### Analysis of Voluntary Behavior to Interrogate Neural Function

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Analysis of Voluntary Behavior to Interrogate Neural Function

A dissertation presented

by

David P. Roberson

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

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in the subject of

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Analysis of Voluntary Behavior to Interrogate Neural Function

Abstract

Mice and rats are critical to our understanding of human biology. They share most of our genome, are susceptible to most of the same diseases and usually benefit from clinical therapeutics, if only at very high doses. Equally important has been the development of new rodent research tools that allow us to manipulate their genome, to induce new likenesses and illuminate our similarities. However, there remain substantial obstacles to modeling clinically relevant human somatosensory experiences, such as chronic pain, in rodents. Conventional rodent pain assays require robust stimuli to generate brief behavioral readouts and they are able to detect the effects of analgesics only at levels well beyond clinically effective human doses. New technologies are needed that are sensitive to low intensity nociceptive stimuli and are able to detect the effects of analgesic drugs at clinically relevant doses.

It is possible to tell when someone is in pain simply by his or her body language; but rodents and other prey animals do not as readily show behaviors that betray the presence of pain or injury. We propose that ancestral selection pressures have favored propagation of prey that mask outward signs of injury or disease from the predator, and that behavioral indicators of ongoing pain or itch in rodents should therefore be most evident when the appearance of predation risk is minimized and/or from a viewpoint not naturally seen by predators. Based on this hypothesis, we developed new devices and techniques for observation and analysis of voluntary rodent behavior, with careful consideration of their ecological position as nocturnal prey animals. Here, we demonstrate the capability of one of these new technologies to detect the prolonged effects of low intensity nociceptive stimuli in freely behaving mice and rats and to
show that clinically relevant analgesic doses can extinguish pain related behaviors in rodents. Applying the same hypothesis to the study of pruriception (itch), we built another device that optimizes quantification of rodent scratching behavior. We demonstrate its utility by using it to reveal new insights into the cellular basis of pain and itch sensation and advance novel therapeutics for human injury and disease.
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# TABLE OF CONTENTS

Abstract ................................................................................................................................. iii
Acknowledgements ............................................................................................................. v

Table of Contents ................................................................................................................ vi

**CHAPTER 1** .......................................................................................................................... 1

Introduction: Assessment of Pain-Related Rodent Behavior ................................................. 2
Challenges to Detection and Interpretation of Laboratory Rodent Behavior ...................... 5
Behavioral Assessment of Ongoing Pain in Rodents ............................................................. 7
References ............................................................................................................................. 16

**CHAPTER 2** .......................................................................................................................... 20

Introduction: The Palm Reader ............................................................................................ 21
Assay Development .............................................................................................................. 22
Results ................................................................................................................................ 37
Discussion ............................................................................................................................ 49
References ............................................................................................................................. 61

**CHAPTER 3** .......................................................................................................................... 63

Abstract ................................................................................................................................. 66
Introduction ........................................................................................................................... 67
Results ................................................................................................................................ 73
Discussion ............................................................................................................................ 99
Acknowledgements ............................................................................................................. 105
References ............................................................................................................................. 106
CHAPTER 1

Introduction
Introduction: Assessment of Pain-Related Rodent Behavior

The past decades have witnessed great strides in our understanding of the basic mechanisms that underlie aversive somatosensation, like pain and itch, but the record of translating promising preclinical findings from animal models into effective therapies for clinical symptoms like pain and itch, is disappointing. The chief problem is not a lack of novel, druggable pathways, but rather that the compounds designed to target these pathways have failed in clinical trials, most often due to a lack of efficacy compared to placebo. The reasons for recurrent clinical trial failure of compounds that reliably produce anti-nociception in preclinical rodent studies may include species differences in how pain is processed. However, the anatomy and neurophysiology that underlie nociception are largely conserved between humans and animals, so this seems to be a rather weak explanation for the longstanding trend of recurrent clinical trial failures. Another seemingly important clue is that animal models are quite effective in “backward” validation. In other words, clinically effective analgesics nearly always reverse pain-related behaviors in laboratory animals if given at high enough doses. Ultimately, the most important obstacle to translation of somatosensory findings may lie in the distinction between nociception and pain. Simply put, the drugs we are developing to reverse withdrawal from acute nociceptive pain stimuli in rodents may have little effect on chronic pain in humans, resulting in the development of clinically ineffective compounds.

Consider, for example, the in vivo outcomes typically used to select experimental analgesics for clinical testing. A mouse or rat is confined within a small transparent chamber that is suspended in the air and has a floor made of chicken wire. An investigator manually applies something sharp, blunt, hot or cold to the tail or the plantar surface of the animal’s paw to find
the minimum stimulus intensity required to evoke “pain related” or nocifensive withdrawal from
the stimulus (Figure 1.1). Withdrawal thresholds are characteristically reduced at sites of
experimentally induced tissue injury or nerve damage, in a manner similar to the human
experience of allodynia (the perception of pain from a stimulus that is not normally painful) and
hyperalgesia (increased pain perception from a normally painful stimulus). These signs of
hypersensitivity in rodents are almost always reversed when treated with clinically effective
analgesics, but the relative drug doses required are remarkably high, frequently 5-10 times higher
than the highest human dose\(^7\), and therefore lack clinical face validity. Moreover, allodynia and
hyperalgesia are relatively infrequent symptoms among pain patients, whose chief complaint is
usually chronic pain (pain that persists in absence of an evident stimulus)\(^8\). There is great need
for measures of ongoing or spontaneous pain in rodents. Despite considerable effort, however,
identifying and measuring a suitable preclinical surrogate of chronic pain has proven difficult.
Figure 1.1 von Frey assay. Rodents are contained in a plastic chamber and poked from below with plastic filaments to determine the minimum mechanical stimulus threshold required to elicit withdrawal away from the stimulus. These thresholds are reduced when local inflammation or nerve injury is present, but the relevance of these measures to ongoing pain in humans is questioned.
Challenges to Detection and Interpretation of Laboratory Rodent Behavior

In humans, the presence of pain and other neurological features can often be detected by observation of voluntary behavior alone. Disparate postures, movement patterns, physical abilities and inabilities reveal telltale clues and pathognomonic signs that betray the presence of ongoing pain, anxiety, vestibular dysfunction, and specific neurological diseases\(^9\). But laboratory rodents, like many other prey animals, do not demonstrate such overt behavioral changes in response to injury or disease. Ancestral selection pressures common to prey species may help explain our differences. In other prey animals, such as the common squid, *Loligo pealeii*, injury-evoked nociceptor sensitization triggers changes in behavior upon encountering a predator that improve their chances of survival\(^10\). These findings support the possibility that ongoing pain in rodents could enhance their Darwinian fitness by inducing behavioral changes in the face of predation risk that make injury more difficult to detect. Regardless of the role of natural selection, it would not be surprising for behavioral signs of injury and disease to be muted in laboratory rodents considering the selection criteria by which they have been bred for hundreds of generations. Laboratory mice and rats are daily checked by trained caretakers for outward signs of disease or injury. Those with overt behavioral anomalies are routinely euthanized before reaching reproductive maturity, while animals that appear to be healthy are spared regardless of the presence of an actual injury or disease.

The possibility that prey animals reflexively mask signs of injury and disease from predators, specifically humans, is an accepted norm in veterinary educational literature, where the “masking reflex” or “preservation reflex” has been proposed to explain why neurological function is difficult to assess in prey animals like psittacine birds, livestock and equines\(^11-14\). It follows that veterinary diagnostic techniques for prey animals routinely employ measures that
attempt to reduce the appearance of predation risk from the animal’s perspective. For example, subtle changes in gait and foot placement can indicate the presence of neurological diseases in horses and cattle, but these signs can be masked by the splayed posture of “spooked” animals. “Calming” measures are encouraged, such as doing neurological examinations of horses in the open field – their innate place of refuge from predation – instead of while detained in a stable. Rodents, on the other hand, are nocturnal and naturally seek shelter from predators by retreating to small dark hideouts – the proverbial mouse hole. In spite of this, rodent *in vivo* studies are typically conducted in chambers that are transparent, brightly lit or with an open top, and often while the investigator (the presumed predator) is present. Behavioral measures that reduce the appearance of rodent predation risk could improve behavioral outcomes.

Numerous factors have been found to affect rodent behavioral readouts, including time of experimentation, diet, animal husbandry and handling techniques, room temperature, barometric pressure, bedding texture, floor composition, housing density, animal social structure, and the sex of the animal. Additional factors, including environmental noise and visible light sources have been proposed to alter voluntary mouse behaviors, like scratching and cheek wiping. In the case of reflexive withdrawal assays, response threshold values can vary significantly based on the sex and age of any person present in the room during behavioral assessment. Thresholds are elevated when an adult man is in the testing room, compared to the presence of a woman or a prepubescent boy. Elevated response thresholds are also seen when the testing room contains an undershirt previously worn by a man, but no man, suggesting that this effect is related to olfactory detection of male human odors by the test subject. While it is noteworthy that mice behave differently in the presence of men compared to women investigators, it is conceivable that the presence of any human could have a profound impact on experimental rodent behaviors.
Somatosensory rodent behavioral assays routinely require a present investigator, however, so determining the impact of human presence on the measured output of conventional assays is not straightforward. The development of new, more sensitive measures of rodent behavior may therefore benefit from employment of strategies that minimize investigator-rodent interactions and apparent predation risk.

**Behavioral Assessment of Ongoing Pain in Rodents**

Measuring behaviors related to *ongoing* pain in rodents has traditionally been performed manually. A trained observer watches an animal behave, either live or on videotape, and records the timing of all supposed “pain-related behaviors,” such as the paw-licking that reliably occurs for 3 to 5 minutes after plantar injection of capsaicin. A more recent manual scoring approach, the “rat grimace scale” has been developed to characterize “grimacing,” a supposed measure of ongoing pain in rats. Observers look at individual video frames of freely behaving rats to score “grimace features” such as “orbital tightening,” “nose flattening,” “ear changes” and “whisker changes”\(^1\,^18\). Automated software has been developed to assist in identifying and collating video frames that contain a rodent face, but observers still must manually categorize and score the appearance of facial features by observing up to thousands of individual video frames for each study. Moreover, while it is trivial for a human observer to assign an anthropomorphic designation to a particular behavioral state (e.g., “grimacing,” “squinting,” “freezing,” “writhing,” “guarding,” “licking,” “lifting,” “wiping,” etc.), what constitutes a “pain-related behavior” is essentially left to the observer and there are almost certainly relevant behavioral states generated by the mouse that defy simple human categorization. In addition, eliciting these behaviors typically requires robust nociceptive stimuli, more intense than those necessary to elicit changes in reflexive withdrawal\(^1\,^8\).
Another weakness common to observer-identified pain measures is the infrequency and inter-subject variability of the target behavior. Manually scoring infrequent, subjectively defined behaviors demands sustained vigilance and is mentally exhausting, which greatly limits the duration of reliable data collection by an individual observer. Behavioral data for a single experiment can include hundreds of mice, spanning hundreds of hours of video, necessitating a team of observers, which inevitably decreases the reliability and reproducibility of results\textsuperscript{8,19}. These weaknesses could be addressed with technology permitting continuous automated analysis of voluntary behaviors to generate an ethogram (a catalogue or inventory of all objective behaviors or actions exhibited by an animal) of globally integrated, objectively identified pain-related behaviors.

Numerous rodent testing devices are commercially marketed for detection and measurement of spontaneous pain-related mouse and rat behaviors. Measures of gait and weight bearing are often more sensitive to analgesic effects than other approaches, but existing technologies are largely insensitive to clinically relevant analgesic doses and their ability to detect neuropathic pain is questionable\textsuperscript{1,20}. Below are descriptions of commercially available technologies currently promoted for their sensitivity to “pain-related behaviors.”

