eNeuro

Research Article: New Research | Development

Developmental axon degeneration requires TRPV1-dependent Ca^{2+} influx

Aaron D. Johnstone[1,2], Andrés de Léon[1,2], Nicolás Unsain[3], Julien Gibon[2] and Philip A. Barker[2]

[1]*Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec, H3A 2B4, Canada*

[2]*Department of Biology, University of British Columbia Okanagan, Kelowna, British Columbia, V1V 1V7, Canada*

[3]*Instituto de Investigación Médica Mercedes y Martín Ferreyra, INIMEC-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de Córdoba, Córdoba, 5016, Argentina*

https://doi.org/10.1523/ENEURO.0019-19.2019

Received: 15 January 2019

Revised: 23 January 2019

Accepted: 24 January 2019

Published: 1 February 2019

ADJ designed and performed experiments, analyzed data, and wrote the manuscript

ADL designed and performed experiments and analyzed data

NU performed experiments

JG supervised the study and edited the manuscript

PAB supervised the study and edited the manuscript

Funding: Canadian Institute of Health Research
MOP137057

Conflict of Interest: The authors declare no conflicting interests.

Funding: Canadian Institute of Health Research: Philip Amos Barker MOP137057

Correspondence should be addressed to Philip Barker, philip.barker@ubc.ca

Cite as: eNeuro 2019; 10.1523/ENEURO.0019-19.2019

Developmental axon degeneration requires TRPV1-dependent Ca^{2+} influx.

Short title: TRPV1 in developmental axon degeneration

Aaron D. Johnstone[1,2], Andrés de Léon[1,2], Nicolás Unsain[3], Julien Gibon[2] and Philip A. Barker[2,*]

[1]Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec, H3A 2B4, Canada

[2]Department of Biology, University of British Columbia Okanagan, Kelowna, British Columbia, V1V 1V7, Canada

[3]Instituto de Investigación Médica Mercedes y Martín Ferreyra, INIMEC-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de Córdoba, Córdoba, 5016, Argentina

* Correspondences should be addressed to Philip Barker, FIP322-3247 University Way, Kelowna, British Columbia, Canada, V1V 1V7, philip.barker@ubc.ca

Pages: 33
Figures: 6
Abstract: 170 words
Significance statement: 118
Introduction: 644 words
Discussion: 1266 words

Conflict of interest: The authors declare no conflicting interests.

Keywords: TRPV1, nerve growth factor, NGF, TrkA, dorsal root ganglion, DRG, calcium, neurodegeneration, developmental degeneration, axon degeneration

Author contributions:
ADJ designed and performed experiments, analyzed data, and wrote the manuscript
ADL designed and performed experiments and analyzed data
NU performed experiments
JG supervised the study and edited the manuscript
PAB supervised the study and edited the manuscript

Funding: Canadian Institute of Health Research: Philip Amos Barker MOP137057

Abstract

Development of the nervous system relies on a balance between axon and dendrite growth and subsequent pruning and degeneration. The developmental degeneration of dorsal root ganglion (DRG) sensory axons has been well studied in part because it can be readily modeled by removing the trophic support by nerve growth factor (NGF) *in vitro*. We have recently reported that axonal fragmentation induced by NGF withdrawal is dependent on Ca^{2+} and here we address the mechanism of Ca^{2+} entry required for developmental axon degeneration of mouse embryonic DRG neurons. Our results show that the Transient Receptor Potential Vanilloid family member 1 (TRPV1) cation channel plays a critical role mediating Ca^{2+} influx in DRG axons withdrawn from NGF. We further demonstrate that TRPV1 activation is dependent on reactive oxygen species (ROS) generation that is driven through protein kinase C (PKC) and NADPH oxidase (NOX) -dependent pathways that become active upon NGF withdrawal. These findings demonstrate novel mechanistic links between NGF deprivation, PKC activation, ROS generation and TRPV1-dependent Ca^{2+} influx in sensory axon degeneration.

Significance Statement

Neurons are equipped with the genetic means to degenerate, and a subset of peripheral neurons normally degenerate during embryonic development to establish a mature pattern. This beneficial neurodegeneration is regulated by signaling pathways that are only partially understood, yet share components with pathways that mediate pathological degeneration of crucial neural structures during adult diseases such as Alzheimer's and Parkinson's. Here, we identify TRPV1 as a key regulator of Ca^{2+} entry into axoplasm that is required for developmental degeneration modeled by NGF withdrawal from sensory neurons of the dorsal root ganglion *in vitro.*

71 Crucially, we report that the TRPV1-mediated Ca^{2+} flux is prompted by a signaling axis
72 comprised of PKC-dependent NOX complex activation and ROS generation upstream of
73 TRPV1.
74

Introduction

Axons normally degenerate during embryonic development to refine the nervous system into its mature pattern (Patel et al., 2000; Schuldiner and Yaron, 2014). In the dorsal root ganglia (DRG), greater numbers of sensory neurons are generated than will persist, and surviving neurons are those that arrive at their targets and receive adequate neurotrophic support from a limited pool secreted from their targets (Barde, 1989; Vogelbaum et al., 1998; Saxena and Caroni, 2007). Genetically-encoded components of developmentally-required signaling pathways that mediate the removal of neurites (including tumor necrosis factor receptors (TNFRs), MAP kinases, Bax and caspases) also underlie neurodegenerative diseases such as Alzheimer's, Parkinson's and amyotrophic lateral sclerosis (ALS) when they are dysregulated in adulthood (Kirkland and Franklin, 2003; Fischer and Glass, 2007; Saxena and Caroni, 2007; Vickers et al., 2009; Tait and Green, 2010; Kanaan et al., 2013).

We recently reported that sensory axons are rescued from developmental degeneration by Ca^{2+} chelation *in vitro* (Johnstone et al., 2018). Intriguingly, nerve terminals that innervate the skin can be locally ablated in the clinic by activation of Ca^{2+} influx mediated by the cation channel Transient Receptor Potential Vanilloid family member 1 (TRPV1); topical application of the TRPV1 agonist capsaicin is used to alleviate chronic pain and itch in humans (Jancso et al., 1985; Gibbons et al., 2010; Chiang et al., 2015; Şavk, 2016). Since activation of TRPV1 can trigger degeneration in sensory neurons (Wang et al., 2017; Jancso et al., 1985; Sann et al., 1995; Gibbons et al., 2010; Chiang et al., 2015), and because we found that Ca^{2+} is required for developmental degeneration (Johnstone et al., 2018), here we have explored the possibility that TRPV1 is required for developmental degeneration of sensory axons.

TRPV1 was identified through expression cloning designed to find the gene product that mediates Ca^{2+} influxes in response to capsaicin (Julius et al., 1997). In the intervening 20 years, TRPV1 has been confirmed to be activated and/or sensitized by heat, protons, reactive oxygen species, by the endogenous compounds N-arachidonoyl dopamine (NADA) and anandamide, by direct oxidation and by an array of noxious pest-defence compounds produced by invertebrates and plants (Hakim et al., 2015; Fenwick et al., 2017; Geron et al., 2017; Grabiec and Dehghani, 2017).

ROS were initially considered a toxic consequence of aerobic respiration, but a large number of studies published in the past two decades have firmly established ROS as second messengers that regulate several signaling cascades (Li et al., 2006, 2009; Bedard and Krause, 2007; Ibi et al., 2008; Pal et al., 2013). NADPH oxidase (NOX) complexes are membrane-associated protein complexes that reduce NADPH to generate superoxide from molecular oxygen. The canonical NOX complexes are activated by protein kinase C (PKC), which targets the p47phox subunit for phosphorylation. NOX complexes are major sources of ROS species that regulate survival, plasticity and degeneration (Cox et al., 1985; Maher and Schubert, 2000; Li et al., 2006, 2009; Ibi et al., 2008; Petry et al., 2010; Reczek and Chandel, 2014).

