# MOUSE MODELS OF UROGENITAL PAIN:

# CAUSES AND CONSEQUENCES

# OF INFECTION AND INFLAMMATION

Melissa A. Farmer

Department of Psychology

McGill University

Montreal, Canada

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# ABSTRACT

The goal of this thesis was to examine the utility of animal models of urogenital pain to explore the causes and consequences of acute and chronic genital and non-genital pain. In the first review chapter entitled “Animal Models of Dyspareunia,” the validity and reliability of animal models associated with dyspareunia (painful intercourse) is examined in terms of how closely they parallel the clinical presentation of their respective pain conditions. The review reveals that many of these models are limited by the lack of specificity of their pain behaviours and/or poor correlations between pain and tissue pathology. The second chapter entitled “Repeated Vulvovaginal Fungal Infections Cause Persistent Pain in a Mouse Model of Vulvodynia,” describes a mouse model of provoked vestibulodynia developed and used to examine the hypothesis that persistent vulvar pain may result from prolonged vulvovaginal inflammation due to *Candida albicans* infection. We found that a subset of mice exhibited vulvar mechanical hypersensitivity following three fully resolved *Candida* infections, as well as following a single, extended infection and repeated vulvar injections of the inflammatory compound zymosan. Following repeated *Candida* infections, allodynic mice showed significant increases in vulvar innervation, including peptidergic afferent and sympathetic efferent nerve fibers. This model provides evidence for biological mechanisms underlying chronic vulvar pain and sheds doubt on previous assumptions that vulvar pain is merely a consequence of impaired sexual response. In the final

empirical investigation, the hypothesis that sexual motivation can be directly modulated by genital versus non-genital pain was evaluated. Whereas genital and non-genital tonic inflammatory pain did not impact the sexual behaviour of male mice, both types of pain resulted in reduced female sexual motivation and behaviour. Together, this body of work demonstrates the potential utility of novel animal models of urogenital pain to study the etiology and impact of pelvic pain in humans.

# RESUMÉ

L'objectif de cette thèse était par conséquent d'examiner l'utilité des modèles animaux de douleur urogénitale afin d'explorer les causes et les conséquences de la douleur aiguë et chronique au niveau des organes génitaux et non-génitaux. Le premier chapitre intitulé “Des modèles animaux de la dyspareunie,” examine la validité et la fiabilité des modèles animaux de la dyspareunie en ce qui concerne le degré de parallèle de la présentation clinique avec leurs conditions de douleur respectives. La revue de littérature démontre que plusieurs de ces modèles sont limités par le manque de spécificité des comportements de douleur et/ou par de faibles corrélations entre la douleur et la pathologie des tissus. Le deuxième chapitre intitulé “Les infections fongiques repetées de la partie vulvo-vaginale causent une douleur persistante dans un modèle murin de la vulvodynie,” décrit un modèle murin de vestibulodynie provoquée développé et utilisé dans le but d’examiner l'hypothèse que la douleur vulvaire persistante peut résulter d'une inflammation prolongée vulvo-vaginale secondaire à une infection à *Candida albicans*. Nous avons constaté qu'un sous-ensemble de souris a démontré une hypersensibilité mécanique au niveau de la vulve après trois résolutions complètes d’infections à Candida ainsi qu’après une seule infection prolongée incluant des injections vulvaires répétées de zymosan, soit un composé inflammatoire. Suite à des infections répétées à *Candida*, les souris présentant une allodynie vulvaire démontraient une augmentation significative de l'innervation de la vulve ainsi que des

fibres afférentes peptidergiques et des fibres nerveuses efférentes sympathiques. Ce modèle fournit des preuves pour les mécanismes biologiques causantes des douleurs vulvaires chroniques prouvant les hypothèses précédentes que la douleur vulvaire est simplement une conséquence de la reponse sexuelle réduite. La dernière étude empirique, intitulé “Pas ce soir chéri, j'ai un mal de tête: Les différences entre les sexes dans l'effet de la douleur sur la motivation sexuelle,” a évalué l'hypothèse que la présence de douleur génitale contrairement à la douleur non-génitale peut directement influencée la motivation sexuelle. Bien que la douleur inflammatoire tonique au niveau de la région génitale et des régions non génitales n’aient pas eu d'impact sur le comportement sexuel des souris mâles, les deux types de douleur ont entraîné une réduction significative de la motivation sexuelle ainsi que des comportements sexuels chez la souris femelle. L’ensemble de ces travaux démontrent l'utilité potentielle de nouveaux modèles animaux de douleur urogénitale pour étudier l'étiologie et l'impact de la douleur pelvienne chez l'humain.

# CONTRIBUTION OF AUTHORS

This thesis consists of one chapter and two papers, with a brief introduction and discussion. The first chapter is co-authored by myself and Drs. Yitzchak Binik and Jeffrey Mogil. The second paper is coauthored by myself, Anna Taylor, Dr. Andrea Bailey, Alexander Tuttle, Leigh MacIntyre, Zarah Milagrosa, Halley Crissman, and Drs. Gary Bennett, Alfredo Ribeiro-da-Silva, Yitzchak Binik, and Jeffrey Mogil. The third paper is coauthored by myself, Alison Leja, Emily Foxen-Craft, Lindsey Chan, Leigh MacIntyre, Tina Niaki, Mengsha Chen, and Drs. Yitzchak Binik, James Pfaus, and Jeffrey Mogil. The following is a statement regarding the respective contributions of the various authors to the chapter and two papers.

The chapter was conceived, planned, and written by myself. Drs. Binik and Mogil served in an editorial capacity during the writing of the final chapter.

The second paper resulted from a research study that was elaborated, conducted, analyzed, and written by myself. Anna Taylor assisted trouble-shooting the immunohistochemistry process and in the processing of images for immunohistochemistry. Andrea Bailey aided in the interpretation of histology slides. Alexander Tuttle conducted the control experiment that demonstrated a lack of hypersensitivity in the hindpaw following repeated zymosan injections. Leigh MacIntyre, Zarah Milagrosa, and Halley Crissman served as behaviour testers blind to condition. Additionally, Zarah Milagrosa aided in the processing and interpretation of

microbiological information. Gary Bennett aided in the theoretical elaboration of research questions and in an editorial capacity during the writing of the final manuscript. Alfredo Ribeiro-da-Silva served in an advisory capacity for the interpretation of immunohistochemical data and in an editorial capacity during the writing of the final manuscript. Yitzchak Binik served in an advisory capacity during the formulation of research questions, the development of protocol in reference to the clinical presentation of the modeled disease, and in an editorial capacity during the writing of the final manuscript. Jeffrey Mogil served in an advisory capacity during the formulation of research questions, the development of protocol, and in an editorial capacity during the writing of the final manuscript.

The third paper resulted from a research study that was elaborated, conducted, analyzed, and written by myself. Alison Leja was paid to help conduct animal experiments, monitor mating progress, and code data. Assistance in conducting animal experiments and blind data coding was also provided by Emily Foxen-Craft, Lindsey Chan, Leigh MacIntyre, Tina Niaki, and Mengsha Chen. Drs. James Pfaus, Yitzchak Binik, and Jeffrey Mogil served in an advisory capacity during the formulation of research questions, the development of the protocol, and in an editorial capacity during the writing of the final manuscript.

# STATEMENT OF ORIGINAL CONTRIBUTIONS

This dissertation is a manuscript-based thesis comprised of three peer-reviewed publications that provide original contributions to the field of urogenital pain. The first paper entitled “Animal Models of Dyspareunia” was published in 2008 in the first edition of *Female Sexual Pain Disorders*, pp. 199-207. This paper was the first comprehensive review and evaluation of animal models of conditions that can produce dyspareunia in humans, including ureteral calculosis, uterine inflammation, vaginal and uterine distension, endometriosis, interstitial cystitis, colitis, parturition, and yeast infection-induced vulvar sensitivity. The validity of these respective models were examined in terms of behavioural specificity to the condition, reliability, relation to pathology, and reversibility with analgesics. The results of this review indicate that a primary weakness of the majority of these models is the reliance on pain behaviour that is not stimulus- or condition-specific. Indeed, only two animal models inferring pain from vaginal distension and vulvar mechanical sensitivity show the needed stimulus specificity (the latter of which is discussed in this thesis). Furthermore, a correlation between physical pathology and pain behaviour is absent in the majority of these models, which raises questions as to how closely these models mimic conditions in humans that are reliably diagnosed via evidence of pathology in affected tissue. This review strongly suggests that animal models of conditions related to dyspareunia require substantial development

to adequately capture the clinically important characteristics of these disease processes.

The second manuscript entitled “Repeated vulvovaginal fungal infections cause persistent pain in a mouse model of vulvodynia” is currently in review (after invited revision) at *Science Translational Medicine*. This publication is the first to demonstrate a causal relationship between repeated and extended yeast infections and vulvar mechanical hypersensitivity, thereby providing the first empirical evidence of an inflammatory etiology of vulvodynia. Indeed, it suggests that any type of inflammation may be sufficient to induce persistent mechanical hypersensitivity, thereby providing the first evidence that prolonged pain states can be caused by infection and inflammation that is long resolved. The results indicate that only allodynic mice exhibit increased vulvar innervation (including increased peptidergic and sympathetic fiber immunoreactivity), which closely parallels evidence of altered innervations patterns in the painful vulvar tissue of women with localized provoked vulvodynia. These findings provide strong support for the long-held hypothesis in the gynaecological literature that vulvar pain may result from inflammatory insults, and it also constitutes a major contribution to the pain research field as a whole as the first evidence of persistent pain following resolved inflammation.

The third manuscript, entitled “ ‘ Not tonight dear I have a headache’: Sex differences in the effect of pain on sexual motivation,” is in preparation. This experiment

provides the first empirical evidence that the presence of pain—both genital and non-genital—suppresses sexual motivation in female mice, whereas male sexual motivation is unaffected by pain. It is also the first study to use paced mating in mice to evaluate sexual reward in the presence of an aversive inflammatory pain state, which suggests this model may be useful in evaluating the efficacy of a variety of clinical compounds thought to impact female sexual behaviour.

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depth of emotion, and most of all your vulnerability, which you face with honesty and perseverance. Kels, I miss your monkey calls…I respect you, I admire you, and I love the humor you find in everyday life. When I am with you, I appreciate the simplicities in life. And Mom, whether you are now dust or recycled energy, I thank you for trusting me and letting me into your emotional world. I never realized how much you are ingrained in me until we lost you, and I think of you often. Your loss has made me an emotional rock in many ways, and I learned volumes about myself in the past 1.5 years. I miss you, and I wish you could see me graduate, marry, have children, and continue to grow. Fortunately Dad, Kels, and Angela will be with me to feel the joy you would have felt.

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# INTRODUCTION

The field of urogenital pain has expanded dramatically in the last 20 years, as clinicians have struggled to treat a variety of chronic urogenital and visceral pain conditions with unknown etiologies, including vulvodynia, prostatitis, endometriosis, interstitial cystitis, and irritable bowel syndrome. Relying on uncontrolled, cross-sectional, retrospective clinical studies, an increased understanding of these conditions has largely failed to materialize, and clinical efforts to assess and treat these conditions are critically limited by a lack of knowledge about disease mechanisms (Masheb, Nash, Brondolo, & Kerns, 2000). Animal models of disease states—because of their considerable ethical and practical advantages compared to human studies—stand to make a vital contribution to our understanding of how these urogenital pain conditions are initiated and maintained (Zimmerman, 1986). The development of valid and reliable animal models may aid in the early identification of at-risk populations, the refinement of assessment strategies when quantifying the extent of pathology, and most importantly, the generation of novel treatment interventions for pain conditions that are largely considered untreatable at the present time.

The following literature review highlights the numerous limitations in our current understanding of the prevalent urogenital pain condition, vulvodynia, which inspired the experiments presented in this dissertation. A review of the

vulvodynia literature emphasizes the need for an empirical evaluation of the longest-standing etiological hypothesis that vulvodynia results from persistent inflammation (Gerber, Witkin, & Stucki, 2008). Given the clear ethical restraints in testing such a hypothesis in humans, the utility of animal models is considered in the first paper, entitled “Animal models of dyspareunia,” published in the first edition of *Female Sexual Pain Disorders*. This paper examined the criteria for developing valid animal models, reviewed the existing animal models of conditions related to dyspareunia in women, and determined which models have demonstrated optimal validity and reliability in relation to their clinical prototypes. This review clearly shows a paucity of viable animal models related to urogenital pain, with the exception of the model developed as part of this dissertation.

Based on the evaluation of existing animal models of female urogenital pain, it was necessary to develop a novel animal model of vulvar pain, in order to directly test the hypothesis that inflammation can produce persistent vulvar pain. This hypothesis was tested for the first time in the next manuscript entitled “Repeated vulvovaginal fungal infection and inflammation causes persistent pain.,” published in *Science Translational Medicine*. The primary objective of this paper was to test this hypothesis in relation to repeated or extended yeast infections, which are commonly reported in populations of women with localized provoked vulvodynia (Bohm-Starke et al., 2001; Witkin, Gerber, & Ledger, 2002),

and to determine whether any type of inflammation was sufficient to produce a chronic vulvar pain state. The goals of this paper included the development of an animal model of vulvar pain, an examination of pain behaviour associated with active and resolved infection/inflammatory states, and an evaluation of histological correlates of vulvar hypersensitivity to determine whether peripheral pathology results from these experimental manipulations.

The confirmation that vulvar pain can be replicated in an animal model raised the question of whether animal models can also be used to evaluate quality-of-life factors associated with chronic vulvar pain. This question led to the development of the final manuscript of this dissertation entitled " 'Not tonight dear I have a headache': Sex differences in the effect of pain on sexual motivation," which is in preparation. The goals of this paper were to determine if urogenital pain directly reduces sexual motivation in females versus males, whether a potential effect of pain on sexual motivation generalizes to non-genital pain, whether pain-induced reductions in sexual behaviour can be reversed with libido-enhancing drugs, and whether acute and chronic pain affect sexual motivation in similar patterns.

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# LITERATURE REVIEW

The first recorded reference to vulvar pain is embedded in the ancient Egyptian Ramesseum Papyrus (Farmer, Kukkonen, & Binik, 2008). The phenomenon was not described in medical texts until the 19th century, where it was variably called vulvar hyperesthesia, vulvar "supersensitiveness" and heightened vulvar "sensitiv[ity]" (Amalraj, Kelly & Bachmann, 2008). The first attempt to develop formal terminology for vulvar pain occurred in 1976, when the International Society for the Study of Vulvovaginal Disease (ISSVD) introduced the terminology "burning vulva syndrome" based on women's reports of a hot, burning vulvar sensation, and soon thereafter this term was replaced with "vulvodynia." During the 1980s, the early gynecological literature differentiated two subtypes of vulvodynia based on the perceived location of vulvar pain. Localized vulvar pain, or vulvar vestibulitis (VVS), was exclusively elicited by touch or pressure to the vulvar vestibule (Friedrich, 1987), whereas a more diffuse, or "generalized" vulvar pain was termed dysesthetic (or "essential") vulvodynia (McKay, 1989). Despite multiple revisions of the vulvodynia nomenclature, the ISSVD's 2003 vulvar pain terminology has essentially preserved these two subtypes as localized vulvodynia and generalized vulvodynia (Moyal-Barraco & Lynch, 2004). Epidemiological studies have

confirmed that localized provoked vulvodynia (commonly called provoked vestibulodynia, and previously vulvar vestibulitis syndrome) is the most common vulvodynia subtype in premenopausal women, affecting between 9-12% of women (Harlow & Stewart, 2003; Harlow et al., 2001), and recent evidence suggests provoked vestibulodynia (PVD) may also explain a majority of postmenopausal dyspareunia (pain during sex; Kao et al., in press).

