Electrophysiological characterization of mouse pelvic nerve afferent activity in response to mechanical and chemical stimulation of the colon

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Abstract

Functional gastrointestinal disorders are characterized in part by hypersensitivity to distention of the distal colon and rectum, causing normally non-noxious stimuli to be perceived as painful. This feature suggests abnormalities in primary afferent neurons, specifically those of the pelvic nerve (PN). The goal of this work was to become proficient in single-fiber electrophysiological characterization of mouse PN primary afferents using an in vitro colon preparation coupled with a novel electrical search strategy, which allowed for unbiased characterization of all excitable receptive endings (REs) including those of mechanically insensitive afferents (MIAs). Using previously established criteria, mechanosensitive fibers were classified as mucosal, muscular, muscular/mucosal, and serosal based on their responses to three distinct mechanical stimuli: probing (0.4, 1.0, and 1.4 g), circumferential stretch (>2 mm), and mucosal stroking (10 mg). Located MIAs were tested for mechanical sensitization by exposing their REs to inflammatory soup and capsaicin. The proportions of afferent subtypes among the 39 PN fibers investigated were as follows: serosal (33%), muscular/mucosal (23%), muscular (26%), mucosal (10%), and MIA (8%). These proportions do not entirely match previous data. Also differing from previous investigations was the topographic distribution of afferents. Despite these discrepancies, which are most likely associated with limited sample size, the present study largely confirms previous findings, thus demonstrating the reliability of the experimenter with this particular technique. In addition to confirming the remarkable detail and fidelity in which mechanical events in the colon are encoded and transmitted, this project establishes the foundation for future work investigating the role of specific gene products in sensory function and the changes which may occur in peripheral innervation in pathophysiological states such as inflammatory/irritable bowel syndrome.
Introduction

Functional gastrointestinal disorders are characterized in part by hypersensitivity to distention of the distal colon and rectum, causing normally non-noxious stimuli to be perceived as painful (Ritchie, 1973; Camilleri, 2001). In the case of Irritable Bowel Syndrome (IBS), which is the most common of these disorders with a worldwide prevalence of over 7% (Brandt et al., 2009), hypersensitivity to mechanical distention is reported in over 90% of patients (Bouin et al., 2002). While psychosocial factors undoubtedly play a role in the manifestation of hyperalgesia, functional neuroimaging studies in patients with IBS have ruled out a purely higher cortical etiology (Verne et al., 2003). These features of IBS, together with an absence of overt colon pathology or any apparent biochemical or inflammatory etiology, suggest abnormalities at the level of the primary afferent neuron and/or spinal cord.

Mechanical, chemical, and thermal information from the distal colon and rectum is transmitted to the spinal cord through two distinct anatomical pathways: (1) the lumbar splanchnic nerve (LSN) terminating in the thoracolumbar spinal cord and (2) the pelvic nerve (PN) ending in the lumbosacral spinal cord. Previous studies in the cat and rat identified mechanosensitive afferents in both of these pathways, mostly in the form of small-diameter, unmyelinated C-fibers (Blumberg et al., 1983; Sengupta & Gebhart, 1994). These studies also found important differences in the nature and sensitivity of the mechanosensory information encoded by these two nerves. A classification system was developed from work in the rat and mouse, in which afferents are labeled as mucosal, muscular, muscular/mucosal, serosal, or mesenteric depending on their mechanosensory profiles, with mesenteric and muscular/mucosal afferents being exclusive to the LSN and PN, respectively (Lynn & Blackshaw, 1999; Brierley et al., 2004). In the first comprehensive study of the mouse distal colon and rectum, both nerves
were found to contain mechanosensitive fibers, but with major differences in the proportions of each afferent class, the locations of their receptive endings, their mechanical thresholds, and their adaptation profiles (Brierley et al., 2004).

Although mechanosensitive afferents have been most thoroughly studied, the viscera are also innervated by silent nociceptors, which are electrically excitable but insensitive to mechanical stimulation. First documented in the medial/posterior articular nerve innervating the knee joint of the cat (Schaible & Schmidt, 1983; Grigg et al., 1986), these mechanically insensitive afferents (MIAs), as they were later designated, were found to become spontaneously active and mechanosensitive upon experimentally-induced inflammation. MIAs are thus hypothesized to be primary afferents which normally do not respond to or have very high thresholds for mechanical stimulation (Handwerker et al., 1991), but which can become active and sensitized in pathophysiological and/or inflammatory settings where they are thought to contribute to the development of hypersensitization and ultimately hyperalgesia. It has thus been proposed that the altered bowel sensations in IBS arise in part because MIAs in the afferent innervation become activated and sensitized to mechanical stimulation.