In this study, we show that axons deprived of NGF display Ca^{2+} influx prior to fragmentation. This Ca^{2+} influx was prevented by TRPV1 inhibition or TRPV1 genetic knockout. We hypothesized that ROS are required for TRPV1 activation and the resulting Ca^{2+} influx, and in support of this, we found that ROS scavengers and NOX complex inhibitors rescue axons from Ca^{2+} influx and fragmentation. PKC activity was necessary for the NGF deprivation-induced

121 Ca^{2+} influx and degeneration that we observed, and direct activation of PKC was sufficient to
122 drive robust Ca^{2+} influx into axons. We reasoned that PKC, NOX complexes, ROS, TRPV1 and
123 Ca^{2+} comprise a signaling axis in these axons and consistent with this, found that PKC functions
124 upstream of NOX, and ROS to mediate TRPV1-dependent Ca^{2+} influx. Taken together, these
125 results show that NGF deprivation induces a Ca^{2+} influx via TRPV1 downstream of PKC and
126 NOX complex-derived ROS during developmental axon degeneration.
127

Materials and Methods

Dissection, culturing and NGF deprivation of DRG explants

DRG explants were dissected from pregnant CD1 mice with litters of E13.5 embryos (Charles River). Explants were seeded on 6-well plastic cell culture plates (Greiner) or 4-well glass-bottom imaging dishes (CellVis) coated in a three-step process with 1 mg/ml poly-D-lysine (Sigma-Aldrich), 10 µg/ml laminin-entactin complex (Corning) and PurCol bovine collagen 0.1 mg/ml (Advanced Biomatrix). Explants were cultured in neurobasal medium (Invitrogen) supplemented with 2% B-27 serum-free supplement (Invitrogen), 1% L-glutamine (Wisent), 1% penicillin/streptomycin (Wisent), and 10 µM 5-fluoro-2'-deoxyuridine (FDU, Sigma-Aldrich) with 12.5 ng/ml NGF (CedarLane). NGF deprivation was achieved using fresh media as described above but lacking NGF and containing 2.8 µg/ml rabbit anti-NGF antibody (produced in-house). A full description of the anti-NGF antibody (raised against 2.5s NGF), its specificity and its biological validation in survival and cell-surface binding assays has been published (Murphy et al., 1993). All experimental procedures were approved by the Montreal Neurological Institute Animal Care Committee and University of British Columbia animal care committees and were in compliance with Canadian Council on Animal Care guidelines.

Fixation, immunostaining and imaging

DRG cultures were fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature and permeabilized and blocked for immunostaining in TBS-T, 5% skim milk, and 0.3% Triton X-100 for 15 minutes at room temperature. Immunostaining was performed in TBS-T with 5% skim milk and 0.3% Triton X-100 with mouse anti-β-III tubulin (Millipore; 1:10 000) primary

antibody and anti-mouse secondary antibody conjugated to Alexa Fluor 488 (ThermoFisher; 1:5000). Cultures were imaged at 5x magnification using a Zeiss ObserverZ.1 inverted epifluorescence microscope with an automated, motorized stage. Images were stitched automatically with Zen 2 software from Zeiss to produce a master image of all explants on the entire 6-well plate. From this master image, quarter-DRG fields were cropped using NIH ImageJ (FIJI build) to create an image set for quantification as recently described in detail by our group (Johnstone et al., 2018).

Quantification of axon degeneration

Axon degeneration was quantified by implementing the R script Axoquant2.0 recently described in detail and made freely available by our group (Johnstone et al., 2018). For plotting and statistical analysis, each point represents the mean value of measurements from the complement of DRG cultured from a single embryo.

Ca^{2+} chelation

After 60h of growth in NGF, cultures were either maintained in NGF or were deprived of NGF and exposed to anti-NGF antibody (2.8 µg/ml, produced in-house) in the presence of EDTA 6 mM (Sigma-Aldrich) for the final 12 hours or for the entire 24 hours before fixation with paraformaldehyde 4% in PBS, stained as described above and imaged for quantification.

Antioxidant preparation

N-acetylcysteine (NAC, Sigma-Aldrich) was prepared at 20 mM in neurobasal media and pH adjusted to 7.4.

173

174 *Pharmacological PKC, NOX complex and TRPV1 inhibitors*

175 PKC inhibitors Gö6976 and Gö6983, NOX complex inhibitors diphenyliodonium (DPI),
176 apocynin and VAS2870 and TRPV1 inhibitor capsazepine (all obtained from Tocris) were
177 prepared in DMSO and delivered to cultures at 10 μM. DMSO did not exceed 0.1% in final
178 culture in any case.

179

180 *Generation of mixed-genotype TrpV1 embryo litters*

181 C57BL6 mice carrying the $TRPV1^{tm1Jul}$ (targeted mutation 1, David Julius) knockout allele in
182 homozygosity were obtained from Jackson Laboratories and crossed with wild-type C57BL6
183 mice to generate $TRPV1^{-/+}$ animals, and these heterozygotes were bred in timed pregnancies to
184 produce mixed-genotype litters (confirmed by PCR, for primer squences see Table 1) of wild-
185 type and *TRPV1*$^{-/-}$ E13.5 embryos for DRG isolation.

186

187 *End-point* Ca^{2+} *imaging with Fluo-4*

188 30 minutes prior to imaging, cultures were incubated with 5 μM fluo-4 (Invitrogen) in
189 dimethylsulphoxide (DMSO, Sigma, final concentration in media did not exceed 0.1%) for 15
190 minutes at 37°C and then allowed to equilibrate in fresh, room-temperature HBSS (Wisent)
191 supplemented with 2 mM $CaCl_2$ for another 15 minutes. Imaging was performed in fresh HBSS
192 with 2 mM $CaCl_2$ with a Leica DMi8 confocal microscope and LAS X software with a 488 nm
193 laser in 0.3 micrometer z-increments with 63x objective to capture at least two fields of axons
194 per ganglia. Background was corrected from images of sum-z-stacks by averaging the mean
195 pixel intensities within four background regions and subtracting this value from each pixel in the

196 image using ImageJ (FIJI build, NIH). The 2D area occupied by axons in each image was then
197 measured using a binary mask of all axons, and the mean pixel intensity value for each image
198 was divided by the area occupied by axons to provide a measure of fluo-4 fluorescence intensity
199 per unit axon area. Field images of DRG axons from the same embryo were averaged to produce
200 the embryo mean value. In each experiment, measurements were standardized to the NGF
201 control value of 1.0 and the size of the treatment values expressed as fold-change from NGF
202 control.
203
204 *Live Ca^{2+} imaging with fluo-4*
205 30 minutes prior to imaging, cultures were incubated with 5 μM Fluo-4 (Invitrogen) in
206 dimethylsulphoxide (DMSO, Sigma, final concentration in media did not exceed 0.1%) for 15
207 minutes at 37°C and then allowed to equilibrate at room-temperature, then washed with fresh
208 HBSS (Wisent) supplemented with 2 mM $CaCl_2$ (Fisher BioReagents) for 15 minutes. Fields of
209 >100 axons each were acquired at 40x at a rate of one frame every 5 seconds on a Zeiss
210 ObserverZ.1 inverted epifluorescence microscope controlled by ZEN2 software with an
211 atmosphere-controlled incubation chamber (Pecon) and 470 nm Colibri LED light source (Zeiss).
212 Before injection of stimulus (PMA 100 nM) baseline fluorescence was recorded for 1 minute (12
213 frames). Background was corrected from each frame individually in each movie by averaging the
214 pixel intensity value of 4 background regions and subtracting this value from the mean pixel
215 intensity of the uncorrected frame (NIH ImageJ, FIJI build). Fluo-4 responses were standardized
216 for each run as fold-change from the initial frame (set to 1.0).

Live Ca^{2+} imaging with GCaMP6f

At time of seeding, DRG cultures were infected with HSV-hEF1-GCaMP6f (Massachusetts Institute of Technology Viral Core Facility) followed by 24 hours to allow for neurite growth and GCaMP6f expression. Images of NGF-supplied or deprived axons were acquired at a rate of one every 10 minutes on a Zeiss ObserverZ.1 inverted epifluorescence microscope. Multiple field positions were imaged with an automated stage and atmosphere-controlled incubation chamber (Pecon) controlled by ZEN2 software, and 40x objective using a 470 nm Colibri LED light source (Zeiss). Axons were cropped from these movies and background was corrected on each frame using ImageJ (FIJI build, NIH) by averaging background regions immediately adjacent to either side of the axon and subtracting this value from each pixel of the uncorrected frame. To standardize fluorescence intensity to the time of morphological degeneration, the frame where axon collapse accompanied by membrane spheroids was observed was considered time = 0, and time-course values were then standardized to the corrected intensity value of the frame acquired 180 minutes prior and expressed as fold-change from 1.0.