**Advanced Dynamic Weight Bearing (Bioseb, France)** – This device (Figure 1.2) uses a resistive touchpad to measure the forces exerted by a mouse standing in an illuminated transparent test chamber. It detects changes in relative weight borne by each of the four paws while tracking body position with a camera mounted above the animal. It is sensitive to behavioral changes in mouse and rat inflammatory pain models when robust nociceptive stimuli are used, such as with intraplantar Complete Freund’s Adjuvant (10-20 ul) and articular arthritis models where the
range of motion of the affected limb is physically reduced\textsuperscript{20}. Clinically effective analgesics administered at high doses (typically 5-10 times the human dose by weight\textsuperscript{7}) can reverse inflammation-evoked changes in dynamic weight bearing, but clinically effective neuropathic pain drugs do not alter weight bearing in rodent neuropathic pain models\textsuperscript{20, 22}.
Figure 1.2 Advanced Dynamic Weight Bearing test, by Bioseb. Mice are placed in a well illuminated plastic chamber for the duration of testing. A resistive touchpad below the animal detects relative pressures between the paws while a camera above the animal interprets the rodent’s position. Changes in weight bearing are seen in rodent models of inflammatory and neuropathic pain, but the sensitivity of the device is limited and interpretation of data is not straightforward.
DigiGait (Mouse Specifics, Boston, USA) is a motorized treadmill technology for acquisition and analysis of rodent gait data in a forced ambulation task. It is essentially a treadmill with a transparent moving belt that measures changes in gait and foot placement as a mouse or rat walks or runs at fixed speed (Figure 1.3). Video of the ventral aspects of the ambulating animal is captured using a camera and illumination panels positioned below the treadmill. An illuminated panel above the mouse provides contrast that facilitates algorithmic identification of body position while the ventral paw images are used to determine paw placement during forced ambulation. Together these data permit automated quantification of gait features including stance, limb swing, stride, propulsion, cadence, step sequence, regularity and paw area. DigiGait is sensitive to behavioral changes present in inflammatory pain models where the stimulus is robust\textsuperscript{20}. However, it is unable to reliably detect enhanced nociception in neuropathic pain models and is no more sensitive than conventional reflexive avoidance assays in detecting behavioral changes in inflammatory pain models\textsuperscript{21}. 
Figure 1.3 DigiGait by Mouse Specifics. Example video frames showing mouse performance on the DigiGait assay. Here, the control animal (left column) is able to run at a fixed rate of speed (20 cm/s), while the test subject (right column) is not. Video frames from Gong et al., 2011, supplemental video²⁴.
CatWalk (Noldus, Netherlands) is an elevated walkway with an illuminated glass floor and an upward facing camera below (Figure 1.4). These features allow for video acquisition and subsequent computer analysis of the position of the paws and body of a mouse or rat performing a trained task (walking the length of a meter-long corridor). Analysis of task performance is limited to a small range of walking speeds, so before conducting experiments each animal must be trained to reliably walk the length of the CatWalk while maintaining a narrow speed range. A trainer or investigator must be present during data acquisition to initiate experiments and to ensure that the animal traverses the walkway at the appropriate rate. The CatWalk is capable of capturing images of individual footprints and semi-autonomously tracking their movement to determine the duty cycle, paw braking and propulsion, stride length, cadence, step sequence, walking speed, paw print area and other gait-related measures. Through analysis of these gait features, the CatWalk system can detect behavioral changes in rodent models of neuropathic pain. CatWalk has also been shown to detect gait changes in a mouse inflammatory pain model (carrageenan mono-arthritis), but these measures are not normalized after treatment with clinically effective analgesics23.
Figure 1.4 CatWalk, by Noldus. The CatWalk device detects changes in gait rodents walking along an elevated corridor. Animals must be repeatedly trained to perform the task of walking at a fixed rate of speed. Even after training, an observer must be present to place the animal in the device for repeated observation periods lasting 5-20 seconds each. Photograph of image published in Noldus brochure “Noldus Catwalk,” obtained at the Society for Neuroscience Meeting, November 2011.
Observer XT (Noldus, Netherlands) – This technology is essentially an event logging system tailored for scoring videos of the voluntary behavior of laboratory animals. Mice, rats or other laboratory animals are placed in a brightly illuminated open field or maze and video recorded from above. A trained observer then watches videos of the animal and notes the incidence, duration and timing of anthropomorphically defined behaviors.

*In vivo* rodent pain assessment techniques and devices, such as these, have been designed predominantly from the investigator’s point of view. Under the spotlight, mice or rats are forced to complete an anthropomorphically designed task, usually requiring training or force. These approaches lack subtlety and are insensitive to the effects of clinically relevant analgesic doses. Another problem common to existing technologies is that their readouts are typically acquired only during brief snapshots of behavior. We predict that spontaneous pain-related behaviors will be better detected by a system capable of measuring voluntary behaviors over long and repeated periods without human presence, and designed to limit environmental factors known to interfere with pain-related rodent behaviors, such as fluctuations in environmental sights, sounds and smells.
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CHAPTER 2

Building a Better Mousetrap
Introduction: The Palm Reader

To address the need for more advanced technologies for assessment of voluntary rodent behavior, we designed and built a prototype plantar imaging device that permits tracking and quantification of multiple behavioral parameters of freely behaving mice and rats at high spatial and temporal resolution for long and repeated periods and with careful consideration of their ecological position as nocturnal prey animals. We incorporated design features intended to decrease the appearance of predation risk, such as using fully-enclosed animal chambers with no outward-facing transparent panels. Cameras are enclosed in opaque chambers so that stray light cannot enter the chambers through camera view windows. Experiments are designed so that mice and rats walk freely, without need to perform trained tasks, and without investigators present in the testing room. Instead of brief snapshots of behavior, our device facilitates recording of voluntary behavior for long and repeated periods without human intervention. To optimize identification of pain- and disease-related behavioral features, animals are observed from below, a view not naturally seen by predators. We designed a lighting paradigm using only near infrared light, which allows these naturally nocturnal animals to explore an enclosed arena in apparent darkness.

Pilot experiments conducted using this new technology have produced promising preliminary results. In inflammatory and neuropathic pain models, we find that mice shift their weight toward the uninjured side of the body, as evidenced by a relative decrease in the illumination signal generated by the paw nearest the injury. These “pain-like” behaviors extend throughout periods of nociceptive hypersensitivity detectible with standard rodent pain models,
and these pain-like behaviors continue beyond the period during which conventional reflexive avoidance assays are able to detect behavioral changes. Notably, reductions in ipsilateral paw luminance are observed at stimuli intensities below the threshold for detection by existing measures, and these behaviors are reversed by administration of anti-inflammatory drugs at clinically relevant doses. The device also detects robust, long-lasting changes in voluntary behavior from “control” stimuli previously presumed insufficiently painful to evoke a behavioral response. It also reveals spontaneous pain-like behaviors that re-emerge hours after initiation of “acute” pain stimuli, the effects of which were thought to last no longer than 30 minutes. Moreover, this technology has revealed a novel behavior that appears to reflect the animals’ state of vigilance toward a present investigator or other stressors. We adopted the moniker “PalmReader” to describe the device because of its ability to detect apparent changes in animal affect and somatosensory experience by changes in the appearance of the plantar surface of the paws.

**Assay Development**

**Instrument Design**

We designed and built a prototype rodent behavioral assessment instrument comprising a translucent acrylic rodent containment chamber positioned on a glass touch sensor (Figure 2.1), with an upward-facing machine vision video camera positioned below (Figure 2.2). Light emitting diodes (LEDs) positioned along the edge of the glass generate totally internally reflected light within the glass (Figure 2.1). Evanescent light waves at the glass-air interface illuminate ventral body surfaces as they make contact with the glass. This phenomenon, called
frustrated total internal reflection (FTIR), was previously described in behavioral studies of fly larvae\textsuperscript{2,3}, but its use has not previously been described in open field rodent studies.

The dimensions of the animal containment chamber (180mm x 180mm x 150 mm) replicate the height and width of a standard mouse cage, providing a testing space dimensionally similar to the animal’s “nest”, and with presumed dimensional familiarity. A second reason for the chosen dimensions is that they will allow the containment chamber to fit into the rack of a standard industrial cage wash machine, facilitating high-throughput washing of chambers between experiments. While our prototype devices have been constructed of acrylic plastic, which is incompatible with the heat and chemicals used in cage wash systems, production chambers will be made using injection-molded thermoplastics such as RADEL R polyphenylsulfone or UDEL polysulfone, as used in standard laboratory mouse cages, that can withstand the thermal and chemical insults of automated cage wash systems.
Frustrated total internal reflection

Light traveling within a glass sheet suspended in air is totally internally reflected when it strikes the glass surface at an angle larger than the critical angle for the glass-air interface. When this occurs, an evanescent wave extending several wavelengths beyond the glass-air interface is generated. Here, we are using the evanescent wave to illuminate rodents’ ventral body surfaces when they are in close proximity of the sensor, such as plantar paw surfaces. Since the glabrous skin of the mouse paw is compressible, its contact area with the glass increases as the forces pressing down on the paw increase. This provides a luminance readout that varies according the relative weight borne by each paw and the forces exerted within each paw.
Our first prototype device (PalmReader v1) utilized a Point Grey Firefly camera (A) which was modified for enhanced NIR sensitivity and mounted below a horizontally positioned FTIR optical touch sensor built using a 12 mm thick borosilicate glass panel (B) illuminated by NIR-emitting (850 nm wavelength) LED light strips (C). A translucent white containment chamber (D) with a removable lid, and dimensionally similar to a standard mouse cage, contains the animal on the FTIR sensor. A problem arose in that the silhouette of the animal sometimes obscured the appearance of the paws. To address this, we tried mounting LED light strips (E) to illuminate the animal from below. However, we light reflections (F) from the bottom-mounted light strips obscured the camera image. (G) Photograph of first prototype instrument.
Our use of translucent white acrylic panels for the walls and ceiling of our first prototype device allowed visible light to enter the test chamber, generating a silhouette of the animal, a strategy that has been used by Noldus, Mouse Specifics and others. Silhouetting is advantageous for computer vision analysis because it increases the visual contrast between animal and background, allowing more consistent tracking of the position of the animal’s body. This strategy produced clear images of the animal silhouette that contrasted with the illuminated paws, but it was sometimes difficult to identify the position of the paws under the body – particularly when the paw was not in contact with the glass. To address this problem, we first installed LED light strips below the chamber of our prototype device to illuminate the ventral plane of the animal chamber (Figure 2.2 E-F). However, we found that light reflections off of the glass obscured the camera’s view of the animal, even when light diffusers were employed. To address this problem, we devised a strategy for ventral plane illumination without visible light reflections (Figure 2.3). It has been shown that mice are more active when illuminated by red light than by white light\(^4\), so we installed red LED (650 nm wavelength) lighting to illuminate the body from below. This ventral plane illumination strategy allowed us to generate high-resolution video where both the silhouette of the animal body and the positioning of ventral body parts are clear (Figure 2.3).
Figure 2.3 Ventral plane illumination strategy, PalmReader v2. Here, as with the initial PalmReader iteration, a camera mounted below the animal containment chamber (A) is used to image the animal and its footprints. Borosilicate plate glass (B) is filled with totally internally reflected light from LED light strips mounted around the perimeter of the glass (C). Aluminum extrusions housing the LED light strips allow vertical adjustment and fine tuning of the light plane. Opaque black acrylic panels (D) form the walls and ceiling of the chamber, preventing entry of light from the test room. LED lights (E) are positioned below and lateral to the chamber so that the animal is fully illuminated by the light but light rays are not reflected toward the camera. To prevent tertiary reflections (F) and lens flare an opaque non-reflective light baffle (G) is positioned between the LED lights and the camera, effectively acting as a photographic lens hood would. The inner walls of the chamber were constructed with a black acrylic with a glossy inner surface. This causes light rays entering from LED lights below the chamber (H) to be reflected away from the camera, making the chamber walls and ceiling invisible from the camera viewpoint. An opaque alignment (I) plate ensures consistent placement of the chamber on the glass surface and limits stray light rays from entering the test arena,
Data Formatting

After conducting pilot experiments at various lighting levels, we set out to score the videos manually by stopwatch. It soon became evident that the illumination of the animal body by the upward-facing LEDs appeared brightest near the edges of the chamber and dimmest near its center. We found that mice frequently walk around the perimeter of the chamber in a clockwise direction, which caused the left hind paw (the paw nearest the wall when walking clockwise) to appear brighter than the opposing hind paw during clockwise circumnavigation of the chamber walls. To determine the primary source of this “halo effect”, we tested the uniformity of FTIR and ventral plane luminance by applying damp filter paper to the glass surface and taking videos of the paper illuminated by each of the light sources alone, and found that the FTIR signal increased negligibly from the center to the edges of the chamber, and that the ventral plane lighting was the primary reason for the halo effect.

Another problem that became apparent while watching the videos was that light from the upward-facing LEDs drastically reduced the apparent dynamic range of the FTIR signals. This made it difficult to determine, for example, whether a mouse was holding his hind paw above the glass, or resting it lightly on the surface. A member of my Dissertation Advisory Committee (DAC), Michael Do, suggested that we broaden the effective dynamic range of the FTIR signal by increasing the power driving the FTIR signal stepwise on successive video frames. Video frames in which the FTIR signal was brightest would facilitate automated detection of timing of paw contact, while the lower illumination frames would maintain a broader dynamic range of the FTIR signal, which we believed would be beneficial for in automated video analysis. However, we decided that a simpler and more effective approach would be to provide power to the ventral illumination LEDs only on alternating video frames, allowing the FTIR signal to be detected
alone on every other video frame. To adjust lighting levels on defined video frames we wrote an Arduino-based switching program that uses the “shutter open” signal from the video camera to supply a pulse width modulated 12v signal to the ventral plane LEDs only on every other video frame.

The new alternating illumination data was a technical success, but the 50hz “red strobe light effect” from the flickering ventral plane LEDs caused marked behavioral changes in test mice, such as vigorous jumping, and rapid ambulation around the perimeter of the chamber. We addressed this problem by replacing the red (~650nm wavelength) upward-facing LED strips (Figure 2.3 E) with NIR LED strips (~850nm wavelength). This approach extinguished the aberrant mouse behavior, facilitated assessment of voluntary behavior in absence of all visible light, and permitted generation paired video frame sets that can be sequentially analyzed for optimal detection of differences in paw luminance.

We conducted pilot experiments using plantar pain models to generate FTIR-enhanced videos of open field mouse behavior. It was clear from watching these videos that animals with paw injury consistently showed decreased luminance in injured paw compared to the contralateral paw. We then turned to Alex Wiltschko, a Harvard PhD student, and his advisor Robert Datta, at Harvard Medical School who worked with us to devise a data processing workflow aimed at automatically identifying differences in paw luminance between the left and right hind paws in freely behaving animals on a video frame-by-frame basis. Starting with ventrally illuminated frames, the position of the left and right hind paws is identified, then luminance values for the paws are measured on the next video frame illuminated only by the FTIR signal (Figure 2.4 C-D).
This strategy also proved beneficial for rendering enhanced videos for observation and video presentation. By generating colorized heat maps of the FTIR-only frames, overlaying them on the ventrally illuminated frames, then rendering the resulting video (Figure 2.4 E), we found that even slight differences in the luminance of the paws can be readily identified by human observers. By manually observing and scoring these “heat map overlay” videos, we were able to troubleshoot the technology and conduct pilot experiments exploring its capabilities using human observers (see Preliminary Results).
Figure 2.4 Comparison of illumination strategies

(A) A translucent plastic chamber allows ambient light from the test room to illuminate a rat, generating a silhouette detectible from the camera view. Totally internally reflected light in the glass panel illuminates paw contact areas. Note the difficulty in identifying the position of the fore paws. (B) An opaque black chamber allows the mouse to be illuminated from below using LED lights. This strategy permits silhouetting that aids computer vision analysis but also allows continuous visualization of ventral animal features. (C) Mouse lit only by FTIR illumination. Note the difficulty in identifying anatomical features of the mouse. (D) On the adjacent video frame, the lighting from beneath the cage illuminates the mouse. (E) Videos for human-assisted scoring were produced by making colorized heat maps of FTIR-only video frames and overlaying them on ventrally illuminated frames before video rendering using Adobe Photoshop software.
Automated Data Analysis Methods

To develop software for automated analysis of alternating-illumination formatted video data, we collaborated with Harvard Medical School faculty member graduate student, Alex Wiltschko, and his faculty advisor, Robert Datta. Firstly, we trained open source machine vision codes to identify and extract images of mouse paws and tails of mice by labeling these features on 10,000 images (video frames) sampled from ventrally illuminated mouse videos. Using these trained algorithms, Alex developed pattern recognition codes capable of extracting paw images from still individual video frames with high accuracy (Figure 2.5).