The goal of this work was to become proficient in single-fiber electrophysiological characterization of mouse PN primary afferents using a previously established in vitro colon preparation coupled with a novel electrical search strategy, which allows for the identification of MIAs. Mechanosensitive afferents were categorized by their responses to three distinct forms of mechanical stimulation: probing (0.4, 1.0, and 1.4 g), circumferential stretch, and mucosal stroking (10 mg). Located MIAs were tested for mechanical sensitization by exposing their receptive endings to inflammatory soup and capsaicin. Because much of this work has already been done in previous investigations, the focus of this project at this stage was more
methodological than analytical, with the ultimate goal being to acquire the skills necessary for independent use of this technique with all up-to-date modifications.

**Methods**

All protocols were performed in compliance with the Guide for the Care and Use of Laboratory Animals (NIH) and were approved by the Institutional Animal Care and Use Committee (University of Pittsburgh).

*Animal sacrifice and dissection of colon and its nerve innervation*

Wild-type male C57/BL/6 mice (6-8 weeks old, 20-30 g, Taconic, Germantown, NJ) were euthanized by CO2 inhalation followed by exsanguination through perforation of the right atrium. As described previously (Brierley et al., 2004), a StereoZoom 7 dissection microscope (Leica Microsystems, Bannockbum, IL) was used to remove the distal 3 cm of the colon along with the major pelvic ganglion, the PN, and both of its contributing roots, L6 and S1. Dissection was performed in 2-3 hours in freshly-prepared bubbled (95% O2, 5% CO2) ice-cold modified Krebs solution (118 mM NaCl, 4.7 mM KCl, 25 mM NaHCO3, 1.3 mM NaH2PO4, 1.2 mM MgSO4(H2O)7, 2.5 µM CaCl2, 11.1 mM D-glucose, 2 mM sodium butyrate, and 20 mM sodium acetate). Also included in the solution were the L-type Ca2+ channel antagonist nifedipine (4 µM) and the prostaglandin synthesis inhibitor indomethacin (3 µM), which were used to suppress smooth muscle activity and block inhibition of afferent activity, respectively. After completion of dissection and nerve isolation, the colon was opened longitudinally on the dorsal side just lateral to the mesenteric border.

*Preparation of tissue and equipment for electrophysiological recording*
The dissected and opened colon was transferred to a custom-built organ chamber made from clear acrylic and Sylgard (Dow Corning, Midland, MI). The organ was pinned flat, with the luminal surface facing up. The PN and major pelvic ganglion were gently extended from the organ chamber into a separate recording chamber through a small “mouse hole,” which served as the only connection between the two otherwise isolated compartments. Within the recording chamber, the PN and its two roots were laid onto a plain glass cover slip. The recording chamber was then filled with paraffin oil (Fisher Scientific, Pittsburgh, PA), while the organ chamber was continuously perfused with oxygenated Krebs warmed to ~33°C. Using fine forceps, the roots and trunk of the nerve were cleaned of any residual tissue, followed by removal of the perineurium.

Electrical signals generated by afferents were amplified (x10,000) using a low-noise AC-coupled DAM80 differential amplifier (World Precision Instruments, Sarasota, FL). The signal was filtered between 0.3 and 10 kHz, sampled at 20 kHz, fed into a MicroCED1401-II acquisition unit (Cambridge Electronic Design Limited, Cambridge, UK), and stored on a PC. The amplified signal was also sent to a Grass AM10 audio monitoring system (Astro-Med, West Warwick, RI). Action potentials were analyzed off-line using Spike2 (version 5.21) and discriminated as single units on the basis of waveform, amplitude, and duration.

