Targeting intrinsically disordered regions facilitates discovery of calcium channels 3.2 inhibitory peptides for adeno-associated virus–mediated peripheral analgesia

Seung Min Shin a, Justas Lauzadis b, Brandon Itson-Zoske a, Yongsong Cai a,c, Fan Fan d, Gayathri K. Natarajan a, Wai-Meng Kwok a,e, Michelino Puopolo b, Quinn H. Hogan a, Hongwei Yu a,*

Abstract

Ample data support a prominent role of peripheral T-type calcium channels 3.2 (CaV3.2) in generating pain states. Development of primary sensory neuron-specific inhibitors of CaV3.2 channels is an opportunity for achieving effective analgesic therapeutics, but success has been elusive. Small peptides, especially those derived from natural proteins as inhibitory peptide aptamers (iPAs), can produce highly effective and selective blockade of specific nociceptive molecular pathways to reduce pain with minimal off-target effects. In this study, we report the engineering of the potent and selective iPAs of CaV3.2 from the intrinsically disordered regions (IDRs) of CaV3.2 intracellular segments. Using established prediction algorithms, we localized the IDRs in CaV3.2 protein and identified several CaV3.2iPA candidates that significantly reduced CaV3.2 current in HEK293 cells stably expressing human wide-type CaV3.2. Two prototype CaV3.2iPAs (IPA1 and IPA2) derived from the IDRs of CaV3.2 intracellular loops 2 and 3, respectively, were expressed selectively in the primary sensory neurons of dorsal root ganglia in vivo using recombinant adeno-associated virus (AAV), which produced sustained inhibition of calcium current conducted by CaV3.2/T-type channels and significantly attenuated both evoked and spontaneous pain behavior in rats with neuropathic pain after tibial nerve injury. Recordings from dissociated sensory neurons showed that AAV-mediated CaV3.2iPA expression suppressed neuronal excitability, suggesting that CaV3.2iPA treatment attenuated pain by reversal of injury-induced neuronal hypersensitivity. Collectively, our results indicate that CaV3.2iPAs are promising analgesic leads that, combined with AAV-mediated delivery in anatomically targeted sensory ganglia, have the potential to be a selective peripheral CaV3.2-targeting strategy for clinical treatment of pain.

Keywords: T-type/Cav3.2 channels, Peripheral nervous system, Dorsal root ganglia, Adeno-associated virus, Peptide aptamer, Neuropathic pain

1. Introduction

Chronic pain is a critical national health problem for which opioid treatment has numerous risks, including misuse, overdose, and addiction, highlighting the need for new analgesic targets and strategies. The peripheral nervous system is a particularly accessible site for devising new pain treatments because the primary sensory neurons (PSNs) of the dorsal root ganglia (DRG) initiate nociception and have a central role in the development and maintenance of nerve injury–induced painful neuropathy. T-type/calcium channels 3.2 (CaV3.2) in PSNs regulate neuronal excitability, peripheral nociceptive transduction, and excitatory neurotransmission in dorsal horn neurons and are important mediators of pain signaling.

Ample data support a prominent role of peripheral CaV3.2 in pain pathology, including elevated expression and activity in inflammatory and neuropathic pain, diabetic peripheral neuropathic pain, chemotherapy-induced peripheral neuropathy, osteoarthritis pain, and postsurgical pain, as well as itch. However, CaV3.2 is also expressed throughout the body, including endocrine, muscle, and kidney tissues, peripheral motor neurons, and pacemaker cells of the heart, and efforts to date using currently available drugs targeted to CaV3.2 administered systemically have led to inadequate analgesia and significant side effects. Indeed, recent multicenter, double-blind, controlled and randomized clinical trials using the established T-type channel blocker ethosuximide (Zarontin, Pfizer) or the T-type CaV3.2+ channel blocker ABT-639 were...
terminated because of the high number of adverse events, as well as failure to reduce pain, so novel approaches are needed.

Molecular signaling interactions are often mediated by the regions of proteins lacking a defined tertiary structure, known as intrinsically disordered regions (IDRs). These comprise a large part of the eukaryotic proteome and have been established as key facilitators of protein regulatory functionality. Intrinsically disordered regions are common in integral membrane proteins, particularly in the intracellular loops (ICLs) linking transmembrane (TM) structural domains and the protein termini. These intrinsically unstructured regions often contain protein-modulating architectures that consist of multiple domains existing as short linear peptide motifs within IDRs and functioning without a stable three-dimensional structure. These types of protein domains within IDRs are named intrinsically disordered domains (IDDs), which are important players in multiple signaling regulations by engaging in binding to cognate sites of multiple partners and are considered new and promising drug targets.

The CaV3.2 protein consists of 4 highly structured homologous TM domains, connected by ICLs and flanked by intracellular N-termini and C-termini that serve as essential molecular interfaces for CaV3.2 regulatory signaling networks. Understanding of functional domains in CaV3.2 IDRs is currently limited but would be valuable in the delineation of CaV3.2 regulatory mechanisms and for future drug development. In this study, we report identification of highly disordered regions in the CaV3.2 protein and define several CaV3.2 inhibitory peptide aptamers (iPAs) that substantially reduce CaV3.2/T-type current. Adeno-associated virus (AAV)-mediated expression of these prototype CaV3.2 iPAs in PSNs in vivo produces sustained T-type Ca2+ current inhibition and attenuates neuropathic pain behavior in rats, suggesting CaV3.2 iPAs as potential analgesic leads for translational pain therapeutic development.

2. Methods

2.1. Animals

Adult male and female Sprague Dawley rats weighing 100 to 125 g body weight (Charles River Laboratories, Wilmington, MA) were used. All animal experiments were performed with the approval of the Medical College of Wisconsin Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Animals were housed individually in a room maintained at a constant temperature (22 ± 0.5°C) and relative humidity (60 ± 15%) with an alternating 12-hour light–dark cycle. Animals were provided access to water and food ad libitum throughout the experiment, and all efforts were made to minimize suffering. All survival surgeries were completed in a sterile environment under a surgical microscope in animals anesthetized with isofluran (2%-5%). For tissue harvest euthanasia, animals were deeply anesthetized using isofluran, followed by decapitation with a well-maintained guillotine. The estimated numbers of animals needed were derived from our previous experience with similar experiments and the number of experiments needed to achieve a statistically significant deviation (>20% difference at P < 0.05) based on a power analysis.

2.2. Computational (in silico) designs

The rat CaV3.2 full amino acid (aa) sequence was retrieved from the UniProt KB knowledge database (UniProt Knowledgebase release 2018_11). CaV3.2 protein TM domains and intracellular termini and loops were predicted by Phobius (https://www.ebi.ac.uk/Tools/pfa/phobius/). The CaV3.2 protein iPAs were predicted by analyzing the full-length rat CaV3.2 sequence using DEPICTER (DisorderEd Prediction CenTER, http://biomine.cs.vcu.edu/servers/DEPICTER/). Relative IDR aa (%) of multiple ion channels was predicted using PONDR (http://www.pondr.com/cgi-bin). Potential phosphorylation sites in the CaV3.2 full aa sequence were identified using Disorder Enhanced Phosphorylation Predictor (DEPP) (http://www.pondr.com/cgi-bin/depp.cgi). Potentially functional peptides (IDPs) within the iPAs were further analyzed using Motifs (http://molbiol-tools.ca/Motifs.html). Eukaryotic Linear Motif (ELM, http://elm.eu.org) and SLIMPrints (http://bioware.ucd.ie/slimprints.html), which predict short linear motifs (SLIMs) based on strongly conserved SLIMs within IDRs. Peptide structure prediction was analyzed by I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER), and the IDR feature of green fluorescent protein (GFP)-IPA was predicted by the PONDR program.