Vulvodynia is an umbrella term that can refer to pain experienced at any location on the vulva (external genital organs), including the clitoris, labia majora, labia minora, Bartholin's glands, and the vaginal entrance (vulvar vestibule). Pain can be highly localized (e.g., PVD) or more diffuse or radiating (generalized vulvodynia). Clinically, two different groups of patients with PVD have been described: primary provoked vulvodynia is defined as dyspareunia from the first attempt of sexual intercourse (and is often associated with a history of pain with tampon use), whereas in secondary provoked vulvodynia the dyspareunia appears after a period of pain-free sexual intercourse. Different subtypes of vulvodynia are often characterized by unique pain descriptors. For instance, PVD is described as "burning" or "cutting" pain, whereas dysesthetic vulvodynia elicits "tingling" and "shooting" sensations. "Sore" and "aching" sensations may refer to pain originating in the vulvar tissue or underlying muscle, potentially due to pelvic floor tension secondary to vulvar pain. Provoked vulvar pain is initiated with

mechanical stimulation (from clothes, sitting, or sexual activity) and may be of brief or prolonged duration, depending on the intensity of stimulation and the degree of vulvar allodynia. In contrast, unprovoked vulvar pain is unpredictable in onset and duration, although mechanical provocation can at times elicit this spontaneous pain as well. These vulvar pain characteristics are not associated with any evident pathology, meaning that vulvodynia is a diagnosis of exclusion rather than a unitary condition (McKay, 1989).

The current understanding of vulvodynia as a multifactorial chronic pain condition can be traced to small, independent literatures on vulvar pain from a variety of disciplines, including gynecology, neurology, dermatology, physical therapy, and psychiatry. These literatures originally consisted of case studies or uncontrolled, cross-sectional research that conceptualized vulvar pain using the vocabulary of the respective discipline (e.g., Friedrich, 1987; Marinoff & Turner, 1991; McKay, 1989). Accordingly, gynaecologists (Goldstein& Burrows, 2008) theorized that vulvodynia may result from anatomical abnormalities, hormonal imbalances, urogenital disease or infection, inflammatory conditions, or bladder dysfunction (interstitial cystitis, descended bladder). Neurologists suspected peripheral nerve injuries, such as pudendal neuralgia or abnormalities in peripheral pain transmission (Bohm-Starke, 2010). Dermatologists investigated lichen sclerosis, lichen simplex chronicus, lichen planus, allergic contact

dermatitis, and poor vulvar hygiene (McKay, 1990), whereas physical therapists targeted pelvic floor abnormalities, including heightened muscle tension, weakness, and instability (Rosenbaum, 2007). When no medical cause of vulvar pain could be identified, women were referred to psychiatrists who often interpreted the pain as a confabulation of female neuroses (e.g., Whitlock, 1967). This fragmented history of the study of vulvodynia provides some key caveats for the interpretation of past research. First, the term "vulvodynia" likely refers to a symptom with multiple etiological pathways, suggesting the existence of unidentified vulvodynia subtypes. Secondly, approaching vulvar pain from any single perspective will limit an understanding of its multidimensional nature. Finally, successful treatment of the condition that originally caused the vulvar pain may not address the mechanisms that have chronically maintained the pain. In summary, vulvodynia likely consists of multiple subtypes, each with unique etiologies, and therapeutic interventions that target these initiating factors may fail to address the pathological mechanisms underlying the maintenance of chronic vulvar pain.

Etiological speculations about vulvodynia have focused on potential biomedical and psychosocial causes of pain, yet to date no specific theory is supported by empirical evidence (Farmer, Kao, & Binik, 2009). The hypothesis that vulvar pain is the product of repeated or prolonged inflammation was

discussed by Friedrich (1987) in his original description of vulvar vestibulitis syndrome and has since dominated the vulvodynia literature. Variants of this hypothesis have focused on the suspected inflammatory culprits, including vulvovaginal yeast or bacterial colonization, or presence of vaginal human papillomavirus (HPV; Marinoff & Turner, 1991). Other hypotheses assume some type of vulnerability in the host, including abnormal innate and adaptive immune responses to vulvovaginal inflammation or genetic predispositions that facilitate such abnormalities (Gerber, Witkin, & Stucki, 2008). Hormonal factors may play a role in the development of vulvar pain, given that vulvar pain can vary across the menstrual cycle, during pregnancy, and with menopause. Histological findings that women with VVS have reduced estrogen receptors in vulvar vestibular tissue suggests that such effects may occur at the local level (Eva, MacLean, Reid, Rolfe, & Perrett, 2003), although estrogen also regulates factors involved in central pain modulation (i.e., serotonin) (see Chapter 1, “Animal Models of Dyspareunia”). Evidence of vulvar pain following physical trauma, such as surgery, suggests that the pain may originate from nerve injury or compression (as is assumed with pudendal neuralgia), but evidence of nerve injury is rarely found (Goldstein & Burrows, 2008). Increased vulvar vestibule innervation, including the transient receptor potential cation channel, subfamily V, member 1 (TrpV1) immunoreactive afferents, indicates peripheral signs

consistent with neuropathy (Bohm-Starke, 2010), yet the stimuli responsible for this heightened innervation is unknown and could be related to physical trauma or inflammation. Hypotheses that vulvar pain either results from or is maintained by pelvic floor muscle dysfunction focus on heightened muscle tension, greater muscle weakness, and lack of pelvic muscle control (Rosenbaum, 2007). Pain improvement with pelvic floor physiotherapeutic interventions has been reported (Glazer, Rodke, Swencionis, Hertz, & Young, 1995; Bergeron et al., 2001), yet pelvic floor muscle tension is more often assumed to be a factor that contributes to pain chronicity rather than a cause *per se*. Theoretically, mechanical friction caused by a lack of vaginal lubrication, sexual practices, or genital size may contribute to vulvar pain, as well.

Psychosocial etiological hypotheses of vulvodynia are based on cross-sectional data demonstrating higher rates of mood disturbances, personality disorders, and other psychological characteristics in women with genital pain, compared to healthy controls. Such research has suggested that anxiety, depression, pain catastrophizing, pain hypervigilance, and personality disturbances may characterize women with genital pain (Farmer, Kao, & Binik, 2009), but no causal evidence links vulvar pain to a specific psychological etiology.

Despite decades of research, our understanding of the mechanisms underlying the development and maintenance of vulvodynia are tentative at best. The inherent limitations of human research have complicated these efforts. An overreliance on cross-sectional, retrospective data collection, often from biased clinic samples, has reduced the generalizability of published findings. In histological studies, ethical concerns make it unfeasible to obtain true control biopsy tissue from age- and parity-matched women, and thus biopsy tissue from vulvar clinic patients or from non-painful tissue in women with vulvar pain are the only points of comparison. It is obviously unethical to induce infection or chronic inflammation in women to see whether they develop vulvar pain, and such experiments would be necessary to evaluate the etiological hypotheses of vulvodynia. As a result, human research can provide a limited amount of information about the mechanisms underlying vulvodynia.

Animal models of pain conditions avoid many of the confounding factors and methodological limitations that have hindered the progress of other areas of pain research (Le Bars, Gozariu, & Cadden, 2001). An increased focus on translational research has emphasized the importance of creating clinically relevant animal models (Mogil, 2009), which yield findings that can tangibly impact patient assessment and treatment practices. In addition to the typical benefits of animal research, including cost effectiveness, convenience, and

removal of confounding variables, translationally relevant animal models in pain research must straddle the boundary between what is clinically meaningful and what is feasible to produce in the laboratory. Whereas medical animal models have historically been used to dissect physiological and behavioural correlates of disease, an increasing number of models capture altered affective, social, motivational, and quality-of-life characteristics associated with disease (e.g., Mogil, 2009; Page, Blakely, & Kim, 2005; Urban et al., 2011). Importantly, animal models have proved useful in elucidating the pathological mechanisms underlying poorly understood clinical pain conditions, including neuropathic pain states, chronic joint pain, and diabetic neuropathy (e.g. Almarestani, Fitzcharles, Bennett, & Ribeiro-da-Silva, 2011; Piriz, Torres-Aleman, & Nunez, 2009; Ruocco, Cuello, & Ribeiro-da-Silva, 2000).

Given our limited knowledge of the mechanisms and consequences of vulvodynia following three decades of cross-disciplinary research, the development of clinically relevant animal models of vulvodynia may provide significant advances in the assessment, diagnosis, and treatment of vulvar pain. To maximize their validity, the design of such models must be guided by epidemiological data, patient self-report, and expert opinions from the men and women who treat this pain condition. This dissertation represents an attempt to do just this—to determine the strengths and weaknesses of existing animal

models of urogenital pain with the aim of developing a novel mouse model of provoked vestibulodynia, the most common form of vulvar pain, using a hypothesized cause extracted from patient reports and clinician observations. In addition to exploiting an animal model to inform our understanding of vulvodynia etiology, a complementary animal model of pain-induced reductions in sexual motivation is developed to explain a frequent clinical characteristic associated with genital pain—reduced motivation for sexual activity. The close alignment of these animal models with the clinical realities of urogenital pain will maximize their relevance, utility, and scientific contribution to the understanding of chronic urogenital pain.

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# ANIMAL MODELS OF DYSPAREUNIA

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## Abstract

Animal models of dyspareunia are powerful preclinical tools for evaluating the experimental parameters for pain. Such models provide cost-effective strategies for studying the mechanisms of pain induction and maintenance without many of the confounding influences on pain in humans. Rodent models of ureteral calculosis, uterine inflammation, vaginal and uterine distension, endometriosis, cystitis, colitis, parturition, and yeast infection-induced provoked vulvar pain are reviewed in terms of clinical relevance. The pursuit of animal models can enhance our understanding of the hormonal, immunological, and pathophysiological factors involved in pain conditions that result in dyspareunia.

## Introduction

In recent years, the development and application of new animal models of disease processes has been a popular scientific trend (Crawley, 2008). However, few of these models have focused on sexuality, and fewer still have modeled pain conditions that impact sexuality. Urogenital and abdominal pain conditions associated with dyspareunia impact a staggering percentage of women, yet very few of these conditions are well understood. Although imaging studies have greatly advanced human research in this area (Pukall et al., 2005), experimental options using human subjects are still limited. Animal models allow

experimental manipulations to evaluate the causal relationships between pathological causes and physiological effects. These models are convenient and cost-effective, and they permit the testing of hypotheses that are otherwise ethically implausible in humans. The development of viable animal models for conditions that are associated with painful intercourse, such as endometriosis, interstitial cystitis (IC), irritable bowel syndrome (IBS), and provoked vestibulodynia (PVD) might have profound implications for our understanding of the etiology, maintenance, and treatment of these debilitating conditions.

## Evaluation of Animal Models of Pain

Pain in animals is defined as "an aversive sensory experience caused by actual or potential injury that elicits progressive motor and vegetative reactions, results in learned avoidance behaviour, and may modify species-specific behaviour, including social behaviour" (Zimmerman, 1986). Animals cannot verbally rate their pain intensity, quality, or location, nor can they communicate the impact of emotion on pain. Instead, researchers infer the presence of pain from abnormal behaviours that are (hopefully) unique to the experimentally induced nociceptive state. The difficulty in measuring an animal's emotional or cognitive responses to pain suggests that we are largely using *nociceptive models*, rather than true pain models (Le Bars, Gozariu, & Cadden, 2001).

However, just because we cannot measure something doesn't mean it is not there. Nevertheless, the word *pain* will be used throughout this chapter.

Pain can be typified as spontaneous or provoked, depending on whether or not it is elicited by exogenous stimulation. Many existing animal models of pain are limited in their duration; chronic, spontaneous pain—the most clinically relevant form—has proved particularly difficult to model in animals (Mogil & Crager, 2004). Pain conditions can also be visceral or somatic in nature. Visceral pain originates from the internal organs contained within the chest and abdomen, and it is characterized by increased autonomic reactivity, emotional salience, and diffuse pain that may be referred to other visceral or somatic tissue that shares common innervations at the level of the spinal cord (Ness & Gebhart, 1990). Referred pain is perceived in areas distal from the site of injury that receives common spinal input as the region where pain originates (Head, 1893; Gebhart, 2000). The majority of animal models of pain conditions associated with dyspareunia are visceral in nature, including uterine inflammation, vaginal and uterine distension, endometriosis, and abdominal pain (including cystitis and colitis).

In animal models, behavioral responses may reflect the location of the pain in the case of somatic tissue (e.g., withdrawal of a heated hind paw), whereas visceral pain may be manifested as referred somatic pain (Head, 1893).

Patterns of pain behavior can increase in frequency or magnitude with higher levels of noxious stimulation. Abnormal behaviors that show temporal correspondence with tissue injury or inflammation are thought to reflect injury-specific pain, although the correlation may not be strong. For visceral pain in particular, the absence of behavior may be indicative of pain, as evidenced by reduced mobility or motivation to engage in normal activity (Bon et al., 2003). As well, estrous cyclicity may significantly impact some behaviors (Fillingim & Ness, 2000), but many indices of behavioral pain show equal variability when male versus female animals are used (Mogil & Chanda, 2005). Empirical validation that a behavior is specific to pain is often achieved via the administration of known analgesics, such as nonsteroidal anti-inflammatory drugs, lidocaine, or morphine.

Ultimately, many behaviors have been associated with pain in rodents. Table 1 lists behaviors that have been linked to animal models of dyspareunia. Notably, the criteria for dyspareunia vary between models. Whereas some models directly measure vaginal sensitivity to noxious stimuli, other models induce pain conditions associated with dyspareunia. Ideally, pain behaviors are unique to an experimental manipulation, easily quantifiable with minimal need for interpretation, frequent enough to allow for statistical comparisons between groups, and reliably observed in afflicted animals (and rare in healthy animals).

Such behavior should coincide with the duration and severity of injury and be mitigated by analgesics in a dose-dependent manner. Most importantly, the validity of an animal model of pain relies on whether the researchers have accurately identified a pain behavior that closely parallels the clinical characteristics of the condition it is intended to model. This chapter will be limited to reviewing rodent models of female urogenital and abdominal pain that include the measurement of pain behavior, not electrophysiological or electromyographic proxies, as a primary outcome measure (Le Bars et al., 2001).

## Animal Models of Dyspareunia

### Ureteral Calculosis

Women with dysmenorrhea, or painful menstruation, often report dyspareunia and are more likely to experience urinary calculosis (kidney stones). Based on this comorbidity, animal models of ureteral calculosis (UC) may indirectly induce dyspareunia, although this link has never been formally tested. The first detailed behavioral characterization of UC-induced visceral pain was conducted by Giamberardino's laboratory (Giamberardino et al., 1995). Within a day of implantation of an artificial stone into the left ureter, rats displayed a variety of spontaneous pain behaviors including stretching, hunched back, abdominal/flank licking, flank muscle contractions accompanied by ipsilateral

inward hind limb motions, lower abdominal squashing (against the floor), and the adoption of a supine position with the left hind limb retracted into the abdomen. These behaviors slowly decreased in frequency and duration over 4 days post-implantation. Rats with frequent visceral pain behaviors were more likely to vocalize to electrical stimulation of the ipsilateral oblique muscles, indicative of referred pain. These behaviors are similar to the protracted abdominal stretching observed in early visceral pain models (Siegmund, Cadmus, & Lu, 1957; Van der Wende & Margolin, 1956). Pain behaviors were reduced with intraperitoneal 5 mg/kg/day morphine. Giamberardino's model established a typology for abnormal pain behaviors associated with visceral pain that would be replicated or modified by the majority of subsequent abdominal visceral pain models.

