PERIPHERAL MEDIATORS OF COLORECTAL NOCICEPTION AND SENSITIZATION

by

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Several ion channels are thought to facilitate colorectal afferent neuron sensitization, which contributes to abdominal pain in irritable bowel syndrome (IBS). In the present work, I hypothesized that two such channels – TRPV1 and P2X3 – cooperate to mediate colorectal pain and hypersensitivity. To test this, I employed TRPV1-P2X3 double knockout (TPDKO) mice and pharmacological antagonists and evaluated combined channel contributions to whole-organism responses to colorectal distension (CRD) and afferent fiber responses to colorectal stretch. Baseline responses to CRD were unexpectedly greater in TPDKO compared with control mice, but zymosan-produced CRD hypersensitivity was absent in TPDKO mice. Relative to control mice, proportions of afferent mechano-sensitive and -insensitive classes were not different in TPDKO mice. Whereas responses of mucosal and serosal class afferents to mechanical probing were unaffected, responses of muscular (but not muscular/mucosal) afferents to stretch were significantly attenuated in TPDKO mice as was sensitization by inflammatory soup of both muscular and muscular/mucosal afferents. In pharmacological studies, the TRPV1 antagonist A889425 and P2X3 antagonist TNP-ATP, alone and in combination, applied onto stretch-sensitive afferent endings attenuated afferent responses to stretch; combined antagonism

produced greater attenuation. In the aggregate, these observations suggest that: (1) genetic manipulation of TRPV1 and P2X3 leads to reduction in colorectal mechanosensation peripherally and compensatory changes and/or disinhibition of other channels centrally and (2) combined pharmacological antagonism produces more robust attenuation of mechanosensation peripherally than single antagonism. The relative importance of these channels appears to be enhanced in hypersensitivity, highlighting the potential utility of multi-target pharmacotherapy in IBS.

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PREFACE

This thesis was supported financially by NIH awards R01 DK093525 (Gerald F. Gebhart) and T32 NS007433 (Michael E. Kiyatkin). The latter award was administered through the Center for Neuroscience (CNUP) graduate program at the University of Pittsburgh by Dr. Alan F. Sved, Co-Director of CNUP. I would like to thank Michael Burcham for his assistance in preparation of all figures and Bin Feng, Erica S. Schwartz, Jun-Ho La, Michael S. Gold, and Nicole Scheff for instruction in methodological details. I would like to thank my thesis advisor, Gerald F. Gebhart, for his help with conceptual design and planning of experiments. Lastly, I would like to thank Abbott Laboratories for their generous gift of the TRPV1 antagonist A-889425.

1.0 GENERAL INTRODUCTION

Portions of this thesis have been presented in abstract form, including:

Kiyatkin ME, Gebhart GF. "Role of TRPV1 and P2X3 in mechanosensation in colorectal primary afferents." Program No. 473.05. 2012 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2012. Online.

Kiyatkin ME, Feng B, Schwartz ES, Gebhart GF. "Contribution of TRPV1 and P2X3 to mechanosensation in colorectal primary afferents in naive and sensitized states." 14th World Congress on Pain. Milan, Italy: International Association for the Study of Pain, 2012.

Kiyatkin ME, Feng B, Gebhart GF. "TRPV1 and P2X3 are important mediators of mechanotransduction and sensitization in colorectal primary afferents." Program No. 494.06. 2011 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2011. Online.

2.0 DUAL INHIBITION OF TRPV1 AND P2X3 ATTENUATES COLORECTAL PRIMARY AFFERENT MECHANOSENSATION AND SENSITIZATION

2.1 INTRODUCTION

Chronic abdominal pain is a key feature of irritable bowel syndrome (IBS), which is prevalent, costly and difficult to manage. An important contributor to pain in IBS is heightened perception of mechanical events in the bowel (i.e., hypersensitivity). Indeed, patients with IBS typically report greater pain with colorectal distension (Ritchie, 1973; Lembo et al., 1994; Mertz et al., 1995; Trimble et al., 1995; Bouin et al., 2002). Although central processes contribute to colorectal hypersensitivity, the driving force is increased afferent mechanosensitivity (i.e., sensitization). In support, intra-rectal lidocaine reduces pain evoked by colorectal distension in healthy subjects as well as ongoing pain and both visceral and somatic (referred) hypersensitivity in IBS patients (Plourde et al., 1993; Lembo et al., 1994; Verne et al., 2003; Verne et al., 2005). Accordingly, developing drugs that selectively attenuate colorectal mechanosensation and/or sensitization would improve management of IBS pain. Targets include ion channels expressed in colorectal afferents, two of which are transient receptor potential vanilloid 1 (TRPV1) and P2X3.

TRPV1 is a capsaicin-, heat- and proton-gated ion channel expressed in the majority of colorectal afferents (Robinson et al., 2004; Christianson et al., 2006). Expression of TRPV1 in colorectal afferents is increased in patients with IBS (Akbar et al., 2008) and the magnitude of

channel expression often positively correlates with the severity of sensory symptoms. We previously demonstrated that TRPV1 knockout mice show reduced responses of stretch-sensitive afferents (Jones et al., 2005) and decreased behavioral responses to colorectal distension (CRD) (Jones et al., 2007) in both naive and hypersensitive states. TRPV1 antagonists similarly decrease afferent and behavioral responses to CRD (Miranda et al., 2007; De Schepper et al., 2008; Phillis et al., 2009; Wiskur et al., 2010).

P2X3 exists in homomeric P2X3 and heteromeric P2X2/3 configurations and is expressed in ~20% of colorectal afferents (Brierley et al., 2005; Shinoda et al., 2010). Both configurations are activated via pressure-dependent mucosal release of ATP (Wynn et al., 2003; Shinoda et al., 2009), which facilitates distension-evoked mechanosensation (Wynn et al., 2003). In agreement, we found that deletion of P2X3 attenuates colorectal mechanosensation and sensitization (Shinoda et al., 2009). Further, the expression and function of P2X3 is enhanced in rodent models of IBS (Xu et al., 2008; Shinoda et al., 2010).

Appreciating it to be unlikely that a single channel mediates colorectal pain and hypersensitivity, we hypothesized that combined inhibition of these channels should produce greater attenuation of mechanosensation than inhibition of either channel alone. To test this hypothesis, we utilized TRPV1-P2X3 double knockout (TPDKO) mice together with selective pharmacological antagonists to evaluate (1) channel contributions to colorectal nociception and hypersensitivity *in vivo* and (2) afferent mechanosensation and sensitization *in vitro*. Portions of these data have been previously reported in abstract form (Kiyatkin and Gebhart, 2012).

2.2 MATERIALS AND METHODS

2.2.1 Animals

Adult male mice (20-30 g) of the following strains were used: C57BL/6 wildtype control (Taconic, Germantown, NY) and global, non-conditional TPDKO. TPDKO mice were generated by crossing TRPV1 and P2X3 single knockout mice. All knockout mice were backcrossed onto a Taconic C57BL/6 genetic background for >10 generations. TRPV1 single knockout mice were provided by Dr. H. R. Koerber, the University of Pittsburgh. P2X3 single knockout mice were obtained from The Jackson Laboratory (Bar Harbor, ME) with the permission of Dr. D. A. Cockayne, Roche Bioscience, Palo Alto, CA. Genotypes were confirmed by PCR and Southern Blot analysis. We used only homozygous TPDKO mice. All protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.2.2 Labeling, single cell PCR, and calcium-imaging of colorectal neurons

Dorsal root ganglion (DRG) sensory neurons innervating the colorectum were labeled and collected as previously detailed (Shinoda et al., 2010; Schwartz et al., 2011). Briefly, a laparotomy was performed on mice anesthetized with isoflurane (Hospira Inc., Lake Forest, IL) to expose the distal colon. Three to six boluses (2-3 μL) of 2% by weight 1,1'-dioctadecyl-3,3,3',3-tetramethylindocarbocyanine methanesulfonate (DiI; Molecular Probes, Eugene, OR) dissolved in DMSO were injected into the colon wall ~1.5 cm rostral to the anal verge. Mice were allowed two weeks for post-surgical recovery and transport of DiI to DRG somata.