2.3. Molecular cloning and adeno-associated virus constructs

To construct the AAV vector encoding a chimeric monomer GFP (hereafter referred to as GFP) CaV3.2IPA expression cassette, the DNA fragments encoding the CaV3.2IPA peptides were synthesized and subcloned into BsrG I/Sal I sites (Genscript, Piscataway, NJ) of a single-strand AAV expressing plasmid pAAV-chicken β-actin (CBA)-GFP. This generated pAAV-CBA-GFP-CaV3.2IPAs that encode the GFP-CaV3.2IPAs fusion protein downstream a chimeric intron for enhancing transcription, driven by a hybrid human cytomegalovirus (CMV) enhancer/CBA promoter, and the mRNA stabilizing Woodchuck Posttranscriptional Regulatory Element sequence was inserted downstream of the stop codon of GFP-CaV3.2IPAs and upstream of human growth hormone poly A signals. Plasmids were subsequently used in transfection experiments and in AAV vector generation. Transfection of cultured cells was performed by a standard polyethylenimine (PEI, MW 40,000, Polysciences, Inc, Warrington, PA) transfection protocol. To package AAV2/6-GFP-CaV3.2IPAs and AAV2/6-GFP-NP (a Cav3.2 N-terminal inert peptide, see further) as a control (subsequently referred to as AAV6-CaV3.2IPAs and AAV6-CaV3.2NP, respectively) for in vivo injection, AAV vectors were produced and purified in our laboratory by previously established methods. This included AAV particle purification by optiprep ultracentrifugation and concentration using Centricon Plus-20 (Regenerated Cellulose 100,000 MWCO; Millipore, Billerica, MA). The AAV titer was determined by the PicoGreen (Life Technologies, Carlsbad, CA) assay, and final aliquots were kept in 1x phosphate-buffered saline (PBS) containing 5% sorbitol (Sigma-Aldrich, St. Louis, MO) and stored at -80°C. The titers (GC/mL) of AAV6-CaV3.2IPA1, 2, and 3 and AAV6-CaV3.2NP vectors were 2.45 × 1013, 3.05 × 1013, and 2.64 × 1013, respectively. Two lots of viral preparations were used for in vivo experiments.

2.4. Cell culture

Human embryonic kidney 293 (HEK293) cell lines stably expressing human wide-type CaV3.2 (HEK3.2), CaV3.1 (HEK3.1), and CaV3.3 (HEK3.3) (Kerafast, Boston, MA), CaV2.2 (HEK2.2, provided by Dr. Missler at Georg-August University, Germany), and neuronal NG108-15 (ATCC, Manassas, VA) were cultured in Dulbecco modified Eagle medium supplemented...
with glutamax, 10% fetal bovine serum (ThermoFisher, Rockford, IL), and antibiotics using standard techniques. Dissociated DRG neuronal culture for electrophysiology was performed as previously described. In brief, DRG (L4 and L5) from male rats were rapidly harvested from the isolufrane-anesthetized animals and were incubated in 0.01% liberase blendzyme 2 (Roche Diagnostics, Madison, WI) for 30 minutes, followed by incubation in 0.25% trypsin and 0.125% DNase for 30 minutes, both dissolved in Dulbecco modified Eagle medium/F12 with glutaMAX (ThermoFisher, Rockford, IL). After exposure to 0.1% trypsin inhibitor and centrifugation, the pellet was gently triturated in culture medium containing Neural basal media A (ThermoFisher) and 0.5 μm glutamine. Dissociated cells were plated onto 5% laminin-coated glass coverslips (ThermoFisher), maintained at 37°C in humidified 95% air and 5% CO2, and were studied in approximately 6 to 8 hours after harvest in electrophysiological experiments.

2.5. Electrophysiological recordings

Electrophysiological recordings were performed, as previously described with minor modifications, in a blinded manner where the electrophysiologist was not aware of the treatment. Patch pipettes, ranging from 2 to 4 MΩ resistance, were formed from borosilicate glass (King Precision Glass Co, Claremont, CA) and fire polished. Recordings were made with an Axopatch 700B amplifier (Molecular Devices, Downingtown, PA). Signals were filtered at 2 kHz and sampled at 10 kHz with a Digidata 1440A amplifier (Molecular Devices, Madison, WI) for 30 minutes, followed by incubation in 0.25% trypsin and 0.125% DNase for at least 30 minutes. Calcium channel blockers were then applied to the bathing solution. The concentrations used were saturating in preliminary experiments. Any residual high-voltage-activated (HVA) ICa after incubation of HVA calcium channel blockers was eliminated by using fluoride in the internal pipette solution.

The commonly used equation for the steady-state inactivation of ICa is the following:

\[ I_{\text{Ca}}(V) = G_{\text{Ca}}(V) \cdot \frac{1}{1 + \exp \left( \frac{V - V_{1/2}}{k} \right)} \]

where \( G_{\text{Ca}}(V) \) is the conductance, \( V_{1/2} \) is the half-activation potential, and \( k \) is the slope factor. 

2.5.2. Whole-cell voltage-clamp recording on dissociated dorsal root ganglia neurons (male rats)

2.5.3. Sodium channel 1.7 current (INav1.7) in NG108-15 cells

2.5.4. Voltage-gated potassium channel current in NG108-15 cells
glucose, 10 HEPES at a pH of 7.4 with NaOH and an osmolarity of 300 mOsm. The recording pipette solution in all the experiments contained the following (in mM): 95 K-Gluconate, 2 KCl, 2 MgCl₂, 4 MgATP, 0.3 Na₃GTP, 0.2 EGTA, and 10 HEPES at a pH of 7.2 and an osmolarity of 290 mOsm. Voltage protocols consisted of 300-ms square-wave commands from a holding potential of −80 mV for Iᵥ channels to +120 mV, in 10 mV increments with 5-second intervals between steps.

2.5.5. Whole-cell current-clamp recording on dissociated dorsal root ganglia neurons (male rats)

Whole-cell current-clamp recording of dissociated DRG neurons was performed as previously described 59,78,92 to determine the effects of AAV-mediated Caᵥ3.2iPA1 expression on neuronal excitability. Neurons were dissociated from DRG of sham-operated rats, rats with tibial nerve injury (TNI) only, and TNI rats injected with AAV6-3.2NP or AAV6-Caᵥ3.2iPA1 at 6 weeks after TNI and 6 weeks after vector injection (n = 5 rats per group). Selected neurons were small-sized and medium-sized (≤40 μm in diameter) and exhibited clear GFP expression in the AAV6-3.2NP or AAV6-Caᵥ3.2iPA1 group. For whole-cell current-clamp, patch electrodes had a resistance of 0.7 to 1.5 MΩ when filled with the pipette solution, which contained the following (in mM): 140 K-Gluconate, 5 KCl, 2 MgCl₂, 0.2 EGTA, 10 HEPES, 4 Mg-ATP, 0.3 Na₃GTP, and 10 Na₂-phosphocreatine at a pH of 7.2 with KOH and an osmolarity of 296 to 300 mOsm. The extracellular solution contained the following (in mM): 140 NaCl, 4 KCl, 2 CaCl₂, 2 MgCl₂, 10 D-glucose, and 10 HEPES at a pH of 7.4 with NaOH and an osmolarity of 300 mOsm. Whole-cell configuration was obtained in voltage-clamp mode before proceeding to the current-clamp recording mode. The membrane input resistance was calculated by dividing the end amplitude of steady-state hyperpolarizing voltage deflection by the injected current.40 Action potentials (APs) were generated by injection of a series of current pulses (100-1000 pA in steps of 100 pA, 250 ms). The baseline (BL) potential had been recorded for 20 ms before the stimulus pulses were injected into the neurons. We defined the resting membrane potential (RMP) as the mean value of the 20-ms prestimulus potential in the first trial and the AP rheobase as the minimum current required to evoke the first AP. Given the knowledge that nerve injury induces high RMP and low rheobase in DRG neurons,12 the neurons with stable RMP more negative than −40 mV and overshooting APs (>80 mV RMP to peak) were used for additional data collection. Excitability was characterized by determining the rheobase, defined as the minimum current necessary to achieve threshold AP firing, and the AP firing frequency elicited in response to 250 ms depolarizing current injections of progressively larger amperage in 0.1 nA increments, administered at 5-second intervals.

2.6. Measurement of cytoplasmic Ca²⁺ concentration (male rats)

Measurement of cytoplasmic Ca²⁺ concentration ([Ca²⁺]ᵢ) was performed following our previously published protocols.24,77 In brief, DRG neurons plated on coverslips were exposed to Fura-2-AM (5 μM) at room temperature in a solution that contained 2% bovine albumin to aid dispersion of the fluorophore. After 30 minutes, coverslips were washed 3 times with regular Tyrode solution containing the following (in mM): 140 NaCl, 4 KCl, 2 CaCl₂, 10 glucose, 2 MgCl₂, and 10 HEPES, with an osmolarity of 297 to 300 mOsm and pH 7.4, given 30 minutes for de-esterification, and mounted in the recording chamber. Neurons were first examined under bright-field illumination, and those showing signs of lysis, crenulation, or superimposed glial cells were excluded. For [Ca²⁺]ᵢ recording, the fluorophore was excited alternately with 340 nm and 380 nm wavelength illumination (150 W Xenon, Lambda DG-4, Sutter, Novato, CA), and images were acquired at 510 nm using a cooled 12-bit digital camera (Coolsnapsfx; Photometrics, Tucson, AZ) and inverted microscope (Diaphot 200; Nikon Instruments, Melville, NY) through a 20× objective. Recordings from each neuron were obtained as separate regions (MetaFluor, Molecular Devices) at a rate of 3 Hz. After background subtraction, the fluorescence ratio R for individual neurons was determined as the intensity of emission during 340 nm excitation (I₃₄₀) divided by I₃₈₀ on a pixel-by-pixel basis. Transient changes in [Ca²⁺]ᵢ were generated by depolarization produced by microperfusion application of K⁺ (50 mM) for 0.3 seconds, which, as previously shown, selectively activates T-channel specific.59

2.7. Microinjection of adeno-associated virus vectors into dorsal root ganglia

Adeno-associated virus vector solution was microinjected into the right lumbar (L4 and L5 DRG using previously described techniques.24 In brief, the surgically exposed intervertebral foramen was slightly enlarged by removal of laminar bone. Injection was performed through a micropipette that was advanced approximately 100 μm into the ganglion. Rats received L4 and L5 DRG injections of either AAV6-Cav3.2iPA or AAV6-Cav3.2NP (one vector per rat), consisting of 2 μL with adjusted titers containing a total of 2.0 × 10¹⁰ genome viral particles for each DRG. Injection was performed over a 5-minute period using a microprocessor-controlled injector (Nanoliter 2000; World Precision Instruments, Sarasota, FL). Removal of the pipette was delayed for an additional 5 minutes to minimize the extrusion of the injectate. After the injection and closure of overlying muscle and skin, the animals were returned to their housing where they remained as the designed experiments required.