Based on preliminary human evidence linking dysmenorrhea, endometriosis, and UC (Giamberardino et al., 2001), Giamberardino and colleagues (2002) developed a dual rat model of endometriosis with UC to investigate whether abdominal pain behaviors found in either condition are enhanced by the comorbidity. Animals with endometrial autografts plus stone implantations showed significantly longer bouts of pain behavior compared to stone implantation only or sham groups. Although all animals developed some referred hyperalgesia caused by the presence of a ureteral stone, the endometriosis + UC group displayed the greatest magnitude of referred pain as

indicated by reduced vocalization threshold in response to electrical stimulation of the left oblique muscles.

### Uterine Inflammation

Wesselmann and colleagues (1998) characterized pain behavior associated with uterine inflammation in the rat. The pain behaviors they examined were based on the Giamberardino model of UC (Giamberardino et al., 1995). To induce inflammation, 10% mustard oil and a mineral oil vehicle were injected into the uterine lumen and pain behaviors were videotaped for seven days post-surgery. Of animals receiving uterine inflammation, 79% displayed prolonged periods of spontaneous pain behavior, with behavior frequency peaking two days after surgery. Dramatic individual differences were found in the frequency and duration of pain behaviors, and animals with uterine inflammation showed reductions in overall mobility. Of animals displaying spontaneous pain behavior, 66% also showed referred muscle hypersensitivity in the lower back and flanks that actually outlasted the occurrence of spontaneous pain behaviors.

Wesselmann's study was especially significant in that it established that pain from distinct viscera—the ureter and the uterus—resulted in very similar behaviors, including behavioral evidence of referred pain. Although this behavioral similarity may support the validity of these behaviors as being specific

to pain, it also indicates that the behaviors are not specific enough to distinguish between visceral pains of different origins. The poor localization of visceral pain, however, makes it very unlikely that different visceral pains would be manifested in unique behavioral patterns.

### Vaginal and Uterine Distension

Berkley and colleagues (1995) established one of the earliest rat models of reproductive tract pain using vaginal and uterine distension. The elegance of this model relies on the novel operant task devised by the authors, which required rats to learn that a discrete behavioral response (extending the nose to interrupt a photocell circuit) would terminate an aversive stimulus (vaginal or uterine mechanical distension with a latex balloon). The authors argued that the rats' motivation to perform the escape behavior in response to high levels of distension indicated that intense mechanical distension constituted an aversive stimulus to the rats. The intense level of stimulation employed by this model is in contrast to innocuous levels of vaginal stimulation, which have positively reinforcing and analgesic properties in rodents (Komisaruk & Whipple, 2000).

Berkley et al. (1995) validated this behavioral pain model in adult virgin female rats with low levels of ovarian hormones (i.e., metestrus), to control for the potentially confounding effects of estrous cycle hormone fluctuations. Rats

reliably escaped distension with increasing speed and frequency as the vaginal distension volume increased, and this response pattern held throughout the estrous cycle (Komisaruk & Whipple, 2000). The rats' ability to detect and escape from uterine distension, however, was less predictable—many rats produced operant responses during control trials when distension volumes were minimal, and a large minority of animals did not show behavioral discomfort with maximum levels of uterine distension. The authors noted that rats often responded to uterine distension with stretching behavior.

Interestingly, escape behaviors increased in response to higher vaginal and uterine pressures when estrogen levels were low during metestrus and diestrus (Bradshaw et al., 1999). Similarly, ovariectomy (OVX) also induced moderate to high levels of vaginal hyperalgesia that were promptly reversed with 17β-estradiol replacement (Bradshaw & Berkley, 2002). This pattern of estrogen-dependent vaginal sensitivity has adaptive reproductive significance. The increased tolerance to vaginal pressure, such as that induced by penile penetration, would be functionally important during the height of sexual activity in late proestrus, after estrogen and progesterone levels have peaked.

The development of this model exemplifies the successes and hazards of validating reliable behavioral correlates of pain. The authors succeeded in identifying a reliable pattern of behaviors for vaginal distension, yet uterine

distension pain proved more difficult to characterize. Escape responding during uterine distension correlated with a prominent visceral pain behavior, which lends support to the aversive quality of the distension stimulus. One strength of this model is that it relies on an organized motor response that requires cerebral processing, which is thought to more accurately reflect the sensory perception of pain compared to simple reflex responses (Le Bars et al., 2001).

### Endometriosis

Endometriosis is a painful condition defined by dysmenorrhea, dyspareunia, infertility, and chronic abdominal and low back pain (Evans, Moalem-Taylor, & Tracey, 2007). To induce endometriosis, a segment of uterine horn is removed (i.e., hysterectomy), and pieces of endometrial tissue from the uterine horn (or fat for sham-operated controls) are autotransplanted onto blood vessels in the left ovary, the internal lower abdominal wall, or the cascade mesenteric arteries. Cysts rapidly develop at uterine transplant sites. The endometriosis rat model shares important similarities with endometriosis in women, including pelvic pain, infertility, in vitro and in vivo tissue and cell properties, and treatment responses (Sharpe-Timms, 2002).

Berkley and colleagues (2001) combined the distension-induced pain model with the endometriosis rat model. Animals subjected to the endometriosis

surgery showed a significant increase in escape behavior in response to vaginal distension compared to baseline, whereas sham-operated animals without cysts showed no change in behavior. The findings of increased hypersensitivity to vaginal distension in rats with endometriosis have immense clinical relevance given the comorbidity of endometriosis and dyspareunia (Evans et al., 2007).

In a follow-up study, Cason, Samuelson, and Berkley (2003) found time- and estrous cycle-dependent changes in distension-induced vaginal hypersensitivity following endometriosis surgery. When post-surgical data from all stages of the estrous cycle were pooled together, the rate of escape responding to vaginal distension steadily increased for eight weeks in proportion with the growth of endometrial cysts. When specific stages of the estrous cycle were examined, rats with endometriosis increased escape responding from vaginal distension during metestrus, diestrus, and proestrus (but not estrus). This finding is interesting for two reasons: first, the robust impact of endometriosis on vaginal sensitivity is fully reversed for about a day during the estrous cycle; second, this effect appears to be independent of normal patterns of vaginal hypersensitivity wherein moderate and high levels of estrogen during estrus and proestrus enhance tolerance to vaginal pressure (Bradshaw et al., 1999). The difference may be that nonpathological fluctuations in vaginal sensitivity are due to the direct effects of estrogen on vaginal tissue (Pessina et al., 2006; Ting,

Blacklock, & Smith, 2004), whereas the pathological mechanisms underlying endometriosis-induced vaginal hyperalgesia may become centrally mediated (Nagabukuro & Berkley, 2007). Even a profound drop in ovarian hormones due to OVX does not change endometriosis-induced vaginal hyperalgesia (Berkley et al., 2007), suggesting that either a reduction in estrogen levels does not alter the mechanisms underlying the hyperalgesia or that the capacity for both endometriosis plus OVX to produce hyperalgesia is not additive. Estrogen replacement following endometriosis plus OVX reverses the vaginal hypersensitivity, and this reversal may in part be due to central effects of estrogen (Berkley et al., 2007).

### Interstitial Cystitis

Interstitial cystitis is highly comorbid with dyspareunia and may be accompanied by a burning or aching vaginal pain (Bogart, Berry, & Clemens, 2007). Animal models of cystitis use a variety of irritants to induce bladder inflammation, including cyclophosphamide (an antitumor agent), turpentine, and even bacteria.

The cystitis-induced visceral pain model was first developed in the rat (Lantéri-Minet et al., 1995) and then in the mouse (Bon et al., 2003; Olivar & Laird, 1999). Following cystitis induction, spontaneous pain behaviors

progressively increased in frequency and were correlated with increasing severity of bladder inflammation. Cystitis pain behaviors may be more pronounced in the rat compared to the mouse, with the former exhibiting spontaneous abnormal behaviors (i.e., hunched posture), abdominal licking and contractions, reduced locomotion) and the latter exhibiting a general reduction in physical activity (Bon et al., 2003; Lantéri-Minet et al., 1995; Olivar & Laird, 1999), although one study found comparable hunching behaviors in the mouse (Wantuch, Piesla, & Leventhal, 2007). Rat and mouse models show that cystitis produces referred pain to other areas receiving common innervation, such as the tail, hind paw, and abdomen (Bon et al., 1997; Jaggar, Scott, & Rice, 1999; Meen et al., 2001; Wantuch et al., 2007). In both species, cystitis-induced referred mechanical and thermal hypersensitivity were dose-dependently reduced with morphine (Bon et al., 2003; Jaggar et al., 1999; Wantuch et al., 2007). The development of cystitis-induced behaviors does not vary across estrous stages, but interestingly, the onset of pain behaviors progresses more rapidly in female compared to male rats (Bon et al., 1997).

A model of bacterial-induced cystitis demonstrated that mice showed reduced hindpaw-withdrawal latencies to noxious radiant heat for 14 days following *Escherichia coli* administration, whereas otherwise genetically similar mice but with deficient Toll-like receptor 4 (TLR-4) function failed to show this

thermal hypersensitivity (Bjorling et al., 2008). Toll-like receptors are part of the innate immune defence against foreign pathogens, and TLR-4 recognizes bacterial wall components, contributing to nuclear factor-kappa B (NF-κB) activation and subsequent increases in proinflammatory cytokine expression (Tsan & Gao, 2004). Central TLR-4 has also been implicated in behavioral hypersensitivity to neuropathic pain (Tanga, Nutile-McMenemy, & DeLeo, 2005; Wadachi & Hargreaves, 2006). These findings indicate that a bacterium is a sufficient inflammatory irritant to induce experimental, TLR-4-dependent cystitis.

### Colitis

In order to model functional abdominal pain like IBS, an animal model of visceral pain from colitis was developed by the Cervero laboratory which measured behavioral responses to colonic irritation from capsaicin and mustard oil (Laird et al., 2001). Colonic irritation rapidly and dose-dependently produced abdominal pain behaviors (i.e., abdominal licking and hunching postures), as well as increased mechanical sensitivity on abdominal, tail, and hindpaw tissues indicative of referred pain. Abdominal pain behaviors were dose-dependently reduced by morphine. Similar models that correlated colonic irritation with increased acute and chronic abdominal pain behaviors showed no apparent structural damage to colonic mucosa (Al-Chaer, Kawasaki, & Pasricha, 2000;

Bourdu et al., 2005). Furthermore, a minority of animals (about one-quarter) may develop chronic mechanical and thermal hypersensitivity lasting up to 16 weeks after severe colitis, indicating the presence of referred pain long after colitis-associated inflammation has resolved (Zhou et al., 2008). Due to the production of abdominal pain without detectable colonic pathology, these animal models are thought to mimic the clinically important characteristics of IBS, including visceral hypersensitivity and referred somatic pain (Verne, Robinson, & Price, 2001).

Estrogen levels may play an important role in visceral pain. Sanoja and Cervero (Sanoja & Cervero 2005, 2008) demonstrated that OVX mice developed robust mechanical, thermal, and visceral allodynia and hyperalgesia in abdominal, hindpaw, and proximal tail skin within a month of OVX surgery. Compared to control groups, the OVX group showed significantly greater numbers of referred visceral pain behaviors following intracolonic capsaicin (including abdominal licking, stretching, squashing, and retractions). This shift in pain sensitivity was reversed with 17β-estradiol replacement. A potential mechanism for this model involves serotonin, which is implicated in the descending inhibitory modulation of pain (Ito et al., 2004).

## Parturition

One of the most commonly encountered forms of visceral pain occurs during labor, when the lower uterus and cervix are stretched and sometimes even torn to permit passage of the offspring. A rat model of parturition pain found that pain behaviors observed in the 1.5 hours preceding birth are similar to behaviors outlined in other animal models of visceral pain (Catheline et al., 2006). Rats in labor displayed frequent abdominal straining and squashing and an inward turning of the hindpaw, and the rate of these behaviors increased proportionately with labor duration. Systemic oxytocin (10 ug/kg) reduced the labor duration and increased the rate of pain behaviors, which were reduced with epidural morphine (30 ug/10 uL).

## Yeast-Induced Sensitization to Touch (YIST) Model

Provoked vulvar pain—involving somatic tissue—is the most common cause of dyspareunia, and yet the majority of existing animal models of pain conditions that cause dyspareunia in women are visceral. We have developed a method of testing vulvar mechanical sensitivity in order to evaluate a mouse model of PVD. The testing method is an adaptation of the classic von Frey (1922) psychophysical test and involves stimulation of mouse posterior vulvar tissue, located ventrally from the anogenital ridge, with calibrated nylon

monofilaments (0.009 – 2.0 g). Mice display varying intensities of behavior in response to vulvar stimulation, including sniffing or licking of the vulva, body repositioning, or jumps. Because a rapid, full jump (all four paws off the ground) was the behavior most reliably elicited (albeit at high levels of applied force), we adopted this behavior as the criterion for an aversive response to mechanical stimulation.

Based on multiple reports that women with PVD are significantly more likely to have experienced recurrent vulvovaginal candidiasis (RVVC) (Mann et al., 1992; Marinoff & Turner, 1991; Pukall et al., 2002), we developed a mouse model of provoked vulvar pain following three successive vulvovaginal infections with *Candida albicans*. For each infection, mice were vaginally inoculated with yeast, and four days following inoculation the infections were verified and eliminated with seven days of oral fluconazole. Following three weeks of consistently negative cultures from vaginal lavage fluid, mechanical sensitivity measurements were taken and compared to baseline measurements. After three yeast infections, significant differences in vulvar mechanical sensitivity was found between RVVC mice exposed to vulvovaginal yeast compared to fluconazole and saline controls. No changes in hindpaw mechanical sensitivity were found, indicating that increases in sensitivity were specific to the vulvar tissue exposed to yeast. We are hopeful that our model may allow an improved understanding of

the mechanisms underlying provoked vulvar pain, as well as the development of novel treatments for clinical use.

## Validity of Animal Models of Dyspareunia

The animal models we have reviewed do not meet the proposed criteria for accurately modeling clinical symptoms of dyspareunia and its associated disorders, as the visceral pain behaviors used in most models are not specific to any particular pain stimulus (see Table 2). Only vaginal distension and vulvar mechanical sensitivity behaviors are unique to a stimulus. Most models produce reliable and frequent pain behaviors, although much individual variation may exist (Berkley et al., 1995; Giamberardino et al., 1995; Wesselmann et al., 1998). Pain models vary from acute onset (Berkley et al., 1995) to tonic inflammatory (e.g., Olivar & Laird, 1999; Wesselmann et al., 1998) and chronic referred pain (e.g., Zhou et al., 2008). A correlation between behavior and physical pathology is largely absent, with the exception of endometriosis and cystitis models (Berkley et al., 2001; Bon et al., 1997; Cason et al., 2003; Lantéri-Minet et al., 1995; Olivar & Laird, 1999), and most models are reversible with known analgesics. Each of these models requires substantial development, including a refinement of pain behavior patterns, improved understanding of corresponding

physiological pathology, and identification of clinically relevant symptoms specific to dyspareunia.