Subsequently, mice were overdosed with isoflurane followed by removal of L6-S2 (LS) DRGs bilaterally. DRGs were incubated at 37°C for 10 min in Hanks' Balanced Salt solution (Sigma-Aldrich, St. Louis, MO) containing L-cysteine (5.5 mM), papain (60 Units; Worthington Biochemical, Lakewood, NJ) and saturated $NaHCO_3$ (2 mM). Collagenase II (4,320 U; Worthington) and dispase II (14 U; Roche Diagnostics, Indianapolis, IN) were added and cells incubated for an additional 20 min before quenching enzyme activity with 10% fetal bovine serum (Sigma) dissolved in advanced Dulbecco's modified eagle medium/F12 containing 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). The cell mixture was gently triturated, cells plated on poly-D-lysine-coated glass coverslips (Becton Dickinson Labware, Bedford, MA) and incubated (37°C, 95% O_2) for 16-18 hours before beginning experiments. No additional growth factors were added to culture media. Two to three mice were used per preparation.

Dissociated and cultured colorectal DRG neurons (c-DRGs) were identified by DiI content, collected with a ~50 μm diameter glass pipette and expelled into tubes containing reverse transcriptase (RT) mix (SuperScript II; Invitrogen). Negative controls were prepared by omitting RT. The first-strand cDNA was used as template for PCR (equipment and reagents from Promega, Madison, WI). We used a nested primer strategy with external primers for the first round of PCR followed by subsequent PCR with internal primers. Primer sequences are listed in **Table 1**. Final PCR products were electrophoresed and visualized on 2% agarose–ethidium bromide gels.

Table 1: Primer pairs for PCR amplification of TRPV1 and P2X3 mRNA. Single cell PCR was performed in dissociated colorectal afferent somata from LS DRGs. A nested PCR strategy with external and internal cDNA primer pairs was used to amplify reverse transcribed TRPV1 and P2X3 mRNA. Sequences for cDNA primers are indicated with the 5' end on the left. The Genebank reference used for designing primers is indicated at the right. bp, base pairs.

Gene (expected size)	External primers	Internal primers	Genebank Number
TRPV1 (486, 191 bp)	GGGAAGAATAACTCACTGCCTGTG TGGGTCCTCGTTGATGATGC	GGCGAGACTGTCAACAAGATTGC TCATCCACCCTGAAGCACCAC	NM_001001445.1
P2X3 (251, 141 bp)	GCTCCGTAGAAGAAGATGGAGA CTGTGTGACCATGTTAGGGATG	TGTCCTAAGAGGATCCTGTACC GGCATCTAGCACATAGAAGTGG	NM_145526

Other c-DRGs were loaded with 2.5 μM of the fluorescent Ca^{2+} indicator fura-2 acetoxymethyl and 0.025% pluronic acid (both from TEF Laboratories, Austin, TX) as detailed previously (Lu et al., 2006). Treated cells were transferred to a recording chamber continuously perfused with bath solution containing (in mM): 130 NaCl, 3 KCl, 2.5 $CaCl_2$, 0.6 Mg Cl_2, 10 HEPES, 10 glucose and pH adjusted with Tris base to 7.4 and osmolarity with sucrose to 325 mOsmol/l. Ca^{2+}-transients were measured as the ratio of fluorescence emission (510 nm) in response to 340/380 nm excitation controlled by a lambda 10-2 filter changer (Sutter Instruments CA). Data were acquired using Metafluor software (Molecular Devices, Sunnyvale, CA) and a CCD camera (Model RTE/CCD 1300; Roper Scientific, Trenton, NJ)**.** DiI-positive c-DRGs were stimulated in the following sequence: KCl (30 mM KCl in normal bath with 100 mM NaCl to maintain osmolarity), the P2X3 agonist α,β-meATP (100 μM in normal bath; Sigma), and the TRPV1 agonist capsaicin (500 nM in normal bath and <0.01% ethanol; Sigma). All drugs were delivered via a fast-step superfusion system (Model DAD-12; ALA Scientific Instruments, Westbury, NY).

2.2.3 Colorectal distension (CRD)

The visceromotor response (VMR) to CRD was measured to assess colorectal nociception and hypersensitivity (Christianson and Gebhart, 2007). Briefly, mice were anesthetized (isoflurane) and a pair of electrodes implanted into the abdominal musculature and exteriorized at the back of the neck for subsequent electromyographic recording of muscle activity in unanesthetized mice. Contraction in response to CRD was quantified with Spike2 software (Cambridge Electronic Design, Cambridge, UK) as electromyographic activity during distension minus pre-distension resting activity. Polyethylene distension balloons (1.5 cm length, 0.9 cm diameter) were inserted transanally 1 cm beyond the anal verge under isoflurane sedation. Mice were placed inside darkened, sound-attenuated plastic cylinders to minimize movement and stress. A 45-min post-isoflurane recovery period preceded CRD. Distension balloons were inflated for 10-s with pressurized nitrogen to 15, 30, 45 or 60 mmHg. Each pressure was tested three times with 4 min between distensions, starting at 15 mmHg (non-noxious) and ending at 60 mmHg (noxious). Baseline VMRs were recorded four days after surgery (Day 0) after which intracolonic treatment with either 0.1 mL of normal saline vehicle or zymosan (30 mg/mL; Sigma) was given and repeated daily for two additional consecutive days; VMRs to CRD were recorded again one day after the third intracolonic treatment (i.e., Day 3) (Shinoda et al., 2009). Some TPDKO mice were sacrificed after Day 3 CRD for single fiber electrophysiology (described below).

2.2.4 Single fiber electrophysiology

Mice were sacrificed by CO_2 inhalation and the distal 2-3 cm of the colorectum was dissected out with the pelvic nerve (PN) innervation intact (Feng and Gebhart, 2011). Dissection was

performed in ice-cold oxygenated Krebs solution containing 4 μM nifedipine (L-type Ca^{2+} channel blocker to inhibit spontaneous muscle contraction; Sigma) and 3 μM indomethacin (to inhibit cyclooxygenase; Sigma). The dissected colon-nerve preparation was isolated and continually perfused with 31-33°C Krebs solution. The colorectum was opened longitudinally along the anti-mesenteric border and pinned mucosal side up. The PN was threaded into a separate oil-filled recording chamber and progressively teased apart into 6-10 bundles (~10 μm thick) to isolate single fibers (>3:1 signal-to-noise ratio). Recordings were made by laying bundles atop a platinum-iridium wire extracellular electrode ~100mm in diameter. Neural activity was amplified (10,000-fold; DAM80, World Precision Instruments, New Haven, CT), filtered (0.3- 10 kHz) and sampled (20 kHz) using a 1401 interface (CED) and Spike2.

An electrical search strategy was used for unbiased detection of all excitable afferent receptive endings (REs) and measurement of their electrical activation thresholds (Feng and Gebhart, 2011). All REs were tested for mechanosensitivity as follows: mucosal stroking with a fine brush producing ~0.1 mN of perpendicular force; blunt perpendicular probing (1-80 mN; 5-s duration) and uniform circumferential stretch (0-170 mN, equivalent to 45 mmHg CRD; Feng et al., 2010) applied as a ramp (5 mN/s, 34 s) or fast-step (to 80 or 170 mN in 0.2 s). Probing and stretching were performed using a servo-controlled force actuator (Aurora Scientific, Toronto, ON). Colorectal PN afferents were classified as previously described (Feng and Gebhart, 2011). Briefly, all REs responded to blunt probing except mechanically insensitive afferents (MIAs). Muscular afferents also responded to stretch, mucosal afferents also to stroking and muscular/mucosal afferents also to stretch and stroking. Serosal afferents responded only to probing. On average, 1-6 fibers were studied per mouse.

Agonists, antagonists and a sensitizing inflammatory soup (IS) were applied directly atop isolated REs (Feng et al., 2010). Inflammatory soup was composed of bradykinin, prostaglandin E2, serotonin and histamine (all at 10μM) with pH adjusted to 6.0 (Jones et al., 2005). IS was applied for 3-min followed by mechanical testing 3 min later and then every 5 min until washout (i.e., return to baseline). Channel antagonists were applied for 5-min followed immediately by mechanical testing repeated every 5 min until washout. In our hands, a 5 min inter-test interval is sufficient for full recovery of fiber response to mechanical stimulation. To inhibit TRPV1, we utilized A889425 (Abbott Laboratories, Abbott Park, IL), a highly selective competitive antagonist for the capsaicin-binding site with an IC_{50} of ~300nM (McGaraughty et al., 2008; Brederson et al., 2011). A889425 was dissolved in 1-methyl-2-pyrrolidinone (1M2P) and diluted to final concentration (1%) with Krebs solution; solvent control experiments revealed no significant effect of 1% 1M2P on fiber mechanosensitivity (see **Fig. 6** for reference). P2X3 was inhibited with TNP-ATP (Sigma), a competitive antagonist for P2X1, P2X3 and P2X2/3 with an IC_{50} of ~30nM (Gever et al., 2006). The TRPV1 agonist capsaicin (3μM in Krebs and <0.01% ethanol) and P2X agonist α,β-meATP (1mM in Krebs) (Brierley et al., 2005; Feng and Gebhart, 2011) were applied for 2-min and removed sooner if activation occurred to minimize afferent desensitization. Agonist application was followed immediately by mechanical testing as described above.