The next step was to automate determination of paw positional identity (right-front, right-back, left-front, left back). Again, we began by manually labeling mouse tails on 10,000 video frames to train machine vision algorithms. Alex then used these trained machine vision algorithms to automate determination of the animal’s tail and tail base. Once the paws and tail could be identified with >90% accuracy, the tail base was used as a starting point to determine the animal’s nose and body midline. From there, codes were developed to determine which quadrant (front right, front left, back right, back left) the extracted paws belonged in (Figure 2.6). Once specific paws regions are defined using the ventrally illuminated frames, the mean luminance of these areas is measured on the next frame, which is illuminated only by the FTIR signal. This workflow is then repeated for all frames in a video to produce measures of the individual paw luminances over time.
Figure 2.5 Computer-assisted extraction of paw images from video frames
Mouse fore paws and hind paws were extracted using machine vision algorithms trained by manually labeling paws on 10,000 video frames. The algorithm correctly identifies paws, but also occasionally extracts the urogenital region instead of the paw.
Figure 2.6 Automated paw identification scheme
Ventrally illuminated video frames, such as in panel A, permit visualization of the paws, tail and body of mice and rats that are necessary for efficient machine vision identification. However, the added lighting introduces noise into the FTIR signal (B), obscuring differences in FTIR-generated paw luminance. So, to identify the regions of interest, the animal is illuminated from below on every other video frame. This provides the information necessary for the machine vision codes to identify paws and other relevant landmarks such as the tail, which are used to determine the position of the left and right hind paws (C). Once the locations of the paws have been determined, the algorithm is able to measure luminance for the defined regions on the next sequential video frame, illuminated only by FTIR lighting (D).
After collecting pilot data and confirming the utility of the new data format, we constructed a 5-chamber Palm Reader device (Figure 2.7) that permits simultaneous tracking and observation of multiple behavioral parameters of five freely behaving, individually contained mice or rats at high spatial and temporal resolution for long and repeated periods. This device permits high-throughput data acquisition that drastically increases the rate of experimentation.
Figure 2.7 Multi-chamber PalmReader device
High throughput experimentation is facilitated by a multi-chamber rodent behavioral acquisition device (A). Five animal containment chambers on top of the device are matched with 5 cameras housed within the body of the device. Beneath each chamber, a glass panel is illuminated by a NIR LED strips positioned along the front and back edges of the glass. A separate set of NIR LEDs (B), highlighted here in red, is positioned below each chamber to illuminate the animal from below. A second set of chambers below the body of the device provides accelerated habituation of mice to the test chambers. The variance in FTIR-evoked luminance between the center and edges of the chamber was negligible, but present. So, in our 5-chamber device we built the sensors using thinner 4 mm borosilicate float glass, which provides more uniformity in the FTIR signal across the area of the chamber.
It is important to note that these paw detection and analysis algorithms are still “rough,” and will benefit from continued development. For example, there are periods of time when it is clear by human observation that the animal is favoring one paw over the other, but the algorithm does not note a significant difference in luminance. One reason is that the algorithm currently determines mean of luminance values for each paw by averaging the luminance values for all frames captured in 60-second increments. An observer watching the video would intuitively notice the maximal pressure of each step and compare it to the maximal value generated by the opposite paw, whereas our preliminary analysis algorithm does not yet have this capability and instead averages all frames. Another limitation of the algorithm used in analysis of our pilot data is that it generally excludes video frames when the animal is stationary. To correct this issue additional code will need to be developed to dynamically adjust the luminance filter so that paws can be identified even when they are relatively dim compared to the luminance of the body, such as when the animal is resting with its body on or near the glass sensor.

Results

Intraplantar injection of capsaicin is a standard model of acute nociceptive pain (pain due to activation of nociceptors by a known noxious stimulus). To test the sensitivity of our approach to nociceptive pain, we treated the left hind paw of male C57BL/6J mice with a low volume injection (5 μl) of 0.1% capsaicin (Sigma) in normal saline (0.9% NaCl, Sigma). Typically, capsaicin assays are performed using 10 μl injections which produce 4-5 minutes of nocifensive behavior (biting and licking) and <10 minutes of mechanical hypersensitivity in (von Frey reflexive withdrawal test), whereas 5 μl injections do not generate reliable changes in reflexive
withdrawal assays. Immediately after injection, mice were placed in the observation chamber of our PalmReader device and recorded for 30 minutes. Subsequent recordings were conducted 90 and 180 minutes after injection. Automated analysis of data was performed, revealing repeated intervals when the untreated paw was significantly more luminous compared to the left (injected) hind paw, but no periods where the injected paw was more luminous than the uninjected paw (Figure 2.8). Naïve mice did not demonstrate these behaviors (Figure 2.9). Three hours (180 minutes) after injection, mice were once again put in the PalmReader device and recorded for 30 minutes. Automated scoring of these data revealed extended periods when the uninjured right paw was less luminous than the left paw, which had been injected with capsaicin three hours earlier (Figure 2.10).
Figure 2.8  Paw luminance values 0-30 minutes after intraplantar capsaicin injection
FTIR-generated paw luminance values after intraplantar injection of capsaicin (5 μl, 0.1%) to the left hind paw. P-values represent comparison of Back Left value (red circles) to Back Right (black squares). (not significant, P > 0.05; *, P < 0.05). Error bars, SEM. n = 10 mice.
Figure 2.9  Paw luminance values 0-30 minutes after introduction of naïve mice to testing chamber
FTIR-generated paw luminance values in naïve male C57BL/6J mice. P-values represent comparison of Back Left value (red circles) to Back Right (black squares). (not significant, P > 0.05; *, P < 0.05). Error bars, SEM. n = 10.
Figure 2.10  Paw luminance values 180-210 minutes after intraplantar injection of capsaicin
FTIR-generated paw luminance values after intraplantar injection of capsaicin (5 µl, 0.1%) to the left hind paw. P-values represent comparison of Back Left value (red circles) to Back Right (black squares). (not significant, P > 0.05; *, P < 0.05; **, P < 0.01). Error bars, SEM. n = 10 mice.
We next explored whether the PalmReader could detect behavioral changes in a low intensity inflammatory pain model, and analgesic effects at clinically relevant doses. Ten male C57BL/6J mice were injected intraplantary with 2.2 μl of Complete Freund’s Adjuvant (CFA) to the left hind paw. Twenty-two hours later the mice were split into two groups of 5 mice per group. One group received intrascapular subcutaneous injections of diclofenac, a non-steroidal anti-inflammatory drug, at the human daily dose equivalent (2 mg/kg). The control group was administered vehicle (0.9% NaCl). Two hours after treatment with diclofenac or saline (24 hours after CFA injection), mice were placed in the PalmReader and recorded for 30 minutes.

PalmReader video data were scored by human observation of heat-mapped overlay videos with observers blinded to identity of treatment groups. The duration of periods when the animal’s right and left hind paw appeared to be brighter were timed by stopwatch. Differences between left and right paw are most evident when the animal is grooming or otherwise stationary, so observers were instructed to only analyze periods when the animal is stationary, and not when the animal is walking, exploring, rearing, et cetera. The data revealed that the right (uninjured) hindpaw appeared brighter for a significantly longer period of time than the left (CFA-injected) hindpaw 24-hours after CFA injection, suggesting that the animal was favoring (carrying more weight on) the right, uninjured paw (Figure 2.11). There was no significant difference in duration of increased luminance of right (uninjured) and left (CFA-injected) hind paws in the group receiving diclofenac.
Diclofenac treatment reverses pain-related changes in paw luminance in a CFA-evoked mouse model of plantar inflammatory pain.

FTIR-generated hindpaw luminance values in male C57BL/6J mice treated 24 hours before testing with intraplantar injection of Complete Freund’s Adjuvant (CFA) to the plantar surface of the right hind paw. Twenty-two hours after injection of CFA, animals were treated with diclofenac (2 mg/kg) or vehicle (0.9% NaCl) administered subdermally by intrascapular injection. Two hours after diclofenac treatment (24 hours after CFA administration), mice were placed in the PalmReader device and recorded for 30 minutes. The duration of periods of spontaneous behavior when either the right hind paw (red columns) or left hind paw (grey columns) appeared brighter was scored by stopwatch by observers watching heat-mapped videos of animal behavior. All observers were blinded to treatment identity. P-values represent comparison of Back Left paw mean luminance value (red circles) to Back Right (black squares). Error bars, SEM. n = 5.
Manual supervision of heat-mapped plantar luminance videos of naïve and treated mice yielded qualitative insights that have not yet been tested in an objective and automated manner. Nonetheless, these subjective video annotations by blinded observers provide clues to the potential capabilities of the technology. Abdominal pain is modeled in rodents by quantifying writhing bouts induced by intraperitoneal injection of a weak solution of acidic acid. In the acid writhing test, our device shows a shift in luminance from the hind paws (naïve mouse, Figure 2.12 C, representative FTIR frames) toward the fore paws (intraperitoneal acid challenge mouse, Figure 2.12 D) when walking on all four paws, whether or not writhing behavior is present. This shift in luminance persists beyond the period of writing, and is present even in animals treated with a more dilute acid solution that does not elicit characteristic writhing behavior.

In inflammatory or neuropathic pain models, we observe that mice consistently shift their weight toward the uninjured side of the body, as evidenced by a relative decrease in luminance in the paw nearest the injury (Figure 2.12 A-B, representative FTIR frames). These pain-like behaviors persist much longer than the duration of reduction in reflexive withdrawal thresholds, and are reversed following administration of anti-inflammatory and analgesic drugs at much lower doses than reported in other rodent pain models. Even intraplantar injection of saline, which has conventionally been used as a negative control in pain studies, produces transient reduction in weight bearing in the injected paw that is evident after 24 hours (data not shown). A similar effect is seen following intraplantar needle insertion without injection of saline (data not shown), confirming that the observed effect is likely due to pain from the needle stick and not an artifact of injected saline volume.
Figure 2.12  Representative heat-mapped FTIR-only video frames
Panel (A) shows a habituated naïve mouse standing on its hind paws while grooming. Note the similar luminance levels of the hind paws. Panel (B) increased luminance in the back left paw following spontaneous injury to right leg. When walking on all fours, mice typically show increased luminance of hind paws relative to fore paws (C). However in a model of abdominal pain (D), luminance is shifted toward the fore paws. (L=left hind paw, R = right hind paw)
Upon supervision of videos recorded with the first iteration PalmReader device, it became evident that our open field plantar imaging approach had utility beyond its targeted capability of increased nociceptive sensitivity. Midway through the initial 30 minute video of a naïve rat, I realized that the paws appeared brighter, flatter and more uniform in luminance than I remembered them being when I had set up the camera. I re-entered the test room where we were recording a second video of the same rat, and there saw that the paws appeared dim and punctate, as I had remembered, not bright and more uniform as observed midway through the first video. It was not until watching the second video that I realized what I was seeing. The footprints appeared dim and stippled at the beginning of the video (Figure 2.13 A), but became gradually brighter and more uniform in appearance over time (Figure 2.13 B); then, at the point in the video when I had entered the testing room, the appearance of the animal’s paws quite suddenly became dim and stippled again.

We find that when naïve rats and mice are first introduced to the chamber (i.e., immediately after being handled) they walk with the footpads, toes and keratin cuticles (toenails) of each paw extended. Only the cuticles, toe tips and small areas of the footpads are in contact with the floor surface (i.e., on tiptoes) (Figure 2.13 A, C). However, after 20 minutes of habituation to the device, the plantar surfaces of the paws become flattened, increasing the paw surface area in contact with the glass sensor (Figure 2.13 B, D). Upon introduction to the chamber on subsequent days, tiptoeing behavior ceases much sooner, typically within 5 minutes. Automatically scored data reflect this increase in mean luminance over time, corresponding to the shift from tiptoeing to a flat-footed plantar posture that occurs over time while the animal is in the chamber (Figure 2.14).
Figure 2.13  Tiptoeing represents a vigilance- or anxiety-related behavior
Immediately after handling, rats (A, C) and mice (E) exhibit tip-toeing behavior, characterized by FTIR illumination as a dim, stippled appearance of the paw print. After habituation to the chamber, the rats (B, D) and mice (F) assume a more flat-footed paw posture, but tiptoeing often resumes upon entry of an investigator into the test room. Panels A and B are still frames taken 20 minutes apart of the same rat in the first generation PalmReader device, which was backlit using room lighting through translucent top and sides of the containment chamber. Panels C and D are heat-mapped still frames of FTIR-illuminated prints of a rat’s paws, taken immediately after handling and 20 minutes later, respectively. Panels E and F are frames from enhanced heat-map overlay videos of a naïve mouse taken 10 seconds after handling and another naïve mouse 20 minutes after being placed in the chamber.
Figure 2.14 Paw luminance values of habituated, naïve mice 0-30 minutes after being placed in chamber

FTIR-generated paw luminance values in naïve animals (violet circles) increase over the course of experimentation, consistent with the transition in paw confirmation from walking on tiptoes, which produces a dimmer-appearing footprint, to more flat-footed ambulation that produces a brighter-appearing footprint upon FTIR illumination. n = 10 mice per group.
Discussion

Preclinical rodent studies serve an essential role in the drug discovery process by helping to identify promising drug candidates and filtering out suboptimal ones before initiating clinical trials, but the ability of current rodent behavioral assays to predict the clinical efficacy and adverse effects of experimental drugs is limited, particularly in the analgesic drug development space. One experimental analgesic after another has shown great promise in preclinical rodent studies, only to fail in subsequent clinical trials, most commonly due to lack of efficacy versus placebo or due to adverse drug effects\textsuperscript{5,6}. It has been proposed that the failure rate of analgesic drug trials might improve if better measures of pain-related rodent behavior were developed\textsuperscript{6}.