Experimental Design and Statistical Analysis

Data was plotted and analyzed with Prism 6 (GraphPad). One-factor ANOVA with Dunnett's post-hoc comparisons were used to analyze the effects of capsazepine, NAC, VAS2870, Go6976 and Go6983 on Fluo-4 intensity standardized to the mean NGF control value, the effect of capsazepine on axon density after NGF deprivation versus the NGF-deprived control, and the effect of NGF deprivation on GCaMP6f response (RM in the 'time' factor, . Two-factor ANOVA was utilized to test the effect of EDTA on axon density (RM in the 'distance from

soma' factor and Dunnett's post-hoc comparisons with NGF-deprived control) and the effect of NAC, VAS2870, Go6976 and Go6983 on axon density (Tukey's post-hoc comparisons and RM in the 'distance from soma' factor). Two-factor ANOVA was also used to analyze the effect of TrpV1 knockout on axon density after NGF deprivation and to assess the effect of capsazepine on maximum Fluo-4 response to PMA (Tukey's post-hoc comparisons made in each case). A two-way ANOVA (RM in the time factor) with Sidak's multiple comparisons was performed on data collected during time-course imaging of the Fluo-4 response to PMA in wild-type and *TrpV1*-null axons. Unpaired, two-tailed t-tests were used to test the significance of the Fluo-4 response to PMA and NGF deprivation and to test the effect of TrpV1 knockout on PMA responses. Plotted values in each case represent the mean of a single embryo, and the number of embryos *n* in each experiment and condition is described in corresponding figure legends. Full statistical results are available upon request.

Table 1. Materials list

Reagent	Source	Identifier
Antibodies		
Mouse anti-tubulin beta III	EMD Millipore	MAB5564
Goat anti-mouse AlexaFluor 488	Jackson ImmunoResearch	115-545-003
Goat anti-mouse HRP	Jackson ImmunoResearch	115-053-146
Mice		
CD1	Charles River	IMSR Cat# CRL:22, RRID:IMSR_CRL:22
B6.129X1-*Trpv1*^{tm1Jul}/J	The Jackson Laboratories	3834761, RRID:MGI:3834761
Software		

Prism 6	GraphPad	RRID:SCR_002798
Inkscape 0.91	The Inkscape Project	RRID:SCR_014479
FIJI	NIH	RRID:SCR_002285
Axoquant2.0	(Johnstone et al., 2018)	http://www.github.com/BarkerLabUBC
RStudio 1.1.442	RStudio	RRID:SCR_000432
ZEN 2	Zeiss	RRID:SCR_013672
LAS X	Leica	RRID:SCR_013673
Culture reagents		
Neurobasal	Gibco	21103049
B-27	Gibco	17504-044
Penicillin/streptomycin	Gibco	15140122
GlutaMax	Gibco	35050-061
NGF	CedarLane	CLMCNET-001
Poly-D-Lysine	Sigma	P6407-5MG
Laminin/Entactin	Corning	08-774-555
Collagen	PurCol	5005-100ML
Oligonucleotides		
For *TrpV1* genotyping (wild-type forward) TGGCTCATATTTGCCTTCAG	Invitrogen	19922
For *TrpV1* genotyping (mutant forward) TAAAGCGCATGCTCCAGACT	Invitrogen	oIMR1627

For *TrpV1* genotyping (in-common reverse) CAGCCCTAGGAGTTGATGGA	Invitrogen	19923

254

255

Results

Ca^{2+} influx is required for axon degeneration

We previously showed that chelation of extracellular Ca^{2+} by EGTA rescues axons from trophic withdrawal-induced degeneration (Johnstone et al., 2018). To confirm that NGF deprivation induces an increase in axoplasmic Ca^{2+}, DRG axons were withdrawn from NGF and examined by Ca^{2+} imaging using the dark-to-bright Ca^{2+}-responsive dye fluo-4. Figure 1A-B shows that axoplasmic Ca^{2+} is significantly increased at 15 hours of NGF withdrawal. To understand the kinetics of the Ca^{2+} increase relative to the timing of membrane spheroid formation and frank degeneration, axons were infected with herpes simplex virus (HSV) harbouring the genetically-encoded Ca^{2+} sensor GCaMP6f and live-imaged after NGF deprivation to record the timing of Ca^{2+} rise (Figure 1C). Because the degeneration of individual axons is not perfectly synchronized, videos were standardized to the frame in which axons acquired membrane spheroids; this approach revealed that an increase in the GCaMP6f signal is initiated approximately two hours pror to morphological degeneration and peaks in the last 40 minutes prior to frank axon breakdown (Figure 1D). Thus, the Ca^{2+} influx occurs prior to the emergence of gross morphological changes.

Since NGF deprivation induced a significant axoplasmic Ca^{2+} influx proximal to membrane spheroid formation, and we recently reported that Ca^{2+} chelation rescues axons from degeneration (Johnstone et al., 2018), we sought to clarify whether Ca^{2+} signaling is required during the early phase after NGF deprivation to induce degeneration, or whether Ca^{2+} is only required as a late event. DRG neurons were grown in NGF and then either maintained in NGF, deprived of NGF or deprived of NGF in the presence of EDTA added at the beginning of the deprivation phase (EDTA 24 hours) or only after the first 12 hours of deprivation (Figure 1E).

Axon density vs distance was quantified using Axoquant2.0 (Figure 1F). Axons were rescued from degeneration when EDTA was added for the full 24 hours of NGF withdrawal or when added for the final 12 hours (Figure 1G). Intrigingly, axons were more robustly protected when EDTA was added only for the final 12 hours of NGF deprivation, versus deprived axons continuously supplied with EDTA for the entire 24-hour deprivation period, likely indicative of the important role for Ca^{2+} in outgrowth and survival pathways. We conclude that Ca^{2+} influx is a late event in axonal degeneration induced by NGF withdrawal.

TRPV1 *mediates developmental degeneration*

Capsaicin-induced activation of TRPV1 can induce Ca^{2+}-dependent degeneration of sensory nerve fibres (Jancso et al., 1985; Gibbons et al., 2010; Chiang et al., 2015; Şavk, 2016) but to date, the role for TRPV1 in developmental degeneration has not been explored. To address this, we deprived DRG sensory neurons of NGF in the presence or absence of TRPV1 antagonist capsazepine and measured axonal Ca^{2+} levels using fluo-4. Figure 2A shows that the increase in axonal Ca^{2+} concentration that normally occurs following NGF deprivation was lost in the presence of the TRPV1 inhibitor (quantified in Figure 2B). Further, the fragmentation of the axonal tubulin cytoskeleton that is normally observed after 24 hours of NGF deprivation was substantially reduced in axons treated with capsazepine (Figure 2C and 2D) as well as in $TRPV1^{-/-}$ DRG axons (Figure 2E). We conclude that TRPV1-dependent Ca^{2+} entry plays an important role in developmental sensory axon degeneration.

ROS generated by NOX complexes are required for degeneration

ROS are important physiological regulators of TRPV1 (Salazar et al., 2008a; Ding et al., 2016; Hargreaves and Ruparel, 2016; Ogawa et al., 2016). To explore the possibility that ROS are generated following NGF deprivation, we first asked if the rise in axonal Ca^{2+} that occurs after NGF deprivation is blocked in the presence of N-acetylcysteine (NAC), a potent antioxidant. Figure 3A and 3B show that NAC completely blocked the rise in axonal Ca^{2+} influx that normally occurs 15 hours after NGF deprivation.

Under normal physiological circumstances, a major source of intracellular ROS are NOX complexes (Li et al., 2006; Cao et al., 2007; Ibi et al., 2008; Kallenborn-Gerhardt et al., 2014; Spencer and Engelhardt, 2014). To determine if NOX complex activity contributes to the axonal Ca^{2+} influx that occurs in DRG sensory axons after NGF withdrawal, we exposed axons to NOX complex inhibitor VAS2870. Figures 3A and 3B show that VAS2870 blocks the Ca^{2+} influx that normally occurs 15 hours after NGF deprivation. Both NAC and VAS2870 rescue axons from degeneration following NGF withdrawal (Figures 3 C-E). Axon protection was also conferred by two additional NOX complex inhibitors besides VAS2870, apocynin and diphenyliodonium (data not shown). Therefore, we conclude that NOX-dependent ROS generation plays a critical role in developmental axon degeneration.

In several settings, NOX complexes are activated by PKC (Frey et al., 2002; Ibi et al., 2008; Cosentino-Gomes et al., 2012) and we therefore asked if PKC activity mediates Ca^{2+} entry and developmental sensory axon degeneration. First, we performed gain of function experiments to determine whether PKC activation is sufficient to drive Ca^{2+} influx by exposing axons to phorbol 12-myristate 13-acetate (PMA), a potent PKC activator. Figures 4A and 4B show that PMA-mediated PKC stimulation potently induces Ca^{2+} influx in axons Next, we applied PKC

inhibitors to axons during NGF deprivation. Figures 4C and 4D show that PKC inhibitors Gö6976 and Gö6983 each strongly blocked the Ca^{2+} influx induced by 15 hours of NGF deprivation and Figures 4E and 4F show that these compounds significantly improve axon integrity observed after 24 hours of NGF deprivation. These results are consistent with the hypothesis that PKC-dependent NOX activation drives TRPV1 opening following NGF deprivation.