*Fiber splitting and afferent identification using electrical stimulation*

Using custom-sharpened fine forceps, the S1 nerve root was teased apart into four daughter bundles. Each bundle was then placed onto a platinum-iridium recording electrode and individually tested for the presence of fibers by stroking the mucosal surface of the colon with a brush of sufficient stiffness (>2 g) capable of activating all types of mechanosensitive afferents. Following the initial splitting of the S1 root, each daughter bundle was split further to increase
the signal-to-noise ratio and to minimize the chance of overlapping REs. The smallest bundle size achieved was 5-10 microns—further splitting was not possible due to the presence of Remak bundles.

All subsequent daughter bundles were tested for electrically excitable receptive endings (REs) using an electrical search strategy in which current was applied systematically along the entire length of the distal colon by a round-tipped concentric bipolar electrode (FHC, Bowdoin, ME). Current was delivered in pulses of 0.5 ms duration and 0.3 Hz frequency. The tip of the electrode was positioned perpendicular to the mucosal surface. The electrode was attached to a micromanipulator by a custom-built compliant bridge so that the electrode imposed only slight mechanical force on the tissue (<100 mg). A suprathreshold stimulus current (10 mA) was used to identify all afferents within a 3 mm diameter circular area of the electrode tip. Because this current magnitude is capable of exciting axons anywhere along their length, once an afferent was identified, electrode position was carefully adjusted to localize the RE, which was the site of activation requiring minimum stimulus intensity. To avoid including neurons other than primary afferents, endings with thresholds greater than 3 mA were discarded.

**Afferent characterization by mechanical stimulation**

Once identified and localized, REs were tested as described previously (Brierley et al., 2004) using three distinct mechanical stimuli: fine mucosal stroking with a calibrated nylon monofilament (10 mg), perpendicular punctate probing with three different von Frey probes (0.4, 1.0, and 1.4 g), and circumferential stretch (>2 mm, or ~25% of resting colon circumference) applied uniformly using a custom-build claw attached to the colon at millimeter intervals. Force, displacement, and rate of stretch were not quantified in this study; longitudinal stretch was also not tested.
Chemical activation and sensitization of MIAs

If a primary afferent detected by electrical stimulation was found to be insensitive to mechanical stimulation, it was tested for chemical activation and sensitization with inflammatory soup (IS) and capsaicin (Cap). Bronze square tubing (1 cm height, 4x4 mm area) was placed over the RE of interest and the Krebs solution within it was replaced with 167 µL of acidic IS. After three minutes of exposure, the IS and bronze tubing were removed and the RE was tested for spontaneous activity and response to probing (1.4 g), mucosal stroking, and circumferential stretch as performed for mechanosensitive fibers. If the fiber remained silent, it was subsequently exposed to 167 µL of Cap for 3 minutes and tested for activation/sensitization following the same procedure as described above and in a previous study (Brierley et al., 2005). If the fiber was sensitized, mechanical stimulation was retested twice, 15 and 30 minutes later. Following this mechanical sensitization protocol, all afferents regardless of sensitivity were retested with electrical stimulation to verify the viability of the RE.

IS was composed of 5-HT, histamine, bradykinin, and prostaglandin E₂ dissolved in dimethylsulfoxide and diluted in freshly oxygenated Krebs to a final concentration of 10 µM for all mediators (Kessler et al., 1992). The pH of IS was adjusted to 6.0 using HCl. Cap was prepared by dissolving capsaicin powder in ethanol and diluting the solution in freshly oxygenated Krebs solution to a final concentration of 3 µM. pH of Cap was neutral. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).
Results

Localization of afferent receptive endings using electrical stimulation

As described above and illustrated in Figure 1, receptive endings (REs) were identified using an electrical search strategy in which the colon surface was broadly scanned for fibers using suprathreshold electrical stimulation (3-10 mA) followed by pinpoint localization using progressively smaller currents. At a distance greater than 3 mm from the RE, even maximum 10 mA stimulation failed to evoke action potentials unless the electrode was positioned near the axon. Using this technique, a total of 39 PN afferents were identified and studied. The use of varying currents enabled the measurement of electrical thresholds for activation. Thresholds of PN afferents ranged from 0.2 to 1.2 mA, with no obvious differences among the afferent subtypes (data not shown). Firing latencies were consistent per afferent and ranged between 30-100 msec. Both of these findings largely agree with previous work (Feng & Gebhart, 2009). Conduction velocities were not quantified.