2.2.5 Statistics

Data are presented as mean ± SEM. Responses to CRD, probing and ramped stretch are presented as stimulus-response functions. For ramped stretch, stimulus-response functions are presented as binned counts during stretch (0-53, 53-113 and 113-170 mN). To compare CRD and

single fiber stimulus-response functions before and after treatment, responses at each pressure or force were normalized to the pre-treatment response at the final pressure (60 mmHg) or force (113-170 mN). Data were analyzed using one or two-way ANOVAs (Holm- Sidak post-hoc tests), paired or unpaired Student's t-tests or Fischer's exact test (SigmaPlot 9, Systat Software, San Jose, CA) as appropriate. Single fiber spontaneous activity was rare and, when present, was subtracted from stimulus-evoked activity. $P<0.05$ was considered statistically significant.

2.3 RESULTS

2.3.1 Co-expression of TRPV1 and P2X3

As detailed in **Table 2**, the majority of LS c-DRGs from C57BL/6 control mice co-expressed mRNA for TRPV1 and P2X3. The proportion of c-DRGs that expressed TRPV1 mRNA closely paralleled the proportion of neurons that responded to capsaicin (500 nM). About a third of these capsaicin-sensitive neurons also responded to α,β-meATP (100 μM). In contrast, only a minority of c-DRGs responded to α,β-meATP, but the majority of these neurons also responded to capsaicin.

Table 2: Expression of TRPV1 and P2X3 mRNA and response to channel agonists in lumbosacral colon sensory neurons. A total of 14 LS c-DRGs including one control from one preparation were tested for mRNA expression. A total of 31 LS c-DRGs from two preparations were examined for Ca^{2+}-transient responses to α,β-meATP (100 μM) followed by capsaicin (500 nM). For Ca^{2+}-imaging, only cells that initially responded to 30 mM KCl were treated with agonists.

	mRNA expression		Response to channel agonists	
	%	n	%	n
TRPV1 only	77%	(10/13)	74%	(23/31)
P2X3 in TRPV1 positive neurons	80%	(8/10)	30%	(7/23)
P2X3 only	85%	(11/13)	35%	(11/31)
TRPV1 in P2X3 positive neurons	67%	(8/11)	64%	(7/11)

2.3.2 Response to colorectal distension

C57BL/6 and TPDKO mice responded similarly to CRD (**Fig. 1A**). However, responses were significantly greater in TPDKO mice, most apparent at noxious intensities of CRD (i.e., 45 and 60 mmHg). Intracolonic treatment with saline had no effect on responses to CRD in either genotype whereas treatment with zymosan elicited significant colorectal hypersensitivity in C57BL/6 (as previously reported; e.g., Feng et al., 2010) but not TPDKO mice (**Fig. 1B-C**).

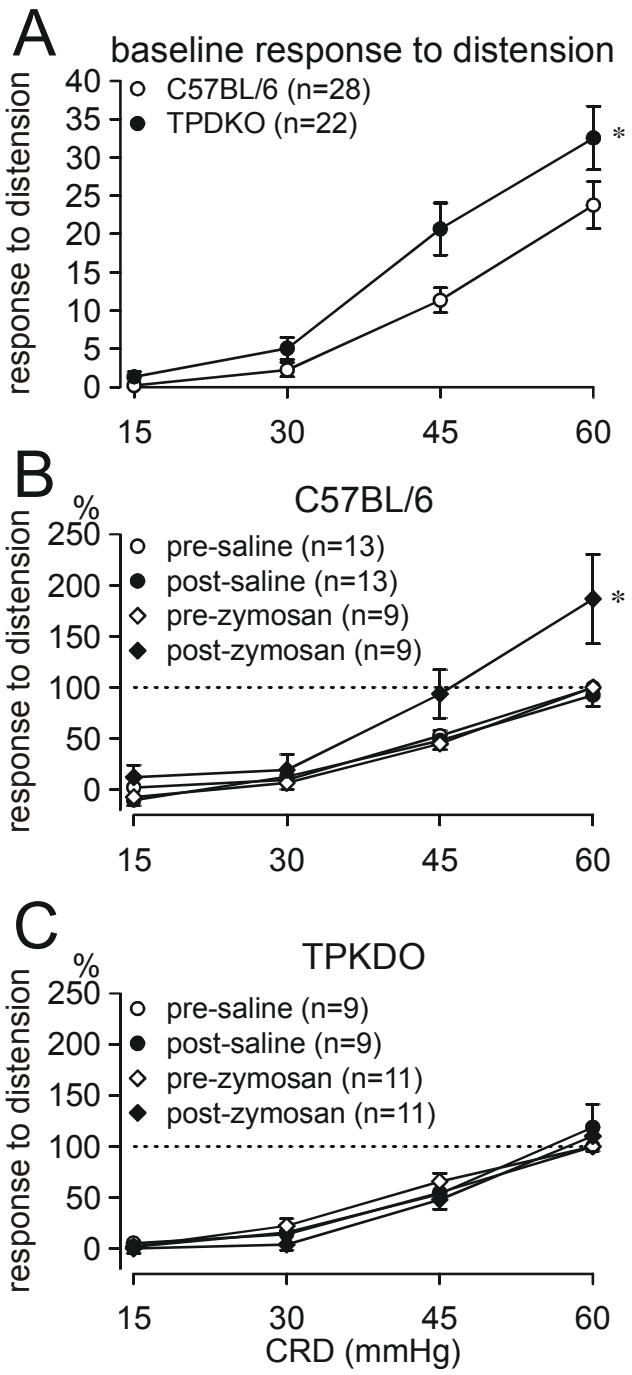

Figure 1: Visceromotor responses to colorectal distension. (**A**) Relative to C57BL/6 controls, TPDKO mice exhibited greater baseline responses to CRD ($F_{1/144}$=5.8, $p<0.05$, Holms-Sidak post-hoc tests, $p<0.01$ at 45 and 60 mmHg CRD). (**B**) Intracolonic zymosan, but not saline, produced colorectal hypersensitivity in C57BL/6 mice ($F_{1/24}$=6.4, $p<0.05$), but not in TPDKO mice (**C**). Visceromotor responses (not normalized) were used to generate stimulus-response curves in panel **A;** normalized responses were used for panels **B-C**. n, number of mice/group.

2.3.3 Characterization of colorectal afferent fibers

To explore the contributions of PN afferents to the CRD phenotype and to study in detail the effects of combined ablation of TRPV1 and P2X3 on colorectal afferent mechanosensation, we compared classes of afferents in C57BL/6 and TPDKO mice. Neither the proportions nor the electrical activation thresholds of the five classes of PN afferents differed between the two genotypes (**Fig. 2**). However, relative to control mice, there was a tendency in TPDKO mice for mucosal and muscular/mucosal afferent REs to be topographically shifted to and concentrated in the distal 1 cm of the colorectum. There were no other differences in topographical distributions of PN afferents between genotypes.