Evoked hypersensitivity is a commonly used endpoint in preclinical rodent trials of experimental therapies for chronic pain, and there are few, if any, more sensitive surrogates of chronic pain in rodents. These investigator-evoked measures have been invaluable in advancing our understanding of the basic mechanisms of nociception, but they have proven inadequate as a rodent surrogate of spontaneous or ongoing pain in rodents. Despite being the primary endpoint for most preclinical \textit{in vivo} validation studies of experimental analgesics, rodent readouts of evoked hypersensitivity poorly predict the clinical efficacy of experimental therapeutics for chronic pain. Indeed, by their very nature, evoked hypersensitivity measures fail to capture the non-evoked aspects of pain that characterize most chronic pain patients. Operant pain models have been shown to be sensitive to some motivational components of chronic pain, but their utility is limited and they are largely insensitive to the spontaneous or ongoing pain that characterize most chronic pain patients. Other more technologically advanced apparatus for detecting rodent pain have been developed and are available commercially (see Figures 1.2, 1.3, 1.4), but they are largely reliant on a present investigator, generally fail to mitigate experimental
factors shown to adversely affect readouts of rodent pain, are typically less sensitive than conventional evoked hypersensitivity measures (Figure 1.1), and none have proven to be reliable indicators of ongoing or spontaneous pain in rodents. As such, evoked withdrawal measures of hypersensitivity to noxious stimuli remain the mainstay of preclinical analgesic development.

Our hypothesis is that behavioral indicators of spontaneous or ongoing pain in rodents will be more evident using assays designed with attention to the ecological position of mice and rats as prey animals, e.g., by observation from below (a view not naturally seen by their predators), in absence of visible light and without present human investigators (to minimize apparent predation risk), over long and repeated intervals. The chief aim of this study was to develop the hardware to enable such measurements and to conduct pilot experiments to enable the subsequent steps, which are: 1) automatic analysis of the data and 2) systematic study of its capabilities and sensitivity to pain-related voluntary rodent behaviors.

Here we introduce such an approach, which is based on the detection and analysis of changes in paw usage of freely behaving laboratory mice or rats, allowing prolonged observation of voluntary behaviors in absence of visible light and without present observers. We show that evanescent light waves, generated by totally internally reflected (FTIR) light within a transparent glass panel, illuminate the plantar surfaces of the paws of laboratory rodents constrained to an open-bottomed chamber placed on the glass panel to reveal changes in voluntary paw usage evoked by experimental rodent models of acute (intraplantar capsaicin injection) and chronic (intraplantar CFA injection) pain. Detected behavioral changes are sensitive to clinically effective analgesics administered at a dose comparable to the human dose by weight, suggesting that this approach may be a more sensitive predictor of analgesic efficacy than many conventional behavioral approaches. Additionally, we demonstrate evidence that this approach
may be useful for the study of anxiety-related behaviors and other behavioral changes that could reflect a rodent’s affective and cognitive functioning.

Automated scoring of paw usage data reveal that mice treated with intraplantar injection of capsaicin demonstrate numerous repeated one-minute time intervals during which their mean left (capsaicin-injected) hind paw luminosity is lower than their right (contralateral; uninjured) hind paw luminosity (Figures 2.8 and 2.10). Naïve mice do not demonstrate repeated periods where the mean luminosity of one hind paw is significantly greater than the contralateral paw (Figure 2.9). In capsaicin-treated animals, these intervals extend well beyond the 5-minute period during which spontaneous flinching and paw-licking behaviors are typically observed. Capsaicin has been shown to evoke action potentials in human sensory afferents intermittently for up to 600 minutes after exposure⁷, and sensory changes following capsaicin challenge can persist for hours⁸, suggesting that the observed changes in voluntary paw usage from 180 to 210 minutes after capsaicin administration (Figure 2.10) may reflect prolonged intermittent effects of the initial capsaicin challenge. These results, therefore, indicate that changes in paw usage detected by automated analysis of FTIR-illuminated plantar behavior may be related to capsaicin-evoked intermittent, spontaneous or paroxysmal pain.

We used manual scoring of heat-mapped FTIR videos to explore the sensitivity of FTIR plantar imaging in a mouse model of chronic pain (Figure 2.11). Twenty-four hours after intraplantar injection of CFA to the plantar surface of the left hindpaw, mice treated with a clinically relevant dose by weight of diclofenac (2mg/kg, intrascapular subcutaneous injection 2 hours before testing) showed no significant difference in the duration of time during which one hind paw appeared brighter than the other. However, mice treated with vehicle (0.9% NaCl solution, intrascapular subcutaneous injection 2 hours before testing) favored the right
(uninjured) hindpaw over the left (CFA-injected) paw (Figure 2.11). These data suggest that observation of voluntary paw usage using FTIR illumination reveals pain-related behaviors that are reversible after treatment with a clinically-relevant analgesic dose, and that this measure may be more sensitive to analgesic effects than other, commonly used preclinical in vivo analgesic development assays.

Manual supervision of FTIR-enhanced videos of rodent plantar behavior revealed other potentially pain-related behaviors that warrant systematic study. For example, in the acid writhing test – a rodent model of abdominal pain – mice appear to shift their weight toward their fore paws as evidenced by a relative increase in apparent brightness of fore paws compared to hind paws (Figure 2.12). Further evidence of the sensitivity of this approach was observed in pilot studies exploring the effects of plantar saline injection, typically used as a negative control in pain studies (data not shown). Together, these observations suggest that our technology is likely a more sensitive measure of acute and chronic nociception and of analgesic effects compared to conventional rodent nociceptive behavioral assays.

Human observation of hundreds of hours of FTIR-enhanced videos of voluntary rodent behavior also revealed unexpected behavioral states. Notably, the appearance of the plantar surfaces of mice and rats appear different during the first 5-15 minutes in the chamber compared to later time points. Upon placement in the chamber by the investigator, mice and rats appear to “tiptoe,” walking on their toes and keratin cuticles, and with a punctate appearance of the footpads (Figure 2.13 A, C, E). Over time, the plantar contact areas change to take on a brighter appearing, more “flat footed” appearance (Figure 2.13 B, D, F). Automatically scored data reflect this increase in mean luminance over time, corresponding to the shift from tiptoeing to a flat-footed plantar posture that occurs over time while the animal is in the chamber (Figure 2.14).
We observed that mice and rats adopt flat-footed walking patterns more quickly upon subsequent introductions to the chamber. Rodents that have been in the chamber long enough to adopt a flat-footed plantar appearance often resume tiptoeing behavior when an investigator enters the testing room. Similarly, a sudden transition from a flat-footed plantar posture to a tiptoe appearance is frequently observed in response to a sudden loud noise, such as a door slamming. Considering the ecological position of mice and rats as prey animals, and presuming investigators are perceived as a predator, these findings support the conclusion that tiptoeing in mice and rats is a novel behavioral indicator of a rodent’s responsiveness (and presumed aversion) to human interaction and may, therefore, represent a measure of predation vigilance (i.e., an anxiety-related behavior).

Together, our automatically scored acute pain data, manually scored chronic pain data, and video observations support our original hypothesis. However, these data and observations are preliminary and lack necessary controls and experimental validation. Additional investigation and statistical analysis is required to validate these observations.

**Planned Experiments**

We will first conduct additional experiments to compare our approach to the outcomes of conventional reflexive withdrawal assays by exploring changes in evoked plantar hypersensitivity using the von Frey (mechanically evoked withdrawal) and Hargreaves (thermally-evoked withdrawal latency) assays, using groups of male C57BL/6J mice (12 weeks old, n=10 per group) treated with intraplantar capsaicin or CFA. For acute (capsaicin) pain assays, mice will be tested immediately after injection of capsaicin or vehicle, and again at 90, 180, 360 and 720 minutes after injection. Additional testing will be conducted at 24 hours after
capsaicin treatment. These results will be compared to baseline von Frey and Hargreaves measurements using appropriate statistical measures described below. Voluntary pain-related behavior will be assessed for 30-minute periods at the same time intervals using matched groups of capsaicin- and vehicle-treated mice. These data will be automatically scored using previously described scoring algorithms. For chronic pain (CFA) experiments, mice will be given intraplantar injections of CFA (CFA mixed in saline) or vehicle (saline alone) and tested using von Frey, Hargreaves or PalmReader assay for at 6 hours, 24 hours, 48 hours, and one week after CFA administration and compared to baseline values.

A separate cohort of mice will be used to test the effects of diclofenac 24 hours after CFA injection. Two hours before testing (22 hours after CFA injection) each test group will be administered one of three different doses (1, 2, or 4 mg/kg) of diclofenac, or vehicle (0.9% NaCl), by subcutaneous intrascapular injection. Two hours later (24 hours after CFA administration) the behavioral response to analgesic treatment will be tested by von Frey, Hargreaves and PalmReader assays. Differences between mean values obtained in the time-courses will be analyzed by 2-way repeated-measures analysis of variance (ANOVA) followed by Bonferroni post hoc test. The effect of analgesic (anti-inflammatory) therapy will be compared to control by 1-way ANOVA followed by Bonferroni post-test. Differences in means will be reported statistically as P values and considered significant when P < 0.05. Dose-response curves will be calculated to determine the diclofenac dose that produces the half maximal effect (ED_{50}) in the CFA-induced behavioral measures. Degree of recovery evoked by drug treatment will be calculated as the percentage of improvement of drug-treated mice over vehicle-treated mice.
We predict that pain-related spontaneous behaviors will persist beyond the period of capsaicin- or CFA-evoked thermal and mechanical hypersensitivity. It is possible that any PalmReader-detected changes in voluntary pain-related behaviors will not exceed the duration of induced thermal or mechanical hypersensitivity or that their time-courses will differ substantially. Such dissociation of evoked hypersensitivity and voluntary behavioral outcomes might indicate that the period of inflammation-evoked hypersensitivity differs from the period of spontaneous or ongoing pain. This outcome would nonetheless be helpful in characterizing the complexities of global changes associated with rodent acute and chronic pain models.

It is also possible that changes in plantar behaviors of freely behaving rodents are yet another surrogate measure of operant pain or hypersensitivity in the injured paw rather than measures of ongoing or spontaneous pain. In any event, any significant changes in voluntary behavior detected by the PalmReader approach will presumably reflect the decision of the animal to adapt its normal paw usage to alleviate or adapt to its awareness of discomfort, and we propose that these outcomes might more realistically represent the human experience of chronic pain, which are frequently characterized by changes in gait and other disturbances in normal life activities.

Based on pilot data showing complete reversal of pain-related plantar behaviors upon administration of a clinically-relevant dose of diclofenac (2 mg/kg), we anticipate that this assay development effort will yield a more sensitive in vivo measure of experimental analgesic efficacy. If validated, this could provide a new preclinical measure of analgesic efficacy based on drug doses that are more in line with human therapeutic doses; but even if this approach is not found to be more sensitive to low analgesic doses of diclofenac or other drugs, we believe that this new technology will prove advantageous for exploration of mechanisms of pain and
analgesia in both academic and industry settings. Conventional rodent pain assays are adversely influenced by human-animal interactions\textsuperscript{9,10}, and susceptible to experimenter subjectivity\textsuperscript{11}. These confounding factors are mitigated in our assay since the investigator is absent from the room during testing. Our approach also benefits from the ability to perform multiple experiments simultaneously rather than testing animals one at a time as with other approaches, greatly increasing experimental efficiency and throughput.

We conclude that FTIR-enhanced plantar assessment of freely behaving rodents is a sensitive and objective experimental measure of acute and chronic pain-related behavior, and that the apparatus developed through this study fulfills our hardware development aims. Our observations and preliminary data using this new technology suggest that this approach is highly sensitive to injury, analgesic effects, and anxiety-like behaviors, and this we argue is just the proverbial tip of the iceberg. We propose that it might also be sensitive to a wide range of central and peripheral nervous system conditions including brain or spinal cord injury, regeneration after nerve injury, neurodevelopmental and neurodegenerative disease models, social behaviors, and aggression. Moreover, we anticipate that it might be capable of detecting behavioral signs of common CNS adverse drug effects including ataxia, vertigo, dizziness, and headache – something not possible at present. We believe this technology will prove to be a transformative improvement in the capacity to assess voluntary behaviors in laboratory rodents.
Interlude: The Itch Detector

A common problem in observational studies of voluntary rodent behavior is that mice and rats, like humans, are easily distracted. I encountered this first-hand when we set out to explore the cellular basis of itch sensation during my research rotation project in Clifford Woolf’s lab and had trouble replicating our own behavioral results. A 2008 study describing a method to differentiate between itch- and pain-related behaviors in the mouse, and a subsequent conversation with the senior author, Bob LaMotte confirmed my suspicion that the variability we had encountered when conducting pilot “itch” experiments in mice could be due to extraneous sensory distractions\(^1\). Shimada and LaMotte reported that virtually any environmental novelty can attract the attention of a caged rodent and alter their behavior, but that results could be improved by recording one mouse at a time (so that they are not distracted by smells or sights of other mice), under infrared lighting, and with a white noise generator to drown out background noises. The setup was complex and recording one mouse at a time seemed inefficient, but it worked. Still, the idea of recording mice one-at-a-time was maddening. In order to increase the rate of experimental throughput, I invented and built a novel, six-chamber mouse enclosure for observation and quantification of itch related mouse behaviors – an “itch detector.”

