12-2018

A Role For Epac1 And Epac2 In Nociceptor Hyperexcitability And Chronic Pain After Spinal Cord Injury

Samantha Berkey

A ROLE FOR EPAC1 AND EPAC2 IN NOCICEPTOR HYPEREXCITABILITY AND CHRONIC PAIN AFTER SPINAL CORD INJURY

By

Samantha Claire Berkey

APPROVED:

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Dr. Carmen W. Dessauer

Advisory Professor

_____

Dr. Xiaodong Cheng

Advisory Professor

_____

Dr. Edgar T. Walters

_____

Dr. Swathi Arur

_____

Dr. Shane R. Cunha

_____

Dr. Juan J. Herrera

_____

Approved:

_____

Dean,

The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences

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A

THESIS

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

Samantha Claire Berkey

Houston, Texas

December, 2018

Dedication

This is for the people I love, who have stood by me through all the ups and downs…

My Dad Richard

My Mother Jackie and Stepdad Joe

Laura and Katie, my two favorite sisters

Shaun, who has seen the grad student process up close and still loves me…

The whole Houston family I am now a part of, especially Helen, Stanley, and Boobie

My Houston friends, who have always risen to the occasion, whether at a bar or hospital

And the new friends I have made here, who have quickly become lifelong

To quote one of you:

“Finally”

Acknowledgements

I would like to thank Dr. Carmen W. Dessauer for her mentorship and expertise. Her incredible support and wealth of knowledge have allowed me to reach this point. She accepts nothing but the best from her students, and that has produced a thesis I am proud to present.

I would also like to thank my co-mentor, Dr. Xiaodong Cheng, for his helpful advice and never-ending support for my project. He is always ready to discuss scientific ideas or provide technical assistance.

Additionally I would like to thank the other members of my committee, Drs. Edgar T. Walters, Swathi Arur, Shane Cunha, and Juan J. Herrera. Besides advising and guiding this thesis project, each has offered continued support and resources. Thank you to Dr. Walters for helping introduce me to the complex field of animal behavior, and allowing me an incredible amount of access to all of his behavior equipment and his SCI rat model. Thank you to Dr. Arur for joining me at the very beginning of this graduate school experience, and sticking with me until the end. Thank you to Dr. Cunha for being an incredible source of knowledge and moral support, as well as for hosting impromptu journal clubs to increase students' exposure to subjects outside their field. And thank you to Dr. Herrera, who not only served on my committee, but without whom this work would have been impossible. Thank you for the massive number of surgeries you performed for this project, as well as your endless supply of assistance with animal care and activity box experiments.

Thank you to Dr. Alexis Bavencoffe, for not only performing all of the electrophysiology experiments, but also instructing me on how to interpret and analyze their meaning.

Thank you to Dr. Fang C. Mei, who always patiently shared her time to help with large and minute aspects of this project, including breeding and sharing the EPAC mice and troubleshooting western techniques with me. Thank you to Dr. Annemieke Kavelaars and Vicky Ma, for taking the time to teach me proper von Frey technique.

I also sincerely thank all of my lab members. Thank you Dr. Yong Li for tirelessly answering my questions, as well as keeping an encyclopedic knowledge of everything in the lab and its location. Thank you Yan Wang for teaching me how to be a mouse whisperer, and for always offering to lend your amazing tissue collection skills. Thank you to Wei Yu, who has made it so I always seem to have exactly what I need when I need it. Thank you to Dr. Anibal Garza Carbajal for sharing your knowledge and advice, especially in regards to signaling pathways and robotic techniques. Thank you to Simran Rahman, who was absolutely indispensable to the animal work. Thank you to Tanya Baldwin, my fellow graduate student, who is a limitless source of help, support, and general happiness.

Thank you to the members of the Cheng lab, Drs. Yingmin Zhu, Hui Chang, Will Robichaux, Upsana Banerjee, Wei Ling, and Wenli Yang, for all of your assistance and help throughout the years. Thank you to the members of the Walters lab, Max A. Odem, Elia Lopez, Guo-Ying Xu, Kendra Wicks, and Emily, who were always willing to share techniques and discuss results. A special thanks to Max, who would endlessly discuss animal behavior, and was my guide in designing the behavioral aspects of this project.

Thank you to Dr. Eric Swindell, who introduced me to the fun of science. He believed in and supported my scientific career when I needed it most, and still does.

Thank you to all the faculty and administrators in the IBP program. You do a fantastic job. Lastly, thank you to all of my fellow students and friends in the program and Houston. You are an amazing support system and I couldn't have done it without you.

A ROLE FOR EPAC1 AND EPAC2 IN NOCICEPTOR HYPEREXCITABILITY AND CHRONIC PAIN AFTER SPINAL CORD INJURY

Samantha Claire Berkey

Advisory Professor: Dr. Carmen W. Dessauer and Dr. Xiaodong Cheng

Chronic pain is a major complaint of those living with spinal cord injury (SCI), affecting 65-80% of the SCI population, but the treatment options remain limited or non-existent. The cAMP sensor EPAC has previously been shown to play a key role in chronic inflammatory and neuropathic pain, though the contribution from each of its two main isoforms, EPAC1 and EPAC2, is unclear. Here I test the hypothesis that both EPAC1 and EPAC2 play a key role in the maintenance of persistent nociceptor hyperexcitability and chronic pain after SCI.

Using both a T9 SCI mouse model and a T10 SCI rat model, we employed behavior assays and electrophysiological techniques to characterize the role of EPAC1 and 2 in SCI-induced pain. We also used three novel methods: the operant mechanical conflict test (MC) behavior test, an electrophysiological measure of ongoing activity (OA), and analysis of the transient depolarizing fluctuations (DSFs) in membrane potential that contribute to nociceptor excitability.

After SCI, we observed a significant increase in the incidence of SA, large DSFs, and OA in WT, Epac2$^{-/-}$, and Epac1$^{-/-}$ mice. The SCI-induced increase in SA and OA incidence could be significantly decreased in nociceptors isolated from Epac1$^{-/-}$ and Epac2$^{-/-}$ mice by inhibition of the complementary EPAC isoform. Behavioral measures of "pain" did not show

significant differences between WT and EPAC2$^{-/-}$ mice in a naïve state or after injury. Additionally, multi-day treatment with ESI-09, an inhibitor of both Epac1 and 2, caused a significant decrease in mechanical sensitivity within WT and Epac2$^{-/-}$ mice; however, *in vitro* treatment with ESI-09 resulted in mass electrophysiological silencing of nociceptors, suggesting the decrease in sensitivity was due to nociceptor cell silencing *in vivo*.

The data shows compensatory and/or redundant functions for EPAC1 and EPAC2. Importantly, future studies and attempts to develop chronic pain therapies may need to account for both isoforms of EPAC and their complementary roles.

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Key Abbreviations

AC	Adenylyl Cyclase
AKAP	A-Kinase Anchoring Protein
AP	Action Potential
ATP	Adenosine Triphosphate
BMS	Basso Mouse Score
cAMP	3’,5’-Cyclic Adenosine Monophosphate
CDC25-HD	CDC25 homology domain
CFA	Complete Freund's adjuvant
CGRP	Calcitonin Gene Related Peptide
CNG	Cyclic Nucleotide Gated ion channel
DEP	Dishevelled–EGL–Pleckstrin homology domain
DRG	Dorsal Root Ganglia
DSF	Depolarizing Small Fluctuation
EPM	Elevated Plus Maze
ESI	EPAC-specific inhibitors
FNE	Free Nerve Ending
GDNF	Glial cell-derived neurotrophic factor
GDP	Guanosine Diphosphate

GPCR	G Protein Coupled Receptor
GRK2	G Protein Coupled Receptor Kinase 2
GTP	Guanosine-5'-triphosphate
HCN	hyperpolarization-activated cation non-selective channel
HTM	High Threshold Mechanosensitive
i.p.	Intraperitoneal injection
i.t.	Intrathecal injection
IB4	Isolectin B4
L4/L5	Lumbar 4/5
MC	Mechanical Conflict Test
NGF	Nerve Growth Factor
OA	Ongoing Activity
PA	phosphatidic acid
PDE	Phosphodiesterase
PGE2	Prostaglandin E2
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PLD	Phospholipase D

PM	Plasma Membrane
POPDC	Popeye Domain Containing Protein
RA	Ras association domain
REM	Ras exchange motif
RMP	Resting Membrane Potential
SA	Spontaneous Activity
SCI	Spinal Cord Injury
SMIR	Skin Muscle Incision Retraction
T10	Thoracic 10
TRPV	Transient Receptor Potential Vallinoid Channel
VF	Von Frey
WT	Wild Type

Chapter 1: Introduction

The ability to feel pain and detect noxious stimuli is essential for survival and well-being. Acute pain serves as a warning system; tissue or nerve damage sensitizes an injured area, helping to promote wound guarding and avoidance behavior during recovery. However, the mechanisms underlying normal pain can become permanently altered, such that hypersensitivity does not resolve after the injury heals, and instead becomes debilitating chronic pain. Chronic pain affects 65-80% of those living with spinal cord injury (SCI), but the treatment options remain limited or non-existent (Siddall et al., 2003).

Spinal cord injury has multiple devastating physiological and psychological impacts, but the management of chronic pain is often cited as the leading factor in determining life satisfaction (van Leeuwen et al., 2012, Finnerup, 2013). Chronic pain after SCI can include nociceptive (Finnerup et al., 2008), neuropathic (Norrbrink Budh et al., 2003, Siddall et al., 2003, Werhagen et al., 2004), or additional "other" or "unknown" pain, as classified by the International Spinal Cord Injury Pain (ISCIP) Classification group (Bryce et al., 2011). The ISCIP attempts to classify SCI by pain type, subtype, and source in attempt to develop better treatment options, and this classification highlights how challenging SCI is to treat, as patients experience different combinations of initial trauma and acute and chronic pain states. Current therapies include attempting to treat secondary symptoms (such as inflammation or muscle weakness), the use of analgesics or opioids, and complementary behavioral programs for coping with pain, all of which simply manage pain (Heutink et al., 2012, Löfgren and Norrbrink, 2012). In order to develop more effective therapies, a deeper understanding of the mechanisms underlying normal, "acute" pain, as well as the transition to and maintenance of chronic pain is required.

1.1 A Brief Introduction to Pain Processing

Nociception is the detection of intense thermal, mechanical, or chemical stimuli by a subpopulation of peripheral nerve fibers, or nociceptors (Basbaum et al., 2009). Nociceptor cell bodies are located in the dorsal root ganglia (DRG in the body; trigeminal ganglion in the face), and innervate their target organ and the spinal cord or brainstem with a peripheral and central axonal branch, respectively. The central and peripheral axonal branches contain similar distributions of proteins generated in the cell body making nociceptors pseudo-unipolar. Importantly, nociceptors are only activated when a stimulus reaches a certain intensity, a threshold meant to ensure only stimuli in the noxious range result in nociception. The biophysical and molecular properties that allow this selective response are important mechanisms in pain signaling, as alterations lead to a lower pain threshold. The pseudo-unipolarity of nociceptors can also affect the threshold, as it allows endogenous molecules, such as inflammatory mediators or neurotransmitters, to target both terminals to affect sensitivity (Basbaum et al., 2009).

Nociceptors can be classified by neuroanatomical and molecular characteristics (Snider and McMahon, 1998). Anatomically, nociceptors consists of two major classes, which are composed of heterogeneous populations. The first class includes the Aδ fibers, which are thinly myelinated, medium diameter afferents that mediate acute, fast pain and can be further subdivided into Type I high threshold mechanical (HTM) and Type II fibers: Type I HTM Aδ Fibers are responsible for the immediate acute response to noxious mechanical stimuli, Type II mediate the immediate response to noxious heat (It is worth mentioning there are also large diameter, fast-conducting, myelinated Aβ fibers, but these mainly respond to innocuous mechanical stimuli). The second class includes unmyelinated, small diameter C-fibers that conduct slow, poorly localized pain. C-fibers are often polymodal, and can be additionally

classified as peptidergic or non-peptidergic. Peptidergic C-fibers synthesize and release neuropeptides, such as substance P and calcitonin-gene related protein (CGRP), and respond to nerve growth factor (NGF) through expression of the neurotrophin receptor TrkA (Snider and McMahon, 1998, Chao, 2003). The non-peptidergic population, which bind isolectin B4 (IB4), responds to glial-derived neurotrophic factors (GDNF) through the c-Ret neurotrophin receptor, and express the purinergic P2X3 receptor, an ATP gated ion channel (Snider and McMahon, 1998).

During normal acute pain responses, a noxious stimulus is detected by the Aδ or C-fiber's peripheral free nerve ending. More specifically, the noxious stimulus is detected by the multiple ion channels and receptors embedded in the plasma membrane of the Aδ or C-fiber's peripheral free nerve ending (Figure 1), which become active allowing ion channels to open and the membrane to depolarize, generating a receptor potential. These receptors include the TRP channels (TRPV1-4, TRPA1, TRPM8); TRPV1 responds to noxious heat, as well as capsaicin, and is most abundantly expressed in nociceptors, which allows a capsaicin positive response to be used to identify nociceptors (Caterina et al., 2000, Lauria et al., 2006). Additional TRP channels are also involved in temperature sensing, such as TRPV2 which responds to higher noxious heat temperatures and is predominantly expressed in Aδ fibers (Leffler et al., 2007, Rau et al., 2007), and TRPA1, which responds to noxious cold and chemical irritants such as mustard oil or garlic (Bandell et al., 2004, Jordt et al., 2004). Identification of mechanoreceptors, which respond to noxious mechanical stimuli such as intense pressure, stress, or visceral pain, has been slightly more challenging, but is likely to include some of the TRP channels and the Piezo2 ion channels (Ranade et al., 2015). Importantly, there are also chemoreceptors, which can factor in acute pain, but play a major

role in responding to endogenous factors released after tissue injury. The presence or absence of certain ion channels or receptors is what allows nociceptors to be poly- or unimodal.

After a receptor potential is generated, the signal is propagated along the axon, through the nociceptor cell body, or DRG, and onward along the axon innervating the spinal cord dorsal horn. The fast acting voltage gated Na^+, K^+, and Ca^{2+} and hyperpolarization-activated, cation non-selective (HCN) channels propagate the action potential, but can also modify the signal to influence nociception. The NaV channels can be tetrodotoxin (TTX) sensitive or resistant, and are expressed predominantly in small-diameter nociceptors. Of interest is the Nav1.7 channel, where a loss of function leads to congenital insensitivity to pain (Cox et al., 2006, Fischer and Waxman, 2010), and the Nav1.8 channel, which plays a role in chronic nociceptor excitability and pain-behavior after SCI (discussed below) (Yang et al., 2014) .

From the DRG, central processes form excitatory synapses with second order neurons in laminae I, II, and V of the dorsal horn, which project via the spinothalamic and spinoreticulothalamic relays to the thalamus and brainstem, and finally to cortical structures, where the noxious signal is processed as the complex emotional experience of "pain" (Millan, 1999). There are also descending, inhibitory pathways that arise in the brainstem, hypothalamus, and cortical structures, that may modulate inputs from primary afferent and second order neurons (Millan, 2002).

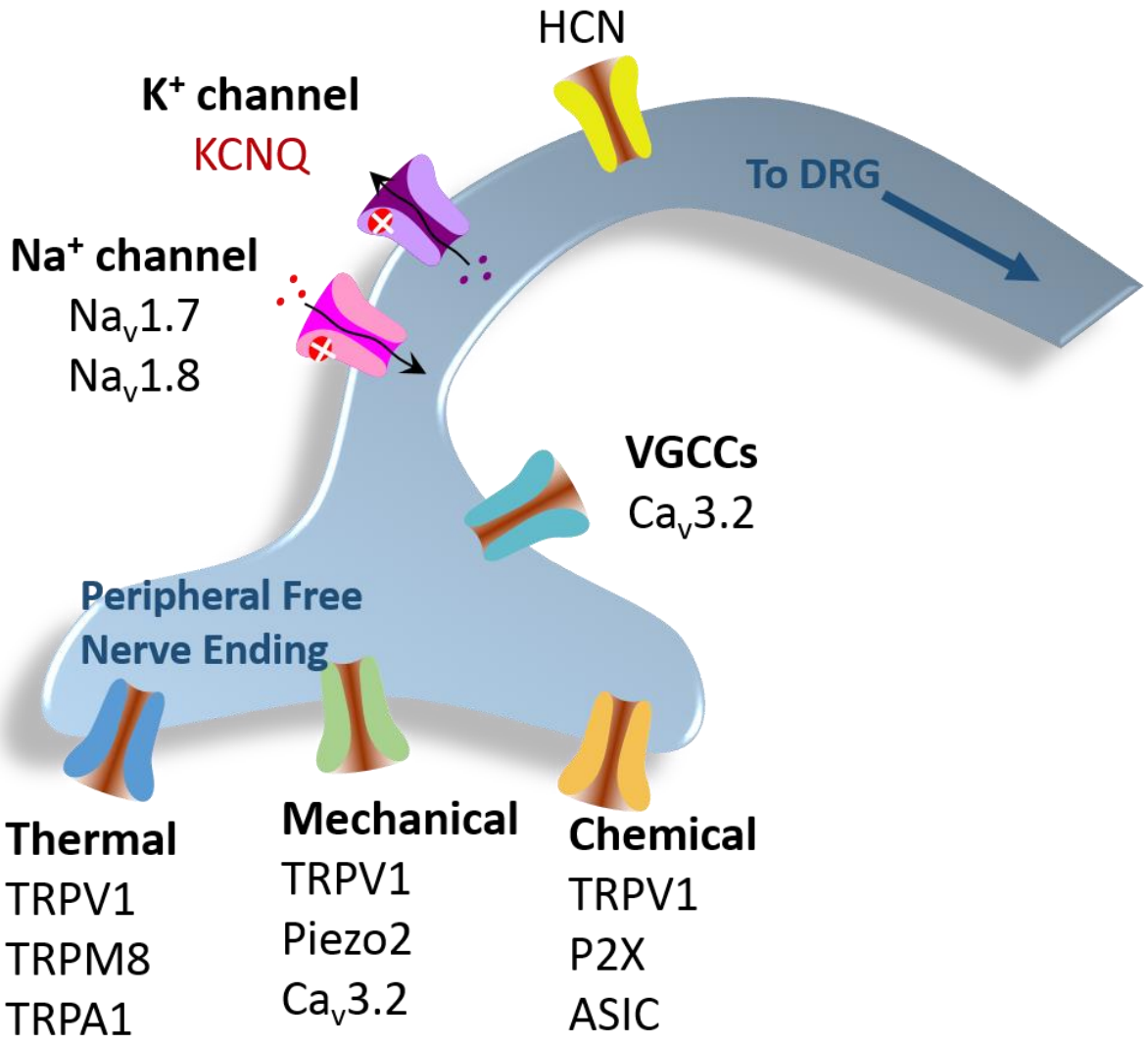


Figure 1. Noxious stimuli are detected by Aδ or C-fibers, specifically by the multiple ion channels and receptors embedded in the plasma membrane of the Aδ or C-fiber's peripheral free nerve ending. Specific ion channels and receptors respond to thermal, mechanical, and/or chemical stimuli, to generate a receptor potential. This signal is propagated by the fast acting voltage gated Na^+, K^+, and Ca^{2+} and hyperpolarization-activated, cation non-selective (HCN) channels along the axon, to the DRG, and onward to the CNS.