Categorization of mechanosensitive afferents

Using previously published criteria (Lynn & Blackshaw, 1999; Brierley et al., 2004), electrically identified colonic afferents were categorized into four mechanosensitive classes (mucosal, muscular, muscular/mucosal, and serosal) and one mechanically-insensitive class (MIA). As illustrated in Figure 2, all mechanosensitive fibers were reproducibly excited by punctate von Frey probing (0.4, 1.0, and 1.4 g). Moreover, responses were graded such that firing rate was highest with 1.4 g and lowest with 0.4 g. Mucosal fibers additionally responded to stroking (10 mg) while muscular fibers responded to circumferential stretch (>2 mm). Fibers responding only to probing were classified as serosal while fibers responding to all three forms of stimulation were labeled as muscular/mucosal. Response to probing and stretch tapered to
pre-stimulus levels over the course of 10-20 seconds (data not shown). The majority of
mechanosensitive afferents were silent at rest. Only 1 of 39 total fibers exhibited spontaneous
activity in the absence of mechanical stimulation. When probed, this single fiber responded with
an accelerated burst of activity (data not shown). It was not uncommon, however, to observe
spontaneous activity without identifiable REs. If the amplitude of a spontaneously firing fiber
resembled and/or interfered with any primary afferents in the bundle being studied, further
splitting was performed to eliminate this problematic fiber. The majority of fibers were
associated with a single RE. Although no formal post-hoc analysis was performed, 2 of the 39
fibers studied were apparently associated with two anatomically distinct REs which had identical
mechanosensory profiles and spike amplitudes. Spike frequencies, stimulus thresholds, and
adaptation profiles were not quantified.

*Population and topographic distribution of afferent subtypes*

As demonstrated in Figure 3A, the proportions of afferent subtypes among the 39 PN
fibers studied were as follows: serosal (33%), muscular/mucosal (23%), muscular (26%),
mucosal (10%), and MIA (8%). These proportions do not match previous data (Feng & Gebhart,
2009); specifically, the proportion of mechanically-insensitive fibers was unusually small in this
study (Fig. 3B). Afferent REs were detected throughout the distal 2.4 cm of the colon including
the rectum and anal canal. They generally clustered along the mesenteric attachment, opposite
the side that was cut longitudinally to open the colon and near the PN innervation zone (Fig. 3C).
In contrast, previous investigations (Brierley *et al.*, 2004; Feng & Gebhart, 2009) found fibers
along the entire width of the distal colon, with no particular preference for one side or the other.
In agreement, however, was an absence of any obvious pattern in the topographic distribution of
specific afferent subtypes.
Chemical activation and sensitization of MIAs

Afferents detected electrically but devoid of mechanosensitivity were labeled as MIAs and tested for chemical activation/sensitization by sequential application of inflammatory soup (IS) and capsaicin (Cap) for 3 minutes each, as diagramed in Figure 4. In the limited sample size tested, application of IS or Cap never triggered spontaneous activity (Fig. 5A). However, IS and Cap successfully sensitized MIAs to 1.4 g von Frey probing (Fig. 5B, C). Interestingly, MIAs were never sensitized to any form of stimulation other than probing. Sensitization usually persisted for 15 minutes after chemical washout (Fig. 5D) and disappeared entirely after 30 minutes (Fig. 5E).

Discussion

Study objectives and evaluation of progress

The objective of this work was to master single-fiber electrophysiological characterization of mouse primary afferents using a previously established in vitro colon preparation coupled with a novel electrical search strategy. Because of time limitations and the technical complexity, the focus of this work was limited to reliable and reproducible characterization of the various afferent subtypes in the PN. Reliability was evaluated in three ways: (1) quantitative comparison with previous results; (2) qualitative assessment of the ability of the experimenter to consistently record well isolated fibers with at least 3:1 signal-to-noise ratios; and (3) acquirement of sufficient knowledge and practical experience to perform this technique independently and/or develop it further as future work dictates.

As is evident from the Results section, mastery of this technique was not fully achieved. The possible reasons for this are discussed below. Regarding the learning process itself, training
proved longer than anticipated, although admittedly this sort of work takes several months for beginners to learn, as noted in a recent review article by the Reeh group (Zimmermann et al., 2009). Training was additionally hindered by several, possibly avoidable technical hurdles including poor-quality recording electrodes, limited magnification of the microscope, and complications resulting from design flaws in the recording chamber. In spite of these obstacles, after two months and close to 35 preparations, significant improvements were made—the experimenter is now capable of consistently recording high quality, well-isolated fibers in record time. Another accomplishment was a marked reduction in the instances of spontaneously firing fibers, which initially occurred in the majority of preparations and greatly hindered afferent characterization. Because this spontaneous firing was usually not associated with detectable REs, it was presumed to be the result of nerve damage stemming from overly forceful nerve splitting and fiber teasing.

