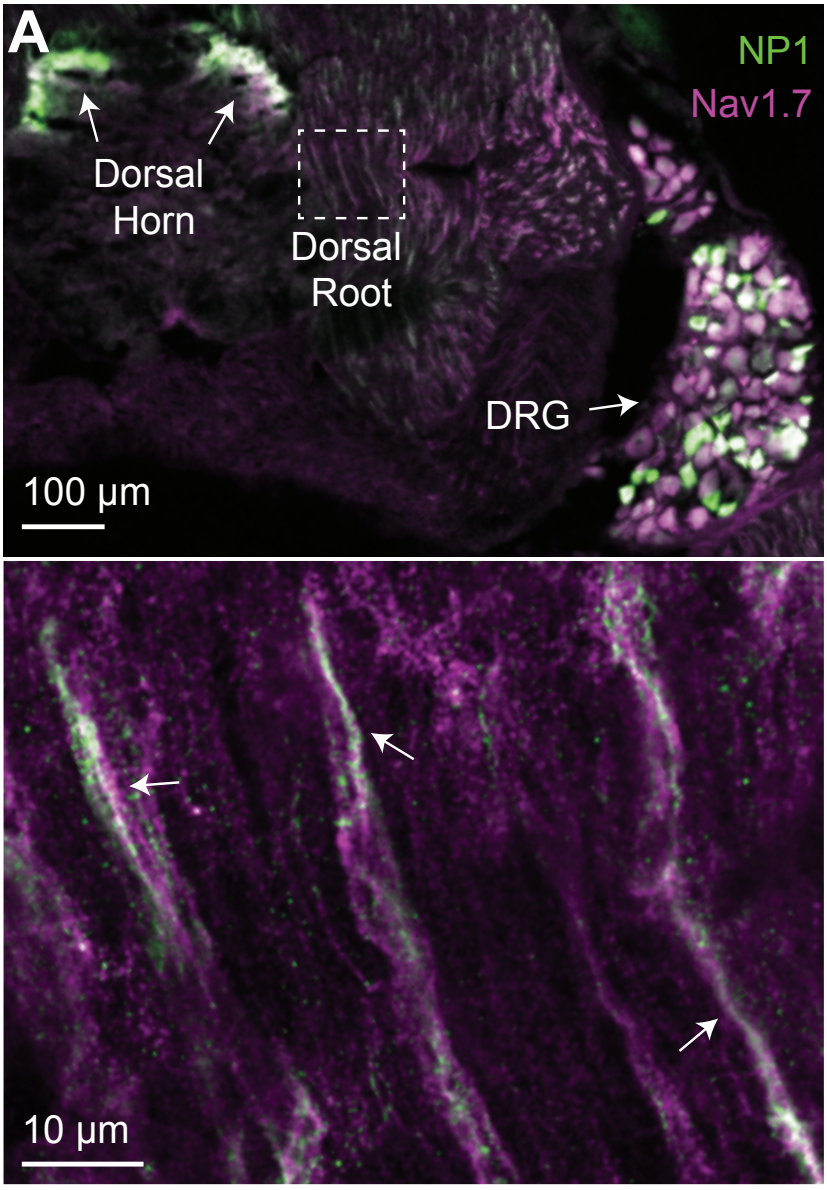

Figure 2. Immunostaining sodium channel in mouse spinal column
Top: Immunofluorescence from $MrgprD^{GFP}$ labeled NP1 nociceptors (AB_300798, green) and $Na_v1.7$ (AB_2877500, magenta) in a mouse L5 spinal section shows colocalization (white) in dorsal horn nociceptor terminals and DRG cell bodies. Bottom: Zoom of dorsal root (dashed box from top)

Discussion

The above image (Figure 2) supports the previously proposed hypothesis that $Na_v1.7$ is not only in the previously identified DRG cell bodies[21], but is also present in the dorsal root in C-type nociceptor fibers as identified by $MrgprD^{GFP}$. $Na_v1.7$ is present in these C-type fibers, which is consistent with previous research[9,27]. Since $Na_v1.7$ is indeed present in this location, it is possible to design blockers that target DRG or dorsal root, meaning that these therapies would not be required to pass through the blood-brain barrier[28].