To determine if any of this PKC-dependent Ca^{2+} influx occurred via TRPV1, axons were exposed to PMA in the absence and presence of capsazepine. Interestingly, the TRPV1 blocker capsazepine strongly reduced the PMA-induced Ca^{2+} entry into axons, suggesting that PKC mediates Ca^{2+} entry into sensory axons mainly via TRPV1 (Figure 5A-C). To confirm this, we examined the effect of PMA on Ca^{2+} entry in DRG sensory axons derived from wild-type and $TRPV1^{-/-}$ embryos; Figure 5D and 5E show that the PMA-induced Ca^{2+} influx that occurs in wild-type axons is virtually absent in $TRPV1^{-/-}$ axons. Together these results suggest that TRPV1 is the predominant Ca^{2+} channel activated by PKC in sensory axons.

In our final experiments, we asked whether PKC-dependent TRPV1 channel activity requires NOX activation and ROS generation. Axons were exposed to PMA in the absence and presence of apocynin (to inhibit NOX complexes) or NAC (a ROS scavenger) and assessed for Ca^{2+} entry. Figure 6 shows that both apocynin (Figure 6A and 6B) and NAC (Figure 6C and 6D) suppress PMA-induced Ca^{2+} entry. Taken together, our data indicate that a direct PKC > NOX > ROS signaling cascade activates TRPV1 in sensory axons and that this cascade plays a critical role in the developmental axon degeneration of DRG sensory axons.

347

348

Discussion

Axon terminals innervating the skin can be induced to degenerate by clinical application of capsaicin, an exogenous TRPV1 agonist, which activates Ca^{2+} influx upstream of mitochondrial dysfunction and cytoskeletal degeneration, indicating that the molecular machinery is present within DRG axons to execute TRPV1-dependent neurite degeneration (Jancso et al., 1985; Gibbons et al., 2010; Chiang et al., 2015). The Ca^{2+}-dependent apoptotic death of retinal ganglia cells, a neuronal subtype, subjected to experimental glaucoma *in vitro* was also found to require TRPV1, as did apoptosis of cortical neurons exposed to oxygen/glucose deprivation (Shirakawa et al., 2008; Sappington et al., 2009). We recently reported that cytoskeletal fragmentation of DRG axons is rescued by the Ca^{2+}-specific chelator EGTA during NGF deprivation *in vitro,* highlighting a key role for Ca^{2+} in developmental degeneration and prompting the current study to understand its source and regulation (Johnstone et al., 2018). NGF withdrawal resulted in formation of spheroid membrane protrusions on axons characteristic of apoptotic-like decoupling of membrane from underlying cytoskeletal structural support, accompanied by elevated axoplasmic free Ca^{2+} concentration.

In the present study, we found that the rise of Ca^{2+} occurred prior to the appearance of membrane spheroids and degeneration of the axon, indicating a late role for Ca^{2+} in axon degeneration induced by NGF deprivation. Cultures of DRG deprived of NGF were rescued from Ca^{2+} influx by TRPV1 antagonist capsazepine, and pharmacological and genetic TRPV1 loss-of-function rescued cytoskeletal integrity. We hypothesized that ROS promote developmental degeneration upstream of TRPV1 and Ca^{2+} influx during trophic withdrawal. Consistent with this, antioxidant NAC rescued axons from Ca^{2+} influx and from degeneration, as did NOX complex and PKC

inhibition. Our results reveal for the first time that a PKC > NOX complex > ROS > TRPV1 > Ca^{2+} signaling axis mediates developmental axonal degeneration in DRG neurons.

Reactive oxygen species mediate developmental degeneration

We sought to place TRPV1 in a signaling context downstream of NGF withdrawal by identifying its mode of regulation. Though TRPV1 is activated by diverse stimuli including protons, heat, lipids and plant and invertebrate defense compounds, we hypothesized that ROS are required for activation of TRPV1 in the context of developmental degeneration for several reasons: 1) intraneuronal Ca^{2+} tone and elevated oxidative status are mutually enhancing in diverse physiological settings and in disease states (Liu et al., 2003; Mattson, 2007; Bezprozvanny, 2009; Marambaud et al., 2009; Tan et al., 2011; Deheshi et al., 2015; Schampel and Kuerten, 2017; Carrasco et al., 2018); 2) ROS are crucial for TRPV1-mediated Ca^{2+} dynamics in nociception and inflammation, two paradigms in which TRPV1 has been extensively studied (Salazar et al., 2008b; Chuang and Lin, 2009; Wang and Chuang, 2011; Ding et al., 2016; Hargreaves and Ruparel, 2016; Ogawa et al., 2016); 3) cultured sympathetic neurons and PC12 cells, which like DRG both require NGF for outgrowth and survival, can be rescued from neurite degeneration and death by an antioxidant (Ferrari et al., 1995), and 4) redox regulation of TRPV1 is well established, and intracellular redox-active cysteine residues that are targeted within TRPV1 have been identified (Ibi et al., 2008; Salazar et al., 2008b; Lin et al., 2015; Ding et al., 2016; Morales-Lázaro et al., 2016; Nazıroğlu, 2017). Based on these findings, we hypothesized that NGF deprivation results in ROS generation that is required for the TRPV1-mediated Ca^{2+} influx in DRG axons and our results clearly demonstrate this to be the case.

NOX complexes generate ROS during trophic factor deprivation

What is the origin of ROS in an NGF-deprived sensory axon? NOX complexes are a family of membrane-associated oxidases that generate superoxide via electron transfer from NADPH that participate in redox regulation of downstream targets (Frey et al., 2002; Geiszt et al., 2003; Hilburger et al., 2005; Cao et al., 2009; Petry et al., 2010). In embryonic sympathetic neurons, which like DRG depend on NGF for survival, genetic deletion of NOX isoform NOX2 rescued axons from degeneration, as did pharmacological NOX complex inhibition (Tammariello et al., 2000; Hilburger et al., 2005). Further, a growing body of literature points to an intimate link between NOX complex activation and TRPV1-mediated Ca^{2+} dynamics in pain and inflammation paradigms (Ibi et al., 2008; Lin et al., 2015; Ding et al., 2016; Nazıroğlu, 2017). It is firmly established that ROS are generated as a consequence of mitochondrial dysfunction during neurite degeneration and apoptosis, and it is likely that mitochondria-derived ROS are generated as axons degenerate (Hajnóczky et al.; Kirkland et al., 2002; Kirkland and Franklin, 2003; Celsi et al., 2009; Prudent et al., 2015). However, in DRG axons stimulated to degenerate with capsaicin, loss of mitochondrial membrane potential is a consequence, not a cause, of TRPV1-mediated Ca^{2+} influx (Czeh et al., 2005; Chiang et al., 2015). Since NOX complex inhibition rescued axons from Ca^{2+} influx and degeneration in our setting, we conclude that NOX complexes are the primary source of ROS that contributes to TRPV1 activation in degenerating axons.

A PKC > NOX > ROS > TRPV1 > Ca^{2+} signaling cascade mediates Ca^{2+} influx in DRG axons.

PKC-dependent phosphorylation of the core activator subunit $p47^{phox}$ plays a critical role in the assembly and activation of several NOX complexes (El-Benna et al., 2008). Typical PKCs are

418 activated by Ca^{2+} and diacylglycerol (DAG) and the pharmacological DAG mimetic PMA is a
419 potent PKC activator (Grimes et al., 1996; Frey et al., 2002; Ibi et al., 2008; Cosentino-Gomes et
420 al., 2012; Lucke-Wold et al., 2015). Our finding that *TRPV1* genetic knockout completely
421 ablated the axonal Ca^{2+} influx response to PMA is consistent with published *in vivo* data
422 indicating that the noxious effect of intraplantar PMA injection was mediated by TRPV1
423 (Bölcskei et al., 2005). Further, our evidence for axonal signaling cassette comprised of PKC >
424 NOX > ROS > TRPV1 > Ca^{2+} is consistent with data from other settings (e.g. inflammation)
425 that have linked extracellular signaling to TRPV1 activation (Ibi et al., 2008; Lin et al., 2015;
426 Ding et al., 2016; Nazıroğlu, 2017).
427
428 *How does TrkA transduce a prodegenerative signal to the* PKC/NOX/TRPV1 *signaling*
429 *cassette?*
430 The apical molecular events that link NGF deprivation to PKC activation remain unknown. In
431 this regard, it is notable that whereas NGF-bound TrkA activates pro-survival signaling (together
432 with the p75 neurotrophin receptor ($p75^{NTR}$), recent studies have suggested that when withdrawn
433 from ligand, TrkA can actually drive pro-degenerative pathways (Barker and Shooter, 1994;
434 Nikoletopoulou et al., 2010; Feinberg et al., 2017). One of the signaling events activated by
435 TrkA is the phospholipase C gamma (PLCɣ) pathway and in subsequent studies it will be
436 interesting to determine if TrkA-dependent PLCɣ activity plays a role in PKC activation after
437 NGF withdrawal. This intriguing possibility would be consistent with results from Calissano and
438 colleagues that show that in NGF-treated hippocampal neurons, TrkA and PLCγ phosphorylation
439 is rapidly lost after NGF withdrawal but both rise several hours later (Matrone et al., 2009).
440 Recent work has indicated a role for TNFR-family member death receptor 6 (DR6) in