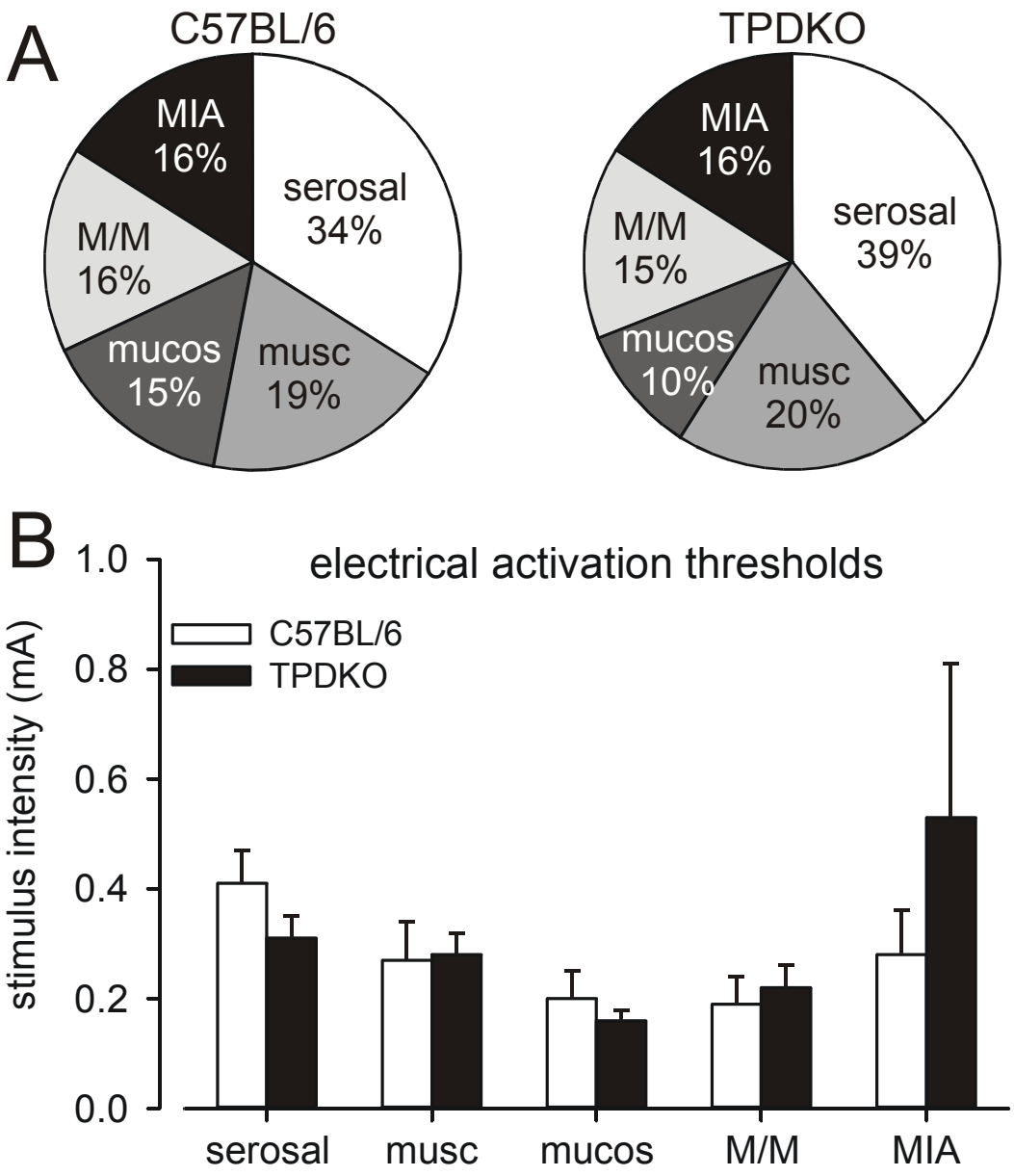

Figure 2: Proportions and electrical activation thresholds of colorectal afferent fibers. Co-deletion of TRPV1 and P2X3 (i.e., TPDKO) had no effect on (**A**) proportions or (**B**) electrical activation thresholds of PN colorectal afferents. A total 116 fibers were studied from 23 C57BL/6 mice and 102 fibers from 24 TPDKO mice. musc, muscular; mucos, mucosal; M/M, muscular/mucosal; MIA, mechanically-insensitive afferent.

Serosal and mucosal REs in both genotypes gave similar responses to probing (**Fig. 3A-B**). Likewise, there was no difference in response to probing between stretch-sensitive muscular and

muscular/mucosal afferents (**Fig. 3C-D**). As noted previously (e.g., Feng et al., 2012), responses of muscular afferents to stretch in C57BL/6 mice were generally more robust than responses of their muscular/mucosal afferent counterparts. In TPDKO mice, responses of muscular (**Fig. 4A**), but not muscular/mucosal afferents (**Fig. 4B**) to stretch were significantly attenuated relative to C57BL/6 mice. Responses to the fast-step stretch protocol did not reveal any genotype differences between muscular and muscular/mucosal afferents (**Fig. 4C-D**).

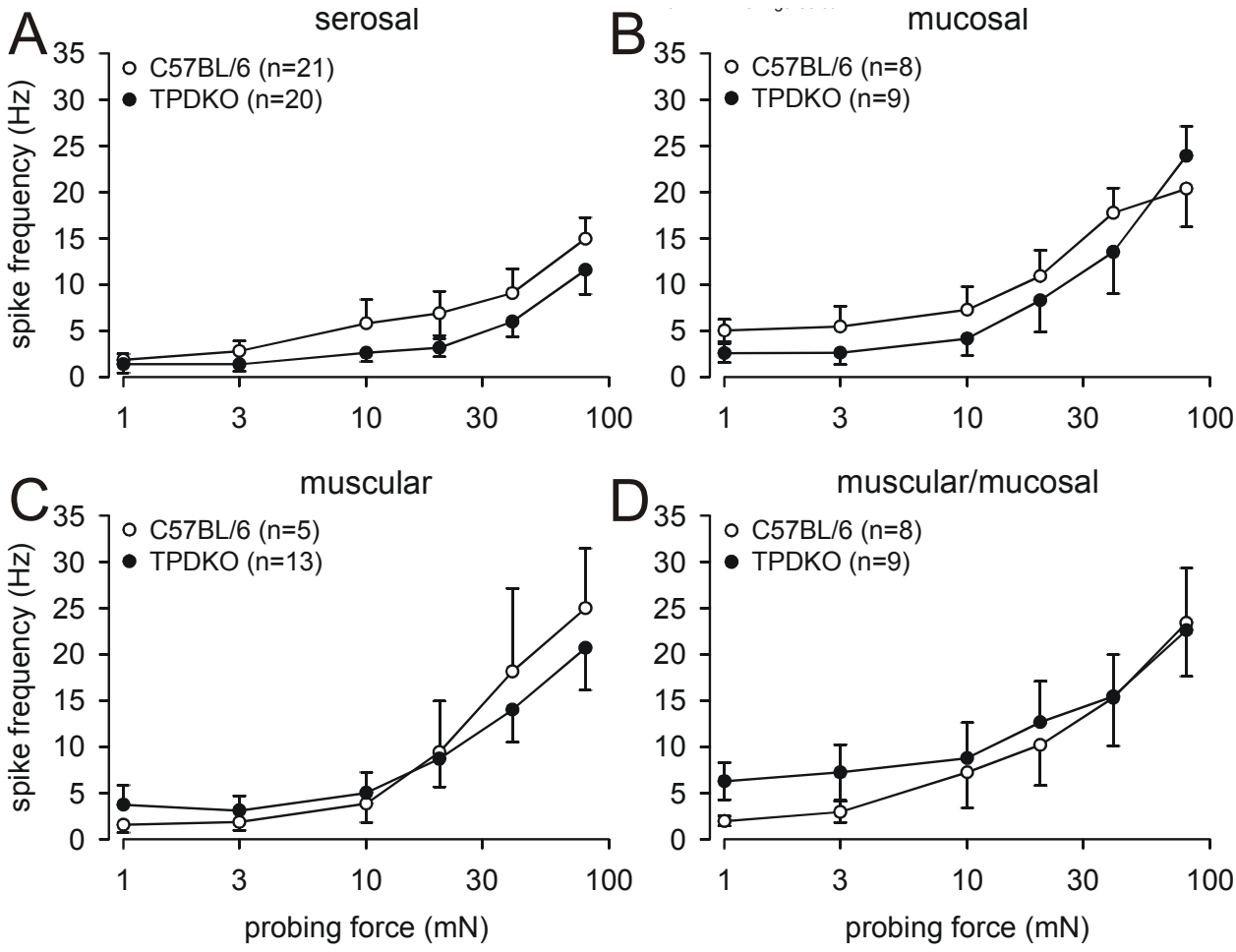

Figure 3: Responses of colorectal afferent fibers to perpendicular probing. Co-deletion of TRPV1 and P2X3 (i.e., TPDKO) had no effect on colorectal afferent responses to blunt probing of receptive endings. Stimulus-response curves are presented for serosal (**A**), mucosal (**B**), muscular (**C**) and muscular/mucosal (**D**) afferents. n, number of fibers/group.