1.2 The Role of Peripheral Sensitization in Chronic Pain after SCI

After SCI, the mechanisms underlying normal pain become permanently altered, such that the hypersensitivity does not resolve, and instead becomes debilitating chronic pain. Many studies focus on alterations in excitability within central neurons at the spinal and supraspinal levels after SCI, such as gliosis (Detloff et al., 2008) or loss of descending, inhibitory (GABAergic) tone (Meisner et al., 2010), which contribute to the maintenance of at-level and below level chronic neuropathic pain after SCI. However, there is increasing recognition that persistent alterations within the peripheral nervous system, specifically a switch in nociceptors within DRG at and below the contusion level to a persistent, hyperexcitable state, contribute to SCI-induced chronic pain (Bedi et al., 2010, Walters, 2012, Yang et al., 2014). This continuous enhanced input from peripheral primary sensory neurons to spinal pain pathways contributes to central sensitization and is correlated with an increase in pain-like behaviors within animal models (Bedi et al., 2010, Walters, 2012, Yang et al., 2014). Interestingly, this same phenomenon is observed in peripheral injury models (Burchiel et al., 1985, Djouhri et al., 2001, Xiao and Bennett, 2007, Xu and Brennan, 2010) and leads to similar central sensitization (Gracely et al., 1992, Xie et al., 2005, Pitcher and Henry, 2008).

The first evidence for the potential contribution of peripheral nociceptors came from multiple pre-clinical models of SCI; the authors found SCI-induced sprouting of $CGRP^{+}$ C-fibers within spinal dorsal horn segments below the injury site (Christensen and Hulsebosch, 1997, Ondarza et al., 2003, Ackery et al., 2007, Zinck et al., 2007, Hou et al., 2009, Lee-Kubli et al., 2016). *In vivo* injection of antibody against the growth factor NGF could inhibit this central expansion of C-fibers, but, more interestingly, also ameliorated the SCI-induced pain (Christensen and Hulsebosch, 1997).

Besides just expanding the number of central peptidergic fibers, *in vivo* work showed SCI enhanced the sensitivity and firing rates of C-fiber neurons, inducing spontaneous activity (SA) within both the peripheral terminals and the somata located within the DRG (Carlton et al., 2009, Bedi et al., 2010). This hyperactive, spontaneously active state can be observed between 3 days and 6 months post injury, and is driven by the combined effects of three nociceptor intrinsic mechanisms: sustained depolarization of resting membrane potential (RMP), a decrease in action potential threshold, and an increase in amplitudes of depolarizing spontaneous fluctuations (DSF) (Figure 2) (Odem et al., 2018). Additionally, these mechanism can potentiate ongoing activity (OA) in nociceptors driven by extrinsic signals (Bedi et al., 2010, Yang et al., 2014, Odem et al., 2018). Compellingly, OA has been observed in recordings from human peripheral nerves, and is correlated with self-reports of ongoing pain in patients with peripheral neuropathy or fibromyalgia (Ochoa et al., 2005, Orstavik et al., 2006, Kleggetveit et al., 2012, Serra et al., 2012, Serra et al., 2014).

Many of the C-fiber neurons exhibiting SA are responsive to the TRPV1 activator, capsaicin, suggesting the hyperexcitable cells are nociceptors (Bedi et al., 2010). In fact, application of capsaicin to isolated DRG neurons leads to repetitive firing patterns reminiscent of SA, and the firing activity is enhanced post-SCI (Wu, 2013). Inhibition of TRPV1 in SCI rats also reverses pain-like behaviors (Rajpal et al., 2007, Wu, 2013). As TRPV1 channels are almost exclusively expressed in nociceptors (Lauria, Cavanaugh et al., 2011), the data taken together suggest that SCI-induced nociceptor SA plays a major role in the maintenance of chronic pain. A study by Yang, et al. (Yang et al., 2014) lends further evidence for the contribution of primary sensory neurons to chronic pain maintenance. Here the authors show that knockdown of the Nav1.8 channel, which is only expressed in peripheral somatosensory,

but not central, neurons, mitigates both the SCI-induced SA and the pain-like behaviors (Yang et al., 2014).

Based on the above studies, one may conclude that SCI-induced chronic pain is at least partially maintained by continuous input from peripheral, hyperactive primary afferent C-fibers, and that this hyperactivity is due to persistent alterations in signaling pathways which affect ion channel function. However, what are the signaling pathways underlying this hyperexcitability and resulting chronic pain? Cyclic adenosine monophosphate (cAMP) to protein kinase A (PKA) signaling has been extensively studied for its role in maintenance of nociceptor hyperexcitability after peripheral inflammation or injury (Taiwo et al., 1989, Ferreira et al., 1990, Villarreal et al., 2009), and a study by our group shows a requirement for cAMP signaling through an AKAP150-AC-PKA signalosome to maintain this hyperexcitability in DRGs isolated from SCI rats (Bavencoffe et al., 2016).

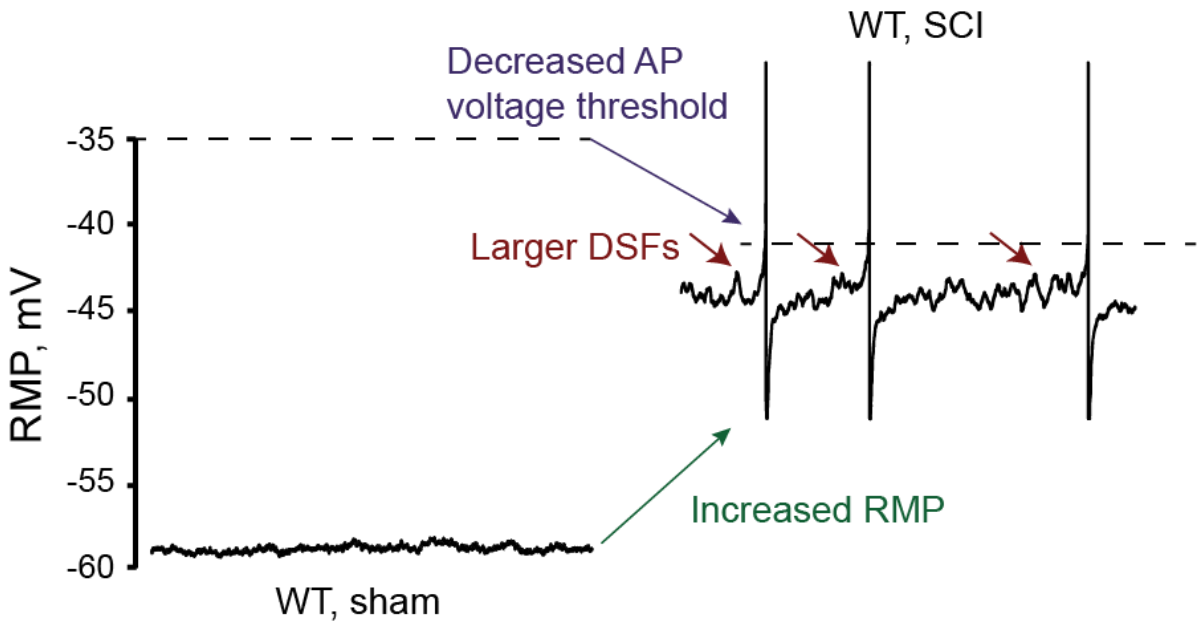


Figure 2. The hyperactive, spontaneously active state is driven by the combined effects of three nociceptor intrinsic mechanisms: sustained depolarization of resting membrane potential (RMP), a decrease in action potential threshold, and an increase in amplitudes of depolarizing spontaneous fluctuations (DSFs).

1.3 cAMP signaling through the Downstream Effectors PKA and EPAC

cAMP and Protein Kinase A (PKA) signaling are often considered synonymous (with a role for cyclic nucleotide gated ion channels, or CNGs and HCNs) (Taiwo and Levine, 1991, Malmberg et al., 1997, Aley and Levine, 1999). cAMP is a prototypic secondary messenger; a cell is stimulated by extracellular ligand binding to G protein coupled receptors (GPCRs) to activate G proteins, which in turn promotes the conversion of ATP to cAMP by adenylyl cyclase (AC). Increased cAMP signals to downstream effectors, which in mammals includes PKA and EPAC (de Rooij et al., 1998, Kawasaki et al., 1998, Taylor et al., 2013), as well as the HCNs and CNGs (Zufall et al., 1997), the popeye domain containing (POPDC) proteins (Schindler and Brand, 2016), and the cyclic nucleotide receptor involved in sperm function (CRIS) (Krähling et al., 2013). For example, the well-studied prostanoid PGE2 interacts with the prostaglandin receptor EP4, a member of a subclass of GPCRs. Multiple studies have shown PGE2 injection into rodent hindpaws leads to transient mechanical hyperalgesia, which is dependent on cAMP/PKA signaling (Ferreira and Nakamura, 1979, Chen et al., 1999, Wang et al., 2007a, Eijkelkamp et al., 2010). However, the ubiquity of cAMP necessitates additional regulatory mechanisms to ensure the extracellular PGE2 signal leads to the appropriate cellular response.

The specificity of cAMP signaling is modulated by its precise localization by ACs, phosphodiesterase (PDEs), and A-kinase anchoring proteins (AKAPs) (Esseltine and Scott, 2013). ACs may be targeted to specific loci within mammalian cells or be tethered to AKAPs, which act as signalosomes to bring together the pertinent cAMP signaling components. In addition, PDEs degrade cAMP to reduce diffusion throughout the cell. The multiple mechanisms allow for precise, localized cAMP signaling, which promotes the appropriate cell

response despite the vast array of possible activating factors and downstream branching signaling cascades.

As mentioned above, a study by our group showed a requirement for an AKAP150-AC-PKA complex to maintain nociceptor hyperexcitability in DRGs isolated from SCI rats, along with a novel alteration in AC signaling (Bavencoffe et al., 2016). RNA sequencing data acquired from the same rat model of contusive SCI suggest that EPAC1 is upregulated after SCI, indicating that the nociceptor hyperexcitabilty and chronic pain behavior maintained by cAMP-PKA signaling might have an additional cAMP-EPAC mediated component (unpublished).

In 1998 the cAMP effector exchange protein activated by cAMP (EPAC) was discovered (de Rooij et al., 1998, Kawasaki et al., 1998) and consequently shown to function in many roles previously ascribed solely to PKA. As suggested by its name, EPAC is a guanine exchange factor for the Ras family members Rap1 and Rap2 (though it can also interact with other small GTPases) and is activated by cAMP (de Rooij et al., 1998, Kawasaki et al., 1998). The major work of this thesis attempts to define a role for EPAC in the maintenance of chronic pain and nociceptor hyperexcitability after SCI in multiple rodent models.

1.4 What is known about the roles of EPAC1 and EPAC2 in pain signaling?

Evidence for a role for EPAC1 and EPAC2 in chronic pain

The first evidence to suggest a role of EPAC in chronic pain proposed that cAMP to PKCε signaling is mediated by EPAC via PI-PLC and PLD, and not by PKA (Hucho et al., 2005). The authors demonstrated that intraplantar injection of EPAC agonist 8-(4-chlorophenylthio)-2′-O-methyl-cAMP (8-pCPT) in male rats leads to long lasting mechanical

hyperalgesia *in vivo*, which can be mitigated through inhibition of PKCε, PI-PLC, or PLD (Hucho et al., 2005). PKCε is known to play a role in nociceptor sensitization due to inflammation and peripheral neuropathies (Khasar et al., 1999, Aley et al., 2000, Dina et al., 2001), and additional work shows a role for EPAC/ PKCε signaling in pain maintenance. DRG neurons previously challenged with the inflammatory mediator complete Freund's adjuvant (CFA) will show an enhanced P2X3-mediated ATP current after another, secondary inflammatory insult. The enhanced response is dependent on EPAC/ PKCε signaling (Wang et al., 2007b). Interestingly, in uninjured DRG neurons the P2X3R mediated current is mediated by PKA only (Wang et al., 2007b). This parallels the change observed in inflammatory priming models used to study the transition from acute to chronic pain; the first inflammatory insult leads to short-lived, acute pain, mediated by cAMP-PKA signaling, while a second insult at least one-week later begets persistent chronic pain, maintained by EPAC signaling (Wang et al., 2013). Treatment with the EPAC specific inhibitor ESI-09 mitigates the inflammatory mechanical hyperalgesia (Wang et al., 2013, Singhmar et al., 2016).

However, the role of EPAC may be slightly more complicated, as there are two main isoforms in vertebrates, with various levels of expression throughout the body. EPAC1 is encoded by Rapgef3 in mouse and EPAC2 by Rapgef4, which can actually give rise to three variants (EPAC2 in this thesis refers to the main isoform EPAC2A; there also exists an adrenal specific EPAC2B and liver specific EPAC2C in mouse; there are validated splice variants in humans as well but the specifics are unknown) (Navegantes et al., 2000, Ueno et al., 2001, Robichaux and Cheng, 2018). EPAC1 is ubiquitously expressed in mice with particularly high mRNA levels in brain, thyroid, kidney, ovary, and skeletal muscle (Kawasaki et al., 1998, Li et al., 2008), while EPAC2 is primarily expressed within the nervous system and adrenal glands (Ueno et al., 2001, Murray and Shewan, 2008). In human tissue, however, EPAC1 and 2

protein is found in almost every tissue examined, with the exception of ovary and oral mucosa, respectively (Robichaux and Cheng, 2018).

The structure of EPAC is highly involved with its function as a guanine exchange factor (GEF) (de Rooij et al., 1998, Kawasaki et al., 1998). Both EPAC isoforms have a C-terminal catalytic domain and a N-terminal auto-inhibitory domain. The catalytic C-terminal contains a CDC-25HD domain, a Ras exchange motif (REM), and a Ras-association domain. The N-terminal contains the high affinity cAMP binding domain (cAMP-B) and a Dishevelled, Egl-10 and Pleckstrin domain (DEP) domain, as well as a second, low affinity cAMP-A binding domain present in only the EPAC2 isoform (Rehmann et al., 2006, Rehmann et al., 2008). Upon cAMP binding conformational changes relieve the auto-inhibition, stabilize the open configuration, and allow for GDP-GTP exchange activity. There is an ionic interaction between the cAMP-B helical bundle and CDC25HD, which is broken after cAMP binding. This allows conformational changes in the hinge region – between cAMP-B and REM – which stabilize the open configuration and allows for the GDP-GTP exchange activity (Rehmann et al., 2006, Rehmann et al., 2008).

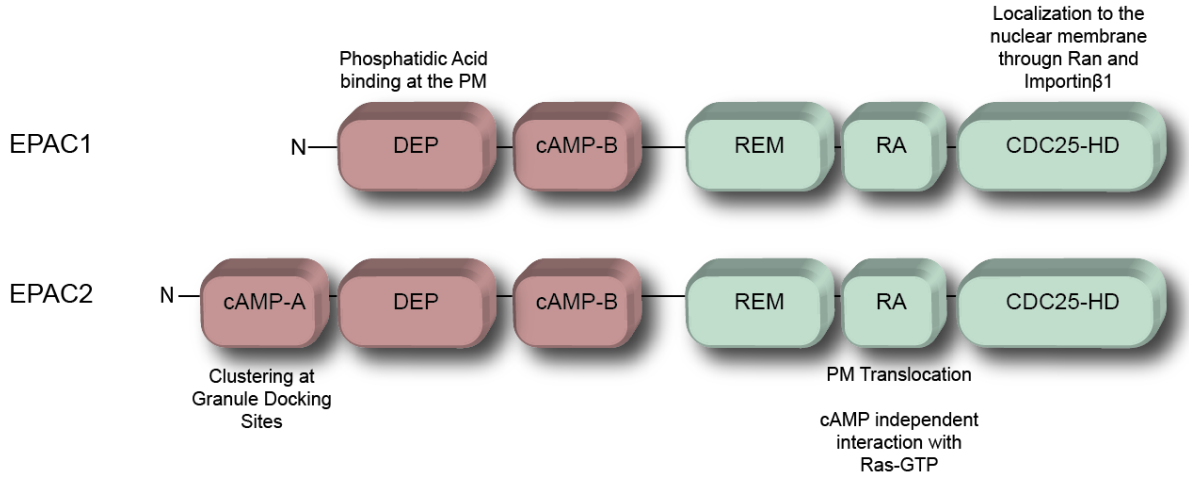


Figure 3. Sequence and structural variations between EPAC1 and EPAC2 impart unique functions to each isoform. Both EPAC isoforms have a N-terminal auto-inhibitory domain containing the high affinity cAMP binding domain (cAMP-B) and a DEP domain, and a C-terminal catalytic domain containing a Ras exchange motif (REM), a RA domain, and a CDC-25HD domain. In EPAC2 the N-terminal also includes a second, low affinity cAMP-A binding domain. Several functions unique to each isoform are highlighted within this figure and in further detail within the text. These include the ability of EPAC1 to translocate to the nuclear membrane, and the potential for EPAC2 to play a role in exocytosis, among others.

Though 50% homologous, the differences between EPAC1 and 2 affect their structure and intracellular localization, which can potentially affect their respective roles in pain signaling (Figure 3). EPAC1's subcellular localization is regulated by cAMP; as cAMP levels and localization fluctuates, so too do those of EPAC1 (Qiao et al., 2002, Ponsioen et al., 2009). Increased cAMP levels promote the plasma membrane association of EPAC1, while decreased levels promote distribution to the microtubule cytoskeleton (Qiao et al., 2002, Ponsioen et al., 2009, Singhmar et al., 2016). After cAMP binding, EPAC1 translocation to the PM is dependent on conformational change in the DEP region, which exposes a 17 amino acid binding motif for phosphatidic acid (PA) (Li et al., 2011, Consonni et al., 2012). At the PM, EPAC1 can also associate with additional factors, such as activated ERM or radixin, for more precise localization (McClatchey and Fehon, 2009, Hochbaum et al., 2011). Here, activated EPAC1 can also sensitize the mechanoreceptor Piezo2, which may increase mechanically evoked currents (Eijkelkamp et al., 2013, Singhmar et al., 2016). This may be prevented by phospshorylation of serine-108 in EPAC1's DEP domain by G protein-coupled receptor kinase 2 (GRK2); as the DEP domain is responsible for EPAC1's translocation to the PM, GRK2 inhibits EPAC1's GEF activity towards Rap1 not through inhibiting the conformational change to the active state but by blocking translocation to the PM (Eijkelkamp et al., 2013, Singhmar et al., 2016). Conversely, EPAC2 translocates proximal to the PM through its RA domain, not the DEP domain (Li et al., 2006). Differences in the EPAC2 RA domain allows EPAC2 to interact with Ras-GTP, independent of cAMP binding. Ras-GTP regulates EPAC2's PM translocation, and is required for cAMP signaling through EPAC2 to Rap (Li et al., 2006, Liu et al., 2008, Alenkvist et al., 2017).

EPAC1 can also localize to the nuclear membrane through interaction with Ran, RanBP2, importin B1, and nucleoporins 98 and 205 (Gloerich et al., 2011). RanBP2 interacts

with the amino acid 764-838 motif in CDC25-HD in EPAC1, and antagonizes EPAC1 by sequestering it at the nuclear membrane (as can importin B1) (Gloerich et al., 2011, Baameur et al., 2016). This interacting amino acid motif is conserved within EPAC2, but sterically blocked by the additional CNB-A domain (Aumo et al., 2010). The EPAC2B isoform, expressed in mouse adrenal glands, does not have the CNB-A domain, and thus localizes to the cytosol and the nuclear pore in a manner similar to EPAC1 (Niimura et al., 2009, Aumo et al., 2010, Parnell et al., 2015).

Additionally, while the second low-affinity cAMP binding domain is not present in EPAC1 and is dispensable for PM trafficking in EPAC2, it is essential for EPAC2 clustering at granule docking sites in β-cells, and possibly in neurons (Alenkvist et al., 2017). While a role for EPAC2 in exocytosis has been more widely studied in the context of insulin release from β cells, it has also been demonstrated in neurons (Shibasaki et al., 2004, Shibasaki et al., 2007). There is evidence that EPAC2 can interact with the small G Proteins Rim1a and Rab3 to bring vesicles closer to the PM, and that EPAC2 can promote redistribution of neurotransmission enhancing factor Munc13-1 (Ferrero et al., 2013, Ferrero et al., 2016).