I configured the device so that the six adjacent isolation chambers could be viewed from a single video camera. This required that the walls of each chamber be aligned with the video camera view angle so that the entirety of each cage chamber was visible from a single viewing point (Figure 2.15).
Figure 2.15 The Itch Detector
Additionally, I included mirrors on opposing sides of each chamber and angled them to permit two side views of each animal when videotaped from above. This was necessary to visually differentiate between different behavioral responses to pain and itch in the mouse cheek. The introduction of video recording to the test environment produced another unintended behavioral distraction where the mice would begin “star gazing” at novel objects such as cameras and lights above. Quality video recordings require adequate lighting, so I redesigned the cage to incorporate semi-opaque acrylic walls in between each chamber with embedded LED bulbs to create soft lighting from two sides of each chamber. Moreover, I incorporated 24 additional near-infrared lamps that are invisible to rodent and human visual spectrum but permit video recording of nocturnal behaviors. In order to limit distractions caused by outside noise I built a custom pink-noise generator that reduces the influence of external and intrachamber sounds.

Previous studies suggested that itch-related behaviors could be altered by the smell of nearby rodents. To address this, I utilized independent filtered air circuits to prevent the recirculation of air between mouse chambers. An additional problem arose when trying to quantify scratches in a cohort of six mice that were unintentionally injected at irregular time intervals. In order to facilitate offset-synchronization of simultaneous temporal behavioral observations, I created an automated “mouse sensing” LED timer in each mouse chamber that detects the presence of the animal, and begins the timer immediately upon the mouse being placed into his chamber. The timers are linked to 7-segment LED outputs that are hidden behind a two-way mirror that permits clear visibility of elapsed time for each cage from the video camera position while at the same time hiding the LED lamps from the viewing range of each mouse.
This device provided the high quality data that served as the foundation for our study demonstrating functionally distinct populations of itch-sensing nerve fibers (the article is included as Chapter 3 of this thesis).
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CHAPTER 3

Analysis of Voluntary Behavior to Reveal Itch Specific Primary Afferents
Author Contributions

D.P.R. conceived, designed and conducted the behavioral experiments, analyzed behavioral data, conceived the silencing strategy and wrote the manuscript. S.G. performed the combined calcium imaging and electrophysiology experiments. J.S. carried out the calcium imaging experiments. H.A.W.P and V.K.R. conducted behavioral experiments and analyzed behavioral data. F.B. contributed to the combined calcium imaging and electrophysiology experiments. B.D. contributed to behavioral experiments and interpretation of behavioral data. S.B.O. gathered behavioral pilot data and gave manuscript advice. B.P.B conceived the silencing strategy, gave technical and conceptual advice, and edited the manuscript. Q.M. provided critical analysis and interpretation of behavioral data, designed behavioral experiments, and contributed to critical revision of the manuscript. A.M.B. conceived the silencing strategy, conceived and designed behavioral experiments and the combined calcium imaging and electrophysiology experiments, gathered behavioral pilot data, supervised the project and wrote the manuscript. C.J.W. conceived the silencing strategy, supervised the project and wrote the manuscript.
At the time of the submission of this dissertation, much of the work presented is this chapter has been published in *Nature Neuroscience* **16**, pages 910–918 (2013) as a manuscript entitled:

**Activity-dependent silencing reveals functionally distinct itch-generating sensory neurons**

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Running Title: Itch Specific Primary Afferents
Abstract

The peripheral terminals of primary sensory neurons detect histamine and non-histamine itch-provoking ligands through molecularly distinct transduction mechanisms. It remains unclear, however, whether these distinct pruritogens activate the same or different afferent fibers. We utilized a strategy of reversibly silencing specific subsets of murine pruritogen-sensitive sensory axons by targeted delivery of a charged sodium-channel blocker and found that functional blockade of histamine itch did not affect the itch evoked by chloroquine or SLIGRL-NH2, and vice versa. Notably, blocking itch-generating fibers did not reduce pain-associated behavior. However, silencing TRPV1+ or TRPA1+ neurons allowed AITC or capsaicin respectively to evoke itch, implying that certain peripheral afferents may normally indirectly inhibit algogens from eliciting itch. These findings support the presence of functionally distinct sets of itch-generating neurons and suggest that targeted silencing of activated sensory fibers may represent a clinically useful anti-pruritic therapeutic approach for histaminergic and non-histaminergic pruritus.
Introduction

Itch is a complex unpleasant cutaneous sensation that in some respects resembles pain, yet is different in terms of its intrinsic sensory quality and the urge to scratch. Histamine-mediated itch, as in patients with urticaria, can be effectively treated using histamine receptor antagonists. However, itch accompanying most chronic pruritic diseases, including atopic dermatitis (eczema), allergic itch and dry skin itch, is not predominantly mediated by histamine. The G-protein coupled receptors responsive to specific itch-generating ligands are distinct, although at a cellular level, there is overlapping responsiveness of trigeminal and dorsal root ganglia (DRG) neurons to itch-producing pruritogens and pain-producing algogens.

Histamine-sensitive H1 receptors (H1Rs) generate histamine itch and are expressed by TRPV1+/phospholipase-β-3 (PLCβ3)+ fibers. Itch evoked by chloroquine is mediated by Mas-related G-protein-coupled receptor (Mrgpr) A3, while MrgprC11 is sensitized in dry skin itch and activated by pruritogens released from mast cells during allergic itch. Notably, co-activation of TRPV1 and H1R is required to produce histamine itch, while MrgprA3- or C11-mediated itch requires co-activation of TRPA1 even though each of these TRP channels are canonical nociceptor transducers. In vitro calcium imaging experiments find that neurons expressing MrgprA3 also respond to histamine, which is interpreted as indicating a single neuronal path for histaminergic and MrgprA3-dependent itch. Supporting this, ablation of neurons expressing MrgprA3 reduces the scratching evoked by histamine, chloroquine, dry skin, and allergic inflammation. However, others report separate neural pathways mediating histamine and certain types of non-histamine itch. Furthermore, while primary sensory neurons of juvenile mice respond to multiple itch mediators, this non-specificity decreases with age. It remains controversial, therefore, if in the adult there are separate afferents that mediate
histamine itch and MrgprA3-dependent non-histamine itch. This distinction is clinically important since therapies targeting histaminergic itch fibers might be ineffective for treating non-histaminergic itch if the neurons mediating the two itches are functionally distinct in the adult.

To study if histaminergic and non-histaminergic itch are functionally distinct we adapted a method originally designed for achieving a pain-specific peripheral nerve block\textsuperscript{19,20} to selectively silence the peripheral terminals of different subsets of pruritogen- and algogen-responsive primary afferents in an activity-dependent manner. To do this we targeted the charged, membrane-impermeable lidocaine derivative N-ethyl-lidocaine (QX-314) (a sodium channel blocker) through large pore ion channels activated specifically by different algogens and pruritogens.

**Materials and Methods**

**Combined Calcium Imaging and Voltage Clamp Recordings**

**Cell Culture.** Trigeminal neuron cultures were prepared from adult (2 to 3-month-old) CD1 mice\textsuperscript{44}. In short, trigeminal neurons were removed and placed into HBSS and 1% penicillin–streptomycin (Sigma), then digested in 5mg/ml collagenase, 1mg/ml Dispase II (Roche, Indianapolis, IN). Cells were triturated in the presence of DNase I inhibitor (50U) and centrifuged through 10% BSA (Sigma). The cell pellet was resuspended in 1ml Neurobasal (Sigma) containing B27 supplement (Invitrogen), penicillin and streptomycin (Sigma), 10\(\mu\)M AraC. Cells were plated onto poly-d-lysine (500ug/ml) and laminin (5 mg/ml) coated 35 mm tissue culture dishes (Becton Dickinson) at 8000-9000 per dish, at 37°C, 5% carbon dioxide.

**Ratiometric calcium imaging.** Cultured adult trigeminal neurons were loaded for 45-60 minutes with 1 \(\mu\)M fura-2 AM (stock in DMSO) in a bath solution composed of (in mM): 145
NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 Glucose, 10 HEPES and then rinsed for 45-60 minutes for de-esterification of intracellular AM esters. Neurons were perfused continuously at 2 ml/min and examined with an inverted microscope (Eclipse Ti) equipped with Epi-Fl attachment; perfect focus system (Nikon, Japan) and Exi Aqua monochromator (QImaging). Intracellular [Ca²⁺]ᵢ was measured fluorometrically as an absorbance ratio at 340 nm and 380 nm (ΔF340/380) (510 nm for emission) (Lambda DG4, Sutter Instruments). Images were taken every 1 second, were monitored online and analyzed offline using Nikon Elements AR Software (Nikon). Histamine (100µM) and chloroquine (100µM) were briefly bath applied (60 s) using a fast-step valve control perfusion system (Harvard Apparatus). In all responsive neurons, the changes in ratio (DF) following application of histamine and chloroquine were larger than 0.1DF and were easily distinguishable from optic noise, which was less than 0.025DF. Whole cell voltage clamp recordings were then performed from the responsive cells (histamine-positive or chloroquine-positive) and non-responsive cells (histamine-negative or chloroquine-negative).

Electrophysiology: For electrophysiological recordings of transmembrane sodium currents, in order to decrease driving force for sodium the solutions were replaced after completing calcium imaging measurements to thereafter contain (in mM): 60 NaCl, 4 KCl, 1 MgCl₂, 2 CaCl₂, 0.1 CdCl₂, 10 HEPES, 10 Glucose, 5 4-AP, 60 Choline chloride, 15 TEA-Cl, (pH, 7.4). Pipette solution contained (in mM): 110 CsCl, 25 CsOH, 2 MgCl₂, 1 CaCl₂, 11 EGTA, 10 HEPES, (pH = 7.4 with CsOH). Recordings were performed using a Multiclamp 700 B amplifier (Molecular Devices) at room temperature (22 ± 2°C). Data were low pass filtered at 1 kHz (-3 dB, 4 pole Bessel filter) and sampled at 10 kHz. Patch pipettes were pulled from thick-walled borosilicate glass capillaries (1.5 mm outer diameter, Sutter Instrument) on a Sutter Instrument P-1000 puller (Novato, CA) and had a resistance of 2-5 MW. Pipette potential was
zeroed before seal formation and membrane potential not corrected for the small liquid junction potential (-2.2 mV). Care was taken to maintain membrane access resistance as low as possible (usually 3-7 MW and always less than 10 MW). Capacity currents were cancelled and series resistance 80% compensated. Linear leakage currents were digitally subtracted on-line using a P/4 procedure. Command voltage protocols were generated on-line with a Digidata 1200 A/D interface (Molecular Devices). Data were digitized on-line using pCLAMP 10.2 (Molecular Devices). Data averaging and peak detection were made using pCLAMP 10.2 software (Molecular Devices). Data were fitted using QPlot.

For these experiments we have used data only from neurons in which input resistance and leak current did not significantly change during 10 minutes of drug application (20 min of recording). Due to the relatively low number of responsive cells and long and complex protocol of subsequent electrophysiological recording we have used data from 3 cells per group (7 groups).

Calcium Imaging (alone) Experiments

All dissections were performed on adult (2 to 4-month-old) male CD-1 mice. The calcium-indicator dye Fura-2 AM was introduced at 2 µg/ml for 30 minutes at room temperature, washed, and analyzed on a Nikon Eclipse Ti inverted microscope with exi-aqua CCD camera with NIS-elements AR 3.10 software. Each cell was given three minutes to recover from each pruritogen exposure, and 8 minutes to recover between exposure to AITC and capsaicin. Each exposure was for sixty seconds, except for capsaicin, which was exposed only for 10 seconds. Pruritogens were locally applied, with the perfusion opening placed approximately 150 microns from the field of view, and preliminary exposure to standard extracellular solution was employed prior to
exposure to reagents made in SES. Pruritogen order was assigned at random (coin toss) for each plate, and no order effects were observed between differently ordered groups.

**Behavioral Studies**

All animal procedures were approved by the Boston Children's Hospital Animal Care and Use Committee. Naïve adult (2 to 4-month-old) male CD-1 mice (Charles River Laboratories) housed in groups of 5 using a normal 12-hour light/dark cycle were used. Animals were fully habituated to handling prior to all experimental procedures and were randomly assigned to experimental groups. The day before beginning itch experiments, mice were briefly anesthetized by inhalation of 1–2% isoflurane and a ~1 cm² area of hair was shaved on the right cheek of each mouse. Capsaicin (Sigma-Aldrich, St. Louis MO) was freshly prepared by dilution in vehicle (20% ethanol, 5% Tween 20 in saline; 10 ml). All other drug solutions were prepared freshly in normal saline (0.9% NaCl).

**Itch Assay.** Mice received intradermal (id) microinjection of pruritogen, algogen, QX-314, vehicle or a combination of pruritogen or algogen together with QX-314 intradermally in the cheek. To ensure proper intradermal injection, needle puncture with a 28g needle was initiated bevel-up at 5° to the plane of taut skin until initial penetration, then inserted horizontally until the needle tip was 0.5 cm beyond the point of insertion before intradermal evacuation of syringe contents. Correct injection was confirmed by presence of a slightly domed bulla immediately following removal of needle. For sequential injection experiments, the intradermal bullae of the conditioning (first) injection were outlined with fine-tip permanent marker to denote the extent of intradermal drug distribution, thus providing visible drug distribution boundaries for subsequent injections. Animals not receiving proper drug injections were noted
and excluded from the study prior to observation. Immediately after conditioning injection, mice were placed in in a custom-built itch observation apparatus and video recorded during the mouse dark cycle as previously described\(^{26}\). Scratches and/or wipes subsequently quantified by blinded observers.