2.8. Animal pain model and behavior testing (male and female rats)

2.8.1. Tibial nerve injury

To model clinical traumatic painful peripheral neuropathy, we performed TNI, an established model of neuropathic pain after peripheral nerve injury.87 Animals were anesthetized using isoflurane at 4% for induction and 2% for maintenance. Under anesthesia, the right sciatic nerve was exposed under aseptic surgical conditions by blunt dissection of the femoral biceps muscle. The sciatic nerve and its 3 branches (sural, common peroneal, and tibial nerves) were isolated. The tibial nerve was then tightly ligated and transected distal to the ligation. The overlying muscle and skin were then sutured after surgery. Sham-operated rats were subjected to all preceding procedures but without nerve ligation and transection.

2.8.2. Evoked behavior testing

Behavioral tests were conducted between 9:00 AM and 12:00 AM. Experimenter were blinded to the treatment. Animals were habituated in individual test compartments for at least 1 hour before each testing. Sensory testing of the plantar skin included eliciting reflexive behaviors induced at threshold intensity by punctate mechanical stimulation (von Frey test [vF]), dynamic mechanical stimulation (brush), noxious mechanical stimulation (pin), cold
2.8.3. Gabapentin injection

Gabapentin (GBP, Sigma-Aldrich) was dissolved in saline immediately before injections and administered intraperitoneally (i.p.) at a volume of 0.5 to 1.0 mL (final dose of 100 mg/kg body weight). The hindpaw vF and Pin tests on the ipsilateral side were performed at 15-minute intervals for 3 hours after GBP injection.

2.8.4. Conditioned place preference

Spontaneous pain, which is a prominent feature of neuropathic pain conditions, can be measured by CPP. The ongoing aversiveness (i.e., affective dimension of spontaneous pain) was determined by GBP-induced CPP test, as previously described with minor modifications. A 3-chamber CPP apparatus was used (Med Associates, St. Albans, VT) in which 2 sliding doors separate the central chamber from the 2 side chambers that have distinct wall stripes and flooring. Animal movement and time spent in each chamber were measured using computer-interface infrared photoembs. The CPP procedure consisted of the following phases: (1) on the preconditioning day, rats were allowed to explore both sides of the chambers for 15 minutes, the time spent in each side was recorded, and the preferred and nonpreferred chambers were identified. Animals that showed a predetermined level of preference for 1 chamber (≥70% of total time) at this stage were excluded for further study; (2) on the conditioning days, place conditioning was conducted using a biased assignment approach to drug pairing: saline was paired with the preferred chamber in the morning, and GBP was paired with the nonpreferred chamber in the afternoon (injections were never paired with the middle grey chamber). Conditioning consisted of the following sequential steps: intraperitoneal injection and restriction of the animal within the nonpreferred chamber for 45 minutes. We used a 45-minute conditioning time based on tests that GBP maximally reduced TNI-induced mechanical hypersensitivity at 30-60 minutes after injection. Animals were conditioned for 2 days because 2-day GBP has been reported sufficient to produce CPP in rodent pain models and (3) for postconditioning testing, the animals were never paired with the middle grey chamber. Conditioning consisted of the following sequential steps: intraperitoneal injection and restriction of the animal within the nonpreferred chamber for 45 minutes. We used a 45-minute conditioning time based on tests that GBP maximally reduced TNI-induced mechanical hypersensitivity at 30-60 minutes after injection. Animals were conditioned for 2 days because 2-day GBP has been reported sufficient to produce CPP in rodent pain models and (3) for postconditioning testing, the animals were never paired with the middle grey chamber. Conditioning consisted of the following sequential steps: intraperitoneal injection and restriction of the animal within the nonpreferred chamber for 45 minutes. We used a 45-minute conditioning time based on tests that GBP maximally reduced TNI-induced mechanical hypersensitivity at 30-60 minutes after injection. Animals were conditioned for 2 days because 2-day GBP has been reported sufficient to produce CPP in rodent pain models and (3) for postconditioning testing, the animals were never paired with the middle grey chamber. Conditioning consisted of the following sequential steps: intraperitoneal injection and restriction of the animal within the nonpreferred chamber for 45 minutes. We used a 45-minute conditioning time based on tests that GBP maximally reduced TNI-induced mechanical hypersensitivity at 30-60 minutes after injection. Animals were conditioned for 2 days because 2-day GBP has been reported sufficient to produce CPP in rodent pain models and (3) for postconditioning testing, the animals were never paired with the middle grey chamber. Conditioning consisted of the following sequential steps: intraperitoneal injection and restriction of the animal within the nonpreferred chamber for 45 minutes. We used a 45-minute conditioning time based on tests that GBP maximally reduced TNI-induced mechanical hypersensitivity at 30-60 minutes after injection. Animals were conditioned for 2 days because 2-day GBP has been reported sufficient to produce CPP in rodent pain models and (3) for postconditioning testing, the animals were never paired with the middle grey chamber.
2.11. Statistical analyses

Statistical analysis was performed with GraphPad PRISM 9 (GraphPad Software, San Diego, CA). Behavioral changes compared with pretreatment BL, and between groups for von Frey and heat measurements, were generated using the repeated measures two-way analysis of variance and Tukey post hoc for within-group analysis and Bonferroni test for between groups. Pin and cold tests produce discrete numerical data without normal distributions, so conservative nonparametric analysis was performed by the Friedman tests and Dunn post hoc analysis. For comparisons between groups, in the pilot in vivo testing of simultaneous TNI operation and AAV injection, the effects of vector injection were characterized by area under the curve (AUC) analysis; in the treatment protocol of established pain, the measures immediately before AAV injection at the 14th day post TNI were used as the treatment BL (tBL) for calculating treatment AUC (tAUC). Calculated AUC and tAUC were compared between vectors by the Student t-test for von Frey and heat and by the Mann-Whitney test for brush, pin, and cold. Differences in the electrophysiological and immunoblot experiments were compared with the 1-way analysis of variance, 2-tailed unpaired t-test, or Mann-Whitney test, where appropriate. Results were reported as mean and SD of the mean (SEM). Significant differences for values were reported using asterisks *, **, and ***, denoting P < 0.05, <0.01, and <0.001, respectively. Exact P values for all
Figure 2. Inhibition of ICa3.2 of HEK3.2 cells by CaV3.2iPA candidates. Shown are results of functional testing of 3.2iPAs in block of ICa3.2 in HEK3.2 cells. In initial screening recordings: Representative Ba2+ current traces elicited by whole-cell voltage-clamp recording for sham-HEK3.2 cells (A) or HEK3.2 cells transfected with plasmids coding GFP (B), 3.2NP (C), 3.2iPA3 (D), 3.2iPA1 (E), 3.2iPA2 (F), and 3.2iPA4 (G), respectively (insets: recording protocol and current/time scales). Comparison of the mean peak current density–voltage (I/V) relationship from different constructs (H) and quantitative analysis of averaged peak ICa3.2 density as indicated (I); *P < 0.05 and ***P < 0.001, 1-way ANOVA and Tukey post hoc test. No effects of expression of 3.2iPA1 and 3.2iPA2 were observed on steady-state activation (J, inset: V50 activation) and inactivation (K, inset: V50 inactivation), compared with sham-transfected and 3.2NP-transfected cells. In replicability tests from an independent external institute: ICa3.2 was elicited from −110 to +60 mV in 10 mV increments. The external solution was in mM: 151 TEA-Cl, 2 CaCl2, 1 MgCl2, 10 HEPES, and 13 Glucose, pH 7.4. The internal solution was in mM: 125 CsCl, 10 NaCl, 1 MgCl2, 10 EGTA, and 10 HEPES, pH = 7.2. Representative ICa3.2 recorded from sham HEK3.2 cells (A1) and from HEK3.2 cells transfected with plasmid containing GFP (B1), 3.2NP (C1), 3.2iPA1 (D1), and 3.2iPA2 (E1) (insets: recording protocol and current/time scales). (F1) I/V curves for ICa3.2 recorded from sham, GFP, 3.2NP, 3.2iPA1, and 3.2iPA2. (H1) quantitative analysis of averaged peak ICa3.2 density; ***P < 0.001, 1-way ANOVA and Tukey post hoc test. 3.2iPA1 and 3.2iPA2 on steady-state activation (G1) (inset: V50 activation) and inactivation (I1) (inset: V50 inactivation), compared with sham-transfected, GFP-transfected, and 3.2NP-transfected cells. ANOVA, analysis of variance; Ca3.2, calcium channels 3.2; ICa, calcium current; G/Gmax, normalized conductance; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; iPA, inhibitory peptide aptamer; I/Imax, normalized current; MCW, Medical College of Wisconsin; SBU, Stony Brook University.
3. Results