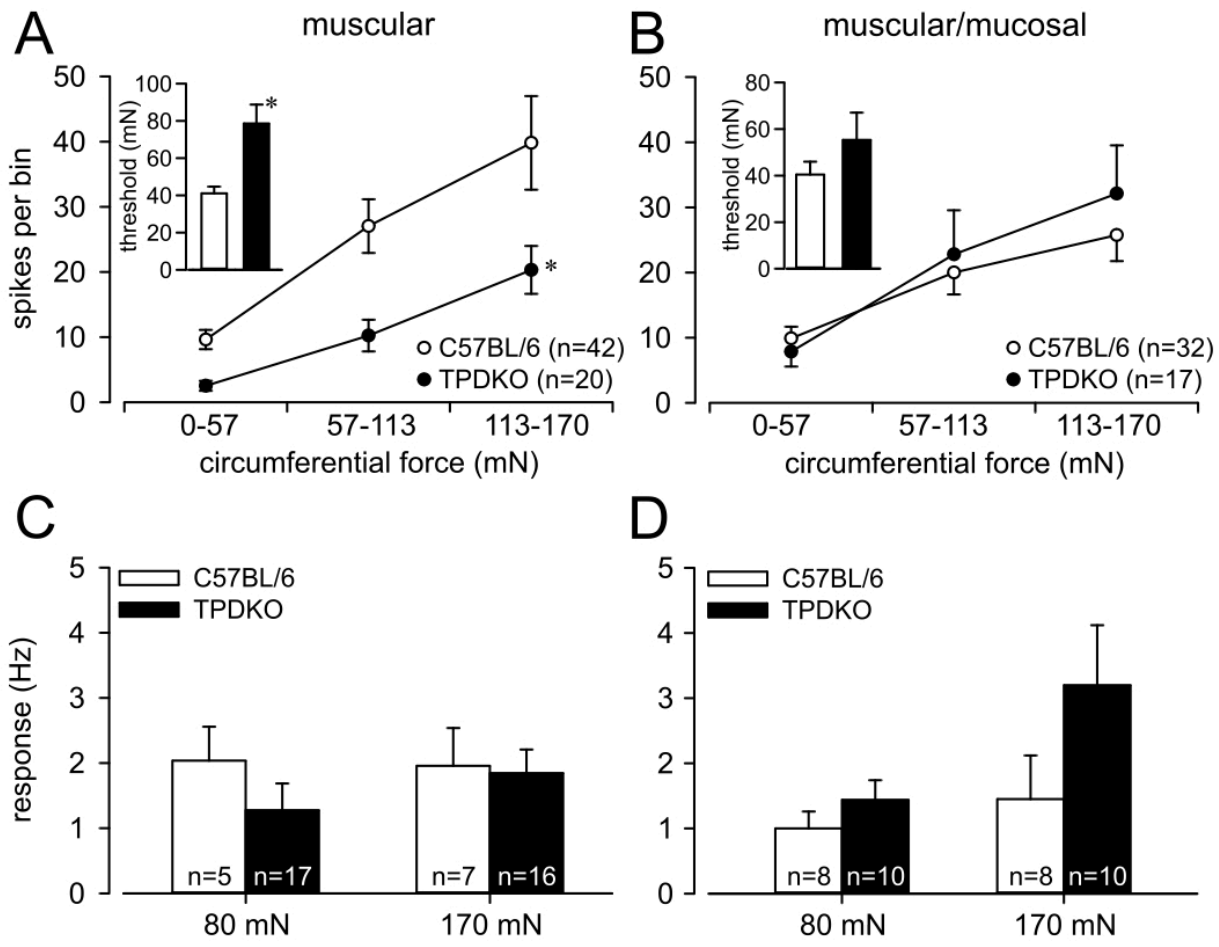

Figure 4: Responses of colorectal afferent fibers to circumferential stretch. Responses of (**A**) muscular, but not (**B**) muscular/mucosal, afferents to circumferential stretch (0-170 mN, 5 mN/sec) were reduced in TPDKO relative to C57BL/6 mice ($F_{1/180}$=11.8, p<0.001). Response thresholds (insets) were increased in muscular (t=4.3, p<0.001), but not muscular/mucosal, afferents. (**C-D**) Responses of muscular and muscular/mucosal afferents to stepped stretch (0.2 s rise time) did not differ between genotypes. n, number of fibers/group.

We next examined IS-induced sensitization of stretch-sensitive afferents. IS significantly increased stretch-response functions and decreased response thresholds (i.e., sensitized) of muscular and muscular/mucosal afferents in both C57BL/6 and TPDKO mice (**Fig. 5**). However, both muscular and muscular/mucosal afferents sensitized to a significantly lesser degree in TPDKO than C57BL/6 mice. All fibers tested, regardless of genotype, were also activated by IS,

revealing RE chemosensitivity; recovery (washout) was typically complete in both genotypes within ~20 min after IS. Single fiber recordings were also performed in TPDKO mice treated intracolonically with saline (36 fibers from 4 mice) or zymosan (26 fibers from 5 mice). There were no differences in either proportions of colorectal afferent classes or sensitization of afferent fiber responses between saline- and zymosan-treated mice (data not shown), consistent with the absence of colorectal hypersensitivity in these same mice (**Fig. 1C**).

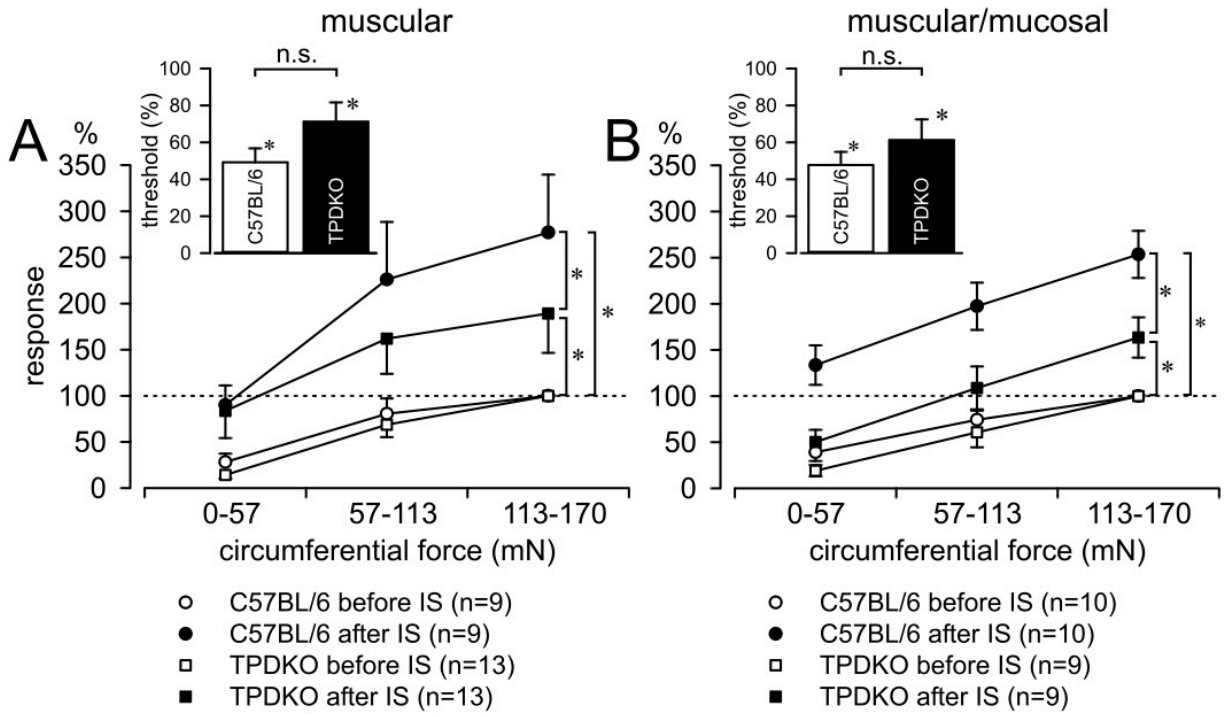

Figure 5: Inflammatory soup-induced sensitization of colorectal afferents fibers. Co-deletion of TRPV1 and P2X3 (i.e., TPDKO) attenuated IS-induced sensitization of stretch-sensitive colorectal afferents. (**A**) IS sensitized stretch-response functions of muscular afferents from C57BL/6 ($F_{1/16}$=9.5, p<0.05) and TPDKO mice ($F_{1/24}$=6.6, P<0.05). IS also decreased response thresholds (inset; n.s., not significant) in C57BL/6 (paired t-test comparing post-IS thresholds normalized to pre-IS thresholds [threshold, %]: t=6.7, p<0.001) and TPDKO mice (t=2.8, p<0.05). (**B**) IS sensitized muscular/mucosal afferents in both C57BL/6 ($F_{1/18}$=37.0, p<0.001) and TPDKO mice ($F_{1/16}$=8.6, p<0.05). Afferent response thresholds were equally decreased after IS (inset) in both C57BL/6 (t=7.3, p<0.001) and TPDKO mice (t=3.5, p<0.05). n, number of fibers/group. The magnitude of sensitization was significantly less in TPDKO mice in both muscular (**A**; p<0.05) and muscular/mucosal (**B**; p<0.001) afferents relative to the sensitization produced in afferents recorded from C57BL/6 mice.