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*Table 1.* Behaviors associated with animal models of dyspareunia

| Pain Behaviors | Type of Pain | Condition Modeled |
|---|---|---|
| Pushing abdomen against floor ("stretch-flat" position) | Spontaneous, visceral | Ureteral calculosis, endometriosis, uterine inflammation, colitis, parturition |
| Lifting abdomen off floor | Spontaneous, visceral | Colitis |
| Sharp back hunch ("lambda" position) | Spontaneous, visceral | Ureteral calculosis, endometriosis, uterine inflammation |
| Abdomen pressed against floor with nose facing toward tail of afflicted side ("alpha" position) | Spontaneous, visceral | Ureteral calculosis, endometriosis, uterine inflammation |
| Lower abdomen pressed against floor while standing/sitting ("squash-pelvic" position) | Spontaneous, visceral | Ureteral calculosis, endometriosis, uterine inflammation, parturition |
| Stretching (back arched) | Spontaneous, visceral, mechanical distension | Ureteral calculosis, uterine inflammation, uterine distension, cystitis, colitis |
| Experimenter observed abdominal contractions | Spontaneous, visceral | Cystitis, colitis |
| Hunched posture | Spontaneous, visceral, mechanical distension | Ureteral calculosis, uterine inflammation, uterine distension, cystitis, parturition |
| Inward turning of hindlimb | Spontaneous, visceral | Ureteral calculosis, uterine inflammation, parturition |
| Jumping or retreating from palpation/pressure | Provoked, mechanical or thermal or electrical, referred | Ureteral calculosis, referred hypersensitivity from: uterine inflammation, cystitis, colitis, ovariectomy, YIST model |
| Operant response | Provoked mechanical distension, spontaneous, referred | Vaginal and uterine distension, endometriosis, ovariectomy |
| Licking afflicted area | Spontaneous or provoked | Ureteral calculosis, uterine inflammation, cystitis, colitis, parturition |
| Writhing | Spontaneous, visceral, tonic | |
| Reduced physical activity | Spontaneous | Cystitis, uterine inflammation, colitis |
| Vocalization | Spontaneous or provoked | Ureteral calculosis + endometriosis, uterine inflammation, uterine distension |
| Piloerection | Spontaneous | Cystitis (rat model only) |
| Abnormal defecation/urination | Spontaneous or provoked | Colitis |
| Facial expression (eye squint, blink) | Spontaneous | Cystitis |

*Table 2.* Evaluation of behavioral and pathological characteristics of animal models of dyspareunia

| Pain Conditions | Behavior Specific to Condition? | Reliable | Frequently Observed? | Behavior Time Course | Related to Pathology? | Reversible with Analgesics? | Human Condition Modeled? |
|---|---|---|---|---|---|---|---|
| Ureteral calculosis | No | Yes | Varies between individuals | Onset in 1st day, reduces within 4 days | Unknown | Yes | Urinary calculosis (kidney stones) |
| Uterine inflammation | No | Yes | Varies between individuals | Onset in 2–4 days of mustard oil | No | N/A | Various uterine pathologies |
| Vaginal distension | Yes | Yes | Yes | Within sec of noxious distension | Yes, in case of endometriosis | N/A | Vaginal dyspareunia |
| Uterine distension | Sometimes | No | Varies | Within sec of noxious distension | Unknown | N/A | Unknown |
| Endometriosis | No | Yes | Yes | Abnormal sensitivity by 1–2 mo | Yes, behavior correlates with cyst growth | Yes | Endometriosis |
| Cystitis | No | Yes | Yes | Gradually increases in 1–4 hrs | Yes, behavior correlates with bladder inflammation | Yes | Cyclophosphamide-induced cystitis |
| Colitis | No | Yes | Yes | Onset within 1 hr, several days referred pain | No | Yes | Irritable bowel syndrome |
| Parturition | No | Yes | Yes | Onset 1.5 hr before birth | N/A | Yes | Labor pain |
| YIST model | Yes | Yes | Yes | Following three infections | Unknown | N/A | Yeast infection–induced provoked vulvar pain |

# TRANSITIONAL TEXT 1

Results from the literature review of animal models of dyspareunia demonstrated that many previous animal models of urogenital pain have failed to mimic the symptoms and pathology of their respective clinical conditions. Although they produced reliable, frequent pain behaviours that could be quantified with observation, most of these models relied on behavioural indices of pain that were not condition-specific, thereby casting doubt on whether they induced pain that paralleled the intended clinical symptoms. Only the endometriosis and cystitis models produced pathological changes in tissue that correlated with pain behaviour. Importantly, none of the existing models were designed to test the vulvar hypersensitivity observed in provoked vestibulodynia, the most common cause of dyspareunia in both pre- and post-menopausal women (Harlow and Stewart, 2003; Kao et al., in press). The chapter briefly touched on the yeast infection-induced, provoked vulvar pain model, which we developed to address the need for an animal model of vulvar pain.

The development of a valid animal model of vulvar pain requires that the model closely parallel the clinical phenomena. Accordingly, pain behaviour has been selected based on the clinical description of exquisite vulvar sensitivity to light touch or pressure (e.g., vulvar mechanical allodynia). Pilot testing in mice led us to define pain behaviour as a reflexive jump response to mechanical (von

Frey hair) stimulation of the vulva. To determine an ecologically valid pain stimulus by which vulvar pain can be experimentally manipulated, we looked to the gynaecological literature that had long advanced the hypothesis that recalcitrant vulvar pain may result from repeated vulvovaginal infection or inflammation (Goldstein & Burrows, 2008). Given the high prevalence of self-reported recurrent vulvovaginal candidiasis in women with PVD (Bohm-Starke, Hilliges, Blomgren, Falconer, & Rylander, 2001; Witkin, Gerber, & Ledger, 2002), we reasoned that this hypothesis could be directly tested by inducing repeated vulvovaginal infections in the mouse. This logic led us to conduct the first study empirically evaluating an etiological hypothesis of PVD, using a) a localized, provoked pain stimulus to evoke pain behaviour, b) a common strain of yeast to induce repeated yeast infections, and c) histological analysis of mouse vulvar tissue to determine whether pain behaviour correlates with tissue pathology.

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REPEATED VULVOVAGINAL FUNGAL INFECTIONS

CAUSE PERSISTENT PAIN IN A MOUSE MODEL OF VULVODYNIA

Reference:

Farmer, M.A., Taylor, A.M., Bailey, A.L., Tuttle, A.H., MacIntyre, L.C., Milagrosa, Z.E., Crissman, H.P., Bennett, G.J., Ribeiro-da-Silva, A., Binik, Y.M., & Mogil, J.S. (2011). Repeated vulvovaginal fungal infections cause persistent pain in a mouse model of vulvodynia. *Science Translational Medicine*, 3, 101ra91.

Abstract

Provoked vestibulodynia (PVD), the most common form of vulvodynia, is a highly prevalent idiopathic pain disorder associated with a history of recurrent candidiasis (yeast infections). We tested whether repeated, localized (vulvar) exposure to a common fungal pathogen can lead to the development of chronic pain. A subset of female mice subjected to recurrent *Candida albicans* infection developed mechanical allodynia localized to the vulva, and only mice becoming allodynic featured hyperinnervation of peptidergic nociceptor and sympathetic fibers (i.e., increased protein gene product 9.5, calcitonin gene-related peptide and vesicular monoamine transporter 2 immunoreactivity in vaginal epithelium). Long-lasting behavioral allodynia in a subset of mice was also observed after a single, extended *Candida* infection, as well as after repeated vulvar (but not hind paw) inflammation induced with zymosan. The hypersensitivity and hyperinnervation were both present long (at least 3 weeks) after the resolution of infection and inflammation, as determined by normal vaginal cultures and tissue histology. As vulvar allodynia and hyperinnervation are prominent features of PVD in women, our model replicates important features of the human disease in the mouse. These data demonstrate that infectious states can cause persistent pain long after their resolution.

## Introduction

Pain is a cardinal feature of the inflammatory response to fungal, bacterial, and viral infections. In most cases, pain rapidly disappears with the resolution of the infection. Pain secondary to previous and resolved infection is suspected to underlie numerous idiopathic chronic pain conditions, including urogenital pain (vulvodynia, endometriosis, prostatitis), interstitial cystitis, and inflammatory bowel syndrome (e.g., Spiller & Garsed, 2009), but a causal relationship between infection and persistent pain has not been demonstrated. To test whether such a causal relationship can exist, we developed a mouse model of provoked vestibulodynia (PVD), a chronic urogenital pain condition suspected to result from repeated infection by a common pathogen, the yeast *Candida albicans*.

Of the idiopathic pain conditions associated with a history of previous infection, vulvodynia (vulvar pain) is the most prevalent, affecting 9–12% of women of childbearing age (Harlow & Stewart, 2003). The predominant form of vulvodynia, PVD (previously known as vulvar vestibulitis), is characterized by burning and cutting pain localized to the vulvar vestibule in response to light touch (vulvar mechanical allodynia), with physical findings limited to occasional erythema (Goldstein & Burrows, 2008). Chronic vulvar pain is associated with significant psychological distress due to its interference with sexual intercourse and nonsexual activities (bike riding, walking, and even standing); as a result,

mood disturbances and reduced quality of life are often reported in this population (Meana, Binik, Khalifé, & Cohen, 1997). Reduced vulvar tactile and pain thresholds in PVD have been experimentally confirmed with standardized mechanical stimuli (Pukall, Binik, Khalifé, Amsel, & Abbott, 2002). Histological changes in vulvar vestibule tissue—including increased density of free nerve endings (Bohm-Starke, Hilliges, Falconer, & Rylander, 1998; Westrom & Willen, 1998)—suggest that neural mechanisms may underlie these clinical symptoms. PVD imaging reveals patterns of brain activity similar to those observed in experimental and clinical pain, as well as neuroanatomical abnormalities suggestive of compensatory central reorganization secondary to chronic pain (Pukall et al., 2005).

No definitive causes of PVD have yet been identified. However, women with PVD have a high prevalence of recurrent vulvovaginal candidiasis (RVVC; or recurrent yeast infections), defined as three or more yeast infections annually, compared to healthy women: 42–60% (Bohm-Starke, Hilliges, Blomgren, Falconer, & Rylander, 2001; Witkin, Gerber, & Ledger, 2002) versus 5–8%, respectively (Sobel, 2007). The commensal yeast, *Candida albicans*, is thought to cause 85–90% of all yeast infections in women (Sobel, 2007). The comorbidity between RVVC and chronic vulvar pain has led to the hypothesis that vulvar hypersensitivity in PVD results from abnormal sensory processing

secondary to past inflammation from prolonged and/or repeated vaginal yeast colonization. This correlational hypothesis remains untested. Here we assess whether persistent vulvar mechanical hypersensitivity can develop in mice following multiple rounds of vulvovaginal infection, or following a single long-lasting infection with *C. albicans*.

## Materials and Methods

### *Subjects*

Female, outbred CD-1® (ICR:Crl; Charles River) mice, 8-10 weeks of age, were housed in facilities equipped with Biohazard Level 2 containment. Mice were maintained on a 12:12 h light/dark cycle (lights on at 07:00 h) and received irradiated food (Harlan Teklad 8604) and autoclaved tap water ad libitum. All procedures, including inoculations, injections and behavioral testing, were conducted within a Class II biological safety cabinet. All procedures were approved by the McGill University animal care and use committee.

### *Microorganism*

A strain of *C. albicans* isolated in a clinical setting (SC5314, a gift of Malcolm Whiteway, National Research Council of Canada) was used for

vulvovaginal inoculations. SC5314 was grown in a phytone peptone broth for 18 h at 25 ºC on an orbital shaker at 7000 rpm. Stationary phase blastoconidia were washed twice and adjusted to 5 x $10^4$ cells/ml. Each inoculum solution was prepared from freshly subcultured SC5314 on the day of inoculation.

### *Vulvovaginal Infection Procedures and Treatment*

Mouse vaginal bacterial and fungal cultures were obtained before testing to ensure that no known pathogenic microorganisms were present. Under non-hormone-primed conditions, murine vaginal *C. albicans* infection resolves without antifungal treatment within 14 days (Fidel, 2007). On Day 0 of each infection, mice were lightly anesthetized with isoflurane/oxygen and inoculated vaginally with either 5 x $10^4$ stationary-phase SC5314 blastoconidia in 20 µl of sterile phosphate-buffered saline (PBS), or saline only. The inoculum was gently pipetted into the vaginal opening and the mouse placed in the supine position to retain the inoculum in the vaginal cavity for 10 minutes. Post-inoculation vaginal lavages were collected daily until infection resolution was confirmed, and weekly thereafter. To minimize tissue irritation unrelated to infection, utmost care was taken to minimize contact between the vulva and the pipette tip during lavages. Vaginal SC5314 burden and infection status were monitored with Gram- and Wright-Giemsa-stained smears prepared from vaginal lavage fluid, which were

examined microscopically for the presence of polymorphonuclear leukocytes and *Candida* morphotypes (blastoconidia and pseudohyphae). Lavage fluid was serially diluted onto Sabouraud dextrose agar (Quelab), incubated for 48 h at 34 ºC, and colony forming units (CFUs) were quantified. Loops of CFUs were submitted to two separate tests to ensure that the isolated yeast was indeed *C. albicans*: the isolated growth was submitted to a germ tube test, and re-plated onto chromogenic agar specific to common *Candida* species (Candida CHROMagar, Hardy Diagnostics) for up to one week at 34 ºC. According to manufacturer's guidelines, emerald green CFUs exhibiting growth characteristics consistent with *C. albicans* were considered positive.

Mice in both the SC5314-infected and control groups were treated with fluconazole (15 mg/kg, once daily for 7 days; LKT Laboratories) administered via oral gavage. These doses are known to be effective in eliminating *C. albicans*-induced vaginitis in mice (Fidel, Cutright, & Sobel, 1997). The treatment regimen was based on the broad use of fluconazole as a first-line treatment for RVVC in humans (Sobel, 2007). The infection was considered to be resolved upon obtaining two successive negative vaginal cultures (see above), and weekly lavages were collected thereafter to ensure the absence of yeast.

### *Repeated Vulvovaginal Infection with* C. albicans

To simulate RVVC, mice received three separate vulvovaginal infections with 5 x $10^4$ *C. albicans* strain SC5314. Infections were allowed to last untreated for 4 days during each round of infection, followed by 7 days of fluconazole treatment (see above). For the second and third rounds of infection, mice were re-inoculated with 5 x $10^4$ SC5314 cells four weeks after clearance of the primary infection (one week following post-infection behavior testing), in order to simulate RVVC. In humans, a new episode of candidiasis can begin from a few days to three months after a previous infection, and the four-week interval between infection resolution and re-infection used here was deemed a valid analog of RVVC. Mice in the fluconazole control group received inoculations of saline and vaginal lavages concurrent to and in the same manner as infected mice.

Tests of baseline vulvar and hind paw thresholds were performed one week preceding initial SC5314 inoculation (Day -7). During each round of infection vulvar sensitivity was tested 4, 11 and 32 days after inoculation (see Fig. 2A). Hind paw sensitivity was measured following each infection, on Day 33.