Localization of afferent receptive endings using electrical stimulation

As discussed in the Introduction and Methods sections, the present study employed a novel search strategy that allowed unbiased detection of all excitable REs, including those of MIAs. This approach differs from the previously used mechanical brushing technique, in which fibers were identified by systemically stroking the luminal surface of the colon with a brush of sufficient stiffness capable of activating all fibers types. In addition to completely missing MIAs, this brushing strategy potentially introduced bias towards low-threshold mechanosensitive afferents as brush stiffness needs to be minimized to avoid injuring the colon by repeated stimulation. Another advantage of the electrical search strategy is the ability to add an additional level of characterization to the study of primary afferents, namely their electrical threshold for activation. In the present work, thresholds ranged from 0.2-1.2 mA. This largely agrees with
recent work in the Gebhart lab which found 97% of afferents to have stimulus thresholds less than 1 mA (Feng & Gebhart, 2009).

**Categorization of mechanosensitive afferents**

As detailed in Figure 2, four types of PN mechanosensitive afferents were identified (mucosal, muscular, muscular/mucosal, and serosal), each of which was tuned to a unique type, magnitude, and duration of mechanical stimulation. Consistent with previous classifications (Lynn & Blackshaw, 1999; Brierley *et al.*, 2004), serosal afferents were activated optimally by probing of their receptive fields. As discussed by Brierley *et al.* (2004), the function of serosal afferents is unclear because their stimulus thresholds exceed physiologic levels. It was thus proposed that serosal afferents signal sharp pain at the onset of spasm or distention during rapid transit of contents or experimental balloon inflation. Muscular afferents were activated by both probing and circumferential stretch. These fibers were hypothesized to detect small changes in intraluminal pressure and encode colonic distension well into the noxious range. Investigating their adaptation profiles in detail, mucosal fibers were found to be exclusively tonic-type, with firing persisting throughout the entire stimulus duration. This feature was confirmed in all 20 muscular fibers characterized in this study. Muscular/mucosal fibers, which were activated by all three forms of mechanical stimulation, were postulated to play a specialized role in the detection of rapidly moving boluses (Page & Blackshaw, 1998; Blackshaw & Gebhart, 2002). Mucosal afferents, which responded only to probing and stretch, were thought to refine the quality of perceived stimuli and to provide fine mucosal input to reflexes controlling motility (Bahr *et al.*, 1986c, b, a). Overall, the PN contains an assortment of specialized primary afferents, which together allow for high fidelity transmission of mechanical information from the colon to the central nervous system.
Population and topographic distribution of afferent subtypes

The most striking discrepancies between this preliminary investigation and previous studies (Brierley et al., 2004; Feng & Gebhart, 2009) are in the proportions and distributions of afferent subtypes. As indicated in Figure 3, whereas serosal afferents were indeed the most common type of mechanosensitive fibers followed by muscular, muscular/mucosal, and mucosal, MIAs were relatively rare, comprising only 8% of all afferents versus 25% reported previously. One possible explanation for this is the limited sample size of this study (39 vs. 159). If more fibers were collected, it would certainly be probable to detect additional MIAs. Other possible explanations include inferior technical skills and slower dissection and characterization times, both of which may expose the MIAs to additional damage sufficient to disable their activity.

Another discrepancy was the distribution of fibers. In this investigation, afferents where clustered along the side of colon closest to the recorded PN. This distribution suggests that the longitudinal cutting used to open the colon severs right PN axons from the left (i.e., anti-mesenteric) portion of the colon and/or afferents from the PN are simply limited to one half of the colon. Regardless of possible reasons, this finding disagrees with previous studies, in which PN afferents were detected along the entire width of the colon. This discrepancy may likewise arise from the comparatively small sample size. Inferior technique may also account for this as the further a RE is from the PN innervation point, the more susceptible it is to damage by tissue manipulation. This explanation is the most likely as REs were in fact detected throughout the width of the colon during the initial scanning of bundles which ultimately needed to be split to isolate single fibers and/or increase the signal-to-noise ratio.