Multiple studies have suggested that EPAC1, but not EPAC2, plays a major role in persistent inflammatory and neuropathic pain in mouse models (Eijkelkamp et al., 2013, Singhmar et al., 2016). A rat postsurgical pain model reached the same conclusion, demonstrating an increase in EPAC1 alone within the DRG, and significant mitigation of pain-like behavior after EPAC1 inhibition (Cao et al., 2016). However, other studies have shown only an increase in EPAC2 expression in rats after CFA induced inflammation (Vasko et al., 2014a). Conversely, work in the same rat CFA model showed an increase in both EPAC isoform expression levels in the DRG, and a reduction in pain-like behavior after inhibition of

either (Gu et al., 2016). To further complicate the literature, work in mice has suggested an ability of EPAC1 and 2 to compensate after knockout (Yang et al., 2012).

1.5 Significance

The roles of EPAC1 versus EPAC2 within chronic pain signaling remain unclear, though studies have argued for a focus on EPAC1, as EPAC1$^{-/-}$ mice do not develop mechanical hyperalgesia after CFA-induced inflammation or after L5 nerve transection (Eijkelkamp et al., 2013, Singhmar et al., 2016). Indeed, a majority of studies within the field attribute inflammatory or neuropathic pain to EPAC1 signaling only, despite increases in both EPAC1 and EPAC2 protein levels within the DRG after CFA or incision-induced inflammation (Wang et al., 2007b). EPAC2 is also more abundant in mature neurons, which control dendrite stability and outgrowth through elevated EPAC2 levels, while EPAC1 is downregulated in neurons after early development (Murray and Shewan, 2008), again suggesting a role for EPAC2 in mature neuron physiology.

I hypothesize that both EPAC1 and EPAC2 contribute to the maintenance and development of SCI-induced chronic pain and nociceptor hyperexcitabilty, and need to be considered in development of future therapies. Thus, within this study we used two SCI rodent models to characterize the roles of EPAC1 and 2 in chronic pain signaling, employing a variety of behavior and electrophysiology tests. Using a T10 (thoracic) contusion SCI rat model, we employed electrophysiological methods to characterize the role of EPAC1 and 2 in promoting nociceptor hyperexcitability. Using a SCI mouse model of T9 contusion, I utilized an array of common behavior tests, including the von Frey filament and Hargreaves thermal test, to test the effect of EPAC2$^{-/-}$ and total EPAC inhibition on SCI pain like behaviors. I also used a

modified testing paradigm within the operant mechanical conflict (MC) test, which may better reflect the complex experience of pain. Electrophysiological techniques were used to study the effects of EPAC isoform specific knockout or inhibition on SCI-induced nociceptor hyperexcitabilty. Additionally, we used two novel, recently published electrophysiological methods, including a measure of ongoing activity (OA) and a analysis method for quantification of transient depolarizing spontaneous fluctuations (DSFs) in membrane potential that contribute to nociceptor excitability (Odem et al., 2018).

Chapter 2: Materials and Methods

2.1 Animals

All procedures followed the guidelines of the International Association for the Study of Pain and were approved by the McGovern Medical School at UT Health and University of Texas MD Anderson Cancer Center Animal Care and Use Committees. Adult male Sprague-Dawley rats (Envigo, USA) were used. After arrival at the McGovern Medical School, the rats (8-9 weeks old, 250-300g, 2 per cage) were allowed to acclimate to a 12-hour reverse light/dark cycle for at least four days before beginning experiments.

Adult male and female C57BL/6 (Charles River, USA) wild-type and EPAC2$^{-/-}$ mice (Pereira et al., 2013) were housed within the McGovern Medical school animal facility and subsequently transferred at 6-8 weeks of age to a controlled environment where they were allowed to acclimate for at least 1 week before beginning experiments.

2.2 Spinal cord injury procedures

Rat SCI surgeries were performed as previously described (Bedi et al., 2010, Wu, 2013, Yang et al., 2014, Bavencoffe et al., 2016). Rats were anesthetized with an intraperitoneal (i.p.) injection of ketamine (60 mg/kg, Henry Schein, Dublin, OH), xylazine (10 mg/kg, Henry Schein, Dublin, OH), and acepromazine (1 mg/kg, Henry Schein, Dublin, OH), or with isoflurane (induction 4-5%; maintenance 1-2%, Isothesia, Henry Schein, Dublin, OH). A T10 vertebral laminectomy was followed by a dorsal contusive spinal impact (150 kdyne, 1 s dwell time) using an Infinite Horizon Spinal Cord Impactor (Precision Systems and Instrumentation,

LLC, Fairfax Station, VA). Sham-operated rats received the same surgical treatment without the contusion. The analgesic buprenorphine hydrochloride (0.02 mg/kg in 0.9% saline 2 ml/kg; Buprenex, Reckitt Benckiser Healthcare Ltd., Hull, England, UK) and the antibiotic enrofloxacin (0.3 ml in 0.9% saline; Enroflox, Norbrook, Inc., Overland Park, KS) were injected i.p. twice daily for 5 days (buprenorphine) or 10 days (enrofloxacin). Manual bladder evacuations were performed twice daily until rats recovered neurogenic bladder voiding. Rats included in this study received a score of 0 or 1 for both hind limbs the day after surgery, as measured on the Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale (Basso et al., 1995).

Mouse SCI surgeries were conducted as previously described (Herrera et al., 2008, Herrera et al., 2010). Briefly, adult female and male mice were anesthetized by inhalation of 4.0% isoflurane and maintained by a mixture of 1.5% isoflurane, 30% oxygen, and air administered through a rodent ventilator (Harvard Apparatus, model 683, Holliston, MA) throughout the surgical procedure. Following laminectomy of the 9th thoracic (T9) vertebrae, a force-controlled contusion (60 kDyne, 1-second dwell time) was performed using an Infinite Horizon Spinal Cord Impactor. Sham-operated animals received the same surgical treatment without the contusion injury. For 2-3 days post injury, mice received twice daily subcutaneous injections of 0.9% saline to ensure proper hydration (0.5 cc) and twice daily subcutaneous injections of buprenorphine (0.02-0.1 mg/kg) for pain management. For 10 days post injury, mice received twice daily subcutaneous injection of Baytril (0.2-0.5 mg/ml) to prevent urinary tract infections. Manual bladder evacuations were performed twice daily for the duration of the study. Mice had free access to food and water. Mice included in this study received a score of 0 or 1 for both hind limbs the day after surgery, as measured by the Basso Mouse Scale for

locomotion (BMS) (Basso et al., 2006). Animals that could not resume a minimum normal activity level after injury were euthanized.

2.3 Western Blot

At 8-10 weeks post-surgery, mice (including naïve litter mates) were euthanized by inhalation of isoflurane followed by cervical dislocation and transcardial perfusion of ice cold PBS (Sigma-Aldrich, St. Louis, MO). 3 months post-surgery, sham and SCI rats, as well as age matched naïves, were euthanized by i.p. injection of an overdose of pentobarbital/phenytoin (0.9 ml of Euthasol, Virbac AH, Inc., Fort Worth TX) followed by transcardial perfusion of ice cold PBS. DRGs from mice and rats were harvested below T9 and T10 levels, respectively, and subsequently frozen in liquid nitrogen. DRGs were homogenized in SDS lysis buffer (1% SDS, 320 mM sucrose, 5 mM Hepes pH 7.4, 1 mM NaF, 1 mM PMSF) with protease inhibitor cocktail P8340 (Sigma-Aldrich, St. Louis, MO). The homogenates were centrifuged at 14,000 rpm for 5 minutes at room temperature and then heated to 100°C for 5 minutes and stored at -80°C. Protein concentrations were determined by the BCA method (Pierce BCA Protein Assay Kit, ThermoFisher Scientific, Waltham, MA). Equal amounts of cell lysates were separated on 9% SDS-PAGE gels and transferred to a PVDF membrane. Membranes were blocked in 5% (w/v) nonfat milk prior to incubation overnight (4°C) in primary antibody. Primary antibodies included EPAC1 (1:1000; Cell Signaling; Cat# 4155), EPAC2 (1:1000; Cell Signaling; Cat# 4156), PKC (1:1000; Santa Cruz; Cat# sc-10800), anti-actin (1:1000; Cytoskeleton; Cat# AAN01); and GAPDH (1:20,000; RDI; Cat#RDI-Trk5G4-6C5). Membranes were incubated with anti-mouse or anti-rabbit IgG for 1 hour (room temperature) and developed using ECL or the Super Signal West Femto Kit (ThermoFisher

Scientific, Waltham, MA). GAPDH, Actin, and Stain Free imaging were used as a loading controls. Protein expression was then quantified by optical density using Image Lab software (Bio-Rad Laboratories, Version 5.2.1 Build 11).

2.4 Dissociation and culture of DRG neurons.

DRGs from mice and rats were harvested below vertebral levels T9 and T10, respectively. Ganglia were cleaned from roots and protective sheath before being transferred in high-glucose DMEM culture medium (Sigma-Aldrich, St. Louis, MO) containing trypsin TRL (0.3 mg/ml, Worthington Biochemical Corporation, Lakewood, NJ) and collagenase D (1.4 mg/ml, Roche Life Science, Penzberg, Germany). After 40 minutes incubation under constant shaking at 34°C, digested fragments were washed by two successive centrifugations and triturated with a fire-polished glass Pasteur pipette. Cells obtained were then plated on 8 mm glass coverslips coated with poly-L-ornithine (Sigma-Aldrich, St. Louis, MO) in DMEM without serum or growth factors and incubated overnight at 37°C, 5% CO2 and 95% humidity.

2.5 Recording from dissociated DRG neurons.

Whole-cell patch clamp recordings were performed at room temperature 18-28 hours after dissociation using a MultiClamp 700B (Molecular Devices, San Jose, CA) or an EPC10 USB (HEKA Elektronik, Lambrecht/Pfalz, Germany) amplifier. Patch pipettes were made from borosilicate glass capillaries (Sutter Instrument Co., Novato, CA) with a horizontal P-97 puller (Sutter Instrument Co., Novato, CA) and then fire-polished with a MF-830 microforge (Narishige, Tokyo, Japan) to a final pipette resistance of 3-8 MΩ when filled with an

intracellular solution composed of (in mM): 134 KCl, 1.6 MgCl2, 13.2 NaCl, 3 EGTA, 9 HEPES, 1 Mg-ATP, and 0.3 Na-GTP, which was adjusted to pH 7.2 with KOH and 300 mOsM with sucrose. Isolated small neurons with a soma diameter ≤ 30 µM were observed at 20x magnification on IX-71 (Olympus, Tokyo, Japan) or 40x on TE2000-U (Nikon, Tokyo, Japan) and Axiovert 200M (Zeiss, Oberkochen, Germany) inverted microscopes and recorded in a bath solution containing (in mM): 140 NaCl, 3 KCl, 1.8 CaCl2, 2 MgCl2, 10 HEPES, and 10 glucose, which was adjusted to pH 7.4 with NaOH and 320 mOsM with sucrose. After obtaining a tight seal (>1 GΩ) when held at -60 mV, the plasma membrane was ruptured to achieve whole-cell configuration. Recordings were acquired with Patchmaster v2x90.1 (HEKA Elektronik, Lambrecht/Pfalz, Germany) and Clampex v10.4 (Molecular Devices, San Jose, CA). The liquid junction potential was calculated at ~4.3 mV and not corrected in the recordings and measurements presented herein.

2.6 Quantifying depolarizing spontaneous fluctuations

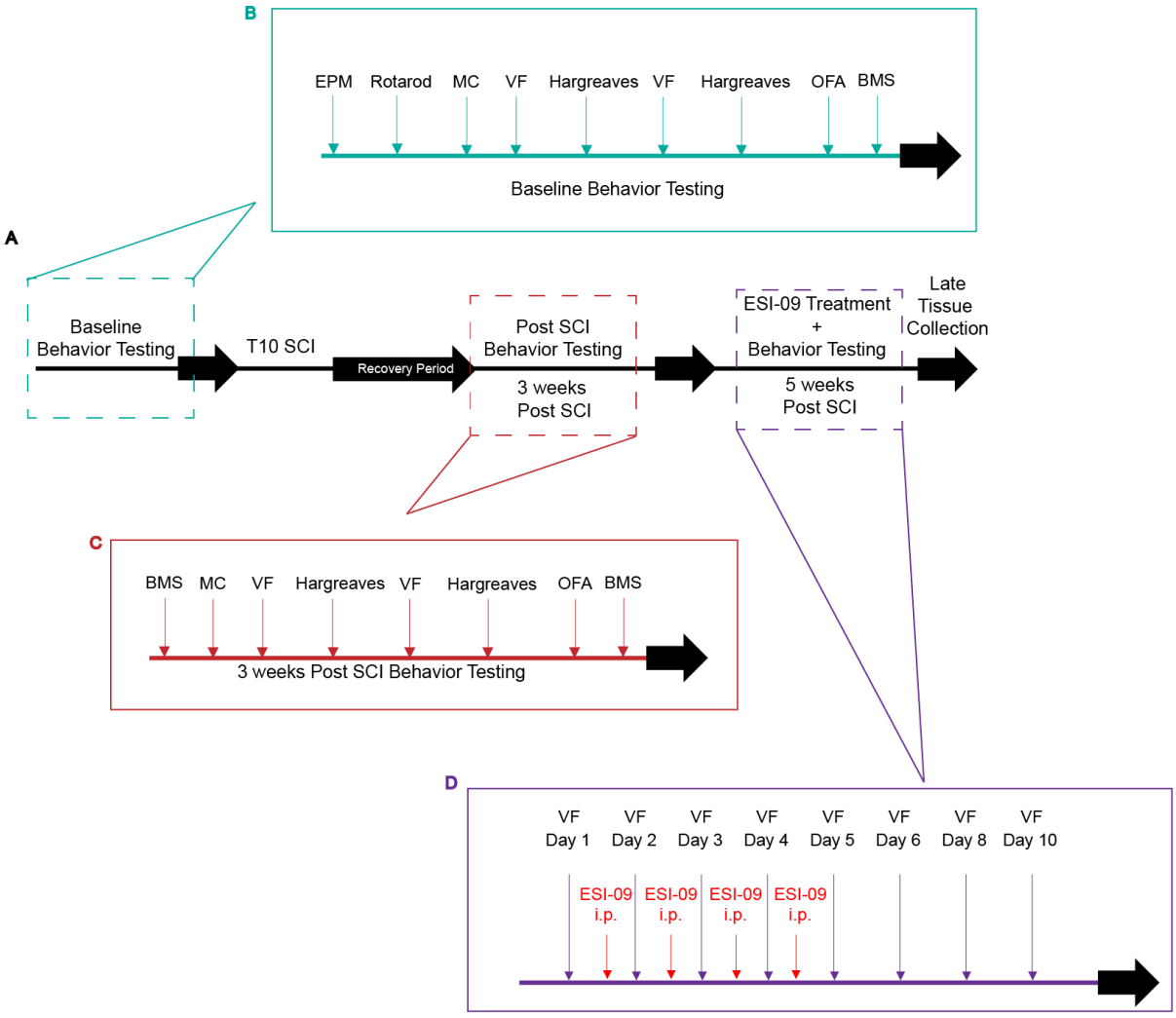
Depolarizing spontaneous fluctuations (DSFs) were analyzed as previously described (Odem et al., 2018). Briefly, we used a custom program (SFA.py) to quantify irregular DSFs in patch recordings, which we imported as time and voltage coordinate data for 30-second periods from recordings obtained with PatchMaster (HEKA Elektronik) sampled at 20 kHz and filtered with a 10 kHz Bessel filter. The program used a sliding median function to calculate resting membrane potential (RMP) at each point and returned coordinates, amplitudes, and durations of identified action potentials (APs) and DSFs (minimum amplitude and duration 1.5 mV and 5 ms), as well as a continuous color-coded plot of membrane potential generated using the matplotlib library (Python) (Odem et al., 2018). Manual inspection of each

plot confirmed that each AP was generated by a suprathreshold DSF. We estimated the amplitude of the suprathreshold DSFs by 1) identifying the point at which the change in membrane potential began to accelerate immediately before the AP and 2) measuring the amplitude of the largest subthreshold DSF at RMP and during rheobase measurements (Odem et al., 2018).

2.7 Pharmacological Agents

EPAC2 inhibitor ESI-05 was synthesized as described in Chen et.al. (Chen et al., 2013). EPAC activator 8-pCPT-2-O-Me-cAMP-AM (007-AM) was purchased from Tocris Bioscience (Bristol, England, UK) and EPAC 1 inhibitor CE3F4 from Cayman Chemicals (Ann Arbor, MI). Drugs were prepared in DMSO (Sigma-Aldrich, St. Louis, MO) at a concentration of 20 mM for ESI-05 and CE3F4 and 10 mM for 007-AM. Reagents were then diluted in extracellular recording solution at a minimum dilution factor of 1/1000.

For *in vivo* studies ESI-09 was dissolved in ethanol (10mg/ml) and diluted 1:10 in 10% Tween80 in PBS. ESI-09 was sterile filtered before intraperitoneal injection at a dose of 10 mg/kg (Gong et al., 2013). For *in vitro* studies ESI-09 was prepared in DMSO at a concentration of 50mM and the diluted in extracellular recording solution to 5 uM.


B
EPM
Rotarod
MC
VF
Hargreaves
VF
Hargreaves
OFA
BMS
Baseline Behavior Testing
A
Baseline Behavior Testing
T10 SCI
Recovery Period
Post SCI Behavior Testing
3 weeks Post SCI
ESI-09 Treatment + Behavior Testing
5 weeks Post SCI
Late Tissue Collection
C
BMS
MC
VF
Hargreaves
VF
Hargreaves
OFA
BMS
3 weeks Post SCI Behavior Testing
D
VF Day 1
VF Day 2
VF Day 3
VF Day 4
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VF Day 6
VF Day 8
VF Day 10
ESI-09 i.p.
ESI-09 i.p.
ESI-09 i.p.
ESI-09 i.p.

Figure 4. Timeline of Mouse Behavior Studies. WT and EPAC2$^{-/-}$ mice underwent a standard set of behavior tests over a 2 month period. (A) 6-8 week-old mice were tested to determine baseline behavioral values before receiving a T10 SCI contusion injury. After a 3 week recovery period, mice were re-tested using the same behavioral assays. At 5 weeks post SCI, mechanical sensitivity was again tested with and without *in vivo* pharmacological inhibition of EPAC by ESI-09. (B) Baseline behavior testing was performed over a 2 week period, and included the Elevated Plus Maze (EPM) anxiety test, rotarod motor coordination test, mechanical conflict (MC) test, von Frey (VF) mechanical sensitivity test, Hargreaves thermal sensitivity test, open field activity test, and evaluation of locomotor ability by the Basso mouse scale (BMS), in the order shown. (C) Three weeks post SCI, mice underwent the same series of behavior tests, with the exception of the EPM and rotarod, in the order shown. (D) Five weeks post SCI, VF was used to evaluate the effect of *in vivo* pharmacological inhibition of EPAC on mechanical sensitivity after SCI. On days 1-4, mice underwent VF testing in the morning and received i.p. injection of ESI-09 in the evening. VF testing continued for 6 additional days after the final treatment.

2.8 Behavioral Testing

Behavior tests were performed by the same blinded investigator throughout (Figure 4). Mice were allowed to acclimate to the behavioral testing room for 30-60 minutes in their home cages before beginning testing. Mice were acclimated with the investigator present.