441 developmental degeneration downstream of NGF deprivation (Nikolaev et al., 2009); whether
442 DR6 enhances PKC activation in this context is not yet known.
443
444 In closing, we have established a novel role for TRPV1 in developmental sensory axon
445 degeneration and established the existence of a PKC > NOX > ROS > TRPV1 > Ca^{2+} signaling
446 cassette in this setting. These studies have been limited to peripheral sensory axons but TRPV1
447 is broadly expressed in the central nervous system, and sporadic evidence has suggested a role
448 for TRPV1 in synapse remodeling (Ramírez-Barrantes et al., 2016). Future studies should test
449 the intriguing possibility that selective pruning of neurites is accomplished by locally-restricted
450 TRPV1 activation, not only in the periphery but also in the brain.
451
452
453
454
455
456
457
458
459
460
461

References

Barde YA (1989) Trophic factors and neuronal survival. Neuron 2:1525–1534 Available at: http://www.ncbi.nlm.nih.gov/pubmed/2697237 [Accessed March 14, 2018].

Barker PA, Shooter EM (1994) Disruption of NGF binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to TrkA on PC12 cells. Neuron 13:203–215 Available at: http://www.ncbi.nlm.nih.gov/pubmed/7519025 [Accessed March 14, 2018].

Bedard K, Krause K-H (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 87:245–313 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17237347 [Accessed September 20, 2014].

Bezprozvanny I (2009) Calcium signaling and neurodegenerative diseases. Trends Mol Med 15:89–100 Available at: http://www.ncbi.nlm.nih.gov/pubmed/19230774 [Accessed March 16, 2018].

Bölcskei K, Helyes Z, Szabó Á, Sándor K, Elekes K, Németh J, Almási R, Pintér E, Petho G, Szolcsányi J (2005) Investigation of the role of TRPV1 receptors in acute and chronic nociceptive processes using gene-deficient mice. Pain 117:368–376 Available at: www.elsevier.com/locate/pain [Accessed September 27, 2018].

Cao X, Dai X, Parker LM, Kreulen DL (2007) Differential regulation of NADPH oxidase in sympathetic and sensory Ganglia in deoxycorticosterone acetate salt hypertension. Hypertension 50:663–671 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17698723 [Accessed October 20, 2014].

Cao X, Demel SL, Quinn MT, Galligan JJ, Kreulen D (2009) Localization of NADPH oxidase in sympathetic and sensory ganglion neurons and perivascular nerve fibers. Auton Neurosci 151:90–97 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2783262&tool=pmcentrez&rendertype=abstract [Accessed October 20, 2014].

Carrasco C, Naziroğlu M, Rodríguez AB, Pariente JA (2018) Neuropathic Pain: Delving into the Oxidative Origin and the Possible Implication of Transient Receptor Potential Channels. Front Physiol 9:95 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29491840 [Accessed March 26, 2018].

Celsi F, Pizzo P, Brini M, Leo S, Fotino C, Pinton P, Rizzuto R (2009) Mitochondria, calcium and cell death: A deadly triad in neurodegeneration. Biochim Biophys Acta - Bioenerg 1787:335–344 Available at: https://www.sciencedirect.com/science/article/pii/S0005272809000826 [Accessed March 16, 2018].

Chiang H, Ohno N, Hsieh Y-L, Mahad DJ, Kikuchi S, Komuro H, Hsieh S-T, Trapp BD (2015) Mitochondrial fission augments capsaicin-induced axonal degeneration. Acta Neuropathol 129:81–96 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25322817 [Accessed March 16, 2018].

Chuang H, Lin S (2009) Oxidative challenges sensitize the capsaicin receptor by covalent cysteine modification. Proc Natl Acad Sci U S A 106:20097–20102 Available at: http://www.ncbi.nlm.nih.gov/pubmed/19897733 [Accessed March 16, 2018].

Cosentino-Gomes D, Rocco-Machado N, Meyer-Fernandes JR (2012) Cell signaling through protein kinase C oxidation and activation. Int J Mol Sci 13:10697–10721 Available at: http://www.ncbi.nlm.nih.gov/pubmed/23109817 [Accessed April 1, 2018].

507 Cox JA, Jeng AY, Sharkey NA, Blumberg PM, Tauber AI (1985) Activation of the human
508 neutrophil nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase by protein
509 kinase C. J Clin Invest 76:1932–1938 Available at:
510 http://www.ncbi.nlm.nih.gov/pubmed/2997297 [Accessed March 16, 2018].
511 Czeh G, Varga A, Sandor Z, Szolcsanyi J (2005) Capsaicin-Induced Changes in the Cytosolic
512 Calcium Level and Mitochondrial Membrane Potential. Neurophysiology 37:76–87
513 Available at: http://link.springer.com/10.1007/s11062-005-0048-9 [Accessed February 4,
514 2016].
515 Deheshi S, Dabiri B, Fan S, Tsang M, Rintoul GL (2015) Changes in mitochondrial morphology
516 induced by calcium or rotenone in primary astrocytes occur predominantly through ros-
517 mediated remodeling. J Neurochem.
518 Ding R, Jiang H, Sun B, Wu X, Li W, Zhu S, Liao C, Zhong Z, Chen J (2016) Advanced
519 oxidation protein products sensitized the transient receptor potential vanilloid 1 via NADPH
520 oxidase 1 and 4 to cause mechanical hyperalgesia. Redox Biol 10:1–11 Available at:
521 http://www.ncbi.nlm.nih.gov/pubmed/27665186 [Accessed March 16, 2018].
522 El-Benna J, Dang PM-C, Gougerot-Pocidalo M-A (2008) Priming of the neutrophil NADPH
523 oxidase activation: role of p47phox phosphorylation and NOX2 mobilization to the plasma
524 membrane. Semin Immunopathol 30:279–289 Available at:
525 http://www.ncbi.nlm.nih.gov/pubmed/18536919 [Accessed November 10, 2014].
526 Feinberg K, Kolaj A, Wu C, Grinshtein N, Krieger JR, Moran MF, Rubin LL, Miller FD, Kaplan
527 DR (2017) A neuroprotective agent that inactivates prodegenerative TrkA and preserves
528 mitochondria. J Cell Biol 216:3655–3675 Available at:
529 http://www.ncbi.nlm.nih.gov/pubmed/28877995 [Accessed October 22, 2018].
530 Fenwick AJ, Fowler DK, Wu S-W, Shaffer FJ, Lindberg JEM, Kinch DC, Peters JH (2017)
531 Direct Anandamide Activation of TRPV1 Produces Divergent Calcium and Current
532 Responses. Front Mol Neurosci 10:200 Available at:
533 http://www.ncbi.nlm.nih.gov/pubmed/28680392 [Accessed March 14, 2018].
534 Ferrari G, Yan CY, Greene LA (1995) N-acetylcysteine (D- and L-stereoisomers) prevents
535 apoptotic death of neuronal cells. J Neurosci 15:2857–2866 Available at:
536 http://www.ncbi.nlm.nih.gov/pubmed/7722634 [Accessed November 5, 2014].
537 Fischer LR, Glass JD (2007) Axonal degeneration in motor neuron disease. Neurodegener Dis
538 4:431–442 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17934327 [Accessed
539 October 29, 2014].
540 Frey RS, Rahman A, Kefer JC, Minshall RD, Malik AB (2002) PKCzeta regulates TNF-alpha-
541 induced activation of NADPH oxidase in endothelial cells. Circ Res 90:1012–1019
542 Available at: http://www.ncbi.nlm.nih.gov/pubmed/12016268 [Accessed November 10,
543 2014].
544 Geiszt M, Lekstrom K, Witta J, Leto TL (2003) Proteins homologous to p47phox and p67phox
545 support superoxide production by NAD(P)H oxidase 1 in colon epithelial cells. J Biol Chem
546 278:20006–20012 Available at:
547 http://www.jbc.org/content/278/22/20006?ijkey=91d5f6e627439953162c9dbb6190b7cb29e
548 e6142&keytype2=tf_ipsecsha [Accessed November 8, 2014].
549 Geron M, Hazan A, Priel A (2017) Animal Toxins Providing Insights into TRPV1 Activation
550 Mechanism. Toxins (Basel) 9:326 Available at:
551 http://www.ncbi.nlm.nih.gov/pubmed/29035314 [Accessed March 14, 2018].
552 Gibbons CH, Wang N, Freeman R (2010) Capsaicin induces degeneration of cutaneous

autonomic nerve fibers. Ann Neurol 68:888–898 Available at: http://www.ncbi.nlm.nih.gov/pubmed/21061393 [Accessed March 16, 2018].