*Von Frey assay.* Mice were assessed manually using the up-down method to determine the median 50% (5/10) withdrawal threshold\(^{45}\).

*Radiant heat (Hargreaves) assay.* Radiant heat withdrawal threshold was determined using the Plantar Analgesia Meter (IITC Life Science, Inc.) according to methods previously described\(^{46}\).

*Focal cold plantar (Brenner) assay.* Latency to remove hindpaw from focally applied cold stimulus was performed according to methods previously described\(^{27}\) only using 3/8” thick (4.78 mm, as measured by electronic caliper) Neoceram\(^{TM}\) N-0 thermal-shock-resistant glass (Nippon Electric Glass Co., Ltd.).

*Adhesive dot plantar tactile (“sticky tape”) assay.* Latency to bite, lick, or attempt removal of a 9 mm diameter circular adhesive Microtube Tough-Spots\(^{®}\) label (Diversified Biotech) was performed according to methods previously described\(^{28}\), only using hindpaw instead of forepaw for application of stimulus.

**Statistical Analysis**

Sample sizes for all experiments were chosen according to standard practice in the field. For electrophysiological experiments the significance of the effect was calculated using two-way ANOVA non-parametric test followed by Bonferroni post-test. Comparison of group means for behavior studies was performed using Student’s t-test. All bar graphs are plotted as mean ±
standard error of the mean (SEM). For behavioral studies, “n” represents the total number of mice used in each group.

Results

Targeted Na\(^+\) current block of pruritogen-activated neurons

Activation of TRPV1 channels permits entry of QX-314 selectively into dorsal root ganglion (DRG) and trigeminal ganglion nociceptors through the TRPV1 pore to produce a selective block of sodium currents only in TRPV1 expressing nociceptors\(^{19-24}\). Here we examined whether histamine-mediated activation of TRPV1 channels\(^ {11,14}\) would allow sufficient QX-314 uptake to suppress sodium channel currents selectively in histamine-responsive trigeminal ganglion neurons. In trigeminal neuron cultures from adult male CD-1 mice we recorded sodium currents using whole cell voltage clamp from small (<25 \(\mu\)m diameter) neurons that showed an increase in intracellular calcium concentration upon a 60 second bath application of 100 \(\mu\)M histamine (Figure 3.1a). In these cells, a subsequent 2.2 minute application of 100 \(\mu\)M histamine together with 5 mM QX-314 significantly and progressively decreased sodium current amplitude with a nearly complete block after 10 minutes (Figure 3.1b,c and Tables 1 and 2). This decrease was prevented by the TRPV1-channel blocker capsazepine (20 \(\mu\)M) (Figure 3.1c). Sodium currents recorded from trigeminal neurons that did not respond to histamine were not affected by co-application of histamine and QX-314 (Figure 3.1b,c), indicating that extracellularly applied QX-314 by itself at this dose has no activity. Together with previous studies demonstrating that histamine produces downstream activation of TRPV1 channels\(^ {14}\), our data indicate that QX-314 enters histamine responsive trigeminal neurons when activated by histamine, likely through TRPV1 channels.
Figure 3.1 Application of pruritogens leads to a QX-314-mediated blockade of sodium currents selectively in pruritogen-sensitive trigeminal ganglion (TG) neurons (a). Photomicrography (left) and representative traces (right) of changes in intracellular calcium concentration recorded from cultured trigeminal ganglia neurons at left. Red arrow indicates a small trigeminal neuron that responded (red trace) to histamine (60 seconds, 100 µM). Blue arrow indicates a cell that did not respond (blue trace) to application of histamine. Dotted line indicates the time point of the photomicrography frame. (b) Representative, normalized traces of sodium currents recorded from histamine-positive (left) and histamine-negative (right) neurons before (black) and 10 minutes after (green) application of 100 µM histamine together with 5 mM QX-314. (c) Time course of the changes in amplitude of peak sodium current. The specific TRPV1 antagonist capsazepine (20 µM) abolished the histamine/QX-314-mediated decrease in sodium current. (d) Photomicrography (left) and representative traces (right, dotted line indicates the time point of the photomicrography frame) of changes in intracellular calcium concentration following application of chloroquine (60 seconds, 100 µM). Color scale represents value of absorbance ratio at 340 and 380 nm (340/380). (e) Representative, normalized traces of sodium current recorded from chloroquine-positive (left) and chloroquine-negative (right) neurons before (black) and 10 minutes after (green) application of 100 µM chloroquine together with 5 mM of QX-314. (f) Time course of changes in amplitude of peak sodium current. The specific TRPA1 antagonist HC-030031 (100 µM) abolished chloroquine/QX-314-mediated decrease in sodium current. Results for all panels are mean ± SEM of peak sodium current relative to control (n = 3 for each group. For numerical values and statistical analysis see Tables 1-3).
We then examined whether we could block sodium currents in chloroquine-responsive trigeminal neurons by co-application of chloroquine and QX-314. TRPA1 channels are activated by chloroquine in these cells\(^\text{12}\). We and others have demonstrated that QX-314 and other cationic organic compounds with a similar molecular weight permeate the TRPA1 pore\(^\text{23,25}\). In trigeminal neurons that showed a robust increase in intracellular calcium after brief chloroquine application (100 µM, 60 seconds) (Figure 3.1d and Tables 3 and 4), subsequent co-application of 100 µM chloroquine and 5 mM QX-314 significantly decreased sodium currents (Figure 3.1e,f). This effect was significantly reduced by pre-incubation with the TRPA1-channel blocker HC-030031 (100 µM) (Figure 3.1f). In cells that did not respond to chloroquine, QX-314 and chloroquine had no effect on sodium current (Figure 3.1e,f).
Table 3.1 The time course of the effect of histamine + QX-314 and histamine + QX-314 + capsazepine on relative amplitude of peak sodium current in histamine-positive (n=3) and histamine-negative (n=3) cells. Note the current rundown when only vehicle was applied (n=4, both histamine-positive and histamine-negative cells). Significance of treatments was obtained by comparison between different treatment groups (see Table 2). Relative Peak Current = treatment/control (%).

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Table 3.2  The time course of the effect of chloroquine+QX-314 and chloroquine + QX-314 + HC-030031 on relative amplitude of peak sodium current in chloroquine-positive (n=3) and chloroquine-negative (n=3) cells. Note, the current rundown when only vehicle was applied (n=4, both chloroquine-positive and chloroquine-negative cells). Significance of treatments was obtained by comparing different treatment groups (see Table 4). Relative Peak Current = treatment/control (%).

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Table 3.3 Comparison of the effects of treatment (groups from Table 1 and 2) on the amplitude of sodium currents in histamine-positive (n=3), histamine-negative (n=3), chloroquine-positive (n=3) and chloroquine-negative (n=3) TG neurons, calculated using two-way ANOVA non-parametric test with post-hoc Bonferroni test. The difference considered significant if p-value is below 0.05. \( t \) = degree of freedom.

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Pruritogen-mediated activation of TRP channels (histamine for TRPV1 and chloroquine for TRPA1) permitted, therefore, sufficient entry of QX-314 selectively into particular pruritogen-sensitive trigeminal neurons to block sodium currents only in these neurons (Figure 3.2).
Pruritogen mediated GPCR activation initiates opening of large-pore ion channels (e.g., TRPV1, TRPA1) to allow entry of the membrane-impermeant sodium channel blocker, QX-314. Once inside the nerve fiber, QX-314 has access to its binding site on the sodium channel and blocks thereby conduction only in fibers activated by the administered pruritogen.
Selective silencing of pruriceptors *in vivo*

To test if we could selectively silence pruriceptors *in vivo* we used intradermal cheek injections of pruritogens together with QX-314, then characterized prurceptor function by quantifying ensuing itch behavioral responses (hindlimb scratching of cheek) or pain (forelimb wiping of cheek)\(^26\). Intradermal injection of histamine (100 µg/20 µl) produced scratching (64.3 ± 7.5 bouts, \(n = 6\)) that fully resolved within 30 minutes. There was no significant change in scratching (56.0 ± 8.3 bouts, \(n = 6; P > 0.05\)) when histamine was injected together with 1% QX-314. Based on the delayed time course of nociceptor block *in vivo* in response to a combination of capsaicin and QX-314\(^19\), and considering the gradual development of full sodium current block *in vitro* after QX-314 and histamine coadministration (Figure 3.1c), we hypothesized that the short duration of histamine-evoked scratching behavior (~25 minutes) was too brief to detect the slow onset blocking effects generated by coadministration of histamine with QX-314. To test this we devised a behavioral model using two sequential intradermal injections, 30 minutes apart, of a pruritogen into the same intradermal cheek-injection site. Identical pruritogen doses were used in each injection. To ensure that both injections were distributed within the same cutaneous area, however, the first dose (conditioning injection) was delivered in 20 µl of vehicle while the second dose (test injection) was administered in 10 µl. Only behavior evoked by the test (second) injection was compared to behavior evoked by other identical test injections, with the experimental variable being the identity of the conditioning (first) injection given 30 minutes earlier.

When histamine alone was given for the conditioning injection (100 µg/20 µl) and again 30 minutes later for the test injection (100 µg/10 µl) at the same site, the amount of scratching evoked by the two injections was not significantly different (Figure 3.3a). However, histamine-
evoked scratching was effectively abolished 30 minutes after a conditioning injection of histamine and QX-314 together (Figure 3.3b), but not when preceded by injection of QX-314 alone (data not shown). We conclude that histamine-mediated activation of large pore channels, such as TRPV1\textsuperscript{14}, permitted uptake of QX-314, to produce a slow (<30 minutes) onset electrical silencing of the histamine-responsive sensory fibers, which then blocked the response to subsequent injection of histamine (Figure 3.3b).
Figure 3.3 Co-administration of QX-314 and pruritogens inhibits subsequent pruritogen-evoked scratching

(a, c, e) Sequential pruritogen cheek injections at 30-minute inter-stimulus intervals (ISI) evoke similar levels of cheek scratching (itch) bouts. (b, d, f) Pruritogen-evoked scratching is inhibited 30-minutes after conditioning injection of pruritogen together with 1% QX-314. (a) Conditioning injection: histamine (100 µg/20 µl), total scratching bouts in 30 minutes (TSB/30 minutes) = 64.3 ± 7.5 bouts. Test injection: histamine (100 µg/10 µl), TSB/30 minutes = 49.2 ± 9.7, P > 0.05, n = 6, degrees of freedom (d.f.) = 10. (b) Conditioning injection: histamine (100 µg/20 µl) + 1% QX-314, TSB/30 minutes = 56.0 ± 8.3. Test injection: histamine (100 µg/10 µl), TSB/30 minutes = 7.5 ± 3.8, P < 0.001, n = 6, d.f. = 10. (c) Conditioning injection: chloroquine (CQ, 50 µg/20 µl), TSB/30 minutes = 103.0 ± 19.1. Test injection: chloroquine (50 µg/10 µl), TSB/30 minutes = 109.0 ± 20.8, P > 0.05, n = 7, d.f. = 12. (d) Conditioning injection: chloroquine (50 µg/20 µl) + 1% QX-314, TSB/30 minutes = 110.0 ± 13.7. Test injection: chloroquine (50 µg/10 µl), TSB/30 minutes = 31.8 ± 13.3, P < 0.01, n = 6, d.f. = 10. (e) Conditioning injection: SLIGRL (50 µg/20 µl), TSB/30 minutes = 80.8 ± 9.6. Test injection: SLIGRL (50 µg/10 µl), TSB/30 minutes = 71.4 ± 12.0, P > 0.05, n=5, d.f. = 8. (f) Conditioning injection: SLIGRL (50 µg/20 µl) + 1% QX-314, TSB/30 minutes = 71.2 ± 16.1. Test injection: SLIGRL (50 µg/10 µl), TSB/30 minutes = 17.7 ± 8.0, P < 0.05, n = 6, d.f. = 10. Figure results are mean ± SEM of total scratching bouts per minute following injection. P-values represent comparison of value of mean total scratching bouts in 30 minutes evoked by test injection to those evoked by conditioning injection.
Based on our *in vitro* results (Figure 3.1d-f) we hypothesized that intradermal administration of chloroquine with QX-314 could block chloroquine itch by permitting selective uptake of QX-314 through chloroquine-mediated activation of TRPA1\(^ {12}\). Co-injection of QX-314 together with chloroquine (50 \(\mu\)g/20 \(\mu\)l) inhibited scratching produced by subsequent chloroquine (50 \(\mu\)g/10 \(\mu\)l) test injection, while injection of chloroquine alone (Figure 3.3c,d) or QX-314 alone (data not shown) did not. We conclude that large-pore channels downstream of chloroquine-evoked MrgprA3 activation, likely TRPA1\(^ {12}\), permitted selective uptake of QX-314 and subsequent electrical silencing of chloroquine-sensitive afferent fibers.

SLIGRL-NH\(_2\) (SLIGRL) acts at MrgprC11 receptors to produce itch\(^ {9}\). MrgprC11-mediated itch also requires TRPA1 activation to generate scratching behavior\(^ {12}\). We next explored, therefore, whether SLIGRL-mediated activation of TRPA1 channels is sufficient to introduce QX-314 and inhibit subsequent SLIGRL-evoked itch. SLIGRL (50 \(\mu\)g) evoked scratching bouts were inhibited 30 minutes after injection of QX-314 together with SLIGRL, but not after injections of SLIGRL alone (Figure 3.3e,f) or QX-314 alone (data not shown). These findings demonstrate that histaminergic and non-histaminergic pruritogen-mediated entry of QX-314 into pruriceptors is sufficient to block activity of itch-generating fibers, as measured by a reduction in scratching behavior on subsequent injection of the same pruritogen.