### *Extended Primary Vulvovaginal Infection with* C. albicans

The extended primary infection with 5 x $10^4$ SC5314 blastoconidia was allowed to last untreated for 14 days, followed by 7 days of fluconazole treatment

(see above). Mice in the fluconazole control group received saline and vaginal lavages concurrent to and in the same manner as infected mice.

Tests of baseline vulvar and hind paw thresholds were performed one week preceding initial SC5314 inoculation (Day -7). Vulvar sensitivity was measured at 14, 21, 42 and 70 days after inoculation (see Fig. 6A). Hind paw sensitivity was measured on Days 43 and 71.

### *Repeated Inflammation with Zymosan*

A new cohort of mice was subjected to repeated subcutaneous injections of zymosan, in the posterior vulva while lightly anesthetized with isoflurane/oxygen. Zymosan is prepared from *Saccharomyces cerevisiae* yeast cell wall and produces sterile inflammation at the site of injection, leading to mechanical allodynia lasting up to 12–24 h without the need for biohazard containment. The dose used (10 mg/ml in 10 µl saline; 0.1 mg) was chosen based on pilot experiments; lower doses produced inconsistent initial allodynia. Injections occurred no more frequently than weekly to allow acute inflammation to subside between successive injections. Mice received zymosan injections immediately after baseline behavior testing and were observed for evidence of vulvar allodynia (defined here as ≥33% reduction in mechanical threshold) 4 h later, corresponding to the temporal peak of vulvar zymosan-induced mechanical

allodynia as defined by our pilot experiments. One week later, each mouse was retested and reinjected with zymosan. Each week thereafter, vulvar von Frey measurements were obtained and additional injections were administered only if a mouse's vulvar sensitivity recovered to nonallodynic levels (defined as > 66% of baseline threshold). If a mouse continued to show evidence of vulvar allodynia 1 week post-zymosan injection, no injection was given, and the mouse was retested 1 week later. Mice were followed for a total of 11 weeks, and received up to and including (but no more than) six zymosan injections. Mice that did not become persistently allodynic (i.e., displaying a ≥33% reduction in threshold for two consecutive weeks) after six injections were classified as non-responders. One control group received weekly vulvar saline injections, with timing matched to a zymosan-treated mouse with equivalent baseline vulvar sensitivity. In a separate experiment, six female mice received zymosan injections in a paradigm similar to that described above except that zymosan (0.25 mg/ml in 20 µl) was injected into the right mid-plantar hind paw. This dose was chosen as it produced equivalent levels of initial mechanical allodynia to the vulvar zymosan.

### *Mechanical (von Frey) Sensitivity Testing*

On each day of behavioral testing, animals were allowed 3 h (11:00 – 14:00 h) to habituate to the testing environment. Each testing session consisted

of two threshold determinations separated by 1 h; these two thresholds were averaged. An observer blinded to experimental condition applied a calibrated series of von Frey filaments (Semmes-Weinstein monofilaments; Stoelting) to the target tissue using the up-down psychophysical method of Dixon (Chaplan, Bach, Pogrel, Chung, & Yaksh, 1994) with pressure applied to each filament until it bowed, and held for 2 seconds. Across species, the vulva is defined as the external female genital organs; this includes the clitoral and preputial glands in the mouse and accordingly, we stimulated the central, hairless posterior aspect of the mouse vulva. A series of eight von Frey filaments (0.06–3.9 g; filaments #4 through #11) were applied to the vulva beginning with the #7 filament. Hind paw mechanical sensitivity was also monitored in all experiments, as a control site. For hind paw testing, a different series of eight filaments (0.015–1.3 g; filaments #2 through #9) were applied to the plantar aspect of the hind paw beginning with the #5 filament. Different ranges of fibers were used for vulva and hind paw testing, as different amounts of force physically lift the stimulated area off the floor (which artificially imposes a ceiling value on testing). Any mouse showing continuous positive or negative responses were assigned ceiling and floor withdrawal threshold values of 4.0 g and 0.025 g, respectively, for vulvar testing, and 2.0 g and 0.01 g, respectively, for hind paw testing. In all other cases the 50% withdrawal threshold was calculated as described (Chaplan et al.,

1994). Aiming accuracy in mouse vulvar stimulation (a 3-mm diameter target) was maximized by shaving anogenital hair the day before testing to improve visibility. von Frey filaments were disinfected with 70% ethanol between each testing session, and independent filament sets were used for each experimental condition to minimize risk of cross-contamination.

The nocifensive endpoint we adopted was a clear reflexive jump (all four paws lifted) in response to vulvar stimulation, chosen because it is most similar to the clear withdrawal response used in hind paw von Frey testing. Note, however, that even the strongest usable von Frey filament (3.9 g, with larger filaments lifting the mouse off the floor without bending) rarely produced this jumping response at baseline, with >75% of mice consistently not responding to the 3.9 g fiber. It is unclear whether "positive" (<4.0 g) responses at baseline for the remaining subjects represent measurement (or testing environment) artefacts or true biological variability.

For practical reasons it was necessary to test all mice together, regardless of their estrous stage, on each scheduled testing day. A pilot experiment confirmed that vulvar mechanical thresholds were invariant of estrous stage. In addition, vaginal lavages taken during the experiment revealed that neither SC5314 nor fluconazole treatment altered normal 4–5-day estrous cyclicity.

Thus, it is highly unlikely that the changes seen in SC5314-infected mice were produced by hormonal alterations.

### *Immunohistochemistry*

Following behavioral testing, mice were deeply anesthetized with sodium pentobarbital (≥50 mg/kg, i.p.) and perfused transcardially with 5% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 at room temperature. The vaginal canal (from the external vulva to the cervix) was excised and postfixed in 5% paraformaldehyde in phosphate buffer for 1 h, and then cryoprotected with 30% sucrose in phosphate buffer for 24 h. Tissue was embedded in an optimum cutting temperature medium (OCT; Tissue Tek) and frozen at -80 °C until cryosectioned. Twelve μm-thick longitudinal sections were cut on a Leica CM3050 S Cryostat at -25 °C and placed directly on poly-L-lysine-treated slides. Slide-mounted sections were rinsed three times with 0.1 M PBS for 10 min, preincubated with 10% normal goat serum diluted with 0.3% Triton X (T) for 60 min, and then incubated for 24 h at 4 °C in one of three primary antibodies: protein gene product 9.5 raised in rabbit (PGP 9.5, 1:2000, Ultraclone), anti-calcitonin gene-related polypeptide (α isoform) raised in sheep (CGRP, 1:1000, Biomol), and vesicular monoamine transporter 2 raised in rabbit (VMAT2, 1:2000, Millipore). The next day slides were washed three times with PBS for 10

min, incubated in Cy3 anti-rabbit and Cy2 anti-sheep secondaries (1:500, Jackson ImmunoResearch Laboratories) in the dark for 2 h, and washed three times for 10 min with PBS. For each reaction, negative controls (processed without the primary antibody) were included.

*Quantitative analysis of RVVC immunohistochemistry*

Immunohistochemical analysis of a subset of post repeated-infection allodynic (n=3-6, depending on the antibody), post-infection nonallodynic (n=5), and fluconazole control (n=4) animals (see below) was based on four randomly selected postmortem vulvar tissue sections per mouse, with a total of 6 non-consecutive pictures per section (i.e., 24 frames per mouse). Pictures were only taken of the lamina propria, as very little innervation was observed in the epithelium. Images were acquired using a Zeiss Axioplan 2 Imaging fluorescence microscope (lenses ranging from 40X to 60X) equipped with a Megaview II CCD camera and processed using AnalySIS 5.0 software (Soft Imaging System). Images were saved in TIFF format and analyzed using an image analysis system (MCID Elite v.7, Imaging Research) by an observer blinded to condition. Fiber density was calculated with functions in the program

configured to measure total fiber length per unit area (Almarestani, Longo, & Ribeiro-da-Silva, 2008).

### *Assessment of Post-Infection Morphology*

Slide-mounted 12 μm thick sections were processed as described above and stained with hematoxylin and eosin (H&E) to identify gross vulvar morphology, epithelial thickness, and inflammation between allodynic (n=4), nonallodynic (n=6), and fluconazole controls (n=5). Four nonconsecutive measurements were taken from the middle third of the posterior vulvar tissue, across 6 sections (24 measurements total). Sections were examined for signs of inflammatory infiltrate in the epithelium and lamina propria, as well as edema and plasma extravasation. Slides were digitally scanned with MIRAX Desk Scanner and visualized with MIRAX Viewer software using the 20x and 40x magnification functions for quantification (Zeiss).

### *Data Analysis*

Normally distributed hind paw threshold data were analyzed using repeated-measures ANOVA. Vulvar threshold data were analyzed using non-parametric $\chi 2$ analysis, with five arbitrarily defined threshold categories (<1.0 g, 1.0–1.99 g, 2.0–2.99 g, 3.0–3.99 g, >4.0 g). Data at particular testing sessions

were compared to within-group baselines, but similar results were obtained when comparing between-group at each testing session. Analysis of percentage of allodynic mice was conducted using a one-tailed Fisher's exact test, based on the a priori hypothesis that previously infected mice would show more allodynia. Normally distributed immunohistochemical and epithelial thickness data were analyzed by one-way ANOVA followed by Tukey's posthoc test. In all cases, a criterion level of $\alpha = 0.05$ was adopted. All statistical tests were two-tailed except as described above.

## Results

### *RVVC Can Cause Vulvar Allodynia*

Mice were tested for baseline mechanical sensitivity of the vulva and hind paws with von Frey filaments. Quantification of vaginal fungal burden confirmed infection status at each time point (Fig. 1A). For each round of infection with *C. albicans* strain SC5314 cells, mice were tested again at 4 days (active infection), 11 days (treated infection), and 32 days (three weeks after infection resolution) after inoculation (Fig. 2A). At baseline the large majority of mice (79%) exhibited vulvar mechanical withdrawal thresholds exceeding 4.0 g, with no between-group differences ($\chi^2 = 5.0$, $p = 0.17$). This was true at every testing session for mice in the fluconazole control group (Saline + FLU; Fig. 2B). By contrast, the group of

mice with repeated infections (Candida + FLU; Fig. 2C) were allodynic during active SC5314 infection (Day 4) and were still allodynic after treatment (Day 11). By Day 32, when the infection was long resolved, evidence of allodynia was absent after the first two rounds of infection. However, after the third round of infection persistent allodynia was present ($\chi^2$ = 9.5, $p$ = 0.02). We obtained similar results when we applied a within-subjects analysis, defining allodynic mice as those displaying a ≥ 33% decrease in vulvar threshold from their own baseline (Fig 2D). On Day 32 after the third round of infection, 40% (6 of 15) of the infected subjects were allodynic by this definition, compared to 5.5% (1 of 18) of the fluconazole control subjects ($p$ = 0.02, one-tailed Fisher's exact test). This allowed the separation of mice in the Candida + FLU group into allodynic and nonallodynic subgroups, from which tissues were obtained for immunohistochemical studies (see below). There were no statistically significant alterations in mechanical sensitivity of the hind paw produced by fluconazole or strain SC5314 over the course of the three rounds of infection (Fig. 3).

### *Lack of Morphological or Inflammatory Effects of RVVC*

Visual inspection of hematoxylin and eosin-stained sections obtained after the third infection revealed no edema and no obvious intergroup differences in inflammatory infiltrate (Fig. 4). The presence of a small number of immune cells

is typical of healthy vaginae, and a few basophils, macrophages, and mast cells were evident throughout the lamina propria and along blood vessel walls in all groups. Inflammatory cells did not penetrate the epithelial layer. No evidence of altered vulvar epithelial morphology was found in Saline + FLU or in Candida + FLU mice (allodynic or nonallodynic) after the resolution of the third infection (Fig. S3). Epithelial thickness at the broad ($F_{3,19}$ = 1.4, $p$ = 0.29) and narrow ($F_{3,19}$ = 0.8, $p$ = 0.50) aspects of the epithelium did not differ between groups, and in all cases the keratin layer was intact across the posterior surface of the vulva (data not shown). Whereas fungal burden covaried with hypersensitivity during acute infection, leukocyte levels showed no such correlation (Table 2).

*Only Allodynic RVVC Mice Show Increases in Vulvar Innervation*

Immunohistochemical analyses after repeated infection in allodynic (n=3-6) mice, after repeated infection in nonallodynic (n=5) mice, and in fluconazole control (n=4) mice were conducted on postmortem vulvar tissue. We observed an almost 300% increase in the density of nerve fibers (as detected by immunoreactivity [IR] for the pan-axonal marker protein gene product 9.5; PGP 9.5) throughout the lamina propria of vulvar tissue taken from allodynic compared to nonallodynic and control mice ($F_{2,10}$ = 12.8, $p$ = 0.002, Fig. 5A–D). The increased density reflected both increased number of fibers and thicker, longer

fibers. A significant, almost 400% increase in the density of peptidergic fibers, as assessed by calcitonin gene-related peptide (CGRP)-IR, was found in allodynic animals, compared to the nonallodynic group ($F_{2,14} = 4.6$, $p = 0.03$, Fig. 5E–H). In all groups, CGRP-IR fibers were observed throughout the lamina propria, but few fibers penetrated the basal cell layer of the epithelium. Allodynic mice displayed increased (over 4-fold higher) sympathetic innervation, as revealed by vesicular monoamine transporter 2 (VMAT2)-IR, compared to nonallodynic and control groups ($F_{2,12} = 8.0$, $p < 0.01$, Fig. 5I–L). Sympathetic fibers in all mice were typically distributed in the deeper layers of the lamina propria; in allodynic mice, there was increased fiber density, with some thin processes seen to penetrate the lamina propria beneath the epithelium (Fig. 5K).

### *Extended Primary Fungal Infection Can Cause Vulvar Allodynia*

In a new experiment, mice were tested for vulvar and hind paw mechanical sensitivity throughout a single but extended-duration (14-day) vulvar *C. albicans* strain SC5314 infection (Fig. 6A). Vaginal fungal burden throughout the extended infection is shown in Fig. 1B. At baseline, 96% of this cohort exhibited vulvar mechanical withdrawal thresholds exceeding 4.0 g, with no between-group differences ($\chi^2 = 0.7$, $p = 0.41$). The fluconazole control group continued to exhibit unchanged thresholds throughout the experiment (Fig. 6B).

In contrast, a significant proportion of extended infection mice became allodynic after the acute phase of SC5314 infection (Day 14; $\chi^2 = 8.1$, $p = 0.005$) (Fig. 6C). This hypersensitivity persisted after completion of anti-fungal treatment (Day 21; $\chi^2 = 5.0$, $p < 0.05$) and three weeks after infection resolution (Day 42, $\chi^2 = 6.9$, $p < 0.01$). A large proportion of mice (86% exhibiting a >66% reduction from baseline threshold) continued to display allodynic behavior up to Day 70, which was 7 weeks after the resolution of the SC5314 infection ($\chi^2 = 14.4$, $p < 0.001$) (Fig. 6C). No alterations in hind paw mechanical sensitivity were observed throughout the experiment (Fig. 7).