Grabiec U, Dehghani F (2017) *N* -Arachidonoyl Dopamine: A Novel Endocannabinoid and Endovanilloid with Widespread Physiological and Pharmacological Activities. Cannabis Cannabinoid Res 2:183–196 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29082315 [Accessed March 14, 2018].

Grimes ML, Zhou J, Beattie EC, Yuen EC, Hall DE, Valletta JS, Topp KS, LaVail JH, Bunnett NW, Mobley WC (1996) Endocytosis of activated TrkA: evidence that nerve growth factor induces formation of signaling endosomes. J Neurosci 16:7950–7964 Available at: http://www.ncbi.nlm.nih.gov/pubmed/8987823 [Accessed November 9, 2014].

Hajnóczky G, Csordás G, Das S, Garcia-Perez C, Saotome M, Sinha Roy S, Yi M Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca2+ uptake in apoptosis. Cell Calcium 40:553–560 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2692319&tool=pmcentrez&rendertype=abstract [Accessed November 19, 2015].

Hakim M, Jiang W, Luo L, Li B, Yang S, Song Y, Lai R (2015) Scorpion Toxin, BmP01, Induces Pain by Targeting TRPV1 Channel. Toxins (Basel) 7:3671–3687 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26389953 [Accessed March 14, 2018].

Hargreaves KM, Ruparel S (2016) Role of Oxidized Lipids and TRP Channels in Orofacial Pain and Inflammation. J Dent Res 95:1117–1123 Available at: http://www.ncbi.nlm.nih.gov/pubmed/27307050 [Accessed March 16, 2018].

Hilburger EW, Conte EJ, McGee DW, Tammariello SP (2005) Localization of NADPH oxidase subunits in neonatal sympathetic neurons. Neurosci Lett 377:16–19 Available at: http://www.ncbi.nlm.nih.gov/pubmed/15722179 [Accessed October 20, 2014].

Ibi M, Matsuno K, Shiba D, Katsuyama M, Iwata K, Kakehi T, Nakagawa T, Sango K, Shirai Y, Yokoyama T, Kaneko S, Saito N, Yabe-Nishimura C (2008) Reactive oxygen species derived from NOX1/NADPH oxidase enhance inflammatory pain. J Neurosci 28:9486–9494 Available at: http://www.ncbi.nlm.nih.gov/pubmed/18799680 [Accessed March 15, 2018].

Jancso G, Király E, Joó F, Such G, Nagy A (1985) Selective degeneration by capsaicin of a subpopulation of primary sensory neurons in the adult rat. Neurosci Lett 59:209–214 Available at: https://www.sciencedirect.com/science/article/pii/0304394085902010 [Accessed March 16, 2018].

Johnstone AD, Hallett RM, de Léon A, Carturan B, Gibon J, Barker PA (2018) A novel method for quantifying axon degeneration Gillingwater TH, ed. PLoS One 13:e0199570 Available at: http://dx.plos.org/10.1371/journal.pone.0199570 [Accessed September 5, 2018].

Julius D, Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389:816–824 Available at: http://www.ncbi.nlm.nih.gov/pubmed/9349813 [Accessed March 14, 2018].

Kallenborn-Gerhardt W, Hohmann SW, Syhr KMJ, Schröder K, Sisignano M, Weigert A, Lorenz JE, Lu R, Brüne B, Brandes RP, Geisslinger G, Schmidtko A (2014) Nox2-dependent signaling between macrophages and sensory neurons contributes to neuropathic pain hypersensitivity. Pain 155:2161–2170 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25139590 [Accessed October 20, 2014].

Kanaan NM, Pigino GF, Brady ST, Lazarov O, Binder LI, Morfini GA (2013) Axonal degeneration in Alzheimer's disease: when signaling abnormalities meet the axonal

599 transport system. Exp Neurol 246:44–53 Available at:
600 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3465504&tool=pmcentrez&ren
601 dertype=abstract [Accessed October 27, 2014].
602 Kirkland RA, Franklin JL (2003) Bax, reactive oxygen, and cytochrome c release in neuronal
603 apoptosis. Antioxid Redox Signal 5:589–596 Available at:
604 http://online.liebertpub.com/doi/abs/10.1089/152308603770310257 [Accessed November 5,
605 2014].
606 Kirkland RA, Windelborn JA, Kasprzak JM, Franklin JL (2002) A Bax-induced pro-oxidant
607 state is critical for cytochrome c release during programmed neuronal death. J Neurosci
608 22:6480–6490 Available at: http://www.ncbi.nlm.nih.gov/pubmed/12151527 [Accessed
609 November 9, 2014].
610 Li Q, Harraz MM, Zhou W, Zhang LN, Ding W, Zhang Y, Eggleston T, Yeaman C, Banfi B,
611 Engelhardt JF (2006) Nox2 and Rac1 regulate H2O2-dependent recruitment of TRAF6 to
612 endosomal interleukin-1 receptor complexes. Mol Cell Biol 26:140–154 Available at:
613 http://mcb.asm.org/content/26/1/140.long [Accessed October 22, 2014].
614 Li Q, Spencer NY, Oakley FD, Buettner GR, Engelhardt JF (2009) Endosomal Nox2 facilitates
615 redox-dependent induction of NF-kappaB by TNF-alpha. Antioxid Redox Signal 11:1249–
616 1263 Available at:
617 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2842115&tool=pmcentrez&ren
618 dertype=abstract [Accessed November 10, 2014].
619 Lin C-S, Lee S-H, Huang H-S, Chen Y-S, Ma M-C (2015) H 2 O 2 generated by NADPH
620 oxidase 4 contributes to transient receptor potential vanilloid 1 channel-mediated
621 mechanosensation in the rat kidney. Am J Physiol - Ren Physiol 309:F369–F376 Available
622 at: https://pdfs.semanticscholar.org/14fd/d5a7cc35aaf2c922b2b69e53c87e42d039a9.pdf
623 [Accessed March 16, 2018].
624 Liu M, Liu M-C, Magoulas C, Priestley J V, Willmott NJ (2003) Versatile regulation of
625 cytosolic Ca2+ by vanilloid receptor I in rat dorsal root ganglion neurons. J Biol Chem
626 278:5462–5472 Available at: http://www.jbc.org/content/278/7/5462.full [Accessed
627 February 4, 2016].
628 Lucke-Wold BP, Turner RC, Logsdon AF, Simpkins JW, Alkon DL, Smith KE, Chen Y-W, Tan
629 Z, Huber JD, Rosen CL (2015) Common mechanisms of Alzheimer's disease and ischemic
630 stroke: the role of protein kinase C in the progression of age-related neurodegeneration. J
631 Alzheimers Dis 43:711–724 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25114088
632 [Accessed April 27, 2017].
633 Maher P, Schubert D (2000) Signaling by reactive oxygen species in the nervous system. Cell
634 Mol Life Sci 57:1287–1305 Available at: http://www.ncbi.nlm.nih.gov/pubmed/11028919
635 [Accessed November 10, 2014].
636 Marambaud P, Dreses-Werringloer U, Vingtdeux V (2009) Calcium signaling in
637 neurodegeneration. Mol Neurodegener 4:20 Available at:
638 http://www.ncbi.nlm.nih.gov/pubmed/19419557 [Accessed March 16, 2018].
639 Matrone C, Marolda R, Ciafrè S, Ciotti MT, Mercanti D, Calissano P (2009) Tyrosine kinase
640 nerve growth factor receptor switches from prosurvival to proapoptotic activity via Abeta-
641 mediated phosphorylation. Proc Natl Acad Sci U S A 106:11358–11363 Available at:
642 http://www.ncbi.nlm.nih.gov/pubmed/19549834 [Accessed September 21, 2018].
643 Mattson MP (2007) Calcium and neurodegeneration. Aging Cell 6:337–350 Available at:
644 http://www.ncbi.nlm.nih.gov/pubmed/17328689 [Accessed March 16, 2018].