Conclusion

This research into channel activity and location using fluorescent markers has led to the following conclusions.

In Section 1 (Contribution to *EVAP: A two-photon imaging tool to study conformational changes in endogenous K_v2 channels in live tissues*), I demonstrated that the probe could target K_v2 channels in living tissue and would bind to endogenous channels. The methodology can also be applied to other types of channels since there are other toxins that can be bound to other types of channels.

From Section 2 data ($Na_v1.7$ in DRG research), it is possible to conclude that $Na_v1.7$ channels can be identified in the nociceptor afferents identified by MrgprD, as well as the dorsal root ganglion cell bodies, which provides evidence that it could be possible to design a fluorescent probe that could target specific channels involved in pain signaling. Future pharmaceutical development can design the $Na_v1.7$ targeted pain attenuator treatment's bioavailability to take into account the location of the channels in

the DRG. Fluorescent tarantula toxin can reveal activation of neuronal ion channels, and if made bioavailable, toxins could bind the $Na_v1.7$ channels along C-type nociceptor fibers. This data provides a basis for future development of methods for ion channel imaging and measurement.

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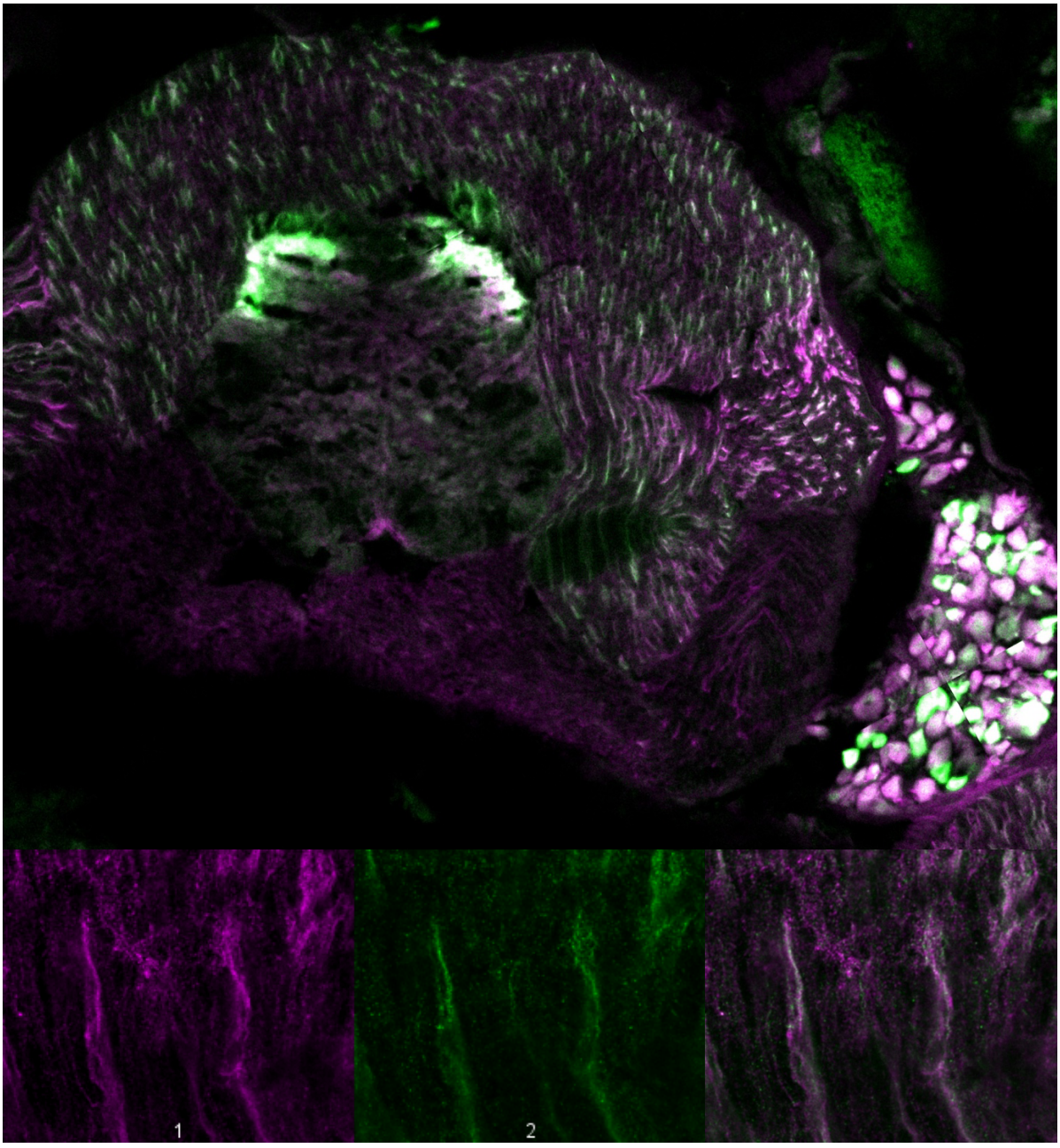
Conjugates, and Horseradish Peroxidase. *Methods Mol. Biol. Clifton NJ* **2367**, 87–103 (2021).