Mechanical Conflict (MC) Test

A mechanical conflict (MC) test was used to assess voluntary avoidance of noxious probes as an operant measure of altered pain sensitivity (Figure 5). The commercially available MC test device (Mechanical Conflict-Avoidance System, Coy Lab Products, Grass Lake, MI, USA) consists of two rectangular chambers (each 16.5 cm wide by 21.5 cm deep by 15.25 cm high) connected by a narrow 30.4 cm long tunnel containing a dense array of sharp probes on the floor (Harte et al., 2016). We modified the tunnel floor for mice to a 30.4 cm by 3.8 cm array of 3 mm, more closely spaced, fine, blunt metal probes (tip diameter ~0. 63 mm). In each test, a mouse was placed in the darkened first chamber with the gate closed to prevent access to the tunnel. After 30 seconds the chamber was illuminated by a bright light emitting diode (LED) with a mildly aversive mean illuminance of 442 foot-candle at the compartment floor, meant to promote escape to the unlit chambers. After at least 15 seconds, when the mouse was facing the gate, the gate was lifted to allow free movement throughout the device for the remaining 3 minutes of the test. The test was video-recorded for subsequent analysis (Sony Handycam, HDR-XR260, recorded in HD). Videos were analyzed by a separate, blinded investigator for latency to the 1st step onto the probe floor, the total time on the probe floor, the latency to the first crossing of the probes (all four paws in the dark chamber), second crossing latency (front-paws re-entering the original chamber), and total number of crossings. Latency measurements always began when the gate was fully raised. In our preliminary testing we

observed that repeated trials significantly decreased the number of crossings and increased the latency to cross. Similar studies with rats suggested that a large component of the motivation to cross the aversive probes is the strong drive of rodents to explore an unfamiliar context. To reduce possible habituation to the MC device, we modified our procedure to allow the mice to experience the MC only twice, with a period of 3-4 weeks separating each exposure, to keep the test novel. Unlike previously published studies using the MC test, the mice had not experienced the probes before their first crossing. When crossing a second time (presumably motivated by innate exploratory drive), the mice had recently experienced the aversive probes and could exhibit voluntary avoidance behavior by delaying or avoiding a second crossing.

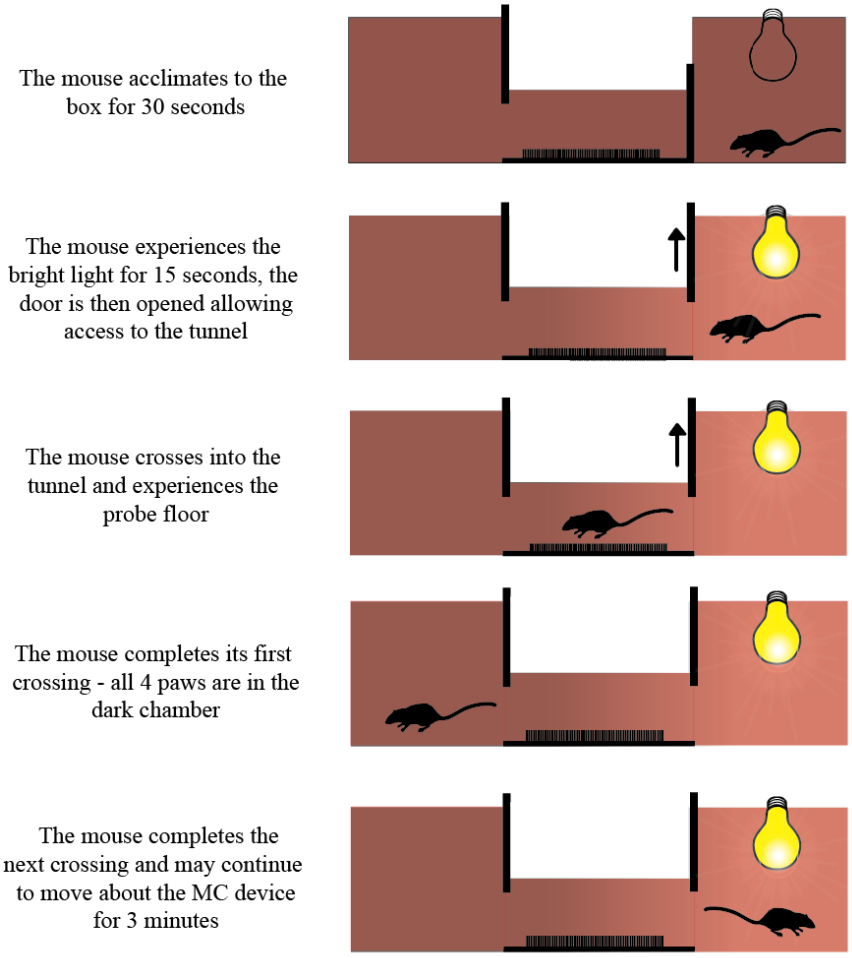


Figure 5. The operant Mechanical Conflict (MC) test procedure maximizes the innate exploratory drive. Mice are placed in the first chamber, where they are allowed to acclimate for 30 seconds with the gate closed and the light off. The LED light is turned on and experienced by the mice for 15 seconds before the gate is lifted (though it may be lifted later to ensure the mice are facing the gate). The mouse crosses the tunnel into the dark chamber, experiencing the mechanical probes for the first time. The mouse may now continue to move freely through the MC device for the next 3 minutes. There are no familiarization pre-trials, thus keeping the test novel.

Von Frey mechanical sensitivity test

Von Frey filaments were used to determine the 50% paw withdrawal threshold using the up and down method (Chaplan et al., 1994). Animals were placed in red plastic testing chambers (8.9 cm wide by 8.9 cm deep by 15.2 cm high) on a wire grid bottom and allowed to acclimate for 30 minutes with the investigator present. Von Frey filaments were applied for < 1 second through the grid perpendicular to the plantar surface of the hindpaw with sufficient force to buckle against the paw, with the tester allowing 30 seconds between stimuli (starting with a bending force of 0.4 g; range: 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4, 2.0, 4.0 g, North Coast Medical, Inc., San Jose, CA, USA). The subsequent filament force was increased following a negative response, or decreased following a positive response, with a total of 10 stimuli per hindpaw. A positive response was recorded if the paw was quickly withdrawn, which was often accompanied by paw grooming or shaking behavior.

Hargreaves heat sensitivity test

The latency to withdrawal from a radiant heat stimulus was measured using the Plantar Analgesia Meter (IITC Life Science Inc., Woodland Hills, CA, USA) as described previously (Hargreaves et al., 1988). Mice were placed in red plastic testing chambers (8.9 cm wide by 8.9 cm deep by 15.2 cm high) on a 30°C heated glass surface and allowed to acclimate for 30 minutes with the investigator present. A light beam, set to an intensity 30% of its maximum value (found to evoke a positive response in 10 seconds at baseline in naïve mice), was aimed at the hindpaw plantar surface until evoking a positive response, or for a maximum of 20 seconds to prevent tissue damage. The latency to evoke a positive response (brisk hindpaw withdrawal) was recorded and each hindpaw was tested 5 times, with a 5 minute interval

between stimuli. The median response latency was recorded after excluding the shortest and longest latencies.

Elevated plus maze anxiety test

The 40 cm high elevated plus maze (EPM) was used to measure differences in anxiety. It consisted of four, 12 cm wide arms: two enclosed by 40 cm high walls and two open (modeled upon the Stoelting Co's EPM model, Wood Dale, IL). Individual mice were placed in the EPM center and allowed to move freely for 5 minutes. The test was video recorded and later analyzed for time spent in the open versus closed arms, as previously described (Pellow et al., 1985, Acharjee et al., 2013, Nyuyki et al., 2018).

Rotarod motor coordination test

The rotarod was used to assess coordination in mice, as described previously (Rozas et al., 1997). Mice were placed on the rotating rod (Model ENV-576M, Med Associates, Georgia, VT) facing the direction opposite to the rotation. Mice underwent 3 preliminary training tests spaced 10 minutes apart. Mice must be able to stay on a rod rotating at 4 rpm for 60 seconds before moving past training. During the actual test, mice were placed on a rotating rod accelerating linearly with time from 4-40 rpm over the course of 5 minutes. The fall latency was recorded automatically by photobeam sensors, with the maximum time being 5 minutes. Mice underwent three trials spaced 15 minutes apart.

Open field activity measure

The open field apparatus (OFA) (ENV-515, Med Associates, Inc., St. Albans, VT) consists of an activity chamber (43.2 cm wide by 43.2 cm deep by 30.5 cm high) with 16 infrared transmitters and receivers evenly positioned around the chamber's periphery. Mice

were placed in the activity chamber and allowed to move freely for 25 minutes, and data were collected by the software provided with the OFA. The OFA software registers movements within the chamber by recording photo beam interruptions. We analyzed the first 5 minutes within the chamber for ambulatory distance traveled and average velocity.

2.9 Statistical Analysis

Data are presented as mean ± SEM or incidence (% of sampled neurons). $P<0.05$ is considered statistically significant. All data sets were tested for normality by the Shapiro-Wilk test. Normally distributed data were tested with the parametric t-test or 1-way ANOVA, followed by the Holm-Sidak method of pairwise comparison. Non-parametric tests included the Mann-Whitney U test or Kruskal-Wallis test, followed by Dunn's test for each pair-wise comparison. Data reported as incidence were compared by Chi square or Fisher's exact test when appropriate. Bonferroni corrections were made after multiple comparisons. Statistical analyses were conducted using SigmaPlot (Systat Software, Inc., San Jose, CA) and Prism v7.04 (GraphPad Software, Inc., La Jolla, CA, USA).

Chapter 3: Results

3.1 Rationale

Chronic pain affects over 25 million Americans yet the underlying mechanisms promoting and maintaining it are not fully understood (Nahin, 2015). Often resulting in a drastic drop in quality of life, chronic pain is a complex syndrome associated with multiple inflammatory and neuropathic pain states (Finnerup and Baastrup, 2012, Finnerup, 2013). While 65-80% of spinal cord injury (SCI) patients report suffering from this condition, treatments remain inadequate, highlighting the necessity of defining new and accessible therapeutic targets (Siddall et al., 2003).

Alterations occurring at the spinal and supraspinal levels have been a focus of multiple studies, with an emphasis on the effects of gliosis (Detloff et al., 2008) or loss of local inhibitory (GABAergic) tone (Meisner et al., 2010), which contribute to the maintenance of at-level and below level neuropathic pain after SCI. However, recent studies indicate an unexpected contribution of the peripheral nervous system, specifically a switch in nociceptors within dorsal root ganglia (DRG) at and below the contusion level to a persistent, hyperexcitable state (Bedi et al., 2010, Walters, 2012, Yang et al., 2014). This hyperactive state can be observed between 3 days and 6 months post injury, and arises from the combined effects of sustained depolarization of resting membrane potential (RMP), a decrease in action potential threshold, and an increase in amplitudes of depolarizing spontaneous fluctuations (DSF) (Odem et al., 2018). These three intrinsic mechanisms promote and maintain spontaneous activity (SA) after SCI and can also potentiate ongoing activity (OA) in nociceptors driven by extrinsic signals (Bedi et al., 2010, Yang et al., 2014, Odem et al., 2018).

Our previous study describing intracellular signaling mechanisms maintaining SCI-induced SA within the somata of small diameter nociceptors showed that the hyperactive state depends on prolonged activation of cAMP-PKA signaling within an intact A-kinase anchoring protein 150 complex, as well as an alteration in adenylyl cyclase regulation (Bavencoffe et al., 2016). Recent work has identified an additional cAMP effector involved in pain development: exchange protein activated by cAMP (EPAC). Peripheral EPAC was shown to mediate hyperalgesia induced by peripheral application of epinephrine or cAMP (Hucho et al., 2005) and to augment sensory neuron excitability and sensitivity *in vitro* (Wang et al., 2007a, Eijkelkamp et al., 2013, Vasko et al., 2014a, Gu et al., 2016), as well as to induce hyperalgesia *in vivo* (Hucho et al., 2005, Gu et al., 2016). However, between injury models, there are diverse changes in EPAC1 and 2 expression levels within the dorsal root ganglion (DRG). The role of EPAC1 versus EPAC2 within chronic pain signaling remains unclear, though many studies have argued for a focus on EPAC1, as EPAC1$^{-/-}$ mice do not develop mechanical hyperalgesia after CFA-induced inflammation or after lumbar 5 (L5) nerve transection (Eijkelkamp et al., 2013, Singhmar et al., 2016).

3.2 Activity of EPAC1 and 2 is required for spontaneous activity generated in dissociated rat nociceptors after spinal cord injury

The major goal of our study was to determine the role of EPAC in maintaining an SCI-induced hyperexcitable state in primary nociceptors. Presumptive nociceptors were selected on the basis of small soma diameter ($\leq$ 30 μm) and nonaccommodating properties during activation by 2-s depolarizing currents at twice the rheobase value (Odem et al., 2018). Previous studies have shown that ~70% of nonaccommodating neurons sampled under our

conditions are nociceptors based on capsaicin sensitivity and/or binding of isolectin B4 (IB4) (Bedi et al., 2010, Bavencoffe et al., 2016). Consistent with these previous studies, 1-8 months after SCI we observed a SA incidence of 72% within small DRG neurons isolated from injured male rats, versus the low 14% incidence found within naïve animals (Figure 6A). The high incidence of SA was associated with a significant depolarization of the RMP (-49 mV in SCI versus -57 in naïve rats, Figure 6B), decrease in AP threshold (Figure 6C), and a lower rheobase (20 pA in SCI versus 60 pA in naïve rats, Figure 6D).

Previous studies have shown that both EPAC1 and EPAC2 can augment excitability in isolated sensory neurons (Eijkelkamp et al., 2013, Vasko et al., 2014b). In nociceptors isolated from SCI rats, we found that pretreatment with either the EPAC1-selective inhibitor CE3F4 (10 µM) (Courilleau et al., 2012, Sonawane et al., 2017) or the EPAC2-specific inhibitor ESI-05 (5 µM) (Tsalkova et al., 2012) for 15 minutes before recording significantly decreased the incidence of SA, by 61% and 66%, respectively, and hyperpolarized RMP (Figure 6B). Action potential voltage threshold in DRG neurons isolated from SCI rats was not significantly affected (Figure 6C), while the rheobase increased after CE3F4 treatment (Figure 6D). Neither drug had a significant effect on the incidence of SA, RMP, rheobase, or AP threshold in isolated DRG neurons from naïve rats (Figure 6). Analysis of DSFs showed that the largest amplitudes occurred at more depolarized RMPs in nociceptors isolated from SCI rats, with the largest amplitudes occurring at -45 mV (Figure 6E), as shown previously by Odem et. al. (Odem et al., 2018). Both CE3F4 and ESI-05 treatment significantly decreased DSF amplitudes at RMPs between -55 and -41 mV (Figure 6E).

ESI-05 inhibits EPAC2 with an IC_{50} of 0.4 ± 0.05 in the presence of cAMP, while it had no effect on EPAC1 (Tsalkova et al., 2012). Previous work suggest ESI-05 may bind the

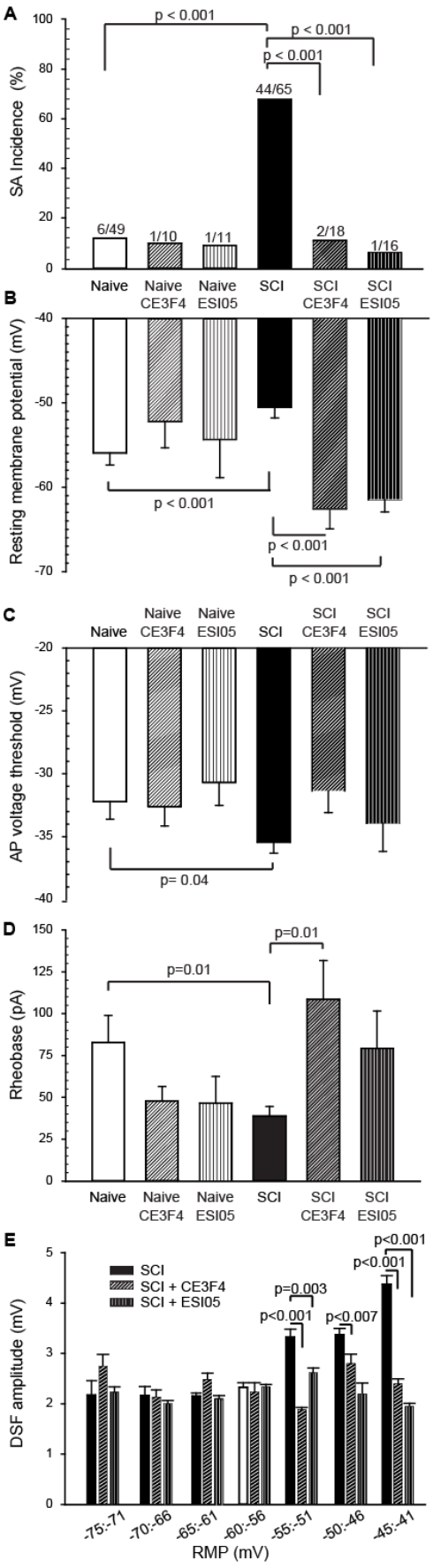

A
SA Incidence (%)
p < 0.001
p < 0.001
p < 0.001
44/65
6/49
1/10
1/11
2/18
1/16
Naive
Naive CE3F4
Naive ESI05
SCI
SCI CE3F4
SCI ESI05
B
Resting membrane potential (mV)
p < 0.001
p < 0.001
p < 0.001
C
AP voltage threshold (mV)
p= 0.04
D
Rheobase (pA)
p=0.01
p=0.01
E
DSF amplitude (mV)
SCI
SCI + CE3F4
SCI + ESI05
p=0.003
p<0.001
p<0.007
p<0.001
p<0.001
-75:-71
-70:-66
-65:-61
-60:-56
-55:-51
-50:-46
-45:-41
RMP (mV)

Figure 6. EPAC1 or EPAC2 activity maintains SCI-induced hyperexcitability in dissociated small diameter rat DRG neurons. Small DRG neurons (diameter ≤ 30μm) harvested from naïve and SCI animals below and at the T10 spinal level were recorded by whole-cell patch clamp 18-30 hours after dissociation. DRG neurons were pretreated with either 10 μM CE3F4 or 5 μM ESI-05 for 15-20 minutes before recording. (A) Inhibition of EPAC1 or 2 attenuated SCI-induced SA incidence. The ratio above each bar denotes the number of neurons with SA/the number of neurons sampled. Statistical comparisons of SA incidence were made with Bonferroni-corrected Fisher's exact tests on the indicated pairs. (B) Inhibition of EPAC1 or 2 reversed SCI-induced depolarization of RMP. (C) Inhibition of EPAC1 or 2 did not reverse SCI-induced depolarization of AP voltage threshold. (D) Inhibition of EPAC1 attenuated the SCI-induced increase in rheobase. Data shown as mean ± SEM. Overall significance determined with one way ANOVA (or Kruskal-Wallis for non-parametric data), followed by multiple comparisons with Dunn's method. Naïve vs SCI rats were compared by paired t-test (or Mann-Whitney U for non-parametric data). (E) Inhibition of EPAC1 or EPAC2 decreased the amplitude of DSFs recorded at rest in DRG neurons from SCI rats, especially at more depolarized initial RMPs. DSFs were quantified by an automated program which estimated RMP via a sliding median function and then identified small depolarizing fluctuations (greater than 1.5 mV in amplitude lasting >5ms). DSFs were binned according to their starting RMP. Data are represented as mean ± SEM. The indicated statistical comparisons were performed with 2-way ANOVA/Dummett test. *Data collected by A. Bavencoffe and M.A. Odem. Analysis by S.C. Berkey and A. Bavencoffe. Figure by S.C. Berkey.*

interface between the two cAMP binding domains to lock EPAC2 into an auto-inhibitory configuration, which could not occur in EPAC1 as only one of these domains is present (Tsalkova et al., 2012). Conversely, CE3F4 has about a 10 fold preference for EPAC1 over EPAC2 , and again acts non-competitively in the presence of cAMP to inhibit EPAC1 GEF activity towards Rap1 (Courilleau et al., 2012, Courilleau et al., 2013).