645 Morales-Lázaro SL, Llorente I, Sierra-Ramírez F, López-Romero AE, Ortíz-Rentería M,
646 Serrano-Flores B, Simon SA, Islas LD, Rosenbaum T (2016) Inhibition of TRPV1 channels
647 by a naturally occurring omega-9 fatty acid reduces pain and itch. Nat Commun 7:13092
648 Available at: http://www.ncbi.nlm.nih.gov/pubmed/27721373 [Accessed March 16, 2018].
649 Murphy R, Acheson A, Hodges R, Haskins J, Richards C, Reklow E, Chlumecky V, Barker P,
650 Alderson R, Lindsay R (1993) Immunological relationships of NGF, BDNF, and NT-3:
651 recognition and functional inhibition by antibodies to NGF. J Neurosci 13:2853–2862.
652 Nazıroğlu M (2017) Activation of TRPM2 and TRPV1 Channels in Dorsal Root Ganglion by
653 NADPH Oxidase and Protein Kinase C Molecular Pathways: a Patch Clamp Study. J Mol
654 Neurosci 61:425–435 Available at: http://www.ncbi.nlm.nih.gov/pubmed/28097492
655 [Accessed April 1, 2018].
656 Nikolaev A, McLaughlin T, O'Leary DDM, Tessier-Lavigne M (2009) APP binds DR6 to
657 trigger axon pruning and neuron death via distinct caspases. Nature 457:981–989 Available
658 at: http://www.nature.com/nature/journal/v457/n7232/full/nature07767.html#online-
659 methods [Accessed October 27, 2014].
660 Nikoletopoulou V, Lickert H, Frade JM, Rencurel C, Giallonardo P, Zhang L, Bibel M, Barde Y-
661 A (2010) Neurotrophin receptors TrkA and TrkC cause neuronal death whereas TrkB does
662 not. Nature 467:59–63 Available at: http://dx.doi.org/10.1038/nature09336 [Accessed
663 October 19, 2014].
664 Ogawa N, Kurokawa T, Fujiwara K, Polat OK, Badr H, Takahashi N, Mori Y (2016) Functional
665 and Structural Divergence in Human TRPV1 Channel Subunits by Oxidative Cysteine
666 Modification. J Biol Chem 291:4197–4210 Available at:
667 http://www.ncbi.nlm.nih.gov/pubmed/26702055 [Accessed March 16, 2018].
668 Pal R, Basu Thakur P, Li S, Minard C, Rodney GG (2013) Real-time imaging of NADPH
669 oxidase activity in living cells using a novel fluorescent protein reporter. PLoS One
670 8:e63989 Available at:
671 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3660327&tool=pmcentrez&ren
672 dertype=abstract [Accessed November 7, 2014].
673 Patel TD, Jackman A, Rice FL, Kucera J, Snider WD (2000) Development of sensory neurons in
674 the absence of NGF/TrkA signaling in vivo. Neuron 25:345–357 Available at:
675 http://www.ncbi.nlm.nih.gov/pubmed/10719890 [Accessed November 9, 2014].
676 Petry A, Weitnauer M, Görlach A (2010) Receptor activation of NADPH oxidases. Antioxid
677 Redox Signal 13:467–487 Available at: http://www.ncbi.nlm.nih.gov/pubmed/20001746
678 [Accessed November 10, 2014].
679 Prudent J, Zunino R, Sugiura A, Mattie S, Shore GC, McBride HM (2015) MAPL SUMOylation
680 of Drp1 Stabilizes an ER/Mitochondrial Platform Required for Cell Death. Mol Cell
681 59:941–955 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26384664 [Accessed
682 February 15, 2016].
683 Ramírez-Barrantes R, Cordova C, Poblete H, Muñoz P, Marchant I, Wianny F, Olivero P (2016)
684 Perspectives of TRPV1 Function on the Neurogenesis and Neural Plasticity. Neural Plast
685 2016:1–12 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26881090 [Accessed March
686 12, 2018].
687 Reczek CR, Chandel NS (2014) ROS-dependent signal transduction. Curr Opin Cell Biol 33C:8–
688 13 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25305438 [Accessed October 12,
689 2014].
690 Salazar H, Llorente I, Jara-Oseguera A, García-Villegas R, Munari M, Gordon SE, Islas LD,

691 Rosenbaum T (2008a) A single N-terminal cysteine in TRPV1 determines activation by
692 pungent compounds from onion and garlic. Nat Neurosci 11:255–261 Available at:
693 http://www.ncbi.nlm.nih.gov/pubmed/18297068 [Accessed March 15, 2018].
694 Salazar H, Llorente I, Jara-Oseguera A, García-Villegas R, Munari M, Gordon SE, Islas LD,
695 Rosenbaum T (2008b) A single N-terminal cysteine in TRPV1 determines activation by
696 pungent compounds from onion and garlic. Nat Neurosci 11:255–261 Available at:
697 http://www.nature.com/articles/nn2056 [Accessed March 16, 2018].
698 Sann H, Jancsó G, Ambrus A, Pierau F-K (1995) Capsaicin treatment induces selective sensory
699 degeneration and increased sympathetic innervation in the rat ureter. Neuroscience 67:953–
700 966 Available at: https://www.sciencedirect.com/science/article/pii/030645229500102O
701 [Accessed March 16, 2018].
702 Sappington RM, Sidorova T, Long DJ, Calkins DJ (2009) TRPV1: contribution to retinal
703 ganglion cell apoptosis and increased intracellular Ca2+ with exposure to hydrostatic
704 pressure. Invest Ophthalmol Vis Sci 50:717–728 Available at:
705 http://iovs.arvojournals.org/article.aspx?doi=10.1167/iovs.08-2321 [Accessed March 11,
706 2018].
707 Şavk E (2016) Neurologic Itch Management. In: Current problems in dermatology, pp 116–123
708 Available at: http://www.ncbi.nlm.nih.gov/pubmed/27578080 [Accessed March 16, 2018].
709 Saxena S, Caroni P (2007) Mechanisms of axon degeneration: from development to disease.
710 Prog Neurobiol 83:174–191 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17822833
711 [Accessed November 6, 2014].
712 Schampel A, Kuerten S (2017) Danger: High Voltage—The Role of Voltage-Gated Calcium
713 Channels in Central Nervous System Pathology. Cells 6:43 Available at:
714 http://www.ncbi.nlm.nih.gov/pubmed/29140302 [Accessed November 29, 2017].
715 Schuldiner O, Yaron A (2014) Mechanisms of developmental neurite pruning. Cell Mol Life Sci
716 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25213356 [Accessed October 24, 2014].
717 Shirakawa H, Yamaoka T, Sanpei K, Sasaoka H, Nakagawa T, Kaneko S (2008) TRPV1
718 stimulation triggers apoptotic cell death of rat cortical neurons. Biochem Biophys Res
719 Commun 377:1211–1215 Available at: http://www.ncbi.nlm.nih.gov/pubmed/18996081
720 [Accessed March 30, 2018].
721 Spencer NY, Engelhardt JF (2014) The basic biology of redoxosomes in cytokine-mediated
722 signal transduction and implications for disease-specific therapies. Biochemistry 53:1551–
723 1564 Available at: http://www.ncbi.nlm.nih.gov/pubmed/24555469 [Accessed June 27,
724 2018].
725 Tait SWG, Green DR (2010) Mitochondria and cell death: outer membrane permeabilization and
726 beyond. Nat Rev Mol Cell Biol 11:621–632 Available at:
727 http://dx.doi.org/10.1038/nrm2952 [Accessed September 20, 2015].
728 Tammariello SP, Quinn MT, Estus S (2000) NADPH oxidase contributes directly to oxidative
729 stress and apoptosis in nerve growth factor-deprived sympathetic neurons. J Neurosci
730 20:RC53 Available at: http://www.ncbi.nlm.nih.gov/pubmed/10627630 [Accessed October
731 20, 2014].
732 Tan AR, Cai AY, Deheshi S, Rintoul GL (2011) Elevated intracellular calcium causes distinct
733 mitochondrial remodelling and calcineurin-dependent fission in astrocytes. Cell Calcium
734 49:108–114 Available at: http://www.ncbi.nlm.nih.gov/pubmed/21216007 [Accessed
735 August 22, 2017].
736 Vickers JC, King AE, Woodhouse A, Kirkcaldie MT, Staal JA, McCormack GH, Blizzard CA,