Pruriceptors are dispensable for non-itch sensations

It is unclear whether fibers that mediate histamine itch contribute to normal chemical, thermal and mechanical pain sensitivity. To explore this, we assessed pain-related behavioral responses to noxious chemical, thermal, and mechanical stimulation after co-injection of QX-314 together with chloroquine (50 \(\mu\)g), histamine (100 \(\mu\)g), capsaicin (0.1%), or AITC (0.15%).
Injection of capsaicin in the cheek evoked robust forelimb wiping (indicative of TRPV1-evoked pain) 30 minutes after vehicle (0.9% NaCl) injection (Figure 3.4a). Administration of QX-314 together with capsaicin, as expected, abolished subsequent capsaicin-evoked wiping (Figure 3.4a). However, capsaicin-evoked wiping was unchanged 30 minutes after injection of QX-314 together with either histamine or chloroquine (Figure 3.4a), even though such injections block scratching generated by these pruritogens (Figs. 2 and 4). Injections of histamine, chloroquine, or QX-314 alone did not reduce subsequent capsaicin-evoked wiping (data not shown).

Test injection of AITC (0.15%) evoked robust forelimb wiping (indicative of TRPA1-evoked pain) 30 minutes after vehicle (0.9% NaCl) injection (Figure 3.4b). AITC-evoked wiping was blocked after a conditioning injection of QX-314 together with AITC (0.15%), but not after conditioning injections of QX-314 together with chloroquine (50 µg), or histamine (100 µg), or by prior administration of chloroquine, or histamine alone (Figure 3.4b).

We next asked whether blocking histamine- or chloroquine-responsive fibers affects mechanical or thermal pain sensitivity. Intraplantar co-injection of QX-314 (20 µl) together with histamine (100 µg) or chloroquine (50 µg) had no effect on mechanical sensitivity to von Frey filaments, while co-application of capsaicin (0.1%) and QX-314 abolished mechanical pain responses (Figure 3.4c). Likewise, intraplantar injection of QX-314 (20 µl) together with histamine (100 µg) or chloroquine (50 µg) did not alter responses to noxious heat (52° C). Noxious thermal sensitivity was, however, abolished when tested after coadministration of capsaicin (0.1%) and QX-314 (Figure 3.4d).
Figure 3.4 Silencing pruritogen-responsive neurons does not alter behavioral responses to non-itch stimuli

Forelimb wipes (pain-related behavior) evoked by (a) capsaicin (0.1%, 10 µl) and (b) AITC (0.15%, 10 µl) 30 minutes after cheek conditioning injections of vehicle or 1% QX-314 together with pruritogens (histamine, chloroquine (CQ)) or algogens (capsaicin, AITC). Hindpaw withdrawal to (c) punctate mechanical (von Frey) stimulus, (d) a radiant heat (52° C) stimulus, or (e) focally applied cold stimulus 30 minutes after intraplantar conditioning injections of vehicle or 1% QX-314 together with pruritogens or algogens. (f) Latency to bite, lick or attempt removal of an adhesive dot on the plantar surface of the hindpaw 30 minutes after intraplantar conditioning injections of vehicle, 1% QX-314 together with pruritogens or algogens was compared with a group receiving saline or intraplantar injection of 5% lidocaine (after 5 minutes). P-values represent comparison to vehicle (white column) value (not significant, P > 0.05; *, P < 0.05; ** P < 0.01). Error bars, SEM. n = 5-7 for all groups.
To determine whether histamine and chloroquine itch-generating fibers are dispensable for sensitivity to cold or tactile stimuli, we performed the focal cold plantar assay and the adhesive dot plantar tactile assay thirty minutes after intraplantar injections of pruritogens and algogens together with QX-314 (Figure 3.4e,f). Cold sensitivity was unaltered 30 minutes after injections of QX-314 together with histamine (100 µg), chloroquine (50 µg), or capsaicin (0.1%). However, cold sensitivity was significantly reduced after co-injection of AITC (0.15%) and QX-314 (Figure 3.4e). We next used latency to attend to an adhesive dot placed on the plantar paw surface as a measure of tactile sensitivity. The latency to attend was unchanged 30 minutes after injections of QX-314 with histamine (100 µg), chloroquine (50 µg), capsaicin (0.1%), or AITC (0.15%), while intraplantar injection of lidocaine (5%) significantly increased the latency (Figure 3.4f).

We propose that while histamine and chloroquine activate TRPV1 and TRPA1 expressing pruriceptors, respectively, these fibers are not essential for acute thermal, mechanical and chemical pain sensitivity, which likely involve different or additional subsets of afferents to generate pain-related behavior. Likewise, we conclude that chloroquine- and histamine-sensitive itch fibers are not essential for normal tactile sensitivity.

**Distinct fibers mediate histamine and non-histamine itch**

We next used the silencing strategy to reveal whether different pruritogens activate the same or unique peripheral afferent pathways by administering test injections (10 µl) of histamine (100 µg), chloroquine (50 µg) or SLIGRL (50 µg), 30 minutes after conditioning injections (20 µl) of histamine (100 µg), chloroquine (50 µg) or SLIGRL (50 µg) with and without QX-314. We hypothesized that if histaminergic and non-histaminergic itch are transmitted by different
subsets of afferent fibers, application of histamine together with QX-314 will block histaminergic itch while chloroquine or SLIGRL itch will remain intact, and *vice versa*.

We first examined whether histamine-evoked scratching is affected by the targeted silencing of chloroquine-activated pruriceptors. Histamine-evoked scratching was unchanged 30 minutes after a conditioning injection of chloroquine and QX-314 together (Figure 3.5a), even though this treatment blocked subsequent chloroquine itch (Figure 3.3b and 5b). Similarly, conditioning injection of SLIGRL with QX-314 (which reverses later SLIGRL-evoked scratching; Figure 3.3c and 5c) did not reduce subsequent histamine (100µg/10µl) itch (Figure 3.5a).

We then asked if chloroquine itch is affected upon silencing of histamine- or SLIGRL-sensitive itch fibers by injecting chloroquine thirty minutes after administration of QX-314 together with either histamine or SLIGRL. Chloroquine-evoked itch was blocked after coinjection of SLIGRL and QX-314, but not by coadministration of histamine and QX-314 (Figure 3.5b). Likewise, SLIGRL-evoked itch was unaffected 30 minutes following injection of histamine with QX-314, but was significantly reduced after injection of chloroquine and QX-314 (Figure 3.5c).

It appears that a common afferent population mediates chloroquine and SLIGRL itch, but that this population is functionally distinct from neurons responsible for histamine itch. These findings contrast, however, with prior *in vitro* data showing that all chloroquine-responsive DRG neurons respond to histamine. A possible explanation for this discrepancy is that peripheral terminals of trigeminal neurons differ from DRG neuron cell bodies in terms of their responsiveness to multiple pruritogens. To explore this, we repeated the same silencing strategy
on the back below the neck. Treatment with histamine and QX-314 together did not reduce subsequent chloroquine-evoked scratching when compared to treatment with vehicle, while intradermal injection of chloroquine and QX-314 together inhibited subsequent chloroquine-evoked scratching (data not shown) indicating that facial and somatic itch appear to be similar in terms of the functional independence of histamine and non-histamine itch.

Another possibility is that different pruricceptor subsets exist with high or low sensitivity to either histamine or non-histamine pruritogens. To investigate this we varied the dose of pruritogen given with 1% QX-314 (20 µl) for conditioning injections. Application of a lower dose of histamine (10 µg) together with QX-314 inhibited subsequent histamine-evoked scratching without reversing chloroquine (50 µg) scratching (Figure 3.5a,b), essentially identical to the effects following a higher dose of histamine (100 µg) with QX-314. However, increasing the concentration of histamine to 108.7 mM (400 µg/20 µl) with QX-314 for conditioning injections blocked scratching evoked by both histamine and chloroquine (Figure 3.5a,b) indicating that an overlap manifests only at very high doses of histamine.

Higher doses of chloroquine (200 µg or 400 µg) together with QX-314 blocked later chloroquine itch behavior, but not scratching evoked by histamine. However, using 96.9 mM chloroquine (1 mg/20 µl) together with QX-314 for conditioning injections blocked subsequent histamine- and chloroquine-evoked scratching (Figure 3.5a,b).
Figure 3.5  Distinct primary afferents mediate histaminergic itch and non-histaminergic itch

Pruritogen-evoked cheek scratching (itch) bouts 30 minutes after different conditioning injections indicated at the x-axes. (a) Intradermal test injection of histamine (100 µg/10 µl) alone, (b) chloroquine (CQ, 50 µg/10 µl) alone or (c) SLIGRL (50 µg/10 µl) alone 30 minutes after conditioning injection of vehicle or pruritogens together with 1% QX-314. P-values represent comparison to vehicle (white column) value (not significant, $P > 0.05$; *, $P < 0.05$; ** $P < 0.01$). Error bars, SEM. $n = 5-7$ for all groups.
These data suggest that histamine and chloroquine generally activate functionally distinct pruriceptor populations, but that these fibers can respond to multiple pruritogens, either directly or indirectly, when they are presented at very high, presumably non-physiological concentrations (histamine, 107.8 mM; chloroquine, 96.9 mM).

**Histamine and chloroquine act on different sets of neurons**

The responsiveness of DRG neurons to multiple pruritogens decreases with age. In juvenile mice, 60-100% of all chloroquine responsive DRG cells also respond to histamine, while only half of chloroquine-responsive DRG neurons respond to histamine in adolescent mice. We therefore asked whether distinct chloroquine and histamine-sensitive neuronal populations could be revealed in adult (2 to 4-month-old) mice by their sensitivity to pruritogens in vitro. MrgprA3 receptors are activated by 10 µM chloroquine and maximally activated by 1 mM chloroquine in HEK293 cells. Murine DRG neurons respond to histamine at doses as low as 10 µM. Here, we examined coincident calcium responses of 564 cultured trigeminal neurons to 10 µM histamine and 10 µM chloroquine. Histamine activated 4.1% (23 of 564) of trigeminal neurons, while 3.0% (17 of 564) responded to chloroquine (Figure 3.6a). A majority of chloroquine- and histamine-responding trigeminal neurons responded to only one pruritogen. Among chloroquine-activated cells, 76.5% (13 of 17) responded to chloroquine but not histamine, while the remaining 23.5% (4 of 17) responded to both 10 µM histamine and 10 µM chloroquine. Likewise, trigeminal neurons responding to histamine were largely unresponsive to chloroquine: 82.6% (19 of 23) responded to histamine but not chloroquine, and 17.4% (4 of 23) responded to both histamine and chloroquine.
Figure 3.6 Proportional representation of coincident trigeminal cell responses to low dose chloroquine and histamine, and their overlapping responsiveness with capsaicin and AITC

Venn diagram of calcium responses of 564 cultured trigeminal (TG) neurons to 10 µM histamine, 10 µM chloroquine (CQ), 1 µM capsaicin, and 100 µM AITC. (a) Histamine (10 µM) activated 23 of 564 TG neurons, while 17 responded to 10 µM chloroquine. Among histamine-activated cells 19 of 23 responded to histamine but not chloroquine, and 4 of 23 responded to both histamine and chloroquine. For TG cells responding to chloroquine 13 of 17 responded to chloroquine but not histamine. (b) Capsaicin activated 235 of 564 cells. Among histamine-responsive cells, 16 of 23 also responded to capsaicin. (c) AITC activated 161 of 564 TG neurons, and more than half of chloroquine-responding cells (9 of 17) responded to AITC. (d) AITC activated 9 of 23 of histamine-responding cells. (e) Capsaicin activated 5 of 17 of chloroquine-responsive TG neurons.
To investigate whether responsiveness of adult trigeminal neurons to chloroquine or histamine is dependent upon pruritogen dose, we further explored coincident calcium responses to 100 µM histamine and 100 µM chloroquine. Most trigeminal neurons that responded either to 100 µM histamine (4.8% of all cells) or 100 µM chloroquine (4.3% of all cells) did not respond to both (0.9% of all cells). Moreover, we also found in DRG neurons from adult mice that the populations of fibers responding to chloroquine and histamine are, as in the trigeminal ganglia, largely distinct (data not shown). These data demonstrate that primary afferent populations responding to histamine and chloroquine are largely distinct in adult mice.

The requisite expression of TRPV1 for histaminergic itch\textsuperscript{14}, and of TRPA1 for chloroquine-evoked itch\textsuperscript{12} raises the question: do relative expression patterns of TRPA1 and TRPV1 differ among chloroquine- and histamine-sensitive neuronal populations? To answer this, we exposed cultured trigeminal neurons to 100 µM AITC and 1 µM capsaicin and measured coincident responses of neurons to both agents. Consistent with the described receptor expression patterns for neurons that mediate histamine itch (e.g., H1R and TRPV1)\textsuperscript{10,14} and chloroquine itch (e.g., MrgprA3 and TRPA1)\textsuperscript{6,12}, we found that a majority (16 of 23, 69.6%) of histamine-responsive cells responded to capsaicin (Figure 3.6b), and most (9 of 17, 52.8%) chloroquine-activated neurons were sensitive to AITC (Figure 3.6c). The inverse relationships were also true: most (14 of 23, 60.8%) histamine-responsive trigeminal cells did not respond to AITC (Figure 3.6d), and capsaicin failed to activate a majority (12 of 17, 70.6%) of chloroquine-activated neurons (Figure 3.6e).
Roles of TRP channels in histamine and non-histamine itch

The different expression patterns of TRPV1 and TRPA1 among histamine and chloroquine-sensitive neurons in vitro (Figure 3.6) suggest that targeted silencing of TRPV1 or TRPA1 fibers may differently affect histamine itch versus chloroquine itch. To explore this we administered 20 µl conditioning injections of capsaicin (0.1%) or AITC (0.15%) with or without QX-314 and then administered 10 µl test injections of the pruritogens histamine (100 µg), chloroquine (50 µg) or SLIGRL (50 µg) 30 minutes later at the same site. Injection of capsaicin alone did not significantly reduce subsequent histamine-, chloroquine-, or SLIGRL-evoked scratching (data not shown). However, when the conditioning injection of capsaicin was administered together with QX-314, the scratching evoked by subsequent histamine injection was abolished (Figure 3.7a). Administration of capsaicin with QX-314 also significantly reduced but did not eliminate scratching produced by subsequent injections of chloroquine or SLIGRL (Figure 3.7b,c) even though only a third of chloroquine neurons are capsaicin sensitive (Figure 3.6e), implying that the TRPV1+ subset of chloroquine-sensitive neurons may have a particularly prominent role in eliciting behavioral itch responses.