3.1. In silico prediction of calcium channels 3.2 intrinsically disordered regions and design of candidate CaV3.2iPAs

To identify specific IDRs, we analyzed the full-length rat CaV3.2 protein sequence using DEPICTER, which combines 10 popular algorithms for IDRs and IDR functional predictions within the primary sequence, based on aa physical features for the protein’s disordered ensemble. This returns a score between 0 and 1 for each residue, indicating the degree to which a given residue is part of an ordered or disordered region (residues with scores >0.5 are considered as disordered). Results revealed clear order-to-disorder transitions where CaV3.2 TM domains and intracellular portions join, and scores indicate a disordered nature of CaV3.2 intracellular and terminal regions (Figs. 1A and B). Specifically, the most extensive IDRs are in the ICLs and C-terminus, while protein TM domains are highly ordered. Owing to their extended conformation, IDRs are more exposed to other proteins and are preferred posttranslational modification sites, including sites for methylation, ubiquitination, and especially for phosphorylation, which are not only most prevalent but also serve as critical signaling nodes. Potential phosphorylation sites in CaV3.2 full-length aa sequence were identified using DEPP. Results showed that most potential phosphorylation residues (serine, threonine, and tyrosine with high DEPP scores) reside in CaV3.2 IDRs and particularly in the IDRs within the ICL1, ICL2, and C-terminus (Fig. 1C). CaV3.2 IDRs feature as potential protein–protein interaction (PPI) binding sites, suggesting these short linear peptides are key binding motifs and domains of the CaV3.2 regulatory signaling interactome. These observations predict that focusing on the CaV3.2 IDR could be an avenue for identifying short peptides effective in CaV3.2 functional regulation. A comparison of the components of CaV3.2 IDRs to the IDRs in a number of known nociception-related ion channels (Fig. 1D) showed that CaV3.2 is particularly enriched with IDRs that can be acted upon by a diverse array of regulatory proteins.

Because of difficulty in direct experimental identification and validation of the numerous possible functional sequences within CaV3.2 IDRs, the potentially functional domains within the CaV3.2 IDRs (ie, short linear peptides defined as functionally intrinsically disordered domain) were further analyzed using Motifs, Eukaryotic Linear Motif, and SLiMPrints. These programs predict SLiMs based on strongly conserved primary aa sequences, followed by filtering based on the prediction scores. SLiMPrints specifically identifies the relatively overconstrained proximal groupings of residues within IDRs, indicative of putatively functional IDDs. The enumerated motifs predicted within CaV3.2 IDRs suggest many possible functional peptides as hot-spots of functional IDDs, including proteolytic cleavage sites, ligand binding sites, posttranslational modification sites, and subcellular targeting sites. Notably, CaV3.2 IDRs contain several linear polybasic peptide (PBP) sequences composed of approximately 20 aa that are enriched with positively charged arginine (R) and lysine (K), other disordered aa, and phosphorylation sites that are clustered in ICL1-3 and C-terminus and can be considered as potential regulatory IDDs. Several previous studies report that polybasic sequences in protein IDRs are crucial in the functional regulation of proteins, and positively charged polybasic domains can be essential for recruiting multiple signaling proteins. These CaV3.2iDR PBP peptides and a 20mer non-PBP peptide from the N-terminal IDR of CaV3.2 (NP) were designed computationally and were the focus as CaV3.2iPA candidates for further testing (Fig. 1E). Notably, candidate CaV3.2iPA3 sequence located within the proximal peptide of CaV3.2-ICL1 that regulates CaV3.2 gating, and the CaV3.2iPA2 sequence is overlaid to a peptide in CaV3.2-ICL3 that interacts with nuclear-expressed deubiquitinating enzyme USP5.

3.2. Expression of CaV3.2iPAs

To allow stable and functional occupancy of CaV3.2 channels by candidate CaV3.2iPAs, we first constructed AAV expression plasmids containing transgene expression cassettes encoding various GFP-CaV3.2iPA chimeras with which we expressed GFP-CaV3.2iPAs (3.2iPAs) by transfection. Specifically, the sequences for interchangeable peptides for testing were cloned with a linker sequence (GLRRAQASNSAVDGTAGPGS), as we described, to form a chimeric transgene in a GFP-linker-3.2iPA orientation driven by CBA promoter to generate pAAV-CBA-GFP-3.2iPA (pAAV-3.2iPA) expression plasmids in which the oligonucleotides encoding the interchangeable iPAs are inserted at the 3′ end of GFP (Fig. 1F). The stable expression of each construct was verified by transfection into HEK293 cells (Figs. 1G and I). The crystal structure analysis of GFP3.2iPA1 and GFP3.2iPA2 by I-TASSER tool shows an unfolded and extended, highly flexible structural ensemble of linker-3.2iPA1, which is compatible with a well-exposed mode to bind to targets (Fig. 1H, I-TASSER for other iPAs not shown).

3.3. Inhibition of calcium channels 3.2 current (ICa3.2) by 3.2iPAs

Whole-cell voltage-clamp recordings of ICa3.2 in HEK3.2 cells transfected with plasmids encoding different 3.2iPAs were performed to characterize functional inhibition of CaV3.2 channels by designed 3.2iPAs. Application of the T-type calcium channel blocker TTA-P217 in HEK3.2 cells reduced peak ICa3.2 density to approximately 5% of BL level (Fig. 2), consistent with T-type calcium current. Transfection followed by patch clamp results showed that 3.2iPA1, 3.2iPA2, and 3.2iPA4 produced approximately 70%, 60%, and 40% reduction of peak ICa3.2 density, respectively. Transfection with plasmids expressing the GFP-linker, 3.2iP, and 3.2iPA3 showed no significant effects on peak ICa3.2 density compared with sham-transfected HEK3.2 cells. These experiments thus identified 3.2iPA1 and 3.2iPA2 as effective iPAs (>50% ICa3.2 inhibition). In addition, their effects on CaV3.2 biophysical properties were examined, using sham-transfected cells and 3.2iP-transfected cells as the controls. Results revealed no significant shifts of the steady-state activation and inactivation curves nor on voltage-activated half activation or half inactivation (Fig. 2), suggesting that 3.2iPA1 and 3.2iPA2 reduced the conduction of Ca2+ through CaV3.2 channels but did not change
channel activation and inactivation properties. To test the replicability of these findings, the experiments testing the effects of 3.2iPA1 and 3.2iPA2 expressions on ICa3.2 in HEK3.2 cells were examined independently by a separate research team at a second institution (Stony Brook University), whose results were comparable with the findings generated by the Medical College of Wisconsin laboratory (Fig. 2). Taken together, these data confirm the efficacy of our discovery approach and indicate that signaling through PBP sequences in CaV3.2 IDRs is important in regulating CaV3.2 channel function. The findings also suggest that 3.2iPA1 and 3.2iPA2 successfully inhibit CaV3.2 channels, thereby justifying further studies of their potentials as therapeutic agents. Potent ICa3.2 inhibition by 3.2iPA1 was confirmed in neuronal NG108-15 cells that naturally express CaV3.2, which can be detected in subcellular locations (Suppl. Fig. 1, available at http://links.lww.com/PAIN/B621). Because the aa sequences of CaV3.2iPA1 and 2 have significant homology to the corresponding aa sequences of CaV3.1 and CaV3.3, we further tested effects of presence of CaV3.2iPA1 and CaV3.2iPA2 on CaV3.1 and CaV3.3 currents using HEK3.1 and HEK3.2 cells, respectively. Using sham transfection and GFP-3.2NP-transfected cells as the controls, illustrations show the ICa current traces recorded in each transfection, IV curves, and comparison of peak ICa density from HEK3.1 (B-E), HEK3.2 (C-E), and HEK3.3 (D-F) of different transfections, respectively. Inset: current/time scales and recording protocols. *, **, and *** denote P < 0.05, 0.01, and 0.001, respectively. One-way ANOVA and the Tukey post hoc test. aa, amino acid; ANOVA, analysis of variance; CaV3.2, calcium channels 3.2; ICa3.1, CaV3.1 current; ICa3.2, CaV3.2 current; ICa3.3, CaV3.3 current.