2.3.4 Pharmacological antagonism of TRPV1 and P2X3

The efficacy of the TRPV1 antagonist A889425 varied considerably and effects on responses of stretch-sensitive afferents were not apparent at all concentrations tested; 300nM was most effective in reducing responses of muscular afferents to stretch and 3μM most effective in

reducing responses of muscular/mucosal afferents (**Fig. 6A-B**). Because of variability in responses to stretch after exposure to vehicle and because TRPV1 is not expressed in all colorectal afferents (**Table 2**), we set as an effect criterion a reduction ≥15% post-treatment in afferent response to stretch. Using this criterion (denoted by $^{>15}$ in **Figs. 6 & 7**), ~65% of both muscular (9/14) and muscular/mucosal (8/12) afferents exhibited a significant reduction in their response to stretch after treatment with 300nM and 3μM A889425, respectively. To pharmacologically probe TRPV1 expression in these experiments, we applied capsaicin (3μM) to eight of the above stretch-sensitive afferents after multiple exposures to A889425. Although washout after A889425 was apparent within ~10 min (i.e., responses to stretch returned to baseline) in the majority of afferents tested, application of capsaicin to REs failed to excite any of the REs tested, but did increase response threshold in 6/8 afferents tested. In contrast to the effect of capsaicin on response threshold, response thresholds were unaffected by A889425, with or without the effect criterion invoked (**Fig 6C-D**).

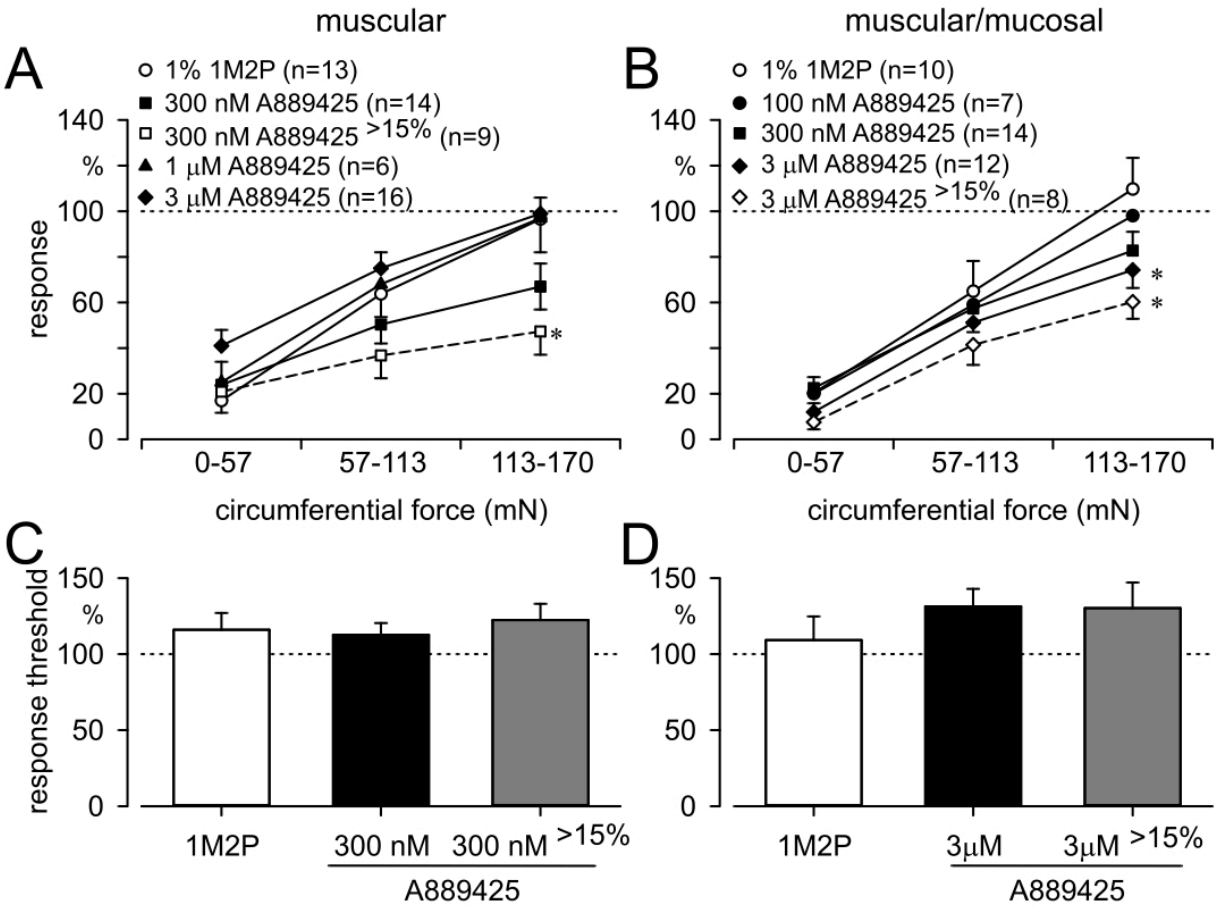

Figure 6: Antagonism of TRPV1 attenuated mechanosensitivity of stretch-sensitive colorectal afferents. (**A**) Relative to vehicle (1% 1M2P), A889425 did not significantly decrease stretch-response functions of muscular afferents at any concentration tested, although 300nM reduced responses to ~60%. Application of an effect criterion of ≥15% post-treatment reduction of stretch-responsive functions (9/14 fibers; ~65% of total) revealed significant attenuation by 300nM A889425 of response to stretch ($F_{3,120}$=8.9, p<0.01, post-hoc comparison, p<0.01). (**B**) 3μM A889425 significantly attenuated muscular/mucosal stretch-response functions relative to vehicle ($F_{3,117}$=2.9, p<0.05, post-hoc comparison, p<0.05). Application of the effect criterion as above (8/12 fibers; ~65%) similarly yielded significant attenuation by 3μM A889425 of response to stretch ($F_{3,105}$=4.8, p<0.05, post-hoc comparison, p<0.05). (**C-D**) Response thresholds of muscular and muscular/mucosal afferents, with or without application of the effect criterion, were unaffected by A889425. For statistical analyses, responses post-A889425 were compared against responses post-1M2P, which were compared against their respective pre-drug baselines. n, number of fibers/group. $^{>15\%}$ denotes application of response criterion.

Compared with vehicle (Krebs solution), 30 - 300nM, 1 and 3μM concentrations of the P2X antagonist TNP-ATP did not attenuate responses of either muscular or muscular/mucosal

afferents to stretch (**Fig. 7A-B**). Applying the same effect criterion as above, approximately 35% of both muscular and muscular/mucosal afferents demonstrated a ≥15% decrease in their stimulus-response functions after exposure to 3μM and 300nM TNP-ATP, respectively. At these concentrations, TNP-ATP significantly attenuated responses to stretch in both muscular and muscular/mucosal afferents. As above with capsaicin, we attempted to pharmacologically establish P2X expression by application of α,β-meATP after multiple exposures to TNP-ATP; 6/13 REs tested exhibited an apparent increase in response to stretch. In contrast to the TRPV1 antagonist, TNP-ATP significantly increased response thresholds of muscular/mucosal afferents (**Fig. 7D**). Antagonist washout was apparent within ~10 min in all muscular and most muscular/mucosal afferents tested.