737 Musgrove REJ, Mitew S, Liu Y, Chuckowree JA, Bibari O, Dickson TC (2009)
738 Axonopathy and cytoskeletal disruption in degenerative diseases of the central nervous
739 system. Brain Res Bull 80:217–223 Available at:
740 http://www.ncbi.nlm.nih.gov/pubmed/19683034 [Accessed October 31, 2014].
741 Vogelbaum MA, Tong JX, Rich KM (1998) Developmental regulation of apoptosis in dorsal
742 root ganglion neurons. J Neurosci 18:8928–8935 Available at:
743 http://www.ncbi.nlm.nih.gov/pubmed/9786998 [Accessed November 9, 2014].
744 Wang DH, Wu W, Lookingland KJ (n.d.) Degeneration of Capsaicin-Sensitive Sensory Nerves
745 Leads to Increased Salt Sensitivity Through Enhancement of Sympathoexcitatory Response.
746 Available at: http://hyper.ahajournals.org/content/hypertensionaha/37/2/440.full.pdf
747 [Accessed March 16, 2018].
748 Wang S, Chuang H-H (2011) C-terminal Dimerization Activates the Nociceptive Transduction
749 Channel Transient Receptor Potential Vanilloid 1 *. Publ JBC Pap Press Available at:
750 http://pubmedcentralcanada.ca/pmcc/articles/PMC3220497/pdf/zbc40601.pdf [Accessed
751 July 5, 2017].
752
753

Figure Legends

Figure 1. Ca^{2+} is required for developmental degeneration *in vitro*.

(A) Cultures of DRG explants were either maintained in NGF or deprived of NGF for 15 hours prior to loading with Ca^{2+} sensor fluo-4. (B) Axons deprived of NGF displayed a significantly elevated axonal Ca^{2+} concentration (data standardized to NGF, n=16, compiled from NGF and deprived controls; analyzed by unpaired two-tailed t-test and indicated are median, min/max and 25/75%). (C) Axoplasmic Ca^{2+} influx reported by GCaMP6f occurred proximal to the time of morphological degeneration of the axon. (D) Axoplasmic Ca^{2+} increase was significantly elevated by 40 minutes prior to membrane spheroid formation but not earlier as compared to intensity 180 minutes prior to spheroids (indicated are mean and SEM; one-factor ANOVA and Dunnett's posthoc). (E) βIII-tubulin staining of DRG explants treated with EDTA after 12 hours of NGF deprivation or for the entire 24 hour deprivation phase. Consistent with a late role for Ca^{2+} in axon degeneration, axons were significantly rescued from cytoskeletal fragmentation even when Ca^{2+} dynamics were left unmodulated by chelation during the first 12 hours of NGF deprivation. (F) Axoquant2.0 output curves are shown with mean and SEM (n=9 embryos from 3 pooled litters). (G) Axon density within 1000 μm bins were analyzed by two-factor ANOVA and Dunnett's post-hoc comparison and plotted with median, min/max and 25/75%. *$p<0.05$, ****$p<0.0001$.

Figure 2. TRPV1 mediates Ca^{2+} flux and cytoskeletal fragmentation during trophic factor deprivation. (A) TRPV1 inhibition by capsazepine rescued axons from Ca^{2+} influx. Axons were loaded with fluo-4 after the indicated treatments and imaged by confocal microscopy. (B) 15

hours of NGF deprivation induced robust activation of Ca^{2+} sensor fluo-4 in axons, but co-application of 10 μM capsazepine (TRPV1 inhibitor, CPZ) ablated the response as compared to deprived controls (n=7 pooled experiments, one-factor ANOVA and Dunnett's post-hoc comparison to the deprived condition). (C-D) NGF deprivation for 24 hours resulted in a significant loss of tubulin-stained axons, but addition of 10 μM CPZ after 12 hours of trophic withdrawal for the final 12 hours resulted in a rescue of axon density to a level not significantly different from healthy controls (n=8 embryos in NGF and capsazepine conditions, n=7 in DMSO; one-factor ANOVA and Dunnett's post-hoc comparison were performed on axon density in a bin between 1000 and 1999 μm from soma. (E) TRPV1 knockout rescued axons from cytoskeletal degeneration; neurons cultured from mixed-genotype litters were deprived of NGF for 24 hours, after which TRPV1-null axons were significantly more dense than axons in cultures derived from wild-type animals. Axon density between 1000 and 1999 μm from soma was compared by two-factor ANOVA and Tukey 's post-hoc comparison. Indicated are median, min/max and 25/75% in each panel. *p<0.05, ***p<0.001, ****p<0.0001.

Figure 3. ROS derived from NOX complexes activate Ca^{2+} flux and axonal degeneration *in vitro*. (A) DRG explants cultured in NGF were either maintained in NGF or withdrawn from trophic factor for 15 hours before staining with fluo-4 and imaged by confocal microscopy. (B) Antioxidant NAC (20 mM) or NOX complex inhibition using VAS2870 (10 μM) significantly impaired axonal Ca^{2+} influx induced by trophic factor withdrawal (n=3 pooled experiments standardized to the NGF condition, one-factor ANOVA and Dunnett's post-hoc comparison; mean and SEM are indicated. (C-E) Antioxidant or NOX complex inhibition significantly rescued DRG axonal cytoskeleton (visualized with βIII-tubulin immunostaining) when added

after 12 hours of NGF deprivation for the final 12 hours. (D) Axoquant2.0 axon density output curves with mean and SEM. (E) Axon density was analyzed within 1000 μm bins using two-factor ANOVA and Tukey's post-hoc analysis. Median, min/max and 25/75% are indicated. *p<0.05, **p<0.01, ****p<0.0001.

Figure 4. PKC mediates Ca^{2+} flux and axonal degeneration *in vitro*. (A) Representative micrographs of DRG axons loaded with fluo-4 and treated with PKC activator PMA (100 nM). (B) Direct PKC stimulation by PMA is sufficient to activate axonal Ca^{2+} influx (n=14 embryos pooled from PMA and buffer controls and compared using an unpaired, two-tailed t-test.) (C-D) Ca^{2+} influx activated by 15 hours of NGF deprivation is significantly rescued when PKC is inhibited by Gö6976 and Gö6983 (10 μM; n=6 embryos each condition standardized to NGF values and tested by one-factor ANOVA and Dunnett's post-hoc comparison. (E-F) PKC inhibition after 12 hours rescues axons from cytoskeletal degeneration induced by 24 hours of NGF deprivation. (E) Axoquant2.0 axon density output curves are presented with mean and SEM (n=9 for all conditions except n=7 for Gö6983). (F) Axon density was analyzed within 1000 μm bins using two-factor ANOVA and Tukey's post-hoc analysis. Median, min/max and 25/75% are indicated. ****p<0.0001.

Figure 5. TRPV1 mediates PKC-dependent axonal Ca^{2+} flux. (A) Representative images of DRG axons loaded with fluo-4 and live-imaged during stimulation with PKC activator PMA in the presence or absence of TRPV1 inhibitor CPZ (10 μM). TRPV1 inhibition abolished the axonal Ca^{2+} response to PKC activation. (B) Time-course of the fluo-4 responses to Ca^{2+} influx during the 15-minute recording period (n=6, mean and SEM are indicated). (C) Maximum

responses during the recording period were analyzed using two-factor ANOVA and Tukey's post-hoc comparison; indicated are median, min/max and 25/75%. (D) The axonal Ca^{2+} response to PKC activation was absent in axons of *TrpV1*-knockout DRG (n=6, mean and SEM are indicated and data was analyzed by two-factor ANOVA and Sidak's post-hoc comparison). (E) The maximum fluo-4 responses to axonal Ca^{2+} were analyzed by an unpaired, two-tailed t-test and indicated are median, min/max and 25/75%. **p<0.01, ***p<0.001, ****p<0.0001.

Figure 6. PKC-dependent Ca^{2+} flux requires ROS from NOX complexes. (A) Representative images of DRG axons loaded with fluo-4 and stimulated with PMA in the presence or absence of apocynin (10 μM). (B) PKC-induced Ca^{2+} influx was significantly reduced by NOX complex inhibition by 10 μM apocynin (n=5 pooled experiments analyzed by two-factor ANOVA and Tukey's post-hoc comparison). (C) Representative images of DRG axons loaded with fluo-4 and stimulated with PMA in the presence or absence of NAC (20 mM). (D) PKC activation with PMA (100 nM) induced axonal Ca^{2+} influx that was abolished by co-application of antioxidant NAC at 20 mM (n=6 pooled experiments analyzed by two-factor ANOVA and Tukey's post-hoc comparison). Plotted are the maximum Fluo-4 responses (relative to baseline values) during the recording period. Indicated are median, min/max and 25/75%. *p<0.05, **p<0.01, ***p<0.001.