### 3.4. No effects of CaV3.2iPAs on sodium channel 1.7 and voltage-gated potassium channels (VGKCs, IKv)

The LVA calcium channel can interact with big conductance (BK, KCa1.1) potassium channels, enabling KCa1.1 activation, and may also affect sodium channels. T-type calcium channel specificity of 3.2iPA1 and 3.2iPA2 action was therefore further examined by using neuronal NG108-15 cells that naturally express VGKCs. Interaction with the NaV1.7 current was examined as well because it is a key element in pain perception. Whole-cell patch-clamp recordings showed that transfection of 3.2iPA1 and 3.2iPA2 have no effects on the INa1.7 and the IKv compared with NG108-15 sham-transfected cells or NG108-15 cells transfected with plasmids encoding GFP or 3.2NP (Figs. 4A and B).
3.5. Inhibition of LVA but not high-voltage–activated calcium channels by 3.2iPA1 and 3.2iPA2 in rat primary sensory neurons

Because no heterologous system or cell lines can fully mimic in vivo conditions of sensory neurons, we further tested the functional inhibition of calcium channels by CaV3.2iPAs (3.2iPAs) in DRG PSNs. AAV6 vectors encoding 3.2NP, 3.2iPA1, 3.2iPA2, and 3.2iPA3 (all fused with GFP) were injected into L4/5 DRG of naïve rats (male), and acutely dissociated sensory neurons from DRG were tested at 4 weeks postinjection. Whole-cell voltage-clamp recordings from small-sized/medium-sized PSNs showed that AAV-mediated expressions of 3.2iPA1 and 3.2iPA2 produced significant inhibition of peak LVA I_{Ca} (pA/pF) by approximately 70% and 60%, respectively, whereas 3.2iPA3 enhanced peak LVA I_{Ca} by approximately 30%, compared with control PSNs. No change of peak LVA I_{Ca} was observed in PSNs expressing 3.2NP compared with that in naive cells (Fig. 4C). To further test the effects of 3.2iPA1 on Ca^{2+} influx through PSN CaV3.2/T-type channels, Ca^{2+} microfluorimetry was performed. Dissociated PSNs were depolarized by exposure to 50 mM KCl for 0.3 seconds to selectively activate T-type channels and in the presence of tetrodotoxin (TTX, 1 mM) to eliminate action potential generation, as previously described. Results showed that AAV-mediated 3.2iPA1 expression in PSNs significantly decreased [Ca^{2+}]_c. Depolarization for 0.3 seconds by 50 mM KCl increased [Ca^{2+}]_c, which was blocked by TTA-P2 in the PSNs of naïve rats, and this effect was precluded by AAV-mediated 3.2iPA1 expression in PSNs; ***P < 0.001, one-way ANOVA and Tukey post hoc test. (D) No effects of 3.2iPAs on HVA I_{Ca} in DRG neurons. Typical HVA I_{Ca} traces in a small-sized neuron from a naïve rat show a threshold for activation approximately ~30 mV and a maximum current amplitude activation at ~10 mV, displaying small inactivation. Typical traces of HVA I_{Ca} recorded at ~10 mV of neurons (<35 μm in diameter) from a sham-operated rat and naïve rats injected with AAV6-encoding GFP, 3.2NP, 3.2iPA1, and 3.2iPA2. Recording protocol is shown on the top of panel C. Current density–voltage (I/V) curves from different constructs (C6) and quantitative analysis of averaged peak HVA I_{Ca} density (C7), *P < 0.05, **P < 0.01, and ***P < 0.001, one-way ANOVA and Tukey post hoc test. (C8, C9) AAV-mediated 3.2iPA1 expression in PSNs significantly decrease [Ca^{2+}]_c. Depolarization for 0.3 seconds by 50 mM KCl increased [Ca^{2+}]_c, which was blocked by TTA-P2 in the PSNs of naïve rats, and this effect was precluded by AAV-mediated 3.2iPA1 expression in PSNs; ***P < 0.001, one-way ANOVA and Tukey post hoc test. (D) No effects of 3.2iPAs on HVA I_{Ca} in DRG neurons. Typical HVA I_{Ca} traces in a small-sized neuron from a naïve rat show a threshold for activation approximately ~30 mV and a maximum current amplitude activation at ~10 mV, displaying small inactivation. Typical traces of HVA I_{Ca} recorded at ~10 mV of neurons (<35 μm in diameter) from a sham-operated rat and naïve rats injected with AAV6-encoding GFP, 3.2NP, 3.2iPA1, and 3.2iPA2 (D1-D6). (D7) HVA I_{Ca} density–voltage (I/V) curves and (D8) quantitative analysis of averaged peak HVA I_{Ca} density, P > 0.05, one-way ANOVA and Tukey post hoc test. AAV, adeno-associated virus; ANOVA, analysis of variance; CaV, calcium channels; DRG, dorsal root ganglia; I_{Ca}, calcium channel current; IPA, inhibitory peptide aptamer; HVA I_{Ca}, high-voltage–activated calcium current; I_{Na1.7}, NaV1.7 current; I_{K}, potassium current; LVA I_{Ca}, low-voltage–activated calcium current; NaV1.7, sodium channel 1.7; PSN, primary sensory neuron.
showed no effect of 3.2iPA1 and 3.2iPA2 on peak HVA I Ca (Fig. 4D). Of importance, AAV-encoded 3.2iPA1 expression in vivo did not affect BL mechanical (vF) and thermal (heat) thresholds in control rats (see further). Because these findings support that 3.2iPA expression in PSNs is a means of selectively inhibiting LVA ICa, further testing was performed in vivo to evaluate their analgesic potential.

3.6. Attenuation of tibial nerve injury–induced hypersensitivity by AAV6-3.2iPA1 in vivo (male rats)

3.6.1. Pilot testing of 3.2iPA1

To detect CaV3.2 protein expression, we first examined the specificity of CaV3.2 antibody by immunoblotting on cell lysates of stable cell lines expressing different calcium channels, which showed detection of Ca V3.2 but not Ca V3.1, Ca V3.3, or Ca V2.2 (Suppl. Fig. 2A, available at http://links.lww.com/PAIN/B621). Immunoblots using this antibody on DRG samples from naive rats revealed that CaV3.2 protein was more abundant in the cytosolic and nuclear fractions than in the PM under our preparation conditions (Suppl. Fig. 2B, available at http://links.lww.com/PAIN/B621). Immunohistochemistry using this antibody revealed Cav3.2 detection preferentially in small-sized and medium-sized PSNs (Suppl. Fig. 2C and D, available at http://links.lww.com/PAIN/B621). These results confirmed the specificity of the Cav3.2 antibody used in this study to detect Cav3.2 expression by IHC and immunoblots.

Pilot in vivo tests were initiated to evaluate whether AAV-mediated CaV3.2iPA1 expression selectively in the PSNs could reduce development of pain behaviors after nerve injury. We first observed that AAV-6 mediated CaV3.2iPA1 expression in vivo did not affect BL mechanical (vF) and thermal (heat) thresholds in control rats (see further). Because these findings support that 3.2iPA expression in PSNs is a means of selectively inhibiting LVA ICa, further testing was performed in vivo to evaluate their analgesic potential.