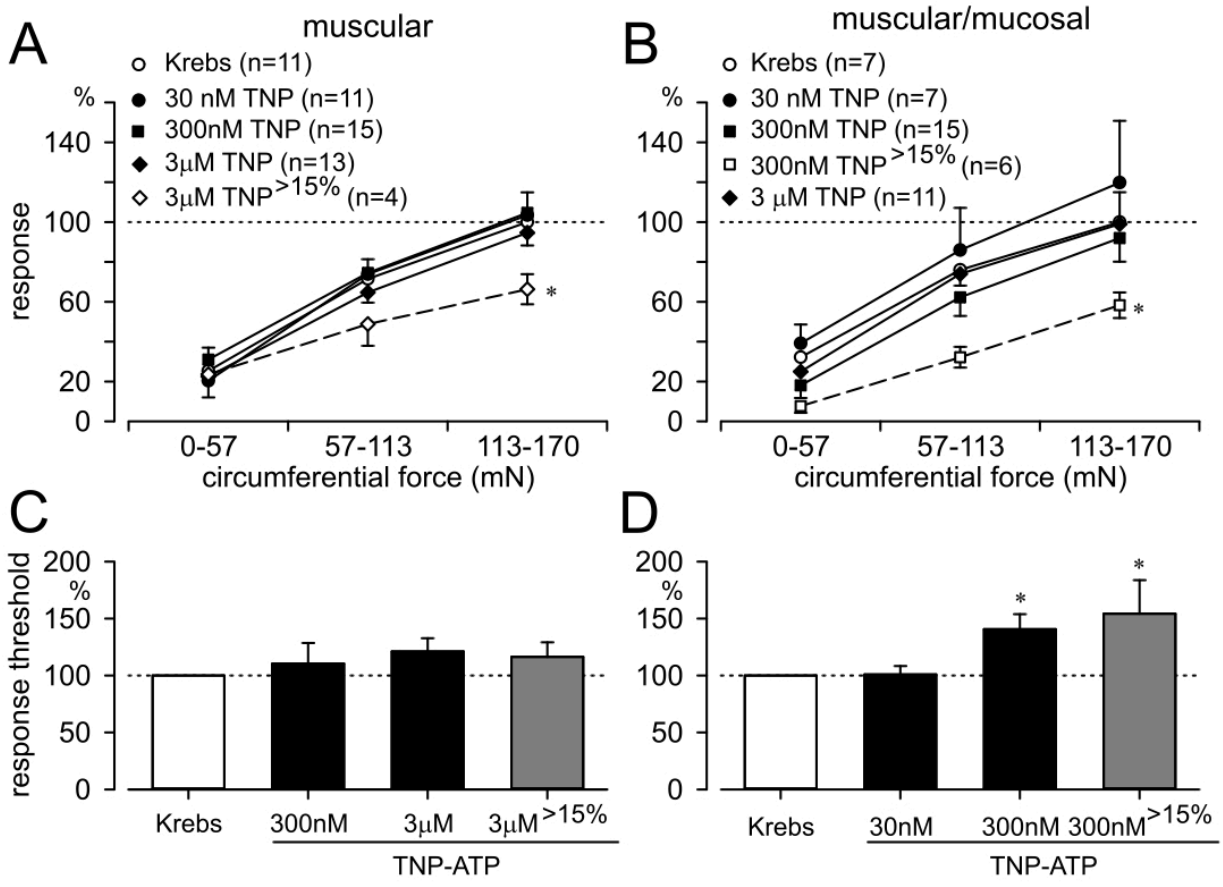

Figure 7: Antagonism of P2X3 attenuated mechanosensitivity in stretch-sensitive colorectal afferents. (**A**) Relative to pre-treatment baseline, TNP-ATP had no effect on stretch-response functions in muscular afferents; application of the ≥15% effect criterion (4/13 fibers; ~31%) revealed a significant effect at the 3μM concentration ($F_{3,111}$=2.9, p<0.05, post-hoc comparison, p<0.05). (**B**) TNP-ATP did not affect muscular/mucosal afferent stretch-response functions, but application of the effect criterion (6/15 fibers; 40%) revealed a significant effect of 300nM TNP-ATP ($F_{3,81}$=5.5, p<0.01, post-hoc comparison, p<0.05). (**C**) TNP-ATP did not affect response thresholds of muscular afferents, but (**D**) did increase response thresholds of muscular/mucosal afferents without ($F_{2,34}$=6.3, p<0.01, post-hoc comparison, p<0.01) as well as with ($F_{2,21}$=4.8, p<0.05, post-hoc comparison, p<0.05) the effect criterion invoked. n, number of fibers/group; TNP, abbreviation for TNP-ATP. $^{>15\%}$ denotes application of response criterion.

In another group of 11 stretch-sensitive afferent endings, we tested combined pharmacological antagonism with A889425 plus ATP-TNP at the most effective concentrations determined above (300nm and 3μM) and also applied the TRPV1 and P2X3 agonists capsaicin (3μM) and α,β-

meATP (1mM), respectively. Four/11 responded to both agonists, five responded to neither agonist and the remainder responded to one but not the other agonist. Interestingly, afferents responding to both TRPV1 and P2X3 agonists showed significantly higher response thresholds to stretch than those responding to neither agonist (71.5±9.1 vs. 21.1±1.6 mN, t-test, $p<0.001$). Combined antagonist application did not affect responses to stretch in those five afferents that did not respond to either agonist (**Fig. 8A**). Co-antagonism with A889425 and TNP-ATP, however, significantly attenuated responses to stretch in those four afferents that responded to both agonists (**Fig. 8B**).

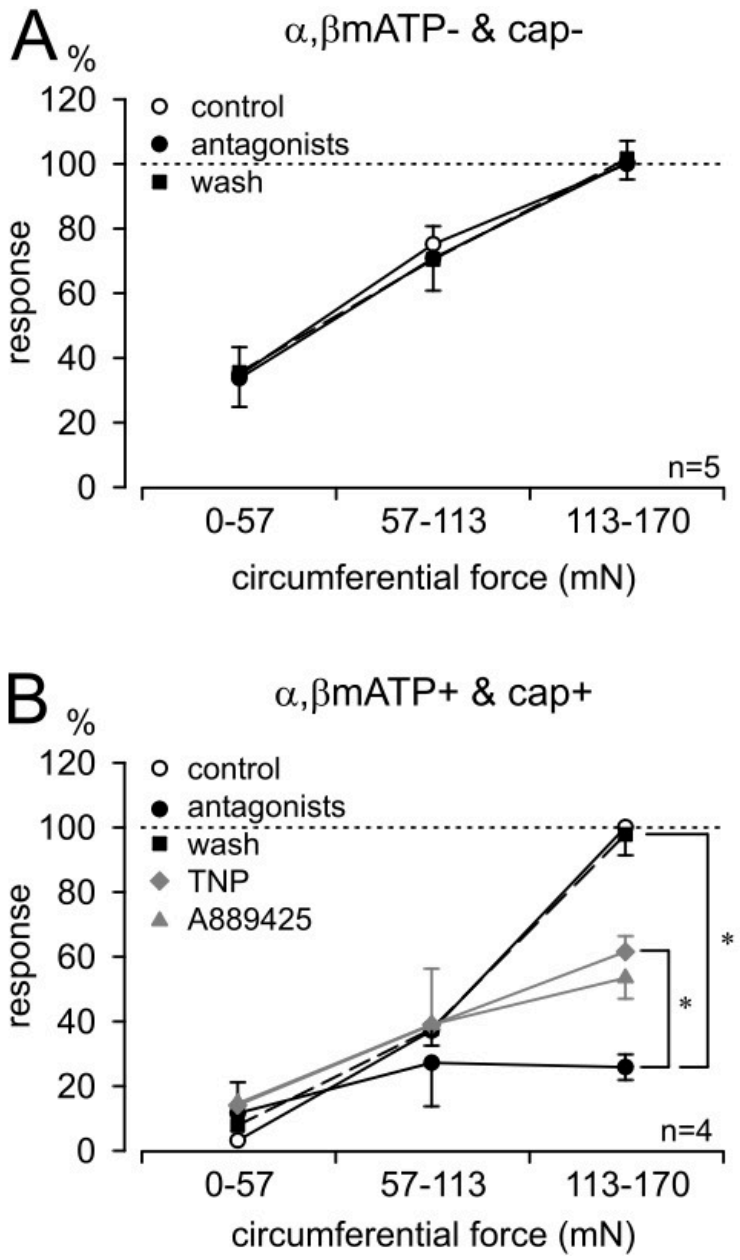

Figure 8: Combined antagonism of TRPV1 and P2X3 attenuated mechanosensitivity of stretch-sensitive muscular and muscular/mucosal colorectal afferents. (A) Combined A889425 and TNP-ATP application to afferent endings that did not respond to either capsaicin (cap) or α,β-methylene ATP (α,βmATP) was without effect on responses to stretch whereas (B) combined A889425 and TNP-ATP application to afferent endings that responded to both cap and α,βmATP significantly attenuated responses to stretch ($F_{2,27}$=7.1, P=0.003, post-hoc comparison, P=0.007 vs. control). For purposes of comparison, muscular and muscular/mucosal stretch-sensitive afferents that met the response criterion (i.e., $^{>15\%}$) were pooled from Figures 6A and B (A889425) and 7A and B (TNP-ATP) and illustrated here in half-tone. Combined antagonism produced a significantly greater attenuation in response than TNP-ATP alone ($F_{1,36}$=7.7, $p<0.01$) and a lower response than A889425 alone (borderline significant, $F_{1,57}$=3.6, P=0.06).