These results suggest that EPAC1 and EPAC2 both play a role in maintaining nociceptor hyperexcitability after spinal contusion. SCI-induced SA is promoted by 3 general electrophysiological alterations known to be intrinsic to the soma (and are likely to also occur in peripheral terminals): depolarization of RMP, decrease in AP voltage threshold, and enhancement in the frequency of large DSFs that bridge the gap between RMP and AP threshold to trigger APs and produce SA (Odem et al., 2018). Two of these alterations appear to involve one or both EPAC isoforms. The SCI-induced depolarization of RMP was reversed by inhibition of either EPAC isoform (Figure 6B). Although a trend for EPAC1 inhibition to reverse the reduction in AP threshold by SCI was seen, this effect was not statistically significant with this sample size and no effect of EPAC2 inhibition was evident (Figure 6C). On the other hand, inhibition of either isoform eliminated the SCI-induced increase in DSF amplitude observed at relatively depolarized RMP levels (Figure 6E).

As EPAC inhibition affected most of the intrinsic mechanisms within nociceptors that lead to hyperexcitability and SA after SCI, we asked whether EPAC activation in DRG neurons isolated from naïve animals would induce hyperexcitability similar to that induced by SCI. Pre-treatment for 10-30 minutes with EPAC activator 007-AM (10 μM) led to a significant depolarization of RMP, hyperpolarization of AP threshold and a trend for rheobase to decrease (Figure 7B, C, D). Moreover, 007-AM treatment significantly increased the DSF amplitudes

in neurons with RMPs between -45 and -41 mV, and when neurons were held artificially at -45 mV (Figure 7F). Nonetheless, EPAC stimulation failed to increase the incidence of neurons with spontaneous activity (SA) at RMP or neurons exhibiting ongoing activity (OA) when artificially depolarized to -45 mV (Figure 7A, E). These data suggest that both EPAC isoforms are necessary for maintenance of nociceptor hyperexcitability after SCI, but their activity is not sufficient in nociceptors from naive rats to produce either SA or substantial OA when combined with extrinsic depolarization.

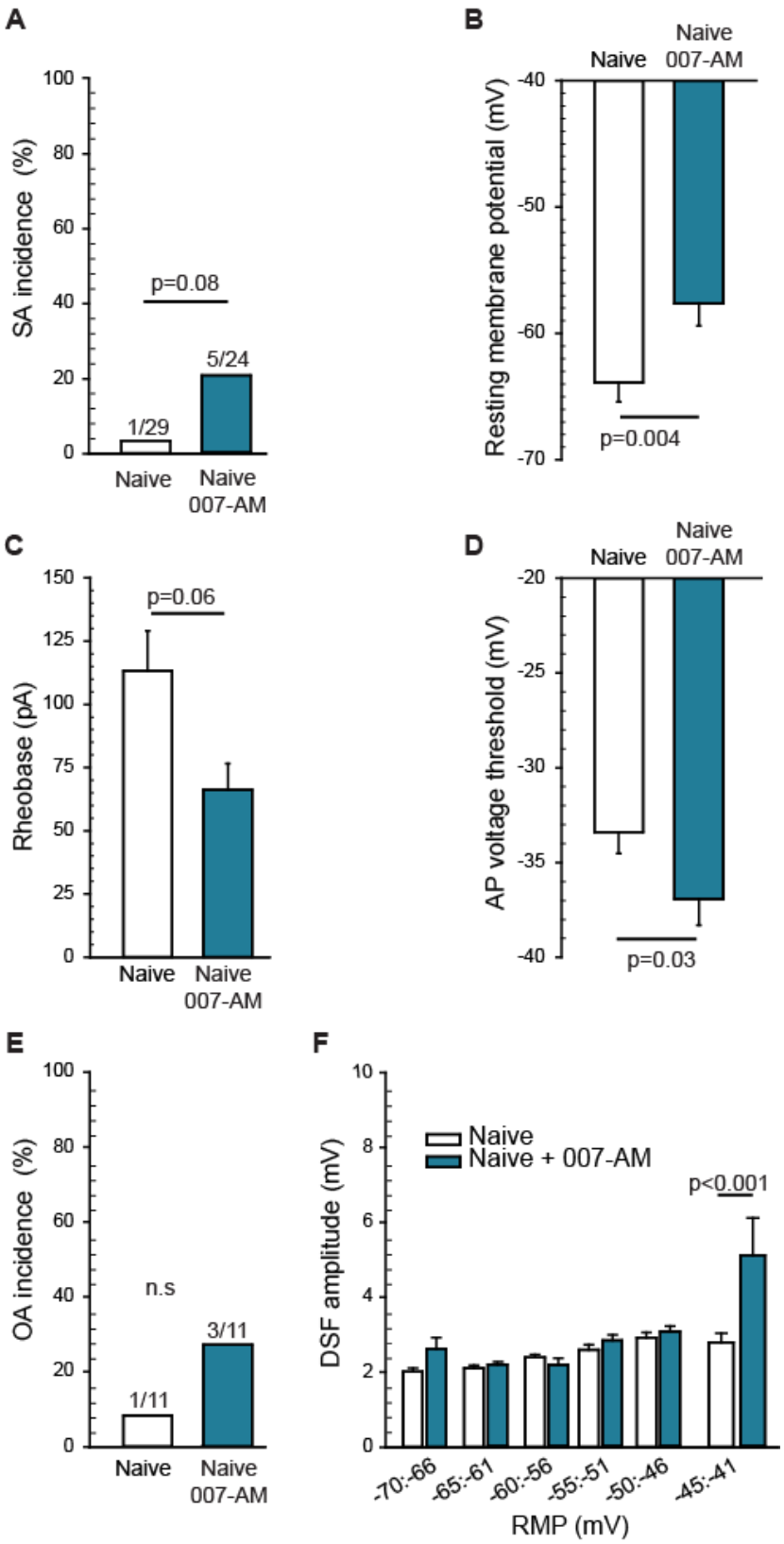

A
SA incidence (%)
100
80
60
40
20
0
p=0.08
5/24
1/29
Naive
Naive
007-AM
B
Naive
Naive
007-AM
Resting membrane potential (mV)
-40
-50
-60
-70
p=0.004
C
Rheobase (pA)
150
125
100
75
50
25
0
p=0.06
Naive
Naive
007-AM
D
Naive
Naive
007-AM
AP voltage threshold (mV)
-20
-25
-30
-35
-40
p=0.03
E
OA incidence (%)
100
80
60
40
20
0
n.s
3/11
1/11
Naive
Naive
007-AM
F
DSF amplitude (mV)
10
8
6
4
2
0
Naive
Naive + 007-AM
p<0.001
-70:-66
-65:-61
-60:-56
-55:-51
-50:-46
-45:-41
RMP (mV)

Figure 7. Activation of EPAC1 and 2 has a slight effect on DRG neurons isolated from naïve rats, depolarizing the RMP and hyperpolarizing AP voltage threshold. DRG neurons harvested from naïve rats were pretreated with 10 μM 8-pCPT-2-O-Me-cAMP-AM (007-AM) for 10-15 minutes before recording. (A) EPAC activation did not significantly affect SA incidence within neurons isolated from naïve rats. The ratio above each bar denotes the number of neurons with SA/the number of neurons sampled. Statistical comparison of SA was performed with Bonferroni-corrected Fisher's exact test. (B) EPAC activation significantly depolarized RMP (C) with a trend toward a reduction in rheobase (D) and a significant hyperpolarization of AP voltage threshold. Statistical comparisons of data (represented were mean ± SEM) were made by Mann Whitney U test. (E) EPAC activation did not significantly affect OA incidence. The ratio above each bar denotes the number of neurons with OA/the number of neurons sampled. Statistical comparison of OA was performed with Fisher's exact test. (F) EPAC activation significantly increased the DSF amplitudes at rest only for cells exhibiting RMP values between -45 and -41 mV. DSFs were binned according to initial voltage. Statistical Comparison was performed by 2-way ANOVA followed by Sidak method. Each point represents a single DSF. *Data collected by A. Bavencoffe and M.A. Odem. Analysis by S.C. Berkey and A. Bavencoffe. Figure by S.C. Berkey.*

3.3 SCI-induced behavioral hypersensitivity is increased after SCI in both wild-type and EPAC2$^{-/-}$ mice

Our finding in rats that SA was reduced by inhibition of either EPAC1 or EPAC2, combined with reports (Wang et al., 2007a, Gu et al., 2016, Matsuda et al., 2017) showing an increase in EPAC2 protein levels in the DRG after peripheral inflammation, and that PGE2-induced nociceptor sensitization involves EPAC2 (Vasko et al., 2014), led us to hypothesize that EPAC2 in primary nociceptors plays a major role in maintaining chronic pain after SCI. To test this hypothesis, we transitioned to a mouse model of EPAC2 knockout (EPAC2$^{-/-}$).

We first confirmed that EPAC2$^{-/-}$ mice did not exhibit significant differences from wild-type mice in general behavioral functions (Srivastava et al., 2012, Lee et al., 2015): anxiety, as tested by time spent in the open arms of the elevated plus maze (EPM); coordination, as tested by latency to fall off the rotarod; and activity level, as tested by average velocity within an activity box (Table 1). Von Frey filaments and the Hargreaves heat sensitivity tests were used to establish basal mechanical and thermal sensitivity thresholds, respectively. The mechanical withdrawal threshold and the thermal withdrawal latency did not differ significantly between genotypes (Table 1).

However, using the enhancement of brisk withdrawal reflexes as a measure of increased pain sensitivity is problematic, especially after SCI, as sensitization of hindlimb reflexes can be part of spastic syndromes and reflexive measures do not capture the motivational/affective dimensions of pain (Baastrup et al., 2010, Yezierski and Vierck, 2010). In contrast, the mechanical-conflict test is a paradigm in which mouse voluntary (operant) behavior reveals the aversiveness of a rough substrate they must cross to explore an unfamiliar setting and to escape from an aversive bright light (Harte et al., 2016, Pahng et al., 2017)(Odem

et al. submitted). When tested with the MC, naïve wild type and EPAC2$^{-/-}$ mice crossed the aversive probes a similar number of times, and spent about the same amount of time on the probes (Table 1). The first and second crossing latency was not significantly different between genotypes. The lack of a difference between the mouse genotypes in their reactions to the aversive probes is consistent with previous studies that found no clear behavioral differences between wild-type and EPAC2$^{-/-}$ mice (Srivastava et al., 2012, Lee et al., 2015), though a thorough analysis of pain-like behavior in EPAC2-/- mice has not been reported.

Because the reflex-based sensitivity tests and the MC depend upon proper plantar placement, weight support, and stepping, we required the injured mice to recover to a BMS score which reflected sufficient motor function to readily cross the probes, defined as ≥3 on the BMS (Basso et al., 2006). One day post-surgery, sham mice exhibited complete locomotor function and received a score of 9, SCI mice exhibited slight to no ankle movements and received a score of 0 or 1 (Figure 8). Unexpectedly, at 3 weeks post-SCI, only the female mice showed sufficient recovery of plantar placement and stepping behavior, as indicated by a BMS score of 3-4. The male SCI mice only showed active movements of the ankle joints and thus received BMS scores of 2, regardless of genotype (Figure 8). Due to this gender difference in locomotor recovery, we only included female test subjects in the following behavioral experiments.

Summary of Baseline Behaviors in Naïve Wild-type and Epac2$^{-/-}$ Mice				
Behavior Test	**Wild-type**	**Epac2 KO**	**Significance**	**Test**
Von Frey (g)	2.5 ± 0.3 (18)	3.1 ± 0.5 (11)	P = 0.281	MW
Hargreaves (s)	9.3 ± 0.5 (18)	8.2 ± 0.4 (11)	P = 0.159	t-test
Rotarod (s)	171.5 ± 18.3 (16)	138.3 ± 14.2 (7)	P = 0.274	t-test
Activity Box Average Velocity (cm/min)	41.8 ± 3.4 (16)	39.7 ± 2.1 (7)	P = 0.699	t-test
EPM (s)	184.1 ± 10.6 (16)	174.5 ± 25.5 (7)	P = 0.867	MW
Mechanical Conflict System				
# of Total Crossings	8.7 ± 0.5 (18)	9.4 ± 1.2 (11)	P = 0.585	t-test
Total Time on Probes (s)	40.6 ± 4.3 (18)	42.4 ± 5.4 (11)	P = 0.805	t-test
Latency to 1st Crossing (s)	38.9 ± 5.6 (18)	42.0 ± 8.6 (11)	P = 0.875	MW
Latency to 2nd Crossing (s)	52.1 ± 6.1 (18)	57.5 ± 9.5 (11)	P = 0.822	MW
MW: Mann-Whitney Rank Sum Test				

Table 1. No behavioral differences were seen between WT and EPAC2$^{-/-}$ mice pre-injury. Behavioral Tests were run pre-surgery with naïve WT and EPAC2$^{-/-}$ mice. Each value is the mean ± SEM followed in parentheses by the number of mice tested. Statistical tests include: paired t-test (t-test) and non- parametric Mann Whitney U test (MW). *Data and Analysis by S. Rahman and S.C. Berkey.*

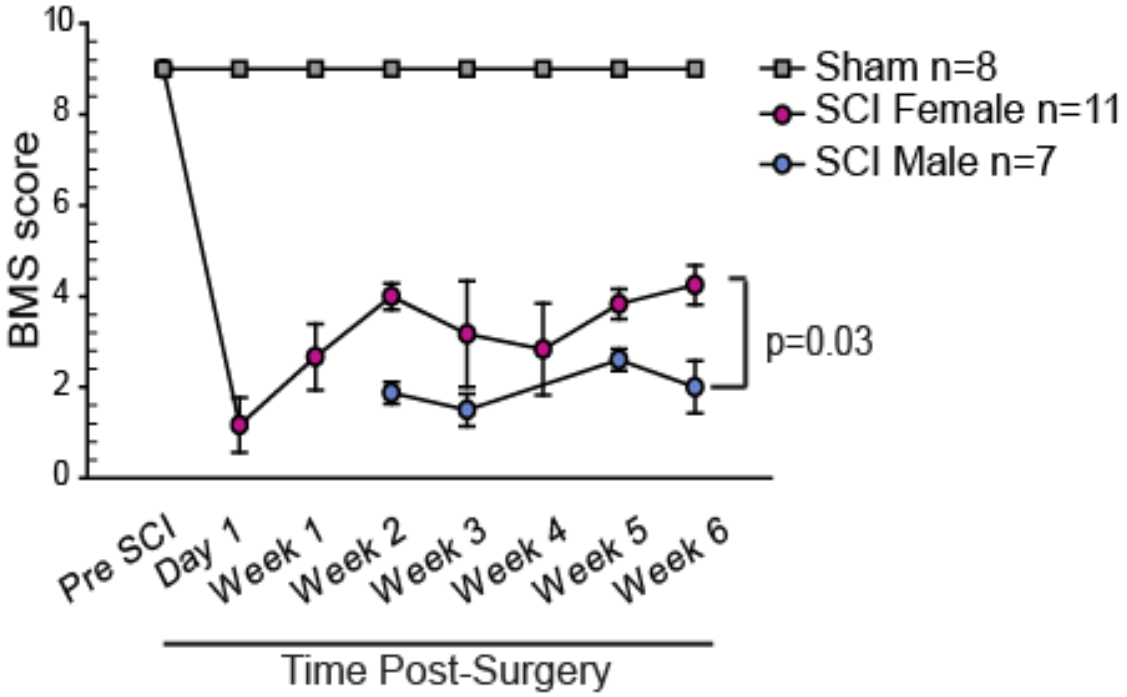


Figure 8. Sex-specific differences in locomotor recovery post-SCI in mice. Gender significantly affected locomotor recovery post-SCI in mice. The BMS experiments were performed weekly after surgery, and significant differences between males and females were observed as early as 2 weeks post-injury. Mice were monitored during 4 minutes observation sessions, during which their movements were assessed according the previously published BMS scale and methodology (Basso et al., 2006). Statistical comparison was made using 2-way RM ANOVA. *Data collected by A. Bavencoffe and S.C. Berkey. Analysis by M.A. Odem and S.C. Berkey. Figure by S.C. Berkey.*

Three weeks after injury, female SCI WT mice showed a significant decrease in mechanical threshold for paw withdrawal as tested by von Frey filaments, but this decrease was not significantly different from the similar decrease seen in the sham control group (Figure 9A). In addition, no change in heat sensitivity was found in SCI WT mice (Figure 9B). Knockout of EPAC2 did not alter the responses of SCI or sham-operated rats to the mechanical or radiant heat test stimuli. In contrast, the operant MC, which reflects affective-motivational and cognitive-evaluative dimensions of pain (Harte et al., 2016, Pahng et al., 2017)(Odem et al., submitted) revealed enhanced pain-avoidance behavior after SCI and no effect of EPAC2 deletion. Both SCI wild-type and EPAC2$^{-/-}$ mice crossed the probes less often than sham animals (Figure 9C), and took significantly longer to complete the second crossing (Figure 9D), indicating similarly increased pain-avoidance behavior in wild type and EPAC2$^{-/-}$ SCI mice. Together, these data indicate that SCI increases sensitivity to noxious but not innocuous mechanical stimuli under our conditions, and that the enhanced pain-avoidance behavior is not prevented by deletion of EPAC2.

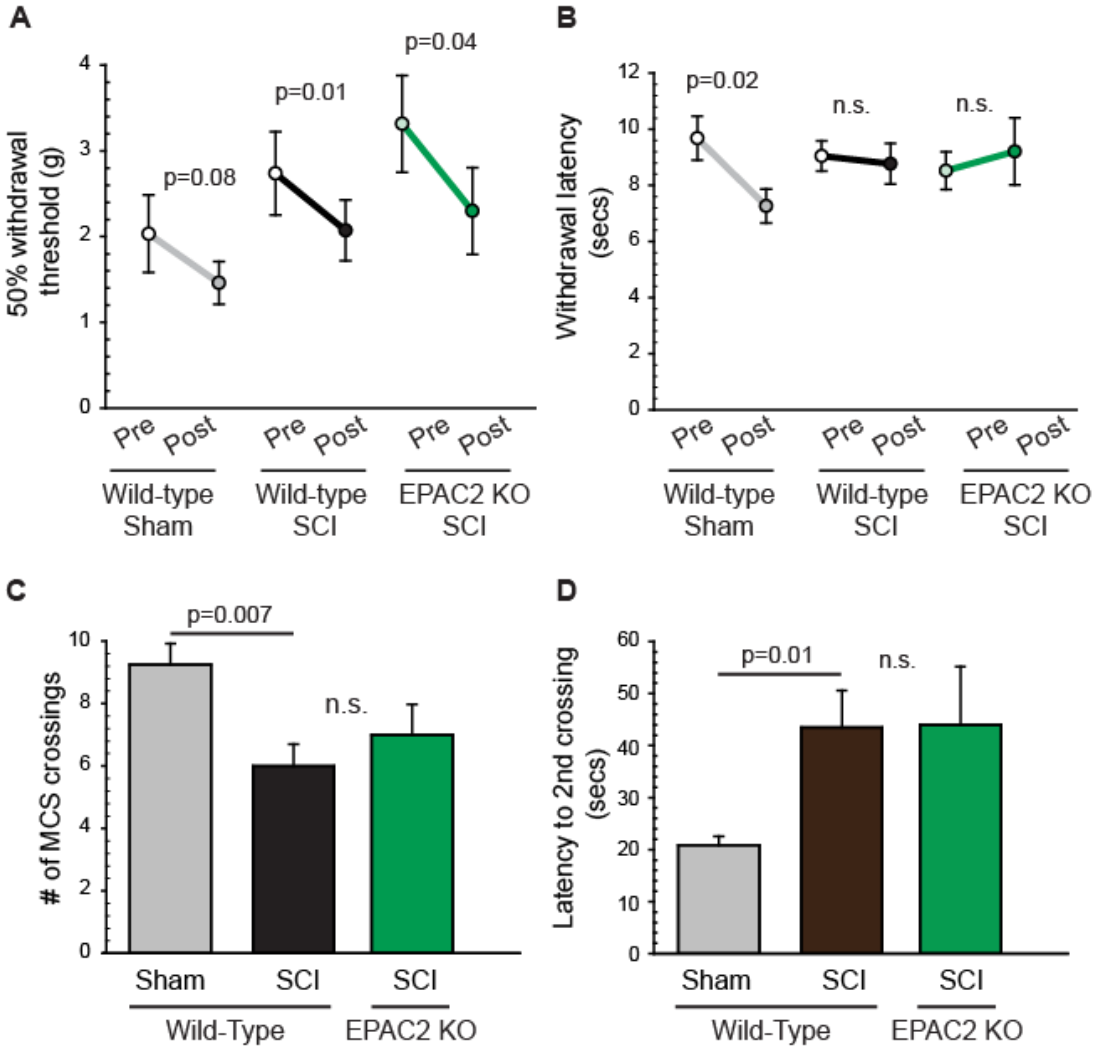

A
50% withdrawal threshold (g)
p=0.08
p=0.01
p=0.04
Pre
Post
Wild-type Sham
Wild-type SCI
EPAC2 KO SCI
B
Withdrawal latency (secs)
p=0.02
n.s.
n.s.
C
# of MCS crossings
p=0.007
n.s.
Sham
SCI
Wild-Type
EPAC2 KO
D
Latency to 2nd crossing (secs)
p=0.01
n.s.