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Pilot in vivo tests were initiated to evaluate whether AAV-mediated CaV3.2iPA1 expression selectively in the PSNs could reduce development of pain behaviors after nerve injury. We first observed that TNI enhanced PSN Cav3.2/T-type channel activity and increased Cav3.2 and GFAP expressions (Figs. 5A–D), as in previous reports. To test potential analgesia, AAVs expressing GFP-fused 3.2iPA1, or 3.2NP as a control, were packaged into serotype 6 (Fig. 5E) because this serotype efficiently transduces nociceptive PSNs, which express CaV3.2. Evoked pain behaviors were tested by sensory
stimuli applied to the lateral margin of the plantar aspect of the paw in the sural area of innervation, as previously described.\(^7\)\(^8\),\(^9\)\(^3\) Initial experiments showed that AAV-3.2iPA1 treatment did not affect mechanical (vF) or thermal (heat) thresholds in control rats (nerve exposure without ligation, Fig. \(5F\)). To test analgesic effects in the context of neuropathic pain, this pilot experimental design involved performing the TNI operation and DRG-AAV injections at the same operation in adult rats (Figs. 5G–K). Rats were randomized to receive intraganglionic vector injection of either AAV6-3.2iPA1 or AAV6-

![Figure 6. Treatment of established neuropathic pain by dorsal root ganglia AAV6-3.2iPA1 (male rats). New lots of purified AAVs (A, silver stain) were prepared for the experiment in an animal protocol schematically outlined (B). AAV6-3.2iPA1 treatment: The time courses for the group averages of sensitivity to vF, pin, heat, and cold before and after DRG injection of either AAV6-3.2iPA1 (n = 7) or AAV6-3.2NP (control, n = 7) (C–F) as indicated; *P < 0.05, **P < 0.0,1 and ***P < 0.001 for comparisons with the TBL within group and #P < 0.01, and ###P < 0.001 for comparisons between groups. Repeated measures parametric two-way ANOVA for vF and heat, followed by the Tukey (within group) and Bonferroni (between group) post hoc tests; and nonparametric Friedman ANOVA for Pin and cold tests and Dunn post hoc analysis as indicated. Right panels of C–F show tAUC calculated using measures 14-day post TNI and before vector injection as TBL. ***P < 0.001, comparisons of tAUC between groups (unpaired, two-tailed Student t tests for vF and heat, and Mann-Whitney U tests for pin and cold). (G) the time courses (3 hours) of sensitivity to vF and pin injected with GBP (100 mg/kg, i.p.) in TNI + AAV-3.2 PN rats, performed at the time point as indicated in (B–D); **P < 0.01 and ***P < 0.001 vs before GBP, repeated measures two-way ANOVA for vF with Tukey post hoc test, and Mann-Whitney U tests for Pin and cold. (H) Comparison of the efficacy between AAV-3.2iPA1 treatment in TNI and GBP (i.p.) anticonception in 3.2NP rats by vF and Pin tests, ***P < 0.001, unpaired two-tailed Student t test for vF and Mann-Whitney U test for Pin. Pin (%, TNI + 3.2iPA1): tBL 87 ± 6 and after treatment 42 ± 4, Pin (%, TNI + 3.2NP); before injection 88 ± 5 and after GBP 59 ± 2. vF (g, TNI + 3.2iPA1): tBL 5 ± 1 and after treatment 15 ± 1. vF (g, TNI + 3.2NP + GBP); before injection 4 ± 1 and after GBP 16 ± 2 (P > 0.05, unpaired two-tailed Student t test for vF and Mann-Whitney U test for Pin). (I) the results of the CPP difference scores (seconds, s) of preconiditioning chamber and of the GBP-paired chamber between AAV-3.2iPA1 (n = 7) and AAV-3.2NP (control, n = 7). ***P < 0.001 (unpaired, two-tailed Student t test), AAV, adeno-associated virus; ANOVA, analysis of variance; BL, baseline; DRG, dorsal root ganglia; GBP, Gabapentin; CPP, conditioned place preference; i.p., intraperitoneal; TNI, tibial nerve injury; tBL, treatment baseline; tAUC, treatment area under curve.

Figure 6.
3.2NP into both the ipsilateral L4 and L5 DRG immediately after TNI surgery. Rats given the control vector (AAV6-3.2NP) developed significant pain behaviors after TNI, which included lowered withdrawal threshold from mild mechanical stimuli (vF and brush testing), more frequent hyperalgesic-type responses (sustained lifting, shaking, and grooming) after noxious mechanical stimulation (Pin testing), reduced withdrawal threshold to heat, and more frequent withdrawals from cold (acetone stimulation), which persisted after injection during the 5 weeks of the observation course. By contrast, rats injected with AAV6-3.2iPA1 showed initial development of similar hypersensitivity at 1 week, but this was followed by a gradual reversal of these changes. These findings suggest that DRG-targeted Ca\textsubscript{v}3.2-iPA1 treatment has analgesic efficacy in reducing peripheral hypersensitivity in TNI rats.

3.6.2. Treatment of established neuropathic pain by dorsal root ganglia-AAV6-3.2iPAs

Having ascertained the potential for in vivo analgesia, we extended our experiments to evaluate the effectiveness of DRG-AAV6-3.2iPAs (iPA1 and iPA2) in a more clinically relevant

Figure 7. Immunohistochemistry characterization of 3.2iPAs expression in vivo (male). Illustration shows representative IHC images of 3.2iPA1 (A–H) and 3.2iPA2 (I–K) on the sections from L4/L5-DRG of TNI rats 6 weeks after treatment. IHC montage images show GFP-3.2iPA1 expression (green), colabeled with Ca\textsubscript{v}3.2 (A, red), Tubb3 (B, red), CGRP (C, red), and IB4 (D, red). An IHC image shows multiple PSN cellular localization of GFP-3.2iPA1; 1, 2, and 3 denote the patterns of localization in PM, cytosol fractions, and nuclear fractions, respectively (E). (F–H) IHC images show GFP-Ca\textsubscript{v}3.2iPA1 expression (green) colabeled with Ca\textsubscript{v}3.2 (red) (F, dashed lines outline DH), and in afferent terminals colabeled with Ca\textsubscript{v}3.2 (red) (H) within the dermis of ipsilateral hindpaw. (I) IHC montage images of GFP-Ca\textsubscript{v}3.2iPA2 expression (green), colabeled with Tubb3 (red), showing colocalization in merged image. (J) IHC montage images of GFP-Ca\textsubscript{v}3.2iPA2 expression (green), colabeled with Cav3.2 (red), showing colocalization in merged image. GFP-3.2iPA2 displays multiple cellular localization in PSNs, including cytosol fractions, nuclear fractions, and PM. Arrowheads point to NKA\textsubscript{1a} (red) and GFP-3.2iPA2 (green) colabeled PM (K, merged image). Scale bar: 50 μm for all images. Ca\textsubscript{v}3.2, calcium channels 3.2; CGRP, calcitonin gene–related peptide; DRG, dorsal root ganglia; GFAP, glial fibrillary acidic protein; IB4, Isolectin IB4; IHC, immunohistochemistry; iPA, inhibitory peptide aptamer; NKA\textsubscript{1a}, sodium/potassium ATPase 1α; PM, plasma membrane; PSN, primary sensory neurons; Tubb3, b3-tubulin; TNI, tibial nerve injury.
design involving reversal of established pain behavior, including both evoked responses as well as spontaneous ongoing pain after TNI (Fig. 6A). New preparations of vectors of AAV6-3.2iPA1, AAV6-3.2iPA2, or AAV6-3.2NP (control), consisting of iPAs or NP fused with GFP, were generated for these experiments. In the experimental design, the sensitivity to mechanical and thermal cutaneous stimulation was assessed at baseline and weekly after TNI for 2 weeks before AAV injection. Thereafter, rats were randomized to receive intraganglionic injection of either AAV6-3.2iPA1, AAV6-3.2iPA2, or AAV6-3.2NP into the ipsilateral L4/L5 DRG, after which sensory behavior was evaluated weekly for 6 weeks. A single-dose GBP (100 mg/kg, i.p.) was injected in animals that had received AAV6-3.2NP 3 weeks previously, as a positive control. The GBP-CCP test was performed in all groups as a terminal experiment to evaluate spontaneous pain.

Behaviors measured before vector injection at the 14th day after TNI were used as a treatment BL (tBL) to evaluate effectiveness of vector treatments (Fig. 6B). Tissues were harvested for IHC characterization of transgene and target gene expression and for whole-cell current-clamp AP recording of neuronal excitability on dissociated DRG neurons.

All rats established multiple modalities of pain behavior 2 weeks after TNI, including lowered threshold for withdrawal from mild mechanical stimuli (vF testing), more frequent hyperalgesic-type responses after noxious mechanical stimulation (Pin testing), and hypersensitivity to heat and acetone stimulation. These behaviors persisted after injection of the control AAV6-3.2NP during the 6 weeks of observation course. By contrast, rats injected with AAV6-3.2iPA1 showed reversal of these changes, which persisted throughout the observation period (Figs. 6C–F). GBP applied between the third and fourth weeks in TNI 1AAV6-3.2NP rats reversed mechanical hypersensitivity at 30 to 60 minutes after GBP, but animals were fully mechanically hypersensitive again 3 hours after injection (Fig. 6G). For animals treated with AAV-3.2iPA1, the averaged (3w 1 4w) allodynia (vF) and hyperalgesia (Pin) showed approximately 60% reversal to tBL, which was comparable with the averaged (30-60 minutes) response to GBP in AAV6-3.2NP group (Fig. 6H). Using a biased CPP paradigm, the effect of AAV-3.2iPA1 treatment on spontaneous pain was evaluated. None of the animals in either group were excluded from study because of their BL preference/avoidance for a chamber. A significant CPP effect of GBP administration was observed in the TNI rats injected with AAV6-3.2NP, whereas there was no significant difference in the time spent in the initially nonpreferred chamber during BL vs testing period in AAV-3.2iPA1–treated TNI animals, indicating AAV-3.2iPA1 treatment relieved ongoing spontaneous pain in TNI rats (Fig. 6I). A comparable analgesic effectiveness for both evoked and spontaneous pain behaviors was observed for AAV-3.2iPA2 treatment in the established pain induced by TNI (Suppl. Fig. 3, available at http://links.lww.com/PAIN/B621), as described earlier for AAV-3.2iPA1. This could be expected on the basis...
that both 3.2iPA1 and 3.2iPA2 displayed similar inhibitory effects on I_{Ca^{3.2}} without changing Cav3.2 biophysical features (Fig. 2).