2.4 DISCUSSION

In agreement with previous studies in rat lumbar DRG neurons (Guo et al., 1999; Ueno et al., 1999; Stanchev et al., 2009), the present work demonstrates that a substantial proportion of mouse PN colorectal afferents co-express TRPV1 with P2X3. Previously, we reported deficits in colorectal mechanosensation and sensitization with single deletion of either TRPV1 or P2X3 (Jones et al., 2005; Jones et al., 2007; Shinoda et al., 2009). Given the co-expression reported here and elsewhere, we expected similar, if not greater, effects in TRPV1-P2X3 double knockout mice. As expected, colorectal hypersensitivity did not develop in TPDKO mice, consistent with the absence of afferent fiber sensitization in zymosan-treated mice and the significantly reduced sensitization by IS of stretch-sensitive afferents. Unexpectedly, basal responses to CRD were greater in TPDKO than C57BL/6 mice despite significantly reduced responses of muscular afferents to stretch and unchanged responses of other afferent classes in TPDKO mice, suggesting enhanced central processing. Greater mechanosensation may be due to disinhibition or compensatory overexpression of ion channels such as TRPV4 or TRPA1 in the peripheral and/or central terminals of primary afferents or in second order spinal neurons.

Of the two channels studied, P2X3 may be most contributory to the greater response to CRD in TPDKO mice because P2X3 single knockout mice have been reported to exhibit pro-nociceptive behaviors, including enhanced avoidance of noxious thermal stimuli (Shimizu et al., 2005). An alternate explanation is the redistribution we noted in TPDKO mice of mucosal and muscular/mucosal afferent endings to the distal 1 cm of the colorectum. In our experience, greater visceromotor responses to CRD are evoked with more distal positioning of the distension balloon. In support, PN afferents in the distal colorectum exhibit greater mechanosensitivity than their more proximal counterparts (Feng et al., 2010). Because the proportions of each class of

afferent were the same in both genotypes, this caudal transposition in TPDKO mice was unlikely a result of conversion of one class of afferent into another. In support, there were no significant differences between genotypes with respect to probing- response functions of any type of afferent.

Co-deletion of TRPV1 and P2X3 reduced responses of muscular afferents to stretch, but had no effect on muscular/mucosal afferents. Responses of muscular and muscular/mucosal afferents to stretch in P2X3-null mice did not differ from responses in C57BL/6 mice (Shinoda et al., 2009), suggesting that central mechanisms contributed to the reduced responses to CRD noted in these mice. In contrast, responses of muscular/mucosal afferents to stretch in mice lacking TRPV1 were significantly attenuated relative to C57BL/6 mice; responses of muscular afferents were ~twice those of control, but not statistically significant (Jones et al., 2005). Collectively, it appears that deletion of either TRPV1 or P2X3 alone does not alter net peripheral input from PN afferents in response to stretch whereas co-deletion of both channels results in a net decrease in peripheral input from stretch-sensitive afferents.

The TRPV1 antagonist A889425 significantly attenuated responses of muscular and muscular/mucosal afferents to stretch in C57BL/6 mice. Consistent with previous (Christianson et al., 2006) and current (**Table 2**) reports of TRPV1 expression in 60-70% of mouse colorectal afferents, A889425 was not uniformly effective on all stretch-sensitive afferents. Baseline mechanosensitivity of A889425-sensitive afferents did not differ from A889425-insensitive afferents, in contrast to a previous report of higher mechanical thresholds in TRPV1-expressing colorectal afferents (Malin et al., 2009). TNP-ATP was not as broadly effective as A889425 on stretch-sensitive afferents; fewer afferent responses met the effect criterion (≥15% reduction), but those that did exhibited attenuated responses to stretch. TNP-ATP did increase response

thresholds in muscular/mucosal afferents, supporting the view that P2X3 and P2X2/3 are important primarily for the initiation, not maintenance, of nociception (Hamilton et al., 2000). In support of a greater role of P2X3 in muscular/mucosal versus muscular afferent mechanosensation and the P2X3 expression proportions reported here (**Table 2**) and elsewhere (Brierley et al., 2005; Shinoda et al., 2010), ~30% of muscular and ~45% of muscular/mucosal afferents were activated by α,β-meATP. Similar proportions of muscular (~30%) and muscular/mucosal (40%) afferents met the TNP-ATP effect criterion.

Combined antagonism of both channels significantly attenuated responses to stretch in afferents that responded to both agonists, but not responses of those afferents that did not respond to both agonists. The results suggest an additive if not greater than additive attenuation of responses to stretch by simultaneous antagonism of both channels. This functional interaction may be mediated through common Ca^{2+}-dependent signaling cascades (Docherty et al., 1996; King et al., 1997; Rosenbaum et al., 2004; Xu and Huang, 2004; Price et al., 2005; Tsuda et al., 2007) or modulatory conformational spread (Bray and Duke, 2004) *via* direct physical association of TRPV1 and P2X3 as demonstrated previously (Stanchev et al., 2009).

To further explore the contributions of TRPV1 and P2X3 to colorectal afferent mechanosensation, we evaluated in TPDKO mice acute sensitization of stretch-sensitive afferents by IS. In TRPV1- and P2X3-null mice, IS sensitized muscular/mucosal, but not muscular afferents (Jones et al., 2005; Shinoda et al., 2009). In TPDKO mice, however, both afferent classes were sensitized by IS, although the magnitude of sensitization was significantly reduced relative to sensitization in C57BL/6 mice. Differences in outcomes between single and double knockout mice may be explained by compensatory changes in protein expression. In muscular/mucosal afferents, sensitization may cause upregulation or enhanced functioning of

TRPV1 and P2X3 (Hamilton et al., 1999) as well as other ion channels. Therefore, if only one channel is deleted, functioning of the remaining channel may be sufficiently enhanced after sensitization such that no loss-of-function phenotype occurs. Only when both channels are eliminated is a reduction in stretch-response functions manifest, and even then sensitization is still present. In muscular afferents, on the other hand, deletion of either or both TRPV1 and P2X3 is sufficient to produce a loss-of-function phenotype, suggesting that both channels are critical for mechanical sensitization in this class of afferent.

We made several additional important observations. First, responses of muscular afferents to stretch were significantly attenuated in TPDKO mice, but responses to stepped stretch or probing were not. This suggests a stimulus-specific contribution of TRPV1 and/or P2X3 to afferent mechanosensation. It is possible that only slow-onset stimuli recruit these channels because this allows sufficient time for biochemical generation of ATP and endogenous lipid ligands of TRPV1 (Hwang et al., 2000; Scotland et al., 2004; Patwardhan et al., 2009). Second, TRPV1 and P2X3 appear to be more important for mechanosensation/sensitization in muscular/mucosal afferents than in other colorectal afferent classes. Coincidently, zymosan-induced hypersensitivity is associated with sensitization of muscular/mucosal afferents (Feng et al., 2012). Thus, TRPV1 and P2X3 may be important in the development and/or maintenance of zymosan-induced colorectal hypersensitivity and, by extension, IBS pain. Third, deletion of both channels abolished zymosan-induced recruitment of MIAs, which has been observed in C57BL/6 mice (Feng et al., 2012). Considering that very few colorectal PN MIAs respond to capsaicin (Feng and Gebhart, 2011), our observation in TPDKO mice suggests that P2X3 may be important for mechanical sensitization of MIAs.

In summary, the present study confirms the importance of TRPV1 and P2X3 for

colorectal mechanosensation and hypersensitivity at the levels of the whole organism and primary afferent. By evaluating inhibition of both channels simultaneously, this work also contributes to the growing appreciation of the functional interaction between distinct ion channels, which is not uncommon (e.g., TRPV1-TRPA1, Staruschenko et al., 2010, and P2X3-$GABA_A$,Toulme et al., 2007) and may prove important for guiding drug development. With regard to limitations, the single fiber work focused exclusively on PN colorectal afferents to build on previous studies in this same afferent population (Jones et al., 2005; Jones et al., 2007; Shinoda et al., 2009). Although the PN is necessary and sufficient for mediating colorectal nociception (Kyloh et al., 2011), the other source of extrinsic colorectal innervation – the lumbar splanchnic nerve (LuSN) – was not evaluated here. Because the LuSN pathway may be relevant for chemosensation (Brierley et al., 2005) and/or central sensitization (Traub, 2000), a TRPV1-P2X3 interaction in LuSN afferents may reveal unexpected contributions to mechanosensation. The results in TPDKO mice are generally supportive of previous studies in single knockout mice as well as pharmacological antagonism. Differences in outcomes between single and double knockout mice could be due to functional redundancy of TRPV1 and P2X3 in some afferents and compensation in others. The relative importance of these channels appears to be enhanced in hypersensitivity, suggesting their importance in colorectal mechanosensation and the potential utility of combined pharmacological antagonism as a treatment for IBS pain and hypersensitivity.

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