Figure 9. SCI-induced increase in pain-like behaviors within both wild-type and EPAC2$^{-/-}$ mice, as shown by novel MC test. Post sham or SCI behavioral experiments were performed 3-4 weeks after surgery. (A) SCI led to a significant decrease in the mechanical 50% withdrawal threshold in tested hindpaws of SCI WT and EPAC2$^{-/-}$ mice, and a trend towards a decrease in sham mice, as shown by von Frey filament testing. (B) Heat sensitivity was not significantly increased in SCI versus sham mice, as tested by the plantar analgesia meter set to 30% its maximum intensity. (C) SCI significantly decreased the total number of crossings made within the MC, and (D) increased the latency to the second complete crossing. Data are shown as mean ± SEM. T-test was used to compare WT sham to WT SCI mice, as well as WT SCI mice to EPAC2$^{-/-}$ SCI mice. *Data and Analysis by S. Rahman and S.C. Berkey*.

3.4 SCI-induced SA and depolarization-dependent OA are prevented by combined inhibition and knockout of both EPAC isoforms

We had expected that EPAC2$^{-/-}$ mice would be protected against pain-like behaviors as an EPAC2 inhibitor was able to mitigate the SCI-induced effects in rat nociceptors. However, EPAC2$^{-/-}$ and WT mice demonstrated a similar enhancement of pain-avoidance behavior after SCI, suggesting that deletion of EPAC2 does not reduce SCI-induced hyperexcitability in mouse nociceptors. To explore potential explanations for this failed prediction in EPAC2$^{-/-}$ mice, we asked if both EPAC2$^{-/-}$ and EPAC1$^{-/-}$ mice exhibit an increased incidence of SA after SCI.

Presumptive nociceptors isolated from WT mice 8-10 weeks after SCI showed a significant increase in the incidence of SA (Figure 10A, B). In addition, each of the three intrinsic electrophysiological alterations that promote SA were found: depolarization of RMP (Figure 10C), hyperpolarization of AP voltage threshold (Figure 10D), and increased DSF amplitude (see below). SCI-induced hyperexcitability also manifested as a decrease in rheobase (Figure 10E).

SCI increased the mean DSF amplitude at RMP or when artificially depolarized (OA) to -45 mV (Fig. 11A). SCI also increased the number of large (≥8 mV) DSFs observed at RMP or when membrane potential was artificially depolarized to -45 mV. Large DSFs were especially prominent in neurons during SA and/or OA, which were common after SCI but not after sham treatment (Fig. 11B). The 8 mV minimum for large DSFs was defined functionally as the difference between the mean RMP and the mean AP voltage threshold (both measured after SCI) – a size that should often but not always trigger APs after SCI. All the APs generated during SA at RMP or during OA at -45 mV appeared to be triggered by

large DSFs, but to ensure that DSF amplitudes were measured precisely, only large subthreshold DSFs were included in this analysis. Deletion of EPAC2 did not alter the effects of SCI on electrical properties as compared to WT, except for the RMP, which was not significantly altered in neurons from EPAC2$^{-/-}$ mice **(**Fig. 10, 11**)**.

Previous work suggested that EPAC1, but not EPAC2, plays a major role in persistent inflammatory and neuropathic pain in rodent models (Eijkelkamp et al., 2013). In contrast, after SCI we found that EPAC1$^{-/-}$ mice showed significant enhancement in the incidence of SA similar to that found in WT and EPAC2$^{-/-}$ mice (Figure 10B). However, EPAC1$^{-/-}$ mice failed to show significant depolarization of RMP or hyperpolarization of AP threshold (Figure 10C, D). Recent work suggests EPAC1 and 2 may play redundant roles and be able to compensate for each other (Yang et al., 2012). This possibility was tested in the mouse SCI model by pharmacological inhibition of the complementary isoform in EPAC1 or EPAC2 knockout mice. Treatment of nociceptors isolated from EPAC2$^{-/-}$ SCI mice with the EPAC1 inhibitor, CE3F4, led to a significant decrease in the incidence of SA (Figure 10B). The converse experiment, using nociceptors from EPAC1$^{-/-}$ SCI mice with the EPAC2 inhibitor ESI-05, also caused a significant decrease in SA (Figure 10B). The AP voltage threshold (Figure 10D) and rheobase (Figure 10E) were not significantly affected by either treatment, although CE3F4 treatment of SCI nociceptors from EPAC2$^{-/-}$ mice did further depolarize the RMP (Figure 10C).

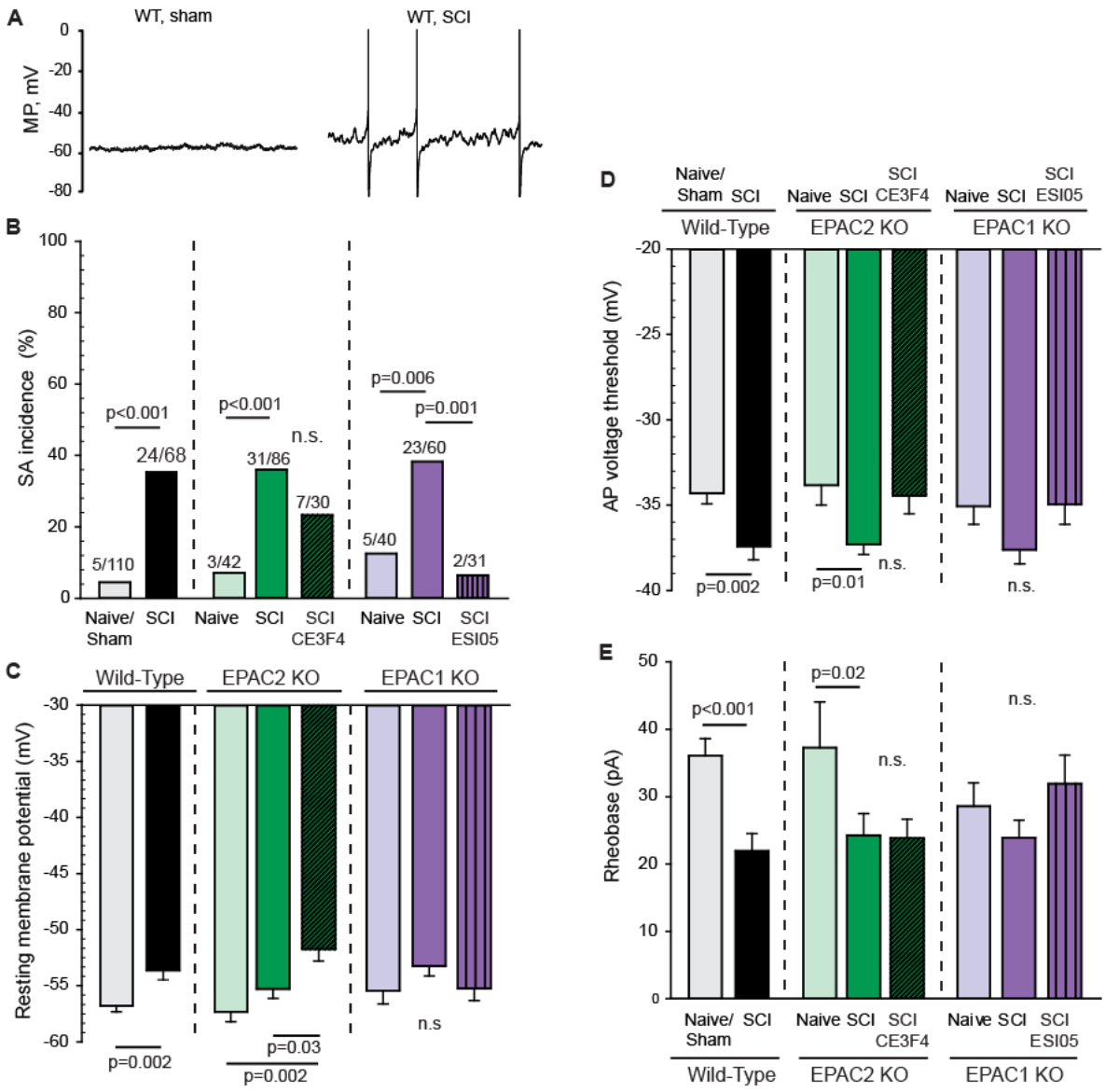

A
WT, sham
WT, SCI
MP, mV
0
-20
-40
-60
-80
B
SA incidence (%)
100
80
60
40
20
0
p<0.001
24/68
5/110
p<0.001
n.s.
31/86
7/30
3/42
p=0.006
p=0.001
23/60
5/40
2/31
Naive/ Sham
SCI
Naive
SCI
SCI CE3F4
Naive
SCI
SCI ESI05
C
Wild-Type
EPAC2 KO
EPAC1 KO
Resting membrane potential (mV)
-30
-35
-40
-45
-50
-55
-60
p=0.002
p=0.03
p=0.002
n.s
D
Naive/ Sham
SCI
Naive
SCI
SCI CE3F4
Naive
SCI
SCI ESI05
Wild-Type
EPAC2 KO
EPAC1 KO
AP voltage threshold (mV)
-20
-25
-30
-35
-40
p=0.002
p=0.01
n.s.
n.s.
E
Rheobase (pA)
50
40
30
20
10
0
p<0.001
p=0.02
n.s.
n.s.
Naive/ Sham
SCI
Naive
SCI
SCI CE3F4
Naive
SCI
SCI ESI05
Wild-Type
EPAC2 KO
EPAC1 KO

Figure 10. Inhibition of both EPAC1 and 2 isoforms is necessary to mitigate SCI-induced hyperexcitability in dissociated mouse DRG neurons. Small to medium-diameter DRG neurons (≤ 30 μm) harvested from below and at the T9 spinal level were recorded by whole-cell patch clamp 18-30 hours after dissociation. Neurons were pretreated with either vehicle, 10-20 μM CE3F4 or 5 μM ESI-05 for 15-20 minutes before recording. (A) Representative 10-second recordings obtained from non-accommodating neurons (presumptive nociceptors) at rest (I = 0). (B) Nociceptors isolated from EPAC1$^{-/-}$ or EPAC2$^{-/-}$ mice were not protected against increased incidence of SCI-induced SA; additional pharmacological inhibition of the complementary isoform was required to bring SA incidence towards a level comparable to neurons isolated from naïve/sham mice. The ratio above each bar denotes the number of neurons with SA/the number of neurons sampled. Statistical comparisons of SA incidence were made with Bonferroni-corrected Fisher's exact tests on the indicated pairs. (C) SCI-induced depolarization of the RMP in wild-type and EPAC2$^{-/-}$ mice; additional inhibition of the EPAC1 isoform was required to mitigate SCI-induced depolarization within neurons isolated from EPAC2$^{-/-}$ mice. (D) SCI-induced significant hyperpolarization of AP voltage threshold in wild-type and EPAC2$^{-/-}$ mice (trending within EPAC1$^{-/-}$ SCI mice); additional inhibition of the EPAC1 isoform did not cause a significant change in the SCI-induced AP voltage threshold hyperpolarization within nociceptors from EPAC2$^{-/-}$ mice. (E) SCI-induced a decrease in rheobase in wild-type and EPAC2$^{-/-}$ mice (trending within EPAC1$^{-/-}$ SCI mice); additional inhibition of the EPAC1 isoform did not cause a further significant change in the SCI-induced decrease in rheobase within nociceptors from EPAC2$^{-/-}$ mice. Comparisons of data (mean ± SEM) were made by t-test (for wild-type data), or Kruskal-Wallis followed by Dunn's method for pairwise comparisons. *Data collected by A. Bavencoffe and M.A. Odem. Analysis by S.C. Berkey and A. Bavencoffe. Figure by S.C. Berkey.*

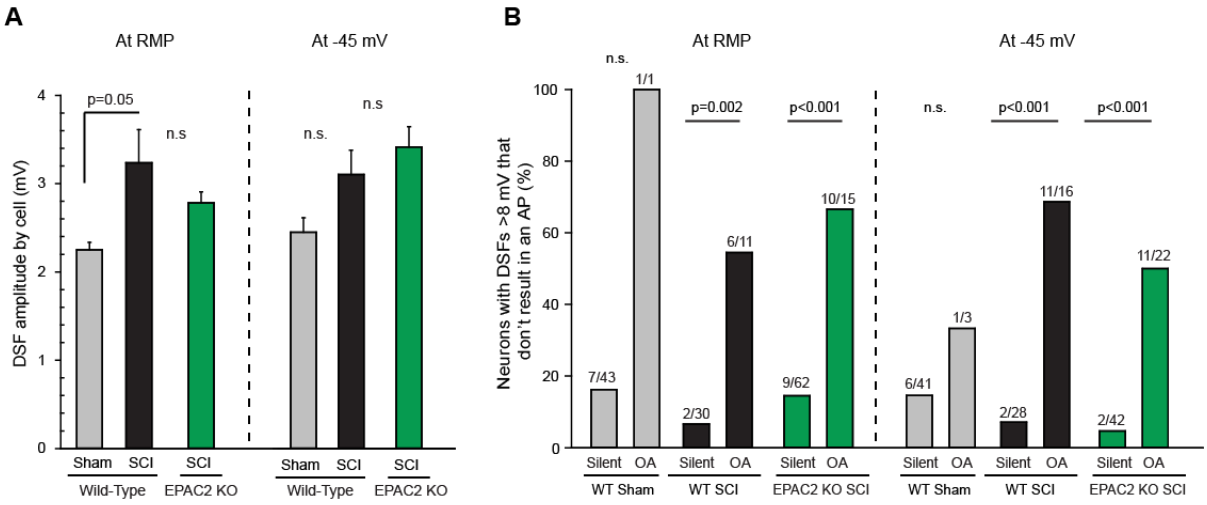


Figure 11. SCI increases the amplitude of DSFs. DSFs were quantified by an automated program which estimated RMP via a sliding median function and identified depolarizing fluctuations >1.5 mV that lasted >5 ms. (A) SCI induced an increase in average DSF amplitudes recorded at RMP and when cells were depolarized to -45 mV in nociceptors isolated from wild-type and EPAC2$^{-/-}$ mice. Data are represented as mean ± SEM. (B) DSFs suprathreshold for generating action potentials have amplitudes ≥8 mV in mice. SCI induced a significant increase in the frequency of non-functional DSFs ≥8 mV in WT and EPAC2$^{-/-}$ mice at rest and when cells were depolarized to -45 mV. Statistical comparisons were performed with Kruskal-Wallis followed by Dunn's method for pairwise comparisons. *Data collected by A. Bavencoffe and M.A Odem. Analysis and figure by S.C. Berkey.*

Interestingly, though there are significant differences in nociceptor hyperexcitability between sham and SCI mice of each genotype, the overall incidence of SA in mice after SCI increased to a lesser degree than in rats; mice of all genotypes showed an SA incidence of ~40%, while rats showed closer to ~75% after SCI. While SA is generated solely by mechanisms intrinsic to nociceptors, SCI-induced OA occurring during artificial depolarization to -45 mV stems from intrinsic changes produced by SCI that only manifest as ongoing discharge when combined with extrinsic depolarizing input, such as occurs during inflammation (Odem et al., 2018). After SCI, the incidence of OA was significantly increased within WT, EPAC1$^{-/-}$, and EPAC2$^{-/-}$ mice (Figure 12A, B). Furthermore, neither EPAC1 nor EPAC2 knockout mice were protected against SCI-induced increases in OA. Again, we asked if there was compensation or redundancy between EPAC1 and EPAC2. Treatment of nociceptors with an EPAC inhibitor in which the opposite isoform was deleted led to a large, significant decrease in the incidence of OA (Figure 12B). Together with the results summarized in Figure 9, this finding provides strong evidence that EPAC1 and 2 either have redundant functions in mouse nociceptors, or can be induced to compensate for the deleted isoform, leading us to examine whether SCI triggered corresponding changes in EPAC1 and 2 protein levels.