When QX-314 was administered together with AITC for the conditioning injection, subsequent SLIGRL-evoked scratching was significantly reduced (Figure 3.7c). Likewise, chloroquine-evoked scratching was virtually abolished 30 minutes after injection of QX-314 together with AITC (Figure 3.7b). In contrast, injection of QX-314 with AITC did not reduce subsequent histamine-evoked scratching (Figure 3.7a). In summary, chloroquine- and SLIGRL-evoked scratching is effectively blocked when TRPA1+ (AITC-responsive) fibers are electrically silenced. Histamine-evoked scratching, on the contrary, is largely abolished when TRPV1+ fibers are electrically silenced but is unaffected when TRPA1+ (AITC-responsive) fibers are blocked.
Histamine itch is predominantly associated with TRPV1$^+$ pruriceptor fibers, and non-histamine itch with TRPA1$^+$ fibers.
Figure 3.7 Selective silencing of nociceptor populations differentially inhibits histamine and non-histamine Itch

Cheek scratching (itch) following intradermal test injection of (a) histamine (100 µg/10 µl) alone (n = 6), (b) chloroquine (CQ, 50 µg/10 µl) alone (n = 6-7), and (c) SLIGRL (50 µg/10 µl) (n = 5-6) 30 minutes after a conditioning injection of vehicle (0.9% NaCl, 20 µl) or 1% QX-314 together with capsaicin or AITC. P-values represent comparison to vehicle (white column) value (not significant, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$). Error bars, SEM.
**TRPV1⁺/TRPA1⁺ afferents are involved in itch inhibition**

Cheek injection of capsaicin normally evokes only a wiping (pain) response without any significant scratching (itch). We found, however, that cheek injection of capsaicin (0.1%, 10 µl) produced significant scratching (itch) together with wiping (pain) when TRPA1-responsive nociceptors were first silenced by conditioning coinjection of AITC (0.15%, 20 µl) with 1% QX-314 (Figure 3.8a). Injection of capsaicin (0.1%, 10 µl) 30 minutes after 20 µl injection of AITC (0.15%), capsaicin (0.1%), QX-314, or vehicle (0.9% NaCl) alone produced only wiping (data not shown). Capsaicin-evoked scratching following coinjection of AITC (0.15%) with QX-314 was prevented when the conditioning combination included histamine (100 µg) (data not shown). These data suggest that a subset of TRPA1⁺ sensory neurons normally mask capsaicin-evoked itch, and that silencing these neurons allows capsaicin to generate itch-related behavior through uninhibited activation of TRPV1⁺ histaminergic itch fibers.

When we repeated the experiments using AITC (0.15%, 10 µl) as the test algogen (Figure 3.8b) we again observed that algogen-mediated electrical silencing, this time by coinjection of capsaicin (0.1%) and QX-314, resulted in both wiping and scratching upon test injection of AITC (Figure 3.8b). The AITC-evoked scratching was largely reversed when chloroquine- and capsaicin-responsive fibers were both silenced by a conditioning injection of chloroquine (50 µg), capsaicin (0.1%) and QX-314 together (data not shown). These findings suggest that a subset of TRPV1-expressing sensory neurons normally mask itch following AITC activation of chloroquine-sensitive itch fibers.
Figure 3.8 TRPV1+/TRPA1+ fibers suppress itch
(a) Hindlimb scratching (itch) evoked by capsaicin (0.1%, 10 µl) thirty-minutes after intradermal injection of vehicle (0.9% NaCl, 20 µl), capsaicin (0.1%) together with 1% QX-314 (20 µl) or AITC (0.15%) together with 1% QX-314 (20 µl). Note the significant increase in capsaicin-mediated scratching following application of AITC with QX-314. (b) Thirty-minutes after injection of vehicle (0.9% NaCl, 20 µl) intradermal cheek injection of AITC (0.15%, 10 µl) generated little hindlimb scratching over the next 30 minutes and this was not changed following a conditioning injection of AITC together with 1% QX-314. However, the AITC evoked scratching increased following intradermal cheek injection of capsaicin (0.1%) together with 1% QX-314 (20 µl). P-values represent comparison to vehicle value (not significant, $P > 0.05$; *, $P < 0.05$). Error bars, SEM. $n = 6$ for all groups.
Thus, silencing TRPA1+ fibers and silencing TRPV1+ fibers allow capsaicin and AITC, respectively, to evoke scratching (itch) responses. Our interpretation is that there is a subset of peripheral TRPV1+/TRPA1+ neurons that when activated normally inhibit or mask itch via central inhibitory interneurons32, and whose silencing then allows either capsaicin or AITC to abnormally evoke itch.

**Discussion**

We selectively inhibited pain- and itch-related behaviors by targeting the membrane-impermeant sodium channel blocker QX-314 into peripheral axon terminals of distinct populations of trigeminal pruriceptors or nociceptors. The specific population silenced was determined by the pattern of activation of particular large pore channels by different pruritogens or algogens; TRPV1 for capsaicin and histamine, TRPA1 for AITC, chloroquine and SLIGRL. We show by electrophysiological recording that activation of these large pore channels by histamine and chloroquine enables sufficient permeation of QX-314 into trigeminal neurons to block sodium currents, as previously shown for capsaicin19, and that this effect is specific; only pruritogen-activated neurons are blocked. The fact that specific TRP-channel blockers (TRPV1 for histamine and TRPA1 for chloroquine) can prevent permeation of QX-314 indicates that it is TRP channel mediated. This has enabled us to exploit the silencing of different afferents to tease out their functional sensitivity to defined stimuli. This approach differs from interventions that only block a particular receptor (e.g., H1R, H4R)33 or channel (e.g. TRPA1)34 in that it targets action potential generation and conduction of the activated axon, and differs from genetic targeted ablation of different sensory neuronal subtypes35 in that it is temporary with no compensatory changes.
Utilizing this selective silencing strategy we reveal that fibers that mediate histamine and non-histamine itch are functionally separable. Moreover we demonstrate that activation of these itch-generating fibers is not required for eliciting normal responses to acute mechanical and thermal stimuli. Our in vitro data confirm the presence of adult sensory neurons that respond only to histamine or only to chloroquine.

Separate afferent lines are described for histamine and cowhage itch\textsuperscript{16,36} and a distinct non-histamine itch pathway activated by β-alanine\textsuperscript{17}. Our data support, in addition, separate functional pathways for histamine itch and itch mediated by the MrgprA3 and C11 ligands chloroquine and SLIGRL, respectively. The separation of these afferents based on the silencing approach was defined for a broad range of pruritogen concentrations for targeting QX-314 into afferent terminals through activated TRP channels. At extremely high concentrations (>90 mM) of pruritogen in vivo, however, an overlap did occur between histamine and chloroquine populations. This cross-activation between the two populations could be secondary to release of endogenous mediators from keratinocytes, mast cells or other non-neuronal cells activated secondary to high-dose pruritogen administration, or it could reflect a very limited sensitivity of the peripheral terminals of histaminergic pruriceptors to chloroquine and SLIGRL, and of the non-histaminergic terminals to histamine. Given that the high concentrations of the opposing pruritogen required to co-activate the separate histamine or non-histamine responsive set of afferents are unlikely to be found in most natural conditions\textsuperscript{29,30}, we consider it probable that the two sets are functionally distinct and normally act independently.

Our calcium imaging data also confirm the existence of distinct trigeminal and DRG neuron populations that respond to either chloroquine or histamine at a range of doses in adult mice. Prior in vitro experiments have shown that MrgprA3 lineage DRG neurons in juvenile (4-
week-old) mice respond both to chloroquine (1 mM) and histamine (50 µM). Similarly, ablation of MrgprA3+ neurons in 5-week-old mice significantly attenuates both chloroquine- and histamine-evoked scratching behavior. However, a recent study that showed that the proportion of DRG neurons responding to both histamine and chloroquine is markedly less in 7 to 9-week-old mice compared to 3 to 4-week-old mice. We interpret these collective data as suggesting that in adult (2 to 4-month-old) mice, Mrgpra3+ neurons are likely composed of pruriceptors with a differential sensitivity to, but not absolute selectivity for chloroquine, and that these afferents normally contribute primarily to non-histaminergic itch. Microneurographic studies in humans have identified a distinct set of histamine-insensitive fibers activated during cowhage-evoked itch. The finding that cowhage spicules activate MrgprA3+ neurons suggests that a common pathway may mediate chloroquine and cowhage itch. However, cowhage spicules act promiscuously on many nociceptor subtypes and may instead trigger itch nonspecifically through focal activation of superficial nociceptor terminals.

H1R and MrgprA3 receptors rely on downstream activation of TRPV1 or TRPA1 channels, respectively, to generate itch behavior, which is somewhat counterintuitive since these TRP channels are also activated by algogens (capsaicin and AITC) that normally produce pain. This raises questions both whether there are different subsets of TRPV1+ or TRPA1+ neurons involved in processing pain or itch, and why pain normally predominates. Our data show that histamine itch is mediated by TRPV1+ fibers that do not express appreciable levels of TRPA1, since histamine itch is inhibited by silencing capsaicin-activated fibers, but not by silencing AITC-activated fibers. This correlates with the coincidence of capsaicin and histamine responsiveness in most histamine responsive trigeminal neurons. Chloroquine or SLIGRL itch, in contrast, appears to be mediated mainly by TRPA1+ fibers. But, using capsaicin-induced
silencing and trigeminal neuron calcium imaging we show that a subset of these fibers also coexpress TRPV1. TRPV1 is therefore promiscuously expressed in nociceptors and both histaminergic and non-histaminergic pruriceptors.

Electrical silencing of either histamine-sensitive or chloroquine-sensitive primary afferents blocks itch but does not alter a wide range of pain-associated behaviors, suggesting that while these primary afferent fibers are required for itch, as are GRPR⁺ neurons in the dorsal spinal cord\(^{35,38}\), they are not necessary for eliciting acute thermal or mechanical pain, as also demonstrated for the MrgprA3⁺ population\(^ {15} \). Our data do not rule out the possibility, though, that activation of some histamine-sensitive or chloroquine-sensitive fibers might be sufficient to produce pain. For example, histamine can evoke pain in rodents and humans\(^ {39,40} \), particularly in bradykinin-sensitized nociceptors\(^ {41} \). We also find a group of peripheral neurons expressing both TRPA1 and TRPV1 that appear to be involved in a functional inhibition or masking of itch, since silencing either TRPA1⁺ fibers or TRPV1⁺ fibers now allows capsaicin or AITC to evoke itch, rather than pain. Consistent with this, when nociceptive sensitivity is reduced by abolishing vesicular glutamate transporter type 2 (VGLUT2)-dependent synaptic glutamate release in nociceptors, intradermal capsaicin injection is also now able to generate scratching\(^ {42} \). We propose, therefore, that the algogen-evoked itch that follows electrical silencing of contra-algogen-responsive fibers may represent a pain-to-itch synesthesia produced only when TRPV1⁺ histaminergic pruriceptors or TRPA1⁺ non-histaminergic pruriceptors are activated and a normally itch-inhibiting subset of TRPV1⁺/TRPA1⁺ nociceptors is silenced (Figure 3.9). In the absence of such silencing, the combination of activation of nociceptive pathways and inhibition of itch by TRPV1 or TRPA1 expressing nociceptors will lead to pain dominating as a sensation.
Our findings suggest that primary afferent itch-generating neurons encode functionally distinct histamine and chloroquine itch pathways (Figure 3.9). In addition to revealing modality specificity and functional specialization of somatosensory afferents, our findings could also help direct development of new treatments for itch. Administration of QX-314 may be an effective treatment for pruritus caused by either histamine or non-histamine pruritogens if they are associated with sufficient activation of TRPV1 or TRPA1. Alternatively, because large-pore channels are present in both histamine- and chloroquine-sensitive pruriceptors, targeting QX-314 broadly into these fibers via co-activation of both TRPV1 and TRPA1 channels, for example by using a non-pungent TRPV1/TRPA1 co-activator\textsuperscript{23,24,43}, may have therapeutic promise for preventing or blocking both histamine-evoked itch and histamine-independent itch, although at the expense of also producing analgesia.
Figure 3.9 Hypothetical model of itch and pain circuitry based on primary afferent neuron subsets associated with transmission of pain and itch

Venn representation of afferents associated with the peripheral transmission of pain and itch are defined by expression of TRPV1 (capsaicin-responsive neurons, red Venn circle), TRPA1 (AITC-responsive neurons, yellow Venn circle), H1R (histamine-responsive neurons, green Venn circle) and MrgprA3 (chloroquine-responsive neurons, blue Venn circle). Our data suggest that peripheral terminals responding to capsaicin and histamine (brown shaded area) mediate histaminergic itch, while non-histaminergic itch is transmitted by two groups of fibers: fibers expressing TRPV1, TRPA1 and MrgprA3 (pink shaded area), and fibers that express only TRPA1 and MrgprA3 (gray shaded area). We propose that distinct populations of primary afferents, histamine itch-generating fibers (H1R+/TRPV1+) and non-histamine itch-generating fibers (MrgprA3+/TRPA1+/-), transmit signals to itch-related sensory neurons located in the spinal cord dorsal horn. Finally, our observation that algogens can evoke scratching (itch) following silencing of contra-algogen-responsive fibers implies existence of a population of TRPV1+/TRPA1+ fibers (from within orange shaded area) that initiate an algogen-mediated inhibition or masking of itch probably via central inhibitory interneurons (IIIn).
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