A histological examination (Fig. 7) determined the in vivo transduction rate for AAV6-3.2iPA1 6 weeks after vector injection. The 3.2iPA1-positive neurons (GFP) comprised 48.5% (971 of 1898 total neuronal profiles) identified by pan-neuronal marker β3-tubulin (n = 3 DRG, 5 sections per DRG, selected as every fifth section from the consecutive serial sections). Transduced DRG neurons included the full-size range of the PSNs that also expressed CaV3.2, and 3.2iPA1 expression showed multiple subcellular localizations, including PSN cytosol, nucleus, and plasma membrane, but not in perineuronal glia cells. The 3.2iPA1-IR signals (GFP) were additionally detected in spinal dorsal horn neuropil, sciatic nerve, and hindpaw afferent terminals. In vivo transduction profile of AAV6-3.2iPA2 showed multiple PSN subcellular expressions (Figs. 7I–K), similar to AAV6-3.2iPA1.

Cumulatively, these findings suggest that treatment with AAV6-mediated expression of Cav3.2iPA1 and 2 targeted to sensory neurons in the DRG has clear analgesic effectiveness in normalizing the established peripheral hypersensitivity for both evoked and spontaneous pain in a rat neuropathy model in a sustained fashion.

3.7. Reversal of primary sensory neuron hyperexcitability by AAV6-3.2iPA1 treatment (male rats)

Cav3.2 channels contribute to nociception by lowering the threshold for excitability in PSNs. We therefore determined whether AAV6-3.2iPA1 treatment reverses the enhanced neuronal excitability of nociceptive PSNs after TNI, using the whole-cell current-clamp AP recording of DRG dissociated neurons from rats after the treatment protocol shown in Figure 6B. Although TNI results in DRG with comingled injured and uninjured axons, nerve injury can induce an increase of voltage-gated ion channel activity in both axotomized neurons and adjacent intact neurons, leading to similar electrophysiological changes and increased discharge frequency in axotomized and neighboring neurons.

Figure 9. Analgesia of dorsal root ganglia-AAV6-3.2iPA2 treatment in female tibial nerve injury rats. Analogous figures to Fig. 6 and suppl. Fig. 3 (available at http://links.lww.com/PAIN/B621) show significant analgesia after DRG delivery of AAV6-3.2iPA2 in established TNI pain behavior in female rats. **P < 0.01 and ***P < 0.001 for comparisons to the treatment baseline (tBL) within group and ###P < 0.001, and ####P < 0.001 for comparisons between groups (A–D). Repeated measures parametric two-way ANOVA for vF and Heat, followed by Tukey (within group) and Bonferroni (between group) post hoc tests; and nonparametric Friedman ANOVA for Pin and Cold tests and Dunn post hoc. Right panels of A to D show tAUC calculated using measures 14-day post-TNI and before vector injection as IBL: ***P < 0.001, comparisons of tAUC between groups (unpaired, two-tailed Student t tests for vF and Heat, and Mann-Whitney U tests for Pin and cold). Conditioned place preference difference scores (t) of preconditioning chamber and of the GBP-paired chamber between AAV-3.2iPA1 (n = 7) and AAV-3.2NP (control, n = 7), **P < 0.01 (unpaired, two-tailed Student t test) (E). Representative IHC overlaid images (GFP3.2iPA2 with Tubb3) show neuronal expression profile 6 weeks after AAV-3.2iPA2 injection (F), multiple cellular localization (G, arrows and arrowheads point to nuclear and PM, respectively), colocalization of GFP-3.2iPA2 with Cav3.2 (H). Scale bar: 50 μm for all images. AAV, adeno-associated virus; ANOVA, analysis of variance; BL, baseline; CaV3.2, calcium channels 3.2; DRG, dorsal root ganglia; IHC, immunohistochemistry; PSN, primary sensory neuron; tBL, treatment baseline; tAUC, treatment area under the curve; TNI, tibial nerve injury.
intact DRG neurons.13 We therefore recorded from randomly chosen small-sized and medium-sized neurons (<40 μm in diameter)77 in the cultures from dissociated L4 and L5 DRGs. Transduced neurons were identified using GFP fluorescence, and excitability was evaluated by measuring rheobase and repetitive firing during 250-ms current injection steps. Results showed that the averaged rheobase in the neurons from TNI rats was significantly decreased, and the frequency of APs evoked in neurons from TNI rats was significantly increased, compared with sham controls. These were normalized in transduced neurons after AAV6-3.2iPA1 treatment, whereas 3.2NP-transduced neurons had no significant effect (Fig. 8). We did not test the effects of 3.2iPA2 on TNI-induced PSN hyperexcitability. However, reversal of TNI-induced PSN hyperexcitability by AAV6-3.2iPA2 treatment is expected, as has been reported that inhibition of Ca3.2/3 channels by a homologous peptide to iPA2 reverses hyperexcitability of peripheral nociceptors and alleviates postsurgical pain.50 These findings indicate that reversal of nerve injury–induced sensory neuron hyperexcitability by Cav3.2iPAs may contribute to its attenuation of neuropathic pain behaviors.

3.8. Analgesia of dorsal root ganglia-AAV6-3.2iPA2 treatment in female tibial nerve injury rats

Sex differences exist in experimental and clinical pain and in responsiveness to interventions.48,51 We therefore next test whether DRG-AAV6-3.2iPA treatment is also effective in attenuating hypersensitivity induced by TNI in female animals. A treatment protocol similar to the tests in male animals (Fig. 6) was conducted. Specifically, vector injection was performed 2 weeks after TNI, and both evoked mechanical and thermal nociception, as well as GBP-CPP were evaluated. The same batch preparation of AAV6-3.2iPA2 tested in male rats was used for testing (AAV6-3.2iPA1 was not applied because these 2 treatment vectors showed comparable antinociceptive effects in male TNI rats, described in Fig. 6 and Suppl. Fig. 3). Results showed that the female rats displayed similar phenotypic development of hypersensitivity after induction of TNI to male rats and both evoked mechanical/thermal hypersensitivity and GBP-CPP responses were normalized after AAV6-3.2iPA2 treatment, demonstrating a comparable analgesic responsivity to interventions.53 We therefore next test whether DRG-AAV6-3.2iPA treatment is also effective in attenuating hypersensitivity induced by TNI in female animals. A treatment protocol similar to the tests in male animals (Fig. 6) was conducted. Specifically, vector injection was performed 2 weeks after TNI, and both evoked mechanical and thermal nociception, as well as GBP-CPP were evaluated. The same batch preparation of AAV6-3.2iPA2 tested in male rats was used for testing (AAV6-3.2iPA1 was not applied because these 2 treatment vectors showed comparable antinociceptive effects in male TNI rats, described in Fig. 6 and Suppl. Fig. 3). Results showed that the female rats displayed similar phenotypic development of hypersensitivity after induction of TNI to male rats and both evoked mechanical/thermal hypersensitivity and GBP-CPP responses were normalized after AAV6-3.2iPA2 treatment, demonstrating a comparable analgesic effect to the male animals (Figs. 9A–E). Immunohistochemistry on the DRG sections from female TNI rats 6 weeks after AAV6-3.2iPA2 injection also revealed GFP-3.2iPA2 expression profile comparable with that of male rats (Figs. 9F–H). Thus, although not rigorously compared, a sexual dimorphism seems not apparent for both pain behavior phenotypes after TNI and in responsivity to DRG-3.2iPA2 treatment in our studies.

4. Discussion

The ongoing opioid crisis in the United States has spurred efforts to develop nonopioid drugs for treating chronic pain, but these efforts have mostly failed.84 New therapeutic approaches are therefore needed. The Ca3.2/3 T-type channels are key elements regulating sensory neuron excitability and shaping multiple modalities of sensory perception and pain signal amplification, and the roles of Ca3.2/3 channels of the peripheral sensory pathways in pain sensation are well established.10 Thus, Ca3.2/3 channels are an important class of targets for drugs that are needed for the clinical treatment of chronic pain.63,83 Not only do Ca3.2/3 channelopathies underlie a critical mechanism for a variety of pain conditions, but modifying Ca3.2/3 function has proved to be a useful preclinical intervention in the treatment of chronic pain.48 In addition, both Ca3.1 and Ca3.3 are also confirmed pain mediators.55,56 These observations underlie our motivation for the experiments reported here.