*Repeated Vulvar Exposure to Zymosan Produces Vulvar Allodynia*

To determine whether the persistent vulvar allodynia observed with SC5314 infection required exposure to a live pathogen, a new cohort of mice was subjected to repeated vulvar injections of the yeast cell wall glucan, zymosan (or saline, using baseline sensitivity-matched controls). Each mouse in the experimental group received two vulvar injections of zymosan, a week apart; each week thereafter mice were only re-injected if they recovered to nonallodynic levels of mechanical sensitivity (>66% of baseline thresholds). This design allowed us to assess individual variability in the number of zymosan injections required to produce persistent vulvar allodynia. Fig. 8A shows the frequency

distribution of the full data set; the number of zymosan injections required to achieve chronic vulvar allodynia in the saline- versus zymosan-treated groups ($\chi^2 = 14.8$, $p < 0.001$). We found considerable individual variation in the number of zymosan injections needed to induce persistent allodynia; data from the first six mice to be tested are shown in Fig. 8B to depict the range of patterns observed. Four hours after the first injection of vulvar zymosan, all mice displayed allodynia; for most mice, this allodynia completely resolved within a week. A single mouse (#1 in Fig. 8B) remained allodynic after the single inflammatory insult. Four hours after the second injection of zymosan, all mice showed robust vulvar allodynia; a week later all mice remained allodynic. However, with each subsequent week some mice maintained the allodynic state (#1,#2 in Fig. 8B), whereas other mice returned to baseline and either required additional injections to achieve persistent allodynia (#2,#3,#5) or never became persistently allodynic (#4,#6). A separately performed experiment in which zymosan was injected into the hind paw revealed no evidence whatsoever of persistent allodynia development in any of the mice tested (Fig. 8A, right). In all cases hind paw hypersensitivity resolved within a week and no chronic allodynia was ever observed even after repeated zymosan injections.

## Discussion

We have observed long-lasting mechanical vulvar hypersensitivity after repeated vulvovaginal infections with the yeast, *Candida albicans*. A single, 14–21 day-long, fully resolved *C. albicans* strain SC5314 infection also induced mechanical allodynia that greatly outlasted the resolution of active inflammation. Finally, comparably long-lasting allodynia was observed in mice receiving multiple vulvar (but not hind paw) injections of zymosan, a mixture of fungal antigens. In all three experiments only a subset of mice developed allodynia. In the RVVC experiment, the allodynic (but not the nonallodynic) mice displayed a significant increase in the density of vulvar nerve fibers, including identified peptidergic sensory and sympathetic fibers. Allodynia was not accompanied by gross morphological changes in the vulvar mucosa (e.g., no reduced epithelial thickness or keratinization). Thus, repeated or extended infection with a common pathogen can induce a pathological pain state that persists long after the resolution of the infection.

As expected, we observed vulvar allodynia during and immediately after each active infection. Onset of vulvar allodynia during the acute infection corresponded with the peak of vaginal fungal burden, suggesting that acute inflammation during active infection can account for acute vulvar allodynia. Clinical reports of vulvovaginal pain during yeast infections are consistent with

this finding (Sobel, 2007). Despite the differences in vaginal and vulvar epithelium morphology (keratinization, thickness, hormonal regulation), few differences have been identified in innate and/or adaptive immune responses throughout the lower genital tract (Quayle, 2002). Innate immunity likely plays a dominant role in the acute response to *C. albicans*, given that changes associated with adaptive immunity are not observed after yeast infections in mice and women (Fidel, 2007). *C. albicans* is recognized by the Toll-like receptors (TLR-) 2 and TLR-4, which are expressed on immune and epithelial cells, and engages the innate immune response, including the upregulation of a yeast-specific pattern of proinflammatory molecules through the nuclear factor-· B pathway (Van der Graaf, Netea, Verschueren, Van der Meer, & Kullberg, 2005). Innate immune cells recruited during acute inflammation (macrophages, mast cells, neutrophils) can interact directly with nerve endings to produce pain hypersensitivity and release inflammatory mediators that contribute to pain (Ren & Dubner, 2010). The presence of TLR4 on primary afferent endings (Wadachi & Hargreaves, 2006) may indicate another potential mechanism by which *C. albicans* activates nociceptors to induce behavioral hypersensitivity (Tanga, Nutile-McMenemy, & DeLeo, 2005). Similarly, zymosan-induced vulvar allodynia may result from acute inflammation mediated by TLR-2 and TLR-6 and/or the

direct sensitization of vulvar mechanoreceptors (Shinoda, Feng, & Gebhart, 2009).

We observe here that mechanical hypersensitivity can persist long after the resolution of the active infection. During the first and second rounds of candidiasis in the RVVC experiment, acute vulvar hypersensitivity resolved after anti-fungal treatment, and was absent 21 days after infection resolution (Day 32). By contrast, during the third round of infection the vulvar hypersensitivity observed during the active stages of infection was maintained long after yeast were absent from the vaginal cavity. Moreover, chronic hypersensitivity was also evident after a single, extended infection with *Candida*, and the phenomenon could still be observed up to 7 weeks after infection resolution. Finally, some mice given as few as two zymosan injections exhibited allodynia lasting at least 11 weeks, indicating that the development of long-lasting hypersensitivity may not require a live pathogen and may be generalizable to fungi other than *C. albicans*. The long delay following the disappearance of detectable inflammation in these paradigms suggests that the chronic hypersensitivity is not inflammatory pain, at least as that term is generally understood. Given our observation of hyperinnervation, it is also problematic to characterize this phenomenon as neuropathic, which requires a neural lesion (Treede et al., 2008).

The fact that only a subset of infected mice developed mechanical allodynia mirrors the clinical situation, as only a minority of women with RVVC develop chronic vulvar pain. Even within the subset of (outbred) mice developing chronic allodynia after zymosan, there was considerable variability in the number of exposures required (see Fig. 8A), strongly suggesting a classic gene-by-environment interaction between as yet unidentified genetic susceptibility factors and inflammatory exposures. Such interactions are well known in the animal pain genetics literature (Mogil, Seltzer, & Devor, 2004). Human genes suggested to be involved in the pathogenesis of vulvodynia include those coding for mannose binding lectin codon 54, the melanocortin-1 receptor, and the interleukin-1 receptor antagonist (Foster, Sazenski, & Stodgell, 2004). Of course, an unknown environmental factor may also be responsible for the susceptibility to chronic hypersensitivity in those that develop it after repeated inflammation. Given that repeated zymosan treatment to the hind paw failed to produce chronic allodynia in any subject, these phenomena may be unique to mucosal tissue (Richardson & Rautemaa, 2009) or the genital tract (Iwasaki, 2010).

Vulvar hypersensitivity after the third infection in the RVVC model was accompanied by increased density of sensory afferents, including increased peptide-containing nerve fibers. Prior work has shown the presence of sensory

hyperinnervation during an inflammatory response; for example, in the mucosa of the urinary bladder during the inflammatory responses evoked by cyclophosphamide (Dickson, Avelino, Cruz, & Ribeiro-da-Silva, 2006) in the upper dermis during the inflammatory response evoked by complete Freund's adjuvant (Almarestani, Longo, & Ribeiro-da-Silva, 2008) and in bone during the inflammatory response evoked via inoculation of prostate cancer cells (Jimenez-Andrade et al., 2010). Sensory hyperinnervation in the upper dermis has also been seen during the regeneration response after a traumatic nerve injury (Grelik, Bennett, & Ribeiro-da-Silva, 2005; Peleshok & Ribeiro-da-Silva, 2011; Yen, Bennett, & Ribeiro-da-Silva, 2006). Significantly, in the current experiment robust sensory hyperinnervation was seen in allodynic mice three weeks after resolution of the infection. This suggests that the pain that persists after the resolution of infection may be due to an abnormal persistence of a hyperinnervation response evoked during the acute inflammatory response. The observations in the animal studies parallel findings of greatly increased vulvar nerve density and peptidergic innervation in the allodynic vulvar vestibular tissue of women with PVD (Bohm-Starke, Hilliges, Falconer, & Rylander, 1998,1999; Bornstein, Goldschmid, & Sabo, 2004; Westrom & Willen, 1998), and suggest that repeated infection is a sufficient cause of altered innervation at the site of infection.

We also observed sympathetic (VMAT2-immunoreactive) hyperinnervation in the vulvae of allodynic mice three weeks after resolution of the infection. Sympathetic hyperinnervation has been observed in the skin during inflammation and following traumatic nerve injury (Almarestani et al., 2008; Grelik et al., 2005; Ruocco, Cuello, & Ribeiro-da-Silva, 2000; Yen et al., 2006). For example, ectopic endometrial cyst growth, which becomes sympathetically innervated, correlates with vaginal hypersensitivity in a rat model of endometriosis (Cason, Samuelson, & Berkley, 2003; Zhang, Dmitrieva, Liu, McGinty, & Berkley, 2008). Sprouting of free nerve endings (including those of peptidergic afferents) and sympathetic efferents suggest the presence of long-term physiological changes that may enhance nociceptive signalling of peripheral tactile input, and promote spontaneous neuronal discharge of affected sensory fibers.

We have developed an etiologically valid and clinically relevant animal model of an idiopathic pain condition. We believe that this model will be useful in the investigation of mechanistic pathways of infection-induced pain, the evaluation of genetic and environmental risk factors, and the preclinical testing of the efficacy of novel treatments for debilitating pain conditions secondary to infection. As the most effective current treatment of PVD is surgical excision of the painful vulvar tissue (Goldstein & Burrows, 2008), new and less-invasive treatments are a clinical necessity.

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Table 1. No changes in epithelium thickness produced by repeated vulvovaginal *C. albicans* strain SC5314 infection.

| Measure | Experimental Group | | |
|---|---|---|---|
| | Saline + FLU | *Candida* + FLU (nonallodynic) | *Candida* + FLU (allodynic) |
| Epithelium Thickness (thickest) (μm) | 200.8 ± 19.3 | 236.0 ± 27.3 | 236.7 ± 9.7 |
| Epithelium Thickness (thinnest) (μm) | 48.6 ± 3.3 | 58.4 ± 3.8 | 66.9 ± 6.4 |

Values represent means ± SEM.

Table 2. Vaginal leukocyte count was not associated with presence of infection following SC5314 inoculation.

*Repeated Infection*

| | | Round 1 | | | Round 2 | | | Round 3 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Group | D0 | D4 | D11 | D32 | D4 | D11 | D32 | D4 | D11 | D32 |
| Saline + FLU | - | - | - | - | + | + | - | - | - | - |
| *Candida* + FLU | - | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + |

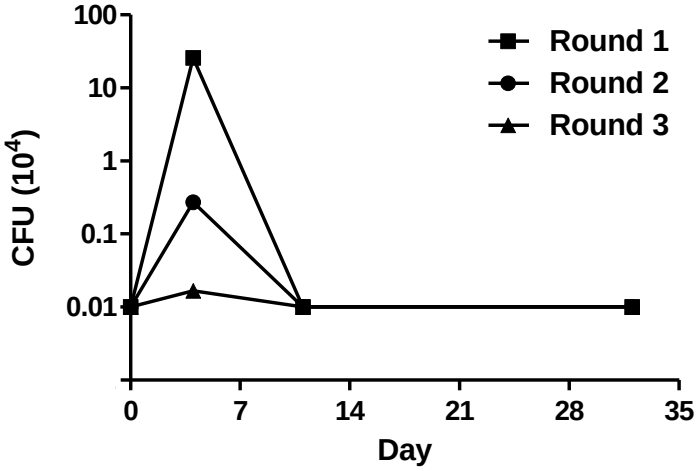
*Extended Primary Infection*

| Group | D0 | D4 | D14 | D21 | D42 |
|---|---|---|---|---|---|
| Saline + FLU | - | + | + | - | + |
| *Candida* + FLU | - | + | + | + | + |

Note: Average leukocyte count per mouse, based on pooled vaginal lavage fluid from each cage. Ranges include <30 (-)*, 30-50 (+), and > 50 (++).

*This definition is based on the normal presence of leukocytes in vaginal fluid in healthy mice. The diestrus phase of the estrous cycle in rodents is characterized by vaginal leukocyte infiltration and thus a low baseline level of white cells can be present in healthy, uninfected mice.

**a. Repeated Infection**




**b. Extended Primary Infection**

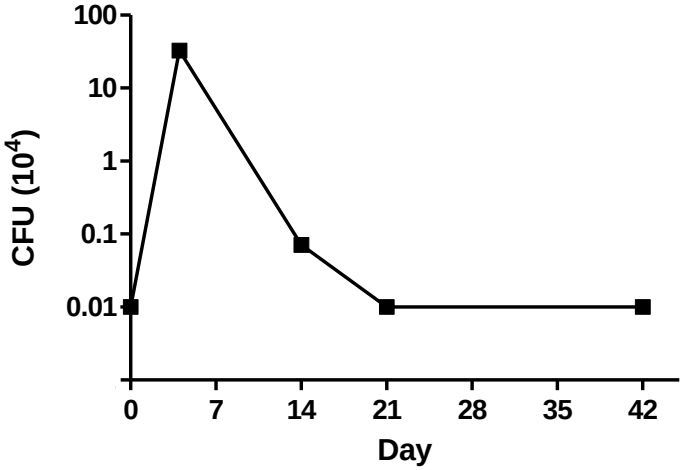


Figure 1. Increased vaginal fungal colonization during acute infection(s) following inoculation with *C. albicans* strain SC5314, and full clearance of yeast following fluconazole treatment. (**a**) Peak vaginal fungal colonization (indicated by colony-forming units; CFUs) occurred on Day 4 of each round of SC5314 infection, with SC5314 CFUs absent after 7 days of fluconazole treatment (Day 11) and three weeks later (Day 32). With each subsequent infection, Day 4 CFU counts were reduced, likely due to local immune protection following the primary infection. (**b**) Peak CFU count occurred on Day 4 of the extended primary infection and were reduced at Day 14, before mice had received fluconazole treatment. On Day 21 (after 7 d fluconazole treatment), vaginal SC5314 levels were undetectable, and vaginal clearance of yeast continued for the remainder of the experiment. Vaginal lavage fluid was pooled for each cage of 4 mice, with average cage CFU count at each time point reported here.