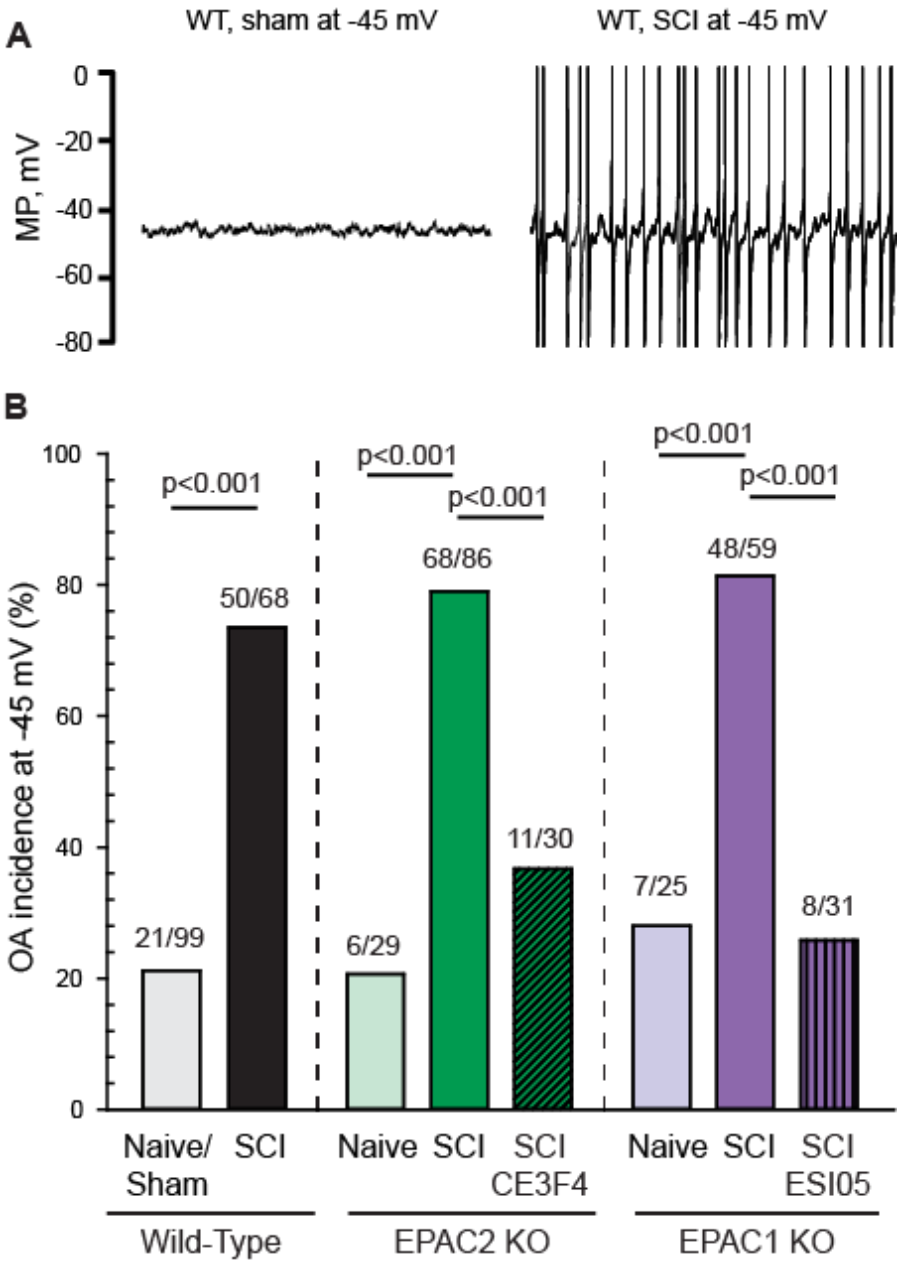

A
WT, sham at -45 mV
WT, SCI at -45 mV
MP, mV
0
-20
-40
-60
-80
B
OA incidence at -45 mV (%)
100
80
60
40
20
0
p<0.001
p<0.001
p<0.001
p<0.001
p<0.001
21/99
50/68
6/29
68/86
11/30
7/25
48/59
8/31
Naive/ Sham
SCI
Naive
SCI
SCI CE3F4
Naive
SCI
SCI ESI05
Wild-Type
EPAC2 KO
EPAC1 KO

Figure 12. Inhibition of both EPAC1 and 2 is necessary to mitigate SCI-induced OA in presumptive mouse nociceptors. To measure extrinsically driven OA, small DRG neurons were artificially depolarized to -45 mV by current injection for 30-60 seconds. DRG neurons isolated from EPAC1$^{-/-}$ or EPAC2$^{-/-}$ mice were not protected against SCI-induced OA; additional inhibition of the complementary isoform was required to reverse the effect of the injury. (A) Representative 10-second recordings obtained from neurons artificially held at -45 mV. (B) The ratio above each bar denotes the number of neurons with OA/the number of neurons sampled. Statistical comparisons of OA incidence were made with Bonferroni-corrected Fisher's exact tests on the indicated pairs. *Data collected by A. Bavencoffe and M.A. Odem. Analysis by S.C. Berkey and A. Bavencoffe. Figure by S.C. Berkey.*

3.5 Treatment with the EPAC inhibitor ESI-09 led to widespread desensitization and electrophysiological silencing of nociceptors

Our data suggests EPAC1 and 2 may play redundant and/or compensatory roles in nociceptor hyperexcitability. While we supported this conclusion *in vitro* using electrophysiological assays, we next attempted to show this mechanism *in vivo*. Previous studies have shown that inhibition of both EPAC isoforms by ESI-09 can mitigate chronic pain-like behaviors after CFA treatment or paclitaxel-induced mechanical allodynia (Singhmar et al., 2016, Singhmar et al., 2018). ESI-09 is a competitive inhibitor for EPAC, and has been shown to inhibit both EPAC1 and 2 *in vitro* (Tsalkova et al., 2009, Tsalkova et al., 2012, Almahariq et al., 2013). We expected that i.p. injection of ESI-09 into WT or EPAC2-/- mice would mitigate SCI-induced chronic pain. Because we saw no significant differences in mechanical withdrawal threshold sensitivity post SCI, we decided to combine all treated animals into one group, regardless of genotype, to increase statistical power.

We found a significant decrease in mechanical sensitivity in all treated mice, regardless of genotype, as measured by von Frey filaments (Figure 13A). The mechanical sensitivity decreased past the baseline seen in naïve mice, in direct disagreement with previous literature, which saw no ESI-09 induced effects on baseline sensitivity (Singhmar et al., 2016). However, use of ESI-09 *in vitro* caused the nociceptors isolated from both rat and mouse DRGs to become electrically inexcitable (Figure 13B); that is, efforts to induce an AP by stepwise increasing current injection into putative nociceptors failed to elicit any AP (representative recordings shown in Figure 13C). Cells were held at -60mV and received successive depolarizing pulses of 5 pA injected current in increments of 2sec, which never resulted in an AP in any neurons tested after ESI-09 treatment. We saw the electrophysiological response in SCI rats as well as SCI mice, regardless of phenotype. Treatment of EPAC1 or 2 knockout

mice with the EPAC1 specific inhibitor CE3F4 or the EPAC2 inhibitor ESI-05 (Figure 13) did not produce inexcitability, suggesting it was an effect due to the ESI-09 drug and not due to EPAC inhibition. As an additional control, we treated DRGs isolated from EPAC2$^{-/-}$ SCI mice with both CE3F4 and ESI-09 and again observed electrophysiological silencing of ESI-09 (Figure 13B). This confirmed ESI-09 has non-specific, off target effects affecting the DRGs, which may have also affected the change in mechanical sensitivity (Figure 13A).

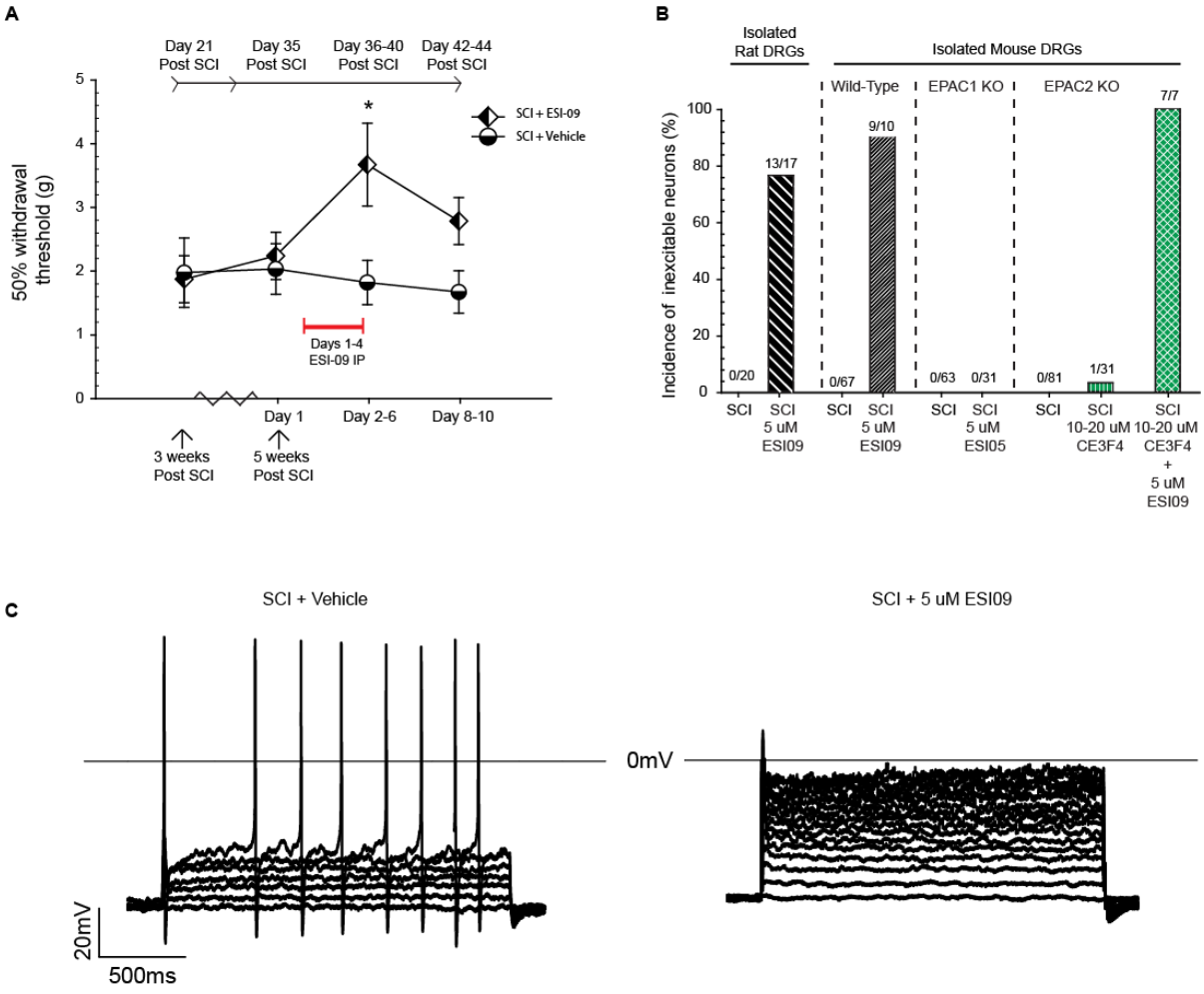

A
Day 21 Post SCI
Day 35 Post SCI
Day 36-40 Post SCI
Day 42-44 Post SCI
50% withdrawal threshold (g)
SCI + ESI-09
SCI + Vehicle
Days 1-4 ESI-09 IP
Day 1
Day 2-6
Day 8-10
3 weeks Post SCI
5 weeks Post SCI
B
Isolated Rat DRGs
Isolated Mouse DRGs
Wild-Type
EPAC1 KO
EPAC2 KO
Incidence of inexcitable neurons (%)
0/20
13/17
0/67
9/10
0/63
0/31
0/81
1/31
7/7
SCI
SCI 5 uM ESI09
SCI 5 uM ESI05
SCI 10-20 uM CE3F4
SCI 10-20 uM CE3F4 + 5 uM ESI09
C
SCI + Vehicle
SCI + 5 uM ESI09
0mV
20mV
500ms

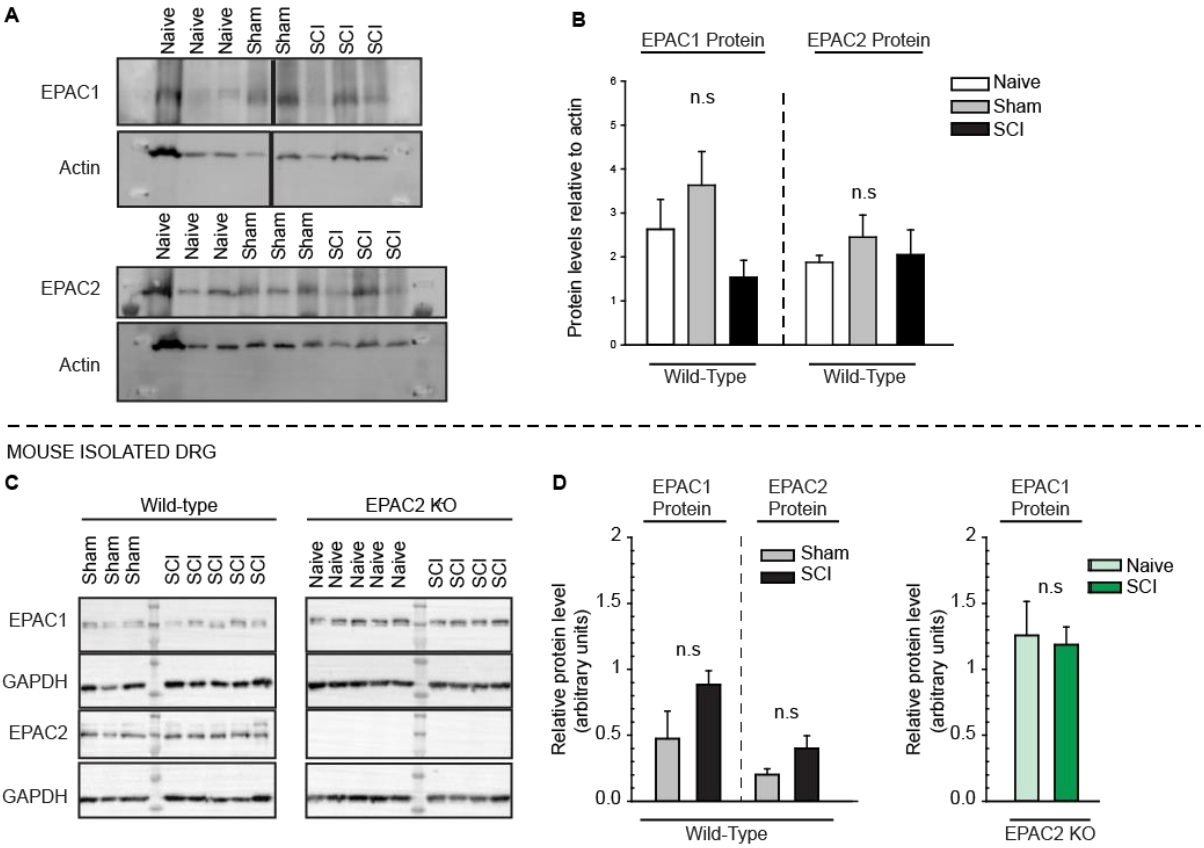
Figure 13. Inhibition of both EPAC1 and 2 by ESI-09 led to electrophysiological silencing of isolated DRGs. Post-SCI behavioral experiments were performed 5 weeks after surgery. (A) Repeated i.p. injection of ESI-09 over a 4 day period led to a significant decrease in mechanical sensitivity. Mechanical sensitivity was measured at 5 weeks Post-SCI by von Frey filament testing. SCI mice then received one i.p. injections of ESI-09 each day for 4 days. Mechanical sensitivity was measured over the 4 days, as well as an additional 6 days after the last treatment. (B) Treatment of isolated DRGs with 5 μm ESI-09 caused inexcitability in SCI rats and mice. There were no significant difference in the inexcitability produced by ESI-09 between species or genotype. The two other inhibitors of EPAC used in this study (CE3F4 and ESI-05) did not silence the DRGs. (C) Representative traces of the rheobase protocol. Cells were held at -60mV and received successive depolarizing pulses of 5 pA injected current in increments of 2sec. In cells treated with ESI-09 the rheobase protocol never resulted in an action potential. *Data collected by A. Bavencoffe. Analysis and figure by A. Bavencoffe and by S.C. Berkey.*

3.6 EPAC expression levels are unchanged in rat and EPAC2$^{-/-}$ mice post SCI

Previous work in rodent models has published conflicting reports regarding EPAC expression levels in DRGs after various injuries: CFA-induced inflammation has been reported to increase only EPAC2 levels (Vasko et al., 2014a) or both EPAC1 and 2 protein levels in the DRG (Gu et al., 2016), while within a skin/muscle incision and retraction neuropathic model only EPAC1 protein is shown to increase within the DRG (Cao et al., 2016). Our electrophysiological data suggested both EPAC1 and 2 expression would be affected by SCI; however, within our neuropathic model of rat SCI, we found no significant change in EPAC1 or 2 protein levels in the DRG between naïve and SCI rats (Figure 14A, B). Sham rats did not show significant differences from either naïve or SCI.

Previous work in a mouse model of spinal nerve transection (SNT) (Eijkelkamp et al., 2013), suggested SCI would result in an increase in EPAC1 expression levels within WT mice. However, again we did not observe significant increases in either EPAC1 or 2 protein levels after SCI in WT or EPAC2$^{-/-}$ mice (Figure 14C, D).

RAT ISOLATED DRG



MOUSE ISOLATED DRG

Figure 14. EPAC1 and 2 expression levels are unchanged after SCI in DRGs isolated from mice and rats. (A) EPAC1 and 2 expression levels were not significantly different in naïve versus SCI rats. (B) Bar graph represents band density for EPAC1 or EPAC2 levels normalized to total actin, with levels in the first lane (Naïve) set to 1. Naïve n= 4; SHAM n=5; SCI n=3. Comparisons of data (mean ± SEM) were made by One-way ANOVA or Kruskal-Wallis followed by Dunn's method for pairwise comparisons. (C) EPAC1 and 2 expression levels are not significantly different in wild-type sham versus SCI mice or EPAC2$^{-/-}$ naïve versus SCI mice. (D) Bar graph represents band density for EPAC1 or EPAC2 levels normalized to total protein as measured by stain free imaging, with levels in the first lane of each blot set to 1. Wild-type SHAM n=4; wild-type SCI n=5: EPAC2$^{-/-}$ naïve n=7; EPAC2$^{-/-}$ SCI n=6 Comparisons of data (mean ± SEM) were made by t-test. Comparisons of data (mean ± SEM) were made by t-test. *Data and Analysis by S.C. Berkey.*

3.7 Summary of Results

Here we demonstrate the importance of both EPAC isoforms (EPAC1 and EPAC2) in maintaining a hyperexcitable state in nociceptors after SCI in two different rodent models.

In SCI rats, inhibition of either EPAC isoform is sufficient to mitigate SCI-induced hyperexcitabilty. We show that while EPAC expression levels were unchanged in rat DRGs after SCI, inhibition of either EPAC isoform significantly mitigates the hyperexcitable state. EPAC inhibition or activation had a significant depolarizing or hyperpolarizing effect, respectively, on RMP. As previously shown in rats (Odem et al., 2018) , SCI significantly increased DSF amplitude, especially at depolarized RMPs. The rise in DSF amplitude after SCI was reversed by inhibition of either EPAC isoform.

In mice, we found EPAC2 knockout was not sufficient to mitigate SCI-induced pain-like behavior in mice (though only female mice were tested). Our behavior testing included a novel design and test procedure within the MC, possibly providing a better measure of the complex experience of pain. EPAC1 or 2 knockout was not sufficient to decrease the significant enhancement in nociceptor hyperexcitability (SA and OA) after SCI. However, this may be due to a redundant and/or compensatory function between the two isoforms, though the mechanism is unclear. Pharmacological inhibition of the complementary isoform in EPAC1 or EPAC2 knockout mice led to a significant decrease in the incidence of SA and OA.

Chapter 4: Discussion and Future Directions

4.1 Summary of Conclusions

Developing effective chronic pain treatment is one of the great challenges facing healthcare today. The cost to the economy as well as individual sufferers is immense, and especially highlighted by the current opioid epidemic. To develop more effective treatment we need to better understand the mechanisms that maintain the chronic pain state. While cAMP signaling has been well studied for its role in chronic pain, most of its effects were thought to be mediated through PKA and cyclic nucleotide gated ion channels (Hingtgen et al., 1995, Kress et al., 1996, Gold et al., 1998, Wei et al., 2002, Kim et al., 2007, Emery et al., 2011). This work focuses on the role of an additional cAMP effector, EPAC, in chronic pain after SCI, with the hope that the mechanisms maintaining pain in this model may be relevant to other chronic pain types.

The data reveal redundancy and/or compensation between EPAC1 and 2 within an SCI mouse model, and that inhibition of either isoform is sufficient in a rat SCI model to mitigate nociceptor hyperexcitability, highlighting the need for careful consideration when choosing a preclinical model. This work additionally reveals gender specific differences in recovery after SCI, as well as the benefits of employing innovative analysis and testing techniques.