Chemical activation and sensitization of MIAs
Inflammatory soup (IS) and capsaicin (Cap) were chosen as sensitizing agents for a number of reasons. Composed of the principal mediators found in inflammatory exudates, IS has been widely used in studies of nociceptor sensitization (Steen et al., 1995; Katz & Gold, 2006). Cap presumably acts on only one receptor, TRPV1, which is present in a high proportion of small-diameter primary afferent neurons (i.e., C-fibers) and which has been strongly implicated in nociception and pain (Caterina et al., 1997; Davis et al., 2000; Caterina & Julius, 2001). Accordingly, brief exposure to IS and Cap sensitized MIAs to 1.4 g von Frey probing for up to 15 minutes, although neither chemical triggered spontaneous activity in the absence of mechanical stimulation. This agrees with Feng & Gebhart (2009), in which 71% of PN MIAs were sensitized upon chemical exposure.

Conclusions and future directions

Despite several discrepancies, which are most likely associated with relatively small sample size, the present study largely confirms previous findings, thus demonstrating the reliability of the experimenter with this particular technique. In addition to confirming the remarkable detail and fidelity in which mechanical events in the colon are encoded and transmitted, this project establishes the foundation for future work investigating the role of specific gene products in sensory function and the changes which may occur in peripheral innervation in pathophysiological states such as inflammatory/irritable bowel syndrome.
Fig. 1. Annotated photograph of electrical search strategy setup after initial suprathreshold scan of colon surface for pelvic nerve primary afferents. This technique allowed for unbiased identification and localization of all fibers including mechanically-insensitive afferents. It also allowed for the determination of electrical thresholds of activation. Photograph reproduced from Feng & Gebhart, 2009. MPG, major pelvic ganglion.
Fig. 2. Five pelvic afferent subtypes classified on the basis of their anatomical location (Lynn & Blackshaw, 1999; Brierley et al., 2004) and their responses to various mechanical stimuli. (A) Muscular/mucosal afferents were activated by punctate probing (0.4 and 1.4 g shown), fine mucosal stroking (10 mg), and circumferential stretch (>2 mm, or ~25% of resting colon circumference). (B) Muscular afferents were activated by probing and stretch but not stroking. (C) Mucosal afferents were activated only by probing and stroking. (D) Serosal afferents responded only to probing. (D) MIAs were identified electrically and failed to respond to any form of mechanical stimulation. Raw electrophysiological traces are presented with horizontal bars representing application of stimulus. Stretch stimulus is presented in the form of a triangular ramp produced by a servo-controlled force actuator. Figure adapted from previous work in the Gebhart lab (Feng & Gebhart, 2009). *, stimulus artifact produced upon delivery of current pulse to tissue, followed by recording of afferent spike.
Fig. 3. Proportions and topographic distributions of PN afferent subtypes. (A) Data obtained in this study (n=39 pelvic afferents). The most common afferent subtype was serosal, followed by muscular, muscular/mucosal, mucosal, and, the least common, MIAs. (B) Data obtained in Feng & Gebhart, 2009 (n=159 pelvic afferents). (C) PN afferent were scattered seemingly at random throughout the distal 2.4 cm of the colon including the rectum, although they were clustered on the side of the mesentery closest to the specific PN studied (right side PN in this case). MPG, major pelvic ganglion
Fig. 4. Setup and protocol for chemical activation and sensitization of MIAs. (A) Photograph of setup showing bronze tubing containing chemical mediator sitting directly atop an afferent receptive endings (RE). (B) Schematic diagram of chemical activation and sensitization protocol. IS, acidic inflammatory soup; Cap, capsaicin. Photograph reproduced from Feng & Gebhart, 2009.
Fig. 5. Representative examples showing responses of MIAs to acidic inflammatory soup (IS) and capsaicin (Cap). Horizontal bars represent application of stimulus. (A) Exposure to IS and Cap did not evoke spontaneous activity. Instead, MIAs were sensitized to punctate mechanical probing by IS (B) and Cap (C). Mechanical sensitization persisted 15 minutes after washout of chemicals (D) but subsided by 30 minutes (E). Figure adapted from Feng & Gebhart, 2009.
References