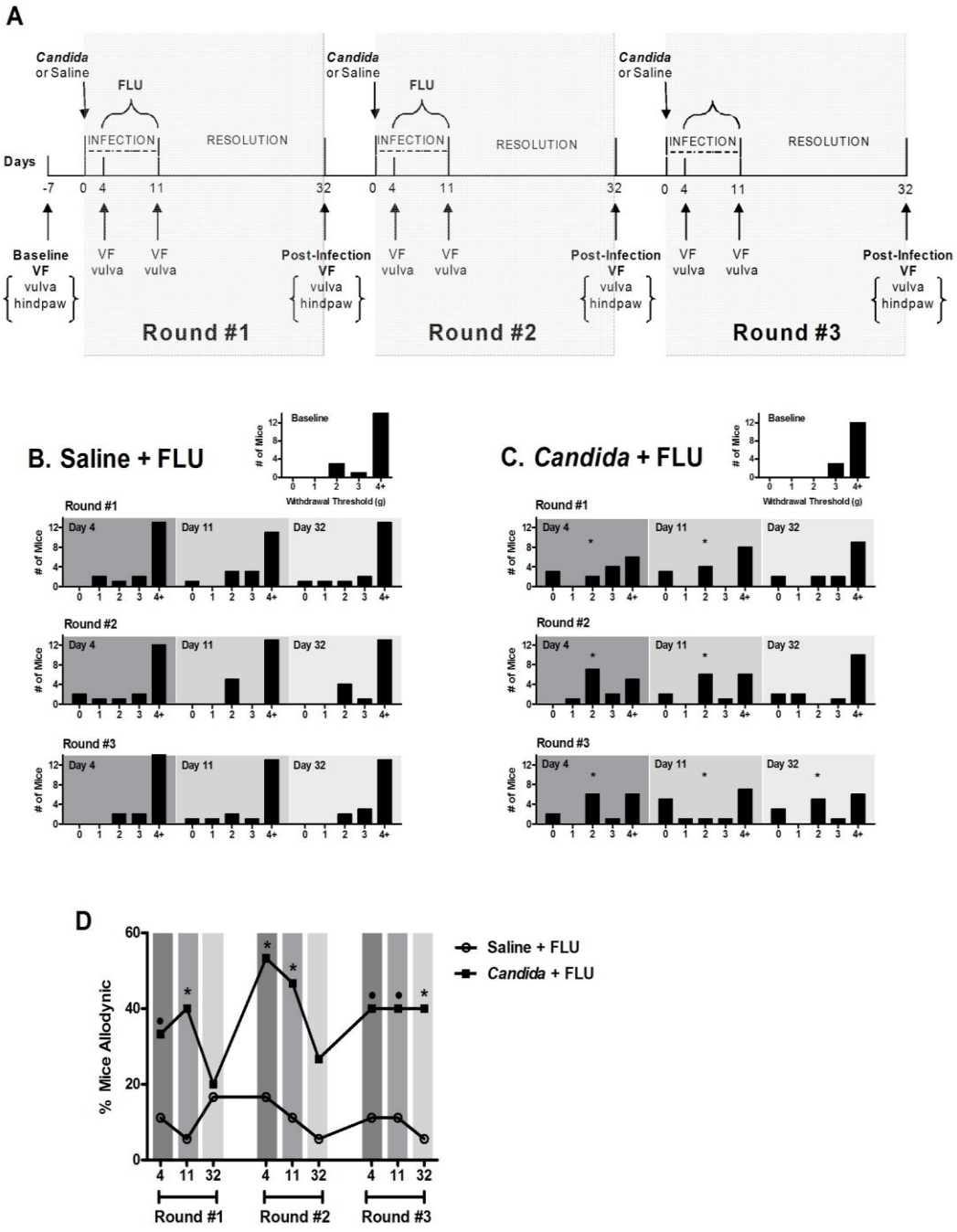

A
Candida or Saline
FLU
INFECTION
RESOLUTION
Days
-7 0 4 11 32
Baseline VF vulva hindpaw
VF vulva
Post-Infection VF vulva hindpaw
Round #1
Round #2
Round #3
B. Saline + FLU
C. Candida + FLU
Baseline
# of Mice
Withdrawal Threshold (g)
Day 4
Day 11
Day 32
D
% Mice Allodynic
Saline + FLU
Candida + FLU

Figure 2. Development of vulvar mechanical allodynia in a subset of mice after multiple rounds of vulvovaginal candidiasis with *C. albicans* strain SC5314. **A**) The experimental timeline illustrates the experimental procedures across three rounds of vulvovaginal infections with SC5314. Inoculations of 5 x $10^4$ cells were given on Day 0 for each of three infections (for second and third inoculations, SC5314was administered no more than 1 week after the previous vulvar and hind paw sensitivity testing). Behavioral measurements of vulvar mechanical sensitivity were taken at baseline and at three points during each infection: Day 4, Day 11, and Day 32. Note that for each Day 32 measurement, yeast was absent from the vaginal cavity for 3 weeks prior to testing. Graphs in **B** and **C** are frequency histograms showing the number of subjects displaying 50% withdrawal thresholds (jumping up with all four paws; see Materials and Methods) in five arbitrarily defined bins (0: 0–0.99 g; 1: 1.0–1.99 g; 2: 2.0–2.99 g; 3: 3.0–3.99 g; 4+: >4.0 g) at each testing session. Mice were inoculated vaginally with saline (**B**) or 5 x $10^4$ SC5314 cells (**C**) on Day 0 of each infection round ($n$=15–18/group); all mice received fluconazole (FLU; 15 mg/kg, p.o., once daily) from Day 4 to Day 11. *$p$<0.05 by $\chi^2$ analysis compared to within-group baseline. In graph **D**, symbols represent percentages of mice displaying ≥33% decreases in withdrawal threshold compared to their own baseline at each testing session. *$p$<0.05 compared to Saline+FLU group by one-tailed Fisher's exact test; •$p$<0.10 compared to Saline+FLU group.

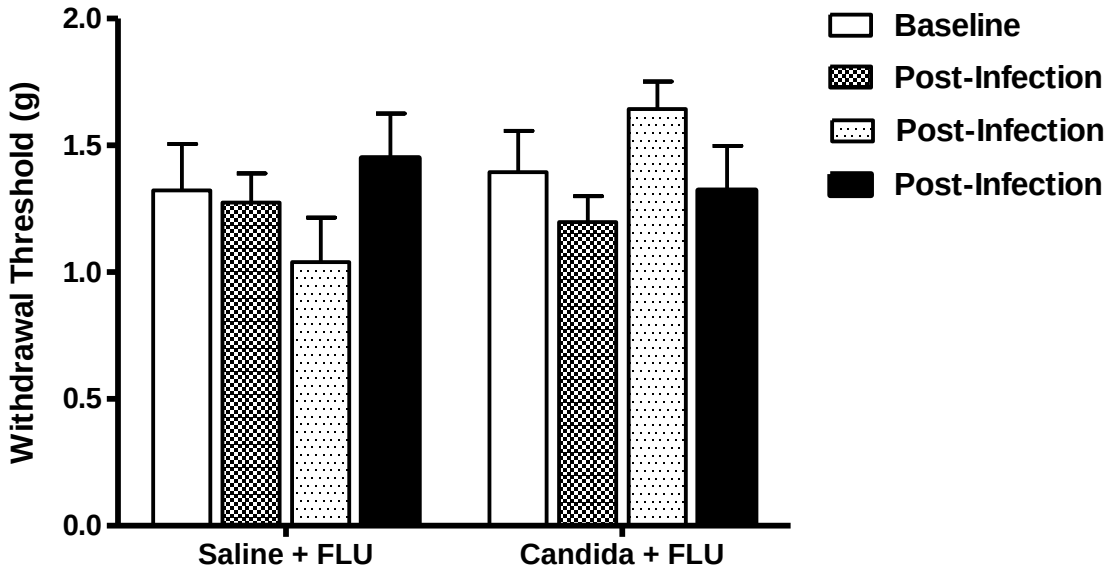

Withdrawal Threshold (g)
2.0
1.5
1.0
0.5
0.0
Saline + FLU
Candida + FLU
Baseline
Post-Infection
Post-Infection
Post-Infection

Figure 3. No effect of repeated vulvovaginal *C. albicans* strain SC5314 infection on hind paw mechanical sensitivity. Mice were inoculated three times with saline (Saline + FLU) or *C. albicans* SC5314 (Candida + FLU) as described in the main text. Hind paw sensitivity to von Frey fiber stimulation was assessed at baseline, and 33 days later (see Fig. 1A) after each of three rounds of infection. Bars represent mean ± S.E.M. withdrawal threshold (g). A repeated-measures ANOVA revealed no significant main effect of condition ($F_{2,126}$ = 0.6, $p$ = 0.53), repeated measure ($F_{3,126}$ = 1.1, $p$ = 0.34), or their interaction ($F_{6,126}$ = 1.9, $p$ = 0.09).

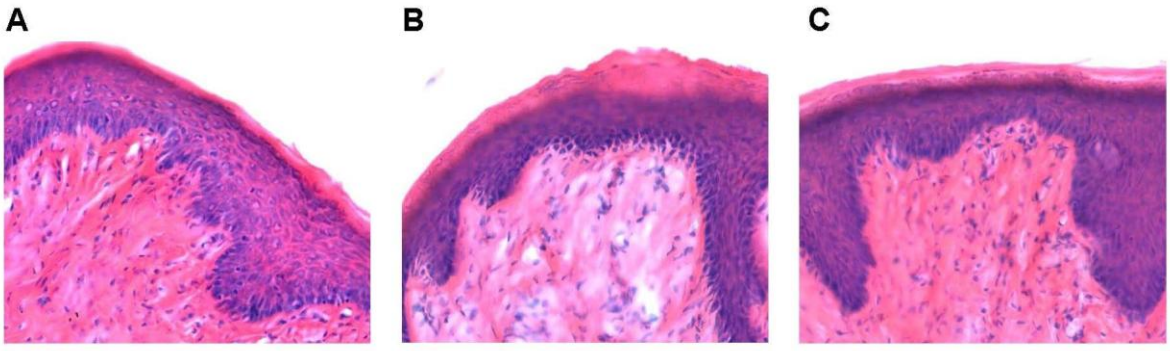

A
B
C

Figure 4. No effect of repeated vulvovaginal *C. albicans* strain SC5314 infection on gross vulvar morphology. Hematoxylin and eosin-stained sections of (**A**) saline +FLU control, (**B**) *Candida* +FLU nonallodynic, and (**C**) *Candida* + FLU allodynic (see main text) mouse vulvar epithelium following the third infection (Day 32) showed no evidence of active inflammation. Groups showed comparable levels of normal inflammatory cells (including few basophils, macrophages, and mast cells), but these cells did not penetrate the epithelial layer or the layer of intact keratin lining the epithelium.

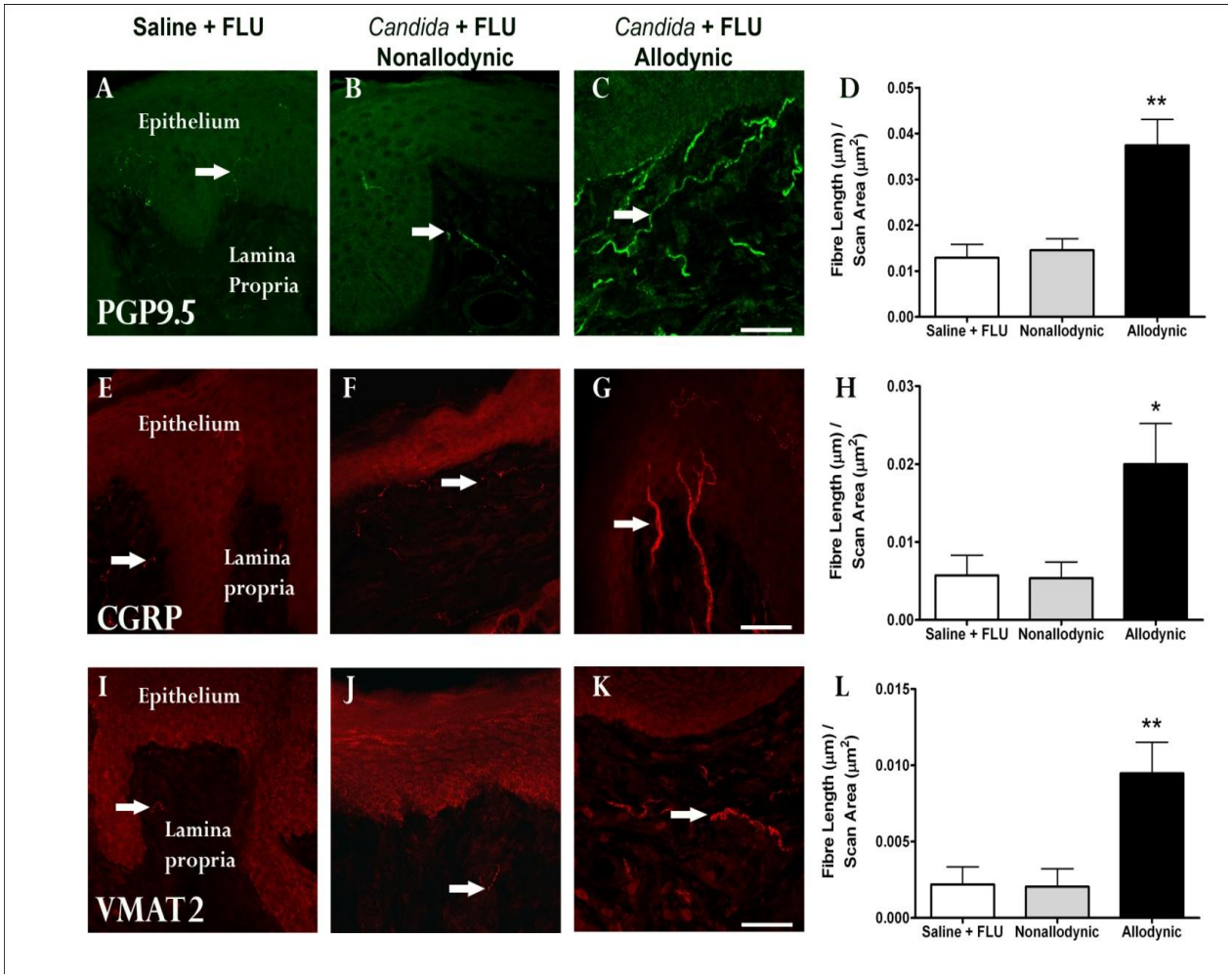
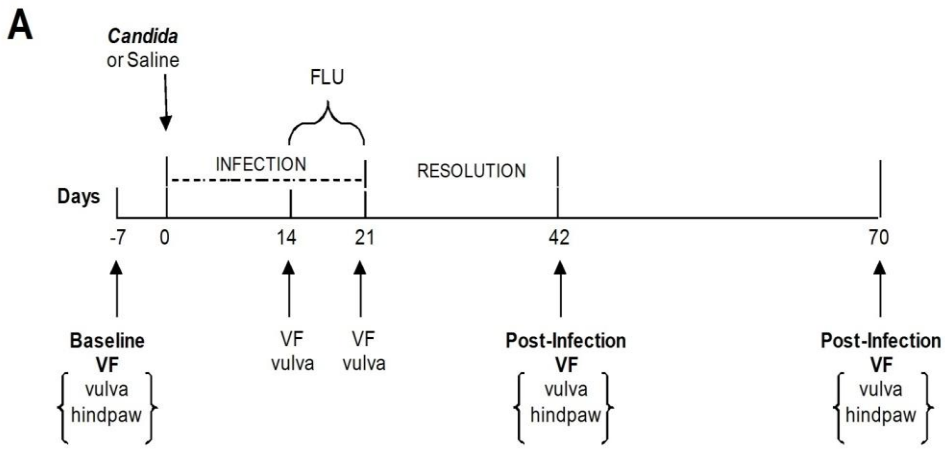
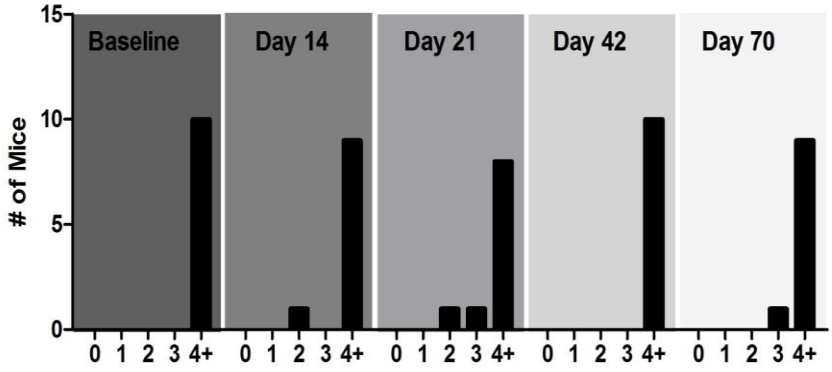

Saline + FLU
Candida + FLU
Nonallodynic
Candida + FLU
Allodynic
A
Epithelium
Lamina
Propria
PGP9.5
B
C
D
Fibre Length (μm) / Scan Area (μm²)
0.05
0.04
0.03
0.02
0.01
0.00
**
Saline + FLU
Nonallodynic
Allodynic
E
Epithelium
Lamina
propria
CGRP
F
G
H
0.03
0.02
0.01
0.00
*
I
Epithelium
Lamina
propria
VMAT2
J
K
L
0.015
0.010
0.005
0.000
**

Figure 5. Allodynic mice previously infected with multiple rounds of vulvovaginal candidiasis have increased expression of total, peptidergic, and sympathetic fibers compared to nonallodynic mice and controls ($n$=4–6 mice/group). The far left column shows the Saline + FLU group (**A**, **E**, **I**, the middle column shows the nonallodynic Candida + FLU subgroup (**B**, **F**, **J**), and the far right column shows the allodynic Candida + FLU subgroup (**C**, **G**, **K**). Bars in graphs **D**, **H** and **L** represent mean ± S.E.M. fiber length (· m) per unit area (· $m^2$). Total nerve fiber density (top row; PGP 9.5-IR) is significantly increased in the allodynic Candida + FLU subgroup, with long fibers lining the lamina propria beneath the epithelium (**C**, **D**). Peptidergic nerve fibers immunoreactive for CGRP, which normally consist of fine processes throughout the lamina propria that occasionally penetrate the basal cell layer of the epithelium, are significantly increased in the allodynic Candida + FLU group (**G**, **H**), and represent approximately half of the total fiber population (compare $y$-axes of **D** and **H**). Sympathetic nerve fibers immunoreactive for VMAT2 sparsely innervate the upper lamina propria in normal and nonallodynic mice, whereas a significant increase in innervation density is observed in the allodynic Candida + FLU group (**K**, **L**). *$p$<0.05, **$p$<0.01 compared to all other groups by one-way ANOVA followed by Tukey's posthoc test. Scale bars in graphs **C**, **G** and **K** represent 50 μm. Arrows point to fibers.