4.2 Compensation and/or redundant functions for EPAC1 and EPAC2 in mice

Behavioral experiments probing a wide spectrum of possible SCI-induced effects, from mechanical and thermal sensitivity to anxiety, did not reveal significant protective effects of EPAC2 knockout in mice. Analysis of electrophysiological data also indicated that knockout

of EPAC2 is not sufficient to protect against SCI-induced nociceptor hyperexcitability. However, neither is knockout of EPAC1. Instead, after knockout of EPAC1 or 2, the Epac2 specific inhibitor ESI-05 or the Epac1 specific inhibitor CE3F4, respectively, significantly decreased the hyperexcitability. The data suggest redundancy and/or compensation between EPAC1 and 2, which is not unprecedented within the literature. A similar result was obtained within mouse hippocampal cells; knockout of both isoforms led to defects in spatial learning and memory, while knockout of only EPAC1 or 2 had no significant effect on behavior (Yang et al., 2012). The authors did not report a corresponding increase in the opposite EPAC isoform after knockout, as seen in our study (Yang et al., 2012).

Portions of the literature point to straightforward mechanisms for compensation; for example, both isoforms are likely present in the affected nociceptor. EPAC1 is ubiquitously expressed while EPAC2 is highly expressed in adult neurons (de Rooij et al., 1998, Kawasaki et al., 1998, Murray and Shewan, 2008). In addition, both isoforms can localize to the PM and the cytoplasm, albeit through different interacting partners, and interact with similar downstream partners once there (de Rooij et al., 2000, Ponsioen et al., 2009, Grandoch et al., 2010). The redundant/compensatory aspect is supported evolutionarily as well, as prokaryotes contain only one EPAC copy (closer to EPAC2), and eukaryotes contain both isoforms seemingly due to a gene duplication event (de Rooij et al., 2000).

However, other aspects are harder to explain. EPAC2 plays a large role in exocytosis through interactions by its low affinity cAMP-A binding domain, which is not present in EPAC1 (Ozaki et al., 2000, Eliasson et al., 2003, Ster et al., 2007). Presumably, the defects in vesicle release would not only be an issue within the nervous system, but also within β-cell

granules and insulin release. Why there is not a larger phenotypic issue from EPAC2 knockout is an interesting question for further studies.

It is also possible that though EPAC1 and 2 appear to be redundant and/or compensating in our assays, there may be some subtle defect that has yet to be parsed out. While EPAC1 and 2 overlap in function within the nociceptor, there may be small differences in their exact roles. This idea is supported by the first study to suggest compensation between EPAC1 and 2; though the authors concluded the EPAC2 KO mouse had no gross phenotype, they did see a subtle change in a measure of reversal learning (Yang et al., 2012). Indeed, closer analysis of our own data suggest a similar slight difference. We observed significant differences post SCI in rheobase and AP voltage threshold only from nociceptors isolated from $EPAC2^{-/-}$ mice, but not within those from $EPAC1^{-/-}$ mice. This suggests EPAC1 KO had a subtle protective effect after SCI, and may help to explain some of the controversy within the pain field regarding the contributions of EPAC1 versus EPAC2. It is possible that within an inflammatory or peripheral nerve injury model, $EPAC1^{-/-}$ is sufficient to protect against the development of chronic allodynia (Eijkelkamp et al., 2013, Singhmar et al., 2016); however, our model suggests that both EPAC isoforms need to be accounted for when designing chronic pain therapies for certain injuries.

I attempted to show *in vivo* redundancy between EPAC1 and 2 using an inhibitor of both EPAC isoforms, ESI-09, in WT and EPAC2 KO mice. While I did not observe a significant rescue of SCI-induced pain-like behavior in treated mice of either genotype, there are multiple possible reasons. Firstly, our study used only female mice (as discussed below), which have been shown in other models to be more susceptible to stress and age induced inflammation. The timeline of an SCI study is long, necessitated by multiple weeks of recovery

from injury and behavioral testing. Multiple animals were lost during recovery or after testing due to secondary SCI-problems (bladder infection, self-harm). It is possible the mice remaining at 5+ weeks post injury were those least affected by the SCI. Thus, the timeline created two confounding issues: a decreased cohort number made up of potentially the least affected/susceptible mice.

However, we were surprised by the large decrease in mechanical sensitivity within all the mice after ESI-09 treatment, regardless of genotype. This was wholly unexpected, and not in line with previous reports. Our methods did differ slightly, as we used i.p. injection versus oral gavage (Singhmar et al., 2016), but that should not have had such a dramatic effect. Our preliminary electrophysiology studies of ESI-09 on isolated SCI nociceptors resulted in complete abolishment of SA. However, closer analysis showed that all treated cells had become inexcitable. This suggests to us that ESI-09 somehow silenced or harmed these nociceptors. The huge decrease in sensitivity seen in the behavior studies may be due to nociceptor silencing or harm, and thus a lack of peripheral sensation or input. Why this was not observed in other studies is unclear, as the dose was similar to other studies (Singhmar et al., 2016, Singhmar et al., 2018). It would be extremely interesting to employ other EPAC1 or 2 specific inhibitors in future behavior studies to evaluate if the same compensation/redundancy is observed *in vivo*.

4.3 Role for EPAC1 and EPAC2 in Rat

Although only the EPAC1 RNA levels were unchanged in rat DRGs post SCI, inhibition of either EPAC1 or 2 ameliorated the nociceptor hyperexcitable state. The observed change in EPAC1 expression adds to the conflicting reports in the literature; in a model of

CFA induced inflammation, Gu et.al. reports increased expression of both isoforms within the DRG (Gu et al., 2016), while previous work within the same rat inflammatory model suggested only EPAC2 levels increased (Vasko et al., 2014a). However, within a skin-muscle incision retraction neuropathic pain model, the authors also demonstrate an increase in only EPAC1 expression within the DRG, and additionally showed EPAC1 inhibition mitigated pain-like behavior (Cao et al., 2016).

Some of the controversy may in fact arise from the clear contribution of both isoforms as seen in our electrophysiology data. Though both EPACs have a role and are normally expressed in nociceptors, how their functions overlap is still unclear. Depending on the severity of the injury, the pain state, and other confounding factors, the expression levels at the chosen end-point might differ. Additionally, EPAC1 has been embraced in the literature as the main isoform involved in chronic pain, possibly causing some studies to not consider or report on EPAC2 (Cao et al., 2016).

One of the advantages of electrophysiology assays is the ability to record data from an individual cell, which can be determined to be a nociceptor. And while done *in vitro*, the observed spontaneous activity and hyperexcitability has been convincingly linked to pain-like behaviors (Bedi et al., 2010, Wu, 2013, Yang et al., 2014). Therefore, the decrease in excitability after EPAC inhibition within nociceptors isolated from SCI rats suggests a role for both EPAC isoforms in SCI chronic pain. The decrease parallels that seen in previously published work examining the effect of PKA inhibition on nociceptors also isolated from SCI rats (Bavencoffe et al., 2016); however, EPAC inhibition resulted in profound depolarization of the RMP, while PKA inhibition had a much smaller effect. Conversely, EPAC activation by 007-AM hyperpolarized nociceptors from naïve rats. Together the data suggest a role for

EPAC in regulation of the membrane potential. How this function for EPAC may crosstalk with PKA signaling to affect overall excitability is discussed further below. EPAC also affects DSF amplitude, another nociceptor intrinsic mechanism contributing to excitability. SCI increased DSF amplitude in rat nociceptors as seen previously (Odem et al., 2018), and this increase could be reversed by inhibition of either EPAC isoform or replicated by EPAC activation in naïve nociceptors held at -45 mV.

4.4 An Operant Mechanical Conflict test reveals subtle SCI-induced effects on pain behavior

Behavior tests measuring brisk withdrawal reflexes are problematic, as reflex behavior has been shown to increase due to spastic syndromes secondary to SCI, not just due to increased pain (Baastrup, Maersk-Moller et al. 2010, Yezierski and Vierck 2010). We addressed this concern with the MC, a conflict paradigm, which may better reflect the affective-motivational and cognitive-evaluative dimensions of pain (Harte et al. 2016, Pahng et al. 2017). As described in the methods, we modified the MC for use with mice; though we are not the first to do so (Zhou S 2012, Shepherd and Mohapatra 2018), we feel our method offered several advantages. Firstly, while our design did not employ different probe heights, it did allow extremely close spacing of the probes, which ensured mice would experience probes with every footfall, eliminating crossing strategies which utilize fitting the paws between the probes. We also believe the probes are causing hyperalgesia, as extensive preliminary testing was required to optimize the probe array to be aversive but not too aversive to naïve animals. For example, in early trials using the mechanical conflict device the probes were arranged in an alternating high low pattern, which resulted in no or only one crossing by 5 out of 6 mice, across multiple trials. Additionally, by narrowing and lowering the crossing chamber width

and height, we made it difficult for mice to turn around on the probes, which can confound the results, and impossible for mice to take a non-linear route, which would inherently increase the cross latency (though, unfortunately, this may frustrate attempts to compare our crossing time data with other studies).

Thirdly, while photophobia in rodents is well documented, we found the aversive light to be only a weak stimulus, as in preliminary tests mice often did not cross from the light chamber at all, or showed no hesitation in crossing back towards the light. The high crossing rate observed suggested the mice were being driven instead by conflict between their innate exploratory drive and the noxious probes. Further, our early tests (employing a prolonged protocol) in which wild-type naive mice (n=5) were exposed to a probe free MC device 3 times within the same day revealed a significant decrease in crossing number that was dependent on the number of times a mouse experienced the test ($p=0.002$, One way RM ANOVA). By using the shortened test procedure described in our methods, we kept the MC novel and maximized the exploratory drive, making latency to the second cross the more informative measure; both WT and EPAC2$^{-/-}$ mice, whether naïve or SCI, experienced the probes, and potentially pain, on the first cross, and then weighed the two conflicting urges before completing the second cross. Preliminary work with EPAC1$^{-/-}$ mice (n=6) showed a similar result, as SCI caused a significant decrease in crossing number ($p=0.005$, t-test), in line with the potentially redundant functions of EPAC 1 and 2 seen in the electrophysiological assays. Previous work has shown an increase in crossing latency may be due to a desire to avoid mechanical stimuli while in a pain state (Harte et al. 2016, Shepherd and Mohapatra 2018). Therefore, the longer crossing times seen here in SCI mice may be indicative of avoidance behavior due to a pain state, which arguably requires higher-level processing and cognition.

4.5 Gender Specific Differences in Pain Signaling

It is worth mentioning that the behavioral results reflect only the performance of female mice, as males do not recover sufficient locomotor function in a reasonable amount of time. We intended to use both sexes, and did so where possible in the electrophysiology and biochemical experiments, as conclusions based on solely male or female data sets are not applicable to half of the population (Mogil and Chanda, 2005). Unfortunately, preclinical studies within the pain field disproportionately rely on male models (Beery and Zucker, 2011), despite females being overrepresented in the human chronic pain patient population (Fillingim et al., 2009).

Chronic pain in males is mediated in part by microglia activation, which act as the principal immune cells for the nervous system. Nerve injury triggers upregulation of purinergic P2X4 receptors, which, after ATP activation, can induce pain behavior and hypersensitivity through well characterized mechanisms. In short, P2X4 activates p38 MAPK, induces Ca^{2+} influx, and releases brain-derived neurotrophic factor to interact with TrkB receptors. This downregulates a neuronal potassium-chloride transporter to disrupt chloride transport, decreases inhibitory tone, and transforms the output of lamina I neurons.

While inhibition or ablation of microglia thus mitigates chronic pain in male models of chronic pain, there is no or little effect on female hypersensitivity (Sorge et al., 2015). Instead it is hypothesized that females employ adaptive immune cells, specifically T cells, rather than microglia to mediate chronic pain signaling (Mapplebeck et al., 2016). Interestingly, immune cells are recruited to the lesion site post SCI, and no doubt circulated throughout the

cerebrospinal fluid, to which the DRGs are exposed. Within female rats, SCI causes macrophage and T-cell infiltration into the DRGs (McKay and McLachlan, 2004).

Further, estrogen was found to affect the cAMP/PKCε induced chronic hyperalgesia in surprising ways. While isoproterenol and 8-pCPT can activate PKCε to induce hyperalgesia (Hucho et al., 2005), estrogen could block this effect, seemingly at or downstream of EPAC (Hucho et al., 2006). Additionally, estrogen application *in vivo* or *in vitro* could activate PKCε, an effect quickly abrogated by EPAC stimulation (Hucho et al., 2006). While this study suggests a male SCI cohort would have been a better model to study EPAC signaling, our results did not show significant differences in nociceptor excitability between genders, suggesting additional mechanisms are at play. Bedi et. al. did show a greater overall incidence of SA after SCI within females versus males, however, the interaction of injury with sex was not significant (Bedi et al., 2010).

Within our study, females recovered locomotor function within 4 weeks post injury, while males did not recover much more than limited ankle movement. The longer recovery time in males may have additional clinical implications, especially as recent studies have highlighted the detrimental effects of analgesic treatments too early or late after injury. Further, the disuse of affected limbs may further heighten the injury: immobilization can disturb mechano- and thermosensitivity in humans, while rat hindlimb immobilization alone can increase mechanical sensitivity (Terkelsen et al., 2008, Trierweiler et al., 2012).

A handful of previous studies have also observed better recovery in female rodents after ischemic stroke (Payan and Conrad, 1977, Alkayed et al., 1998), spinal cord contusion (Hauben et al., 2002, Farooque et al., 2006), hypoxic brain injury (Stupfel et al., 1984), and closed head injury (Roof and Hall, 2000). While the mechanism is still being elucidated,

estrogen has been proposed to confer a protective effect, potentially by increasing expression of the anti-apoptotic genes bcl-2 and bcl-x (Yune et al., 2004, Farooque et al., 2006). However, the protective effect was lost in a model of moderate SCI employing nude mice, suggesting estrogen's protective effect is T-cell dependent (Hauben et al., 2002). How this potential T-cell dependent protective effect of estrogen on recovery may interplay with the role of T-cell dependent pain signaling in females would be a fascinating area for future study.

4.6 EPAC and PKA Signaling in a Chronic Pain Context

The literature suggests that biased cAMP signaling towards either PKA or EPAC/PKCε underlies the transition from an acute to chronic pain state, respectively (Wang et al., 2007b, Eijkelkamp et al., 2010, Huang et al., 2015). For example, PGE2 potentiates P2X3R-mediated ATP currents within DRGs, mediated by PKA signaling downstream of the EP3C receptor coupled Gαs (Gu et al., 2016). However, if PGE2 is given as a secondary inflammatory insult after CFA, EPAC mediates and enhances the PGE2 induced P2X3R current (Gu et al., 2016).

However, our results highlight the possible interplay between the two cAMP sensors in chronic pain; in nociceptors isolated from naïve rats, EPAC inhibition resulted in the same decrease in excitability as seen after PKA inhibition from previous work, but had a greater effect on RMP (Bavencoffe et al., 2016). It is probable that while these two cAMP-dependent signaling pathways can affect the same ligand and voltage gated ion channels, they do so in diverse contexts and through divergent localization mechanism, to affect different components of nociceptor hyperexcitability.

cAMP was the first secondary messenger implicated in nociceptor sensitization, and, besides limited signaling through CNGs, PKA was considered the major cAMP effector.

However, cAMP signaling though either PKA or EPAC can promote sensitization through regulation of voltage and ligand gated ion channels. For example, TRPV1 and TTX-R Na^+ channels are strongly implicated in nociceptor sensitization, and cAMP signaling through PKA or EPAC can influence either channel. PKA may directly phosphorylate TRPV1 to prevent its desensitization (Bhave et al., 2002), while EPAC mediates cAMP to PKCε signaling to indirectly effect sensitization of TRPV1. Other examples of synergy between PKA and EPAC have been published previously (Hewer et al., 2011, Yu et al., 2017), including one proposing cooperative roles in neuron sprouting and neurite extension in the DRG after SCI (Wei et al., 2016). It's easy to imagine how an increase in activated nociceptors resulting in additional excitatory synapses could play a role in promoting chronic pain.

Both PKA and EPAC have also been implicated in the regulation of intracellular Ca^{2+} levels, which markedly change within DRGs after inflammatory or neuropathic injury (Sah and Louise Faber, 2002, Bourinet et al., 2014). Work in pancreatic cells has shown EPAC mediates Ca^{2+} induced Ca^{2+} release (CICR) from intracellular stores, possibly through synergistic EPAC/Rap1 modulation and PKA sensitization of ryanodine receptors (RyRs) (Kang et al., 2003). This could potentially occur within nociceptors as well, as EPAC2 activation in cultured cerebellar granule cells also mobilizes intracellular Ca^{2+} through Rap and p38 activation (Sah and Louise Faber, 2002, Ster et al., 2007). The resulting rise in intracellular levels promotes activation of calcium sensitive big potassium (BK) channels, which play a role in regulation of the AP duration and repetitive firing (Sah and Louise Faber, 2002, Ster et al., 2007). BK channel regulation by PKA has been well characterized previously (Schubert and Nelson, 2001, Wang, 2008), providing another example of the overlap in EPAC and PKA signaling targets involved in nociceptor excitability.

4.7 Future Directions

Here we have shown a role for EPAC1 and 2 in maintaining hyperexcitability of presumptive nociceptors that promotes chronic pain. As knockout of either EPAC1 or 2 does not seems to disrupt normal development, both isoforms are good potential drug targets.

However, to develop the most effective therapies, future efforts should focus on cAMP to EPAC signaling in the context of discrete localization mechanisms. Though some mechanisms of EPAC trafficking to specific loci are known, such as the role of the DEP domain in PM targeting of EPAC1, much remains to be elucidated (de Rooij et al., 2000, Ponsioen et al., 2009, Grandoch et al., 2010). cAMP signaling is well known to depend upon scaffolding of upstream and downstream pathway components by AKAPs, yet in neuronal cells there is almost nothing know about potential EPAC interactions (Nijholt et al., 2008). No doubt some of the differences in mechanisms targeting EPAC1 and 2 to the PM or nuclear membrane result in divergent localization to AKAPs or additional organizing platforms such as lipid rafts.

Our data suggests that EPAC1 and 2 can act in a redundant and/or compensatory manner in mice and within rats. Additional work will be required to realize which model gives a closer estimate of the role of EPAC in human pain pathways. However, understanding which signalosomes EPAC interacts with will help illuminate EPAC1 and EPAC2's respective roles within specific signaling networks. Further studies might also make use of Rap1 KO mice to investigate whether similar SCI-induced effects are observed (Pan et al., 2008). Use of these animals might help to tease apart the specific roles EPAC1 and 2 play, as both isoforms exhibit GEF activity towards Rap1 but also act through additional, unique signaling pathways.

Additionally, distinct roles for EPAC1 and 2 in neuron development and mature repair programs have been suggested, respectively, based on fluctuations in protein expression levels (de Rooij et al., 1998, Kawasaki et al., 1998, Murray and Shewan, 2008). It is likely that the EPAC2 mediated programs for repair and regrowth after SCI are distinct from those involved in development; however, a better understanding of how each isoform acts in it respective role could help to delineate differences between complete neuron growth during development and the partial regeneration observed after injury in adults. Therapies that could account for these differences might help to improve current efforts aimed at regenerating injured nerves or spinal cord.

In conclusion, this work shows an important role for both EPAC1 and EPAC2 in chronic pain pathways, highlighting the need for future studies to consider both isoforms.

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Samantha Claire Berkey is the daughter of Richard Berkey and Jacqueline Gessner. After graduating Pine View High School, Sarasota, Florida in 2007, she entered Rice University in Houston, Texas. She received her Bachelor of Science with a major in Ecology and Evolutionary Biology from Rice in May, 2011. In August of 2011 she entered The University of Texas MD Anderson Cancer Center UT Health Graduate School of Biomedical Sciences.

Permanent address:

2320 East Newton Avenue

Shorewood, WI 53211