Appendix – Supplemental Images



Supplemental Figure 1. Alt. Section 2 – Nav1.7 in DRG image research. Immunostaining sodium channel in mouse spinal column

Top: Immunofluorescence from MrgprD^{GFP} labeled NP1 nociceptors (AB_300798, green) and $Na_v1.7$ (AB_2877500, magenta) in a mouse L5 spinal section shows colocalization (white) in dorsal horn nociceptor terminals and DRG cell bodies.

Bottom: Zoom of dorsal root, Far left is zoom of dorsal root stained against $Na_v1.7$ in magenta, center is MrgprD^{GFP} in dorsal root displayed in green, and far right is overlay composite image where colocalization is displayed in white.

Figure S2, A

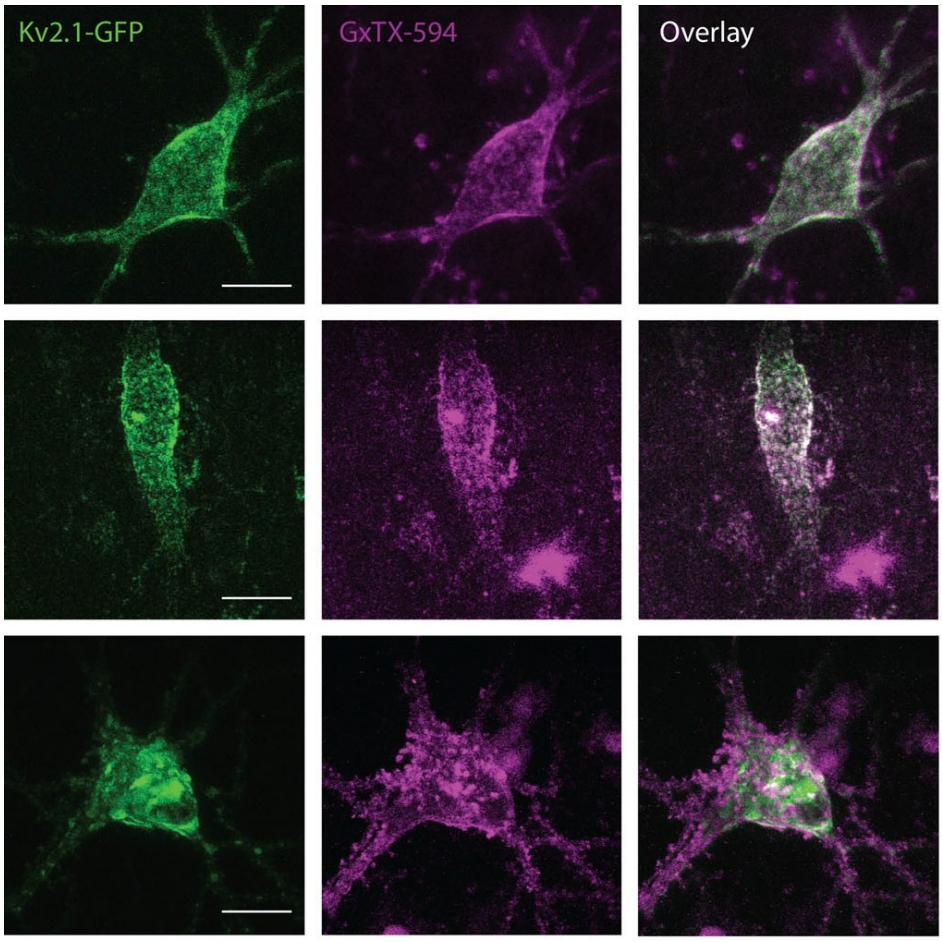


Figure S2, B

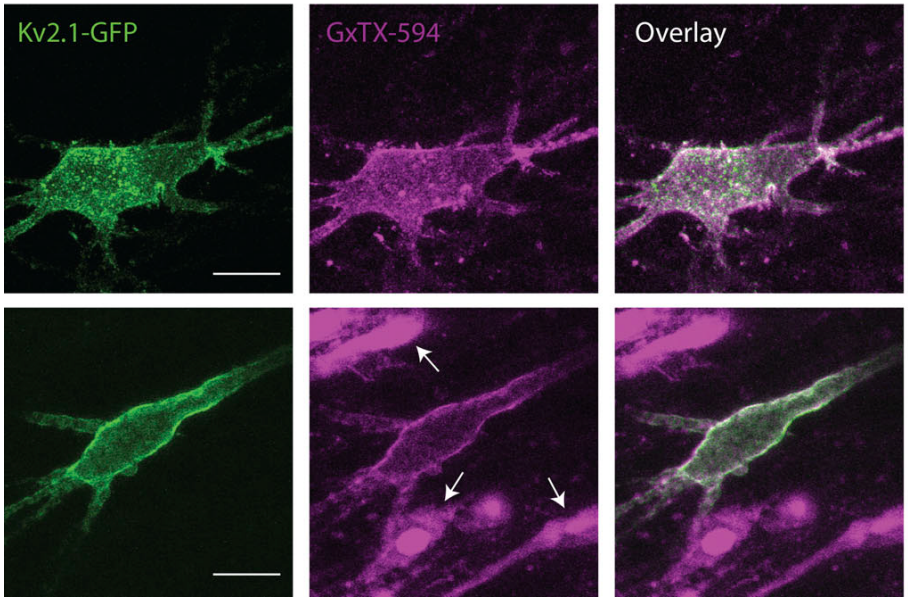
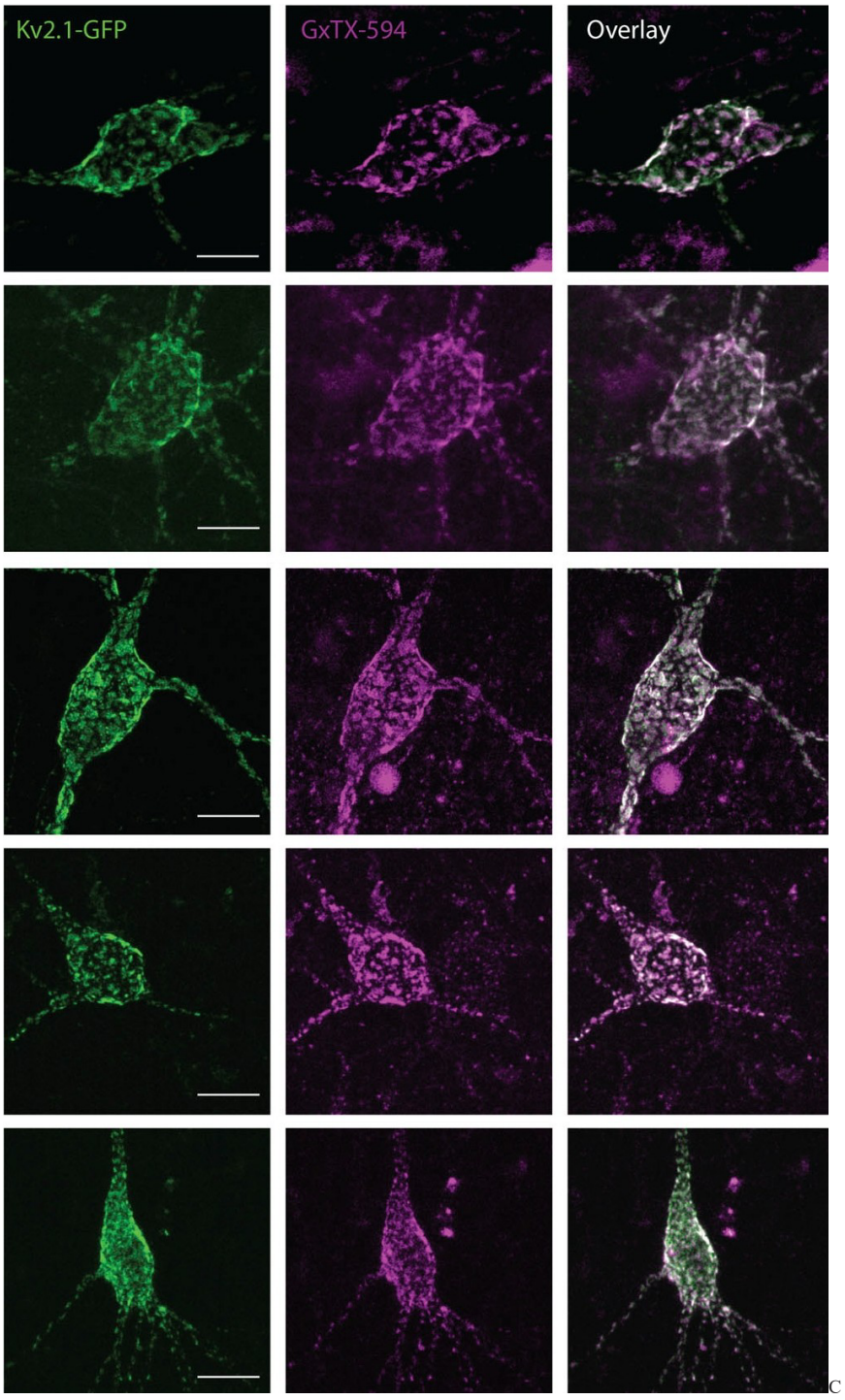


Figure S2, C



C

Supplemental Figure 2. Alt. Section 1 Contribution to* EVAP: A two-photon imaging tool to study conformational changes in endogenous Kv2 channels in live tissues –*GxTX-594 labels CA1 hippocampal pyramidal neurons transfected with K_v channels

Top: Two-photon excitation images of rat CA1 hippocampal pyramidal neurons in brain slices as in Fig. 1. K_v2.1-GFP (left), GxTX-594 (middle), and overlay (right). Scale bars, 10 μm in all panels. (A) Pyramidal neurons 2 days after transfection with K_v2.1-GFP. (B) Pyramidal neurons 4 days after transfection with K_v2.1-GFP. (C) Pyramidal neurons 6 days after transfection with K_v2.1-GFP.