In this study, we used a combined in silico and experimental strategy to design iPAs by targeting Ca3.2/3 IDRs. Promising Ca3.3.iPA candidates were selected and validated using in vitro cell line-based and ex vivo PSN-based methods. Expression of these Ca3.2/iPAs inhibits T-type/Ca3.2 and Cav3.1 currents but not Cav3.3 calcium currents. Because the steady-state properties of Ca3.2/3 channels are not affected, it is therefore possible that iPAs may reduce Cav3.2/Cav3.1 T-type channel conduc-
tance possibly by a direct or indirect channel occupancy or by decreased channel numbers in the membrane due to altered trafficking or transcription/translation. Of importance, AAV-mediated expression of the prototypes Ca3.2.iPA1 and Ca3.2/iPA2 restricted to DRG neurons produces sustained inhibition of ICaL conducted by Ca3.2/Cav3.1 T-type channels in PSNs and attenuates established nerve injury–induced pain behavior in both male and female rats, effective for both evoked mechanical and thermal hypersensitivity and ongoing or spontaneous pain behavior, which are symptoms commonly found in patients experiencing multiple types of painful neuropathy.49 Overall, our results indicate that targeting IDRs facilitates identification of druggable T-type iPAs and Ca3.2iPAs are promising analgesic leads with potentials for translational clinical analgesia.

Research efforts have helped us better understanding the pathobiological role of Ca3.2 in chronic pain. Progress has been made over the past decade to develop more selective and efficacious T-type/Ca3.2 blockers to treat pain.47,89 However, the clinically available small molecule Ca3.2 blockers used to treat pain applied systemically and orally are nonselective across different tissues and contribute to off-site effects because Ca3.2/3 are widely expressed throughout the body of mammals involving in a wide variety of physiological functions. Thus, despite preclinical studies demonstrating that decreased T-type/Ca3.2 activity induces a reduction in pain, few molecules targeting this gene product have reached the final phase of clinical trials,6,42,43,47,81,98 and many small molecules targeting T-type/Ca3.2 have failed to be validated as pain therapy drugs.1,79 Thus, development of novel peripheral acting strategies for T-type/Ca3.2 inhibition would be an ideal approach for clinical pain treatment.

Chronic pain in almost all cases is maintained by ongoing afferent hyperactivity originating from peripheral pathological sources.6,69 Primary sensory neurons not only initiate nociception but also play a central role in the development and maintenance of painful neuropathies.13 Pain-sensing DRG-PSNs can become hyperexcitabale in response to pathological conditions such as peripheral nerve injury, which in turn leads to the development of neuropathic pain. Multiple lines of evidence from both preclinical and clinical studies demonstrate that block of peripheral nociceptive input can effectively relieve pain symptoms, including spontaneous pain.63,81 Therefore, treatments targeting the peripheral neurons both avoid CNS side effects and are likely to succeed. Indeed, a recent expert commentary states, “activity in primary afferent neurons represents a ‘low-hanging target’ in the development of safe therapies” for patients with chronic pain.99 Delivering drugs to the peripheral nervous system is well developed and safe, for instance, as used in clinical anesthesia for regional blockade and by pain physicians for diagnosis and treatment of radiculopathy.66 Injection into the DRG has minimal consequences in preclinical models.24 It has also been demonstrated that unintentional intraganglionic injection commonly accompanies clinical foraminal epidural steroid injection,66 a very common procedure with minimal risk of nerve damage. Thus, the
PSNs are particularly suitable for targeting new analgesic treatments, especially at the level of the associated pathological DRG.

Our approach used here includes a novel strategy in which highly selective and nontoxic CaV3.2iPAs are designed and developed from CaV3.2 IDRs, and these are delivered by using AAV in an anatomically targeted fashion that restricts block of T-type CaV3.2/Cav3.1 to the peripheral nerves. In preclinical models, direct DRG delivery of AAVs encoding analgesic biologics can provide relief in chronic pain, with high transduction efficiency, flexibility for selective segmental localization, and minimal behavioral changes attributable to the surgical procedure. In parallel, injection techniques are being advanced to achieve minimally invasive delivery of biologics for future clinical pain therapy. Small peptides that mimic target protein sequences can serve as decoy molecules to selectively interfere with the function of their target signaling protein by preemptively binding to it. We have successfully used this strategy in rat models to induce analgesia by preventing assembly of functional transient receptor potential cation channel subfamily V member 1 (TRPV1) and by blocking membrane trafficking of CaV3.2 channels by interrupting their interactions with the structural protein collapsin response mediator protein 2 (CRMP2). Here, we extend the applicability of DRG-AAV strategy to the analgesic effectiveness of PSN T-type/CaV3.2 blockade for neuropathic pain. The multifranged feature of Cav3.2iPA1/2 with combined CaV3.2 and Cav3.1 inhibition restricted in PSNs might be an additional analgesic advantage because both are known nociceptive hubs. We believe that our promising results showing effectiveness and tolerability, if proving long-term efficacy and absence of adverse events, suggest the utility of the approach for developing therapeutic reagents. Because activation of T-type/CaV3.2 has been found in various pain conditions, it will be of interest to address the analgesic efficacy of AAV-CaV3.2iPAs for PSN T-type/CaV3.2 inhibition in additional models of other pain etiologies.

Injury-induced ectopic hyperactivity of PSNs causes hypersensitization in multiple sites of the peripheral sensory nervous system, including augmented pain perception in the peripheral terminals, enhanced nociceptive signal transduction in PSN somata and T-junction, and increased neurotransmission in the spinal dorsal horn. At this early stage, our studies did not investigate differential actions by block of T-type/CaV3.2 along the pathway of nociceptors nor did the results rule out the possibility that block of T-type/CaV3.2 reduces pain by inhibiting afferent hyperexcitable input, thus indirectly modulating spinal cord and brain antinociceptive control circuits. Although our studies have initially focused on testing whether targeting IDRs can facilitate discovery of T-type/CaV3.2 inhibitory peptides for AAV-mediated analgesia, how the identified CaV3.2iPA functioning remains unknown. The potential signaling pathways that the CaV3.2iPAs affected could be many because CaV3.2 intracellular segments serve as essential interfaces for many regulatory signaling molecules, including nuclear-localized deubiquitinating enzyme (USP5), calcium/calmodulin-dependent protein kinase II, cyclin-dependent kinase 5, G-proteins, calmodulin, calpain, syntaxin/SNAP25, Kelch-like protein 1, and Stac1. In addition, CaV3.2 can form protein complexes with members of the K+ channel family, such as KV4, KCa3.1, and KCa1.1 (BK); and K+/Na+ hyperpolarization-activated cyclic nucleotide-gated channel 1, as well as with lipids. Alterations of these molecules and ionic channels after nerve injury are essential for ectopic PSN hyperactivity and pain.

In conclusion, our data describe a strategy that addresses a significant hurdle in CaV3.2 inhibitor discovered by targeting CaV3.2 IDRs. CaV3.2iPA1 and 2 are promising analgesic leads that, combined with AAV-targeted gene delivery in anatomically segmental sensory ganglia, have the potentials for future development as novel peripheral CaV3.2-targeting therapeutics in the treatment of intractable pain that cannot be controlled with current medical care. In addition, the IDR approach is also applicable of discovering IPAs to many other pronociceptive ion channels for AAV-mediated sensory neuron-specific analgesia. Finally, many questions are raised by this study, and the precise structural details and molecular mechanisms of CaV3.2iPAs for their I<sub>Ca3.2</sub> blockade remain to be identified. For example, what functional role may be served by CaV3.2iPAs in nuclei? Do CaV3.2iPAs regulate gene expression? Indeed, in searching for functional domains, high-score nuclear localization signals are identified in the sequences of CaV3.2iPAs and CaV3.2 protein (Suppl. Fig. 4, available at http://links.lww.com/PAIN/B621). To account for this intriguing mechanism, it seems plausible that nuclear-localized CaV3.2iPAs may indirectly, at least partly, contribute to I<sub>Ca3.2</sub> inhibition by affecting calcium-regulated transcriptions of genes that are critical for CaV3.2 functions. Forthcoming studies will aim at addressing these questions.

Conflict of interest statement
The authors have no conflicts of interest to declare.

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Appendix A. Supplemental digital content
Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/B621.

Supplemental video content
A video abstract associated with this article can be found at http://links.lww.com/PAIN/B622.

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