A
Candida
or Saline
FLU
INFECTION
RESOLUTION
Days
-7
0
14
21
42
70
Baseline
VF
vulva
hindpaw
VF
vulva
VF
vulva
Post-Infection
VF
vulva
hindpaw
Post-Infection
VF
vulva
hindpaw


B. Saline + FLU
# of Mice
15
10
5
0
Baseline
Day 14
Day 21
Day 42
Day 70
0 1 2 3 4+

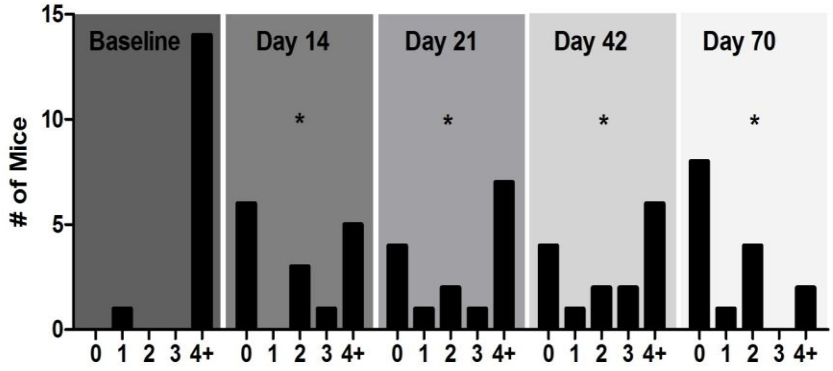

C. Candida + FLU
# of Mice
15
10
5
0
Baseline
Day 14
Day 21
Day 42
Day 70
*
0 1 2 3 4+

Figure 6. Development of vulvar mechanical allodynia in a subset of mice after a single, extended SC5314infection. **A**) The experimental timeline illustrates the experimental procedures. An inoculation of 5 x $10^4$ SC5314 cells was given on Day 0; fluconazole (FLU; 15 mg/kg, p.o., once daily) treatment occurred from Day 14–Day 21. Behavioral measurements of vulvar mechanical sensitivity were taken at baseline (Day -7) and at 14, 21, 42 and 70 days post-inoculation. Graphs in **B** and **C** are frequency histograms showing the number of subjects ($n$=10–15/group) in the Saline +FLU (**B**) and Candida + FLU (**C**) groups displaying 50% withdrawal thresholds in five arbitrarily defined bins (0: 0–0.99 g; 1: 1.0–1.99 g; 2: 2.0–2.99 g; 3: 3.0–3.99 g; 4+: >4.0 g) at each testing session. *$p$<0.05 by $\chi^2$ analysis compared to within-group baseline.

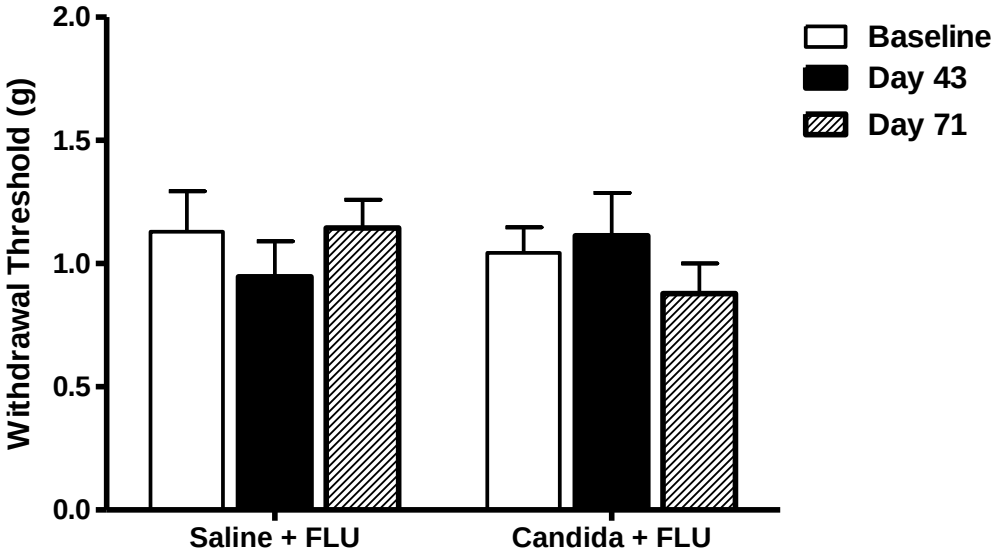
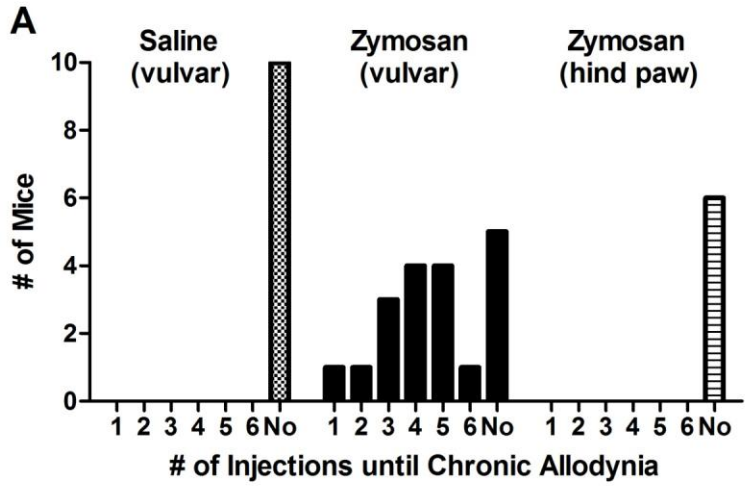

Withdrawal Threshold (g)
2.0
1.5
1.0
0.5
0.0
Saline + FLU
Candida + FLU
Baseline
Day 43
Day 71

Figure 7. No effect of single, extended-duration vulvovaginal *C. albicans* strain SC5314 infection on hind paw mechanical sensitivity. Mice were inoculated once with saline (Saline + FLU) or $5 \times 10^4$ *C. albicans* SC5314 cells (Candida + FLU) as described in the main text. FLU was not administered until 14 days after inoculation. Hind paw sensitivity to von Frey fiber stimulation was assessed at baseline, and 43 and 71 days later (see Fig. 6A). Bars represent mean ± S.E.M. withdrawal threshold (g). A repeated-measures ANOVA revealed no significant main effect of condition ($F_{1,60} = 0.3$, $p = 0.59$), repeated measure ($F_{2,60} = 0.2$, $p = 0.86$), or their interaction ($F_{2,60} = 1.2$, $p = 0.31$).


A
Saline
(vulvar)
Zymosan
(vulvar)
Zymosan
(hind paw)
# of Mice
10
8
6
4
2
0
1 2 3 4 5 6 No 1 2 3 4 5 6 No 1 2 3 4 5 6 No
# of Injections until Chronic Allodynia

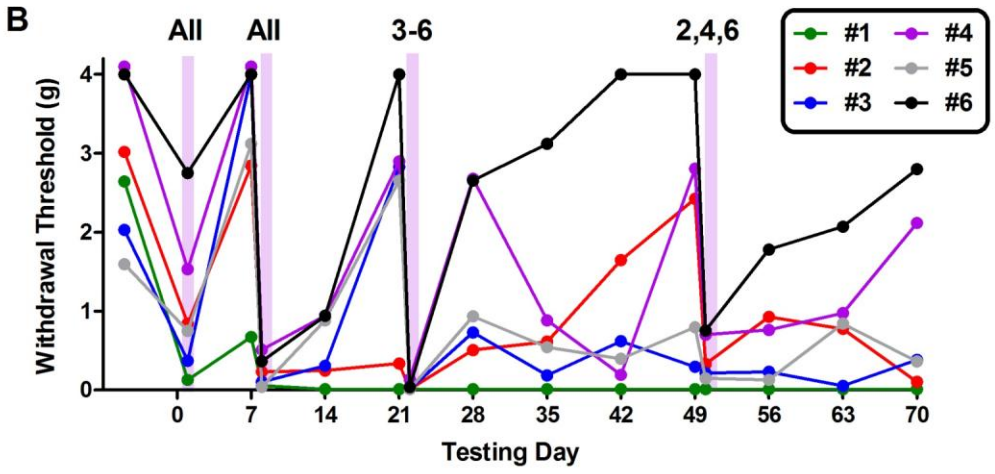

B
All
All
3-6
2,4,6
#1
#2
#3
#4
#5
#6
Withdrawal Threshold (g)
4
3
2
1
0
0 7 14 21 28 35 42 49 56 63 70
Testing Day

Figure 8. Development of vulvar mechanical allodynia in a subset of mice after repeated vulvar injections of zymosan. The frequency histogram in graph **A** shows the proportion of female mice displaying chronic allodynia after 1–6 weekly (or less) injections of vulvar saline (left; $n$=10), vulvar zymosan (middle; $n$=19) or hind paw zymosan (right; $n$=6). "No" indicates that chronic allodynia was never observed, even after six injections. Graph **B** illustrates representative patterns of vulvar mechanical sensitivity over time, using data from the first six mice to be tested (#1–#6). Vulvar zymosan was injected into mice so indicated 4 h prior to the data points highlighted in magenta.

# TRANSITIONAL TEXT 2

Results from the first study to demonstrate a causal relationship between repeated or extended vulvovaginal infection and inflammation provided a powerful tool for future research: the first animal model of PVD. This model allowed us to replicate the primary clinical symptom of PVD—provoked vulvar hypersensitivity in response to mechanical stimulation—using a pathogen that is suspected to cause the condition in a subgroup of women with vulvar pain, and this pain behaviour is correlated with known pathological correlates of PVD in the human literature.

Yet our extensive clinical experience with women who suffer from vulvar pain has taught us that the consequences of pain can be as detrimental to a woman's psychological health as the pain itself. One of the most common reasons women with vulvar pain seek medical and/or psychological help is because intercourse becomes unbearably painful (Basson, 2005). This inspired the question of whether animal models can inform us about the impact of pain on other quality-of-life factors, such as sexual motivation and behaviour. Women with PVD report reduced frequency of sexual behaviours (Meana, Binik, Khalife, & Cohen, 1999; Wiegel, Meston & Rosen, 2005), and thus reduced sexual motivation appears to be another clinical manifestation of vulvar pain. Similarly, the inferred link between pain and sexual motivation has been used to explain

why men with chronic prostatitis/chronic pelvic pain syndrome show reduced levels of sexual motivation compared to healthy controls (Davis, Binik & Carrier, 2009). The assumption that this reduction in sexual motivation results from genital pain is a prevalent but untested hypothesis. Likewise, it is feasible that reduced sexual motivation may not be specific to genital pain *per se*. To address these questions, we have adapted existing behavioural paradigms testing male and female rodent sexual motivation to directly test the hypothesis that genital (and potentially nongenital) pain can reduce sexual motivation.

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Davis, S. N., Binik, Y. M., Carrier, S. (2009). Sexual dysfunction and pelvic pain in men: A male sexual pain disorder? *Journal of Sex and Marital Therapy*, 35, 182-205.

Meana, M., Binik, Y. M., Khalife, S., & Cohen, D. (1999). Psychosocial correlates of pain attributions in women with dyspareunia. *Psychosomatics*, 40, 497-502.

Wiegel, M., Meston, C., & Rosen, R. (2005). The female sexual function index (FSFI): Cross-validation and development of clinical cutoff scores. *Journal of Sex and Marital Therapy*, 31, 1-20.

# “NOT TONIGHT DEAR I HAVE A HEADACHE”:

# SEX DIFFERENCES IN THE EFFECT OF PAIN

# ON SEXUAL MOTIVATION

Publication in preparation for submission.

Farmer, M, A., Leja, A., Foxen-Craft, E., Chan, L. J., MacIntyre, L., Binik, Y. M., Pfaus, J. G., & Mogil, J. S. “Not tonight dear I have a headache”: Sex differences in the effect of pain on sexual motivation. (In preparation).

Abstract

Pain is thought to impact diverse motivational states in humans and rodents. The relationship between pain and sexual motivation remains largely unexplored, despite the frequent comorbidity between clinical pain conditions and impaired sexual desire. Study objectives included the assessment of the negative impact of tonic, inflammatory pain on sexual motivation, a comparison of the sexual impact of genital versus non-genital pain, and a determination of possible sex differences in pain-influenced sexual behavior We compared male and hormonally primed female mice experiencing genital (penile for vulvar, respectively) or non-genital (dorsal aspect of hind paw, dorsal tail, or cheek pad) pain with respective saline controls and a no treatment control group. For pain groups, genital or hindpaw zymosan (0.5 mg/ml in 10 µL volume) or tail / cheek carrageenan (2% in 10 µL volume saline) injections were given before sexual behavior testing. Results indicate that female sexual behavior is equivalently reduced by genital and non-genital pain, whereas male sexual behavior remains unaltered, despite evidence that males and females show comparable levels of zymosan-induced hindpaw hypersensitivity. The sex difference in sexual behavior secondary to pain points to the robustness of male sexual motivation and/or the context-sensitive nature of female sexual motivation.

## Introduction

Darwin acknowledged the centrality of sexual reproduction as a vehicle for biological evolution (Darwin, 1871/1982). The divergence between male and female sexual selection strategies is thought to follow from their differential parental investment (of energy, resources, and time) in the production and maintenance of offspring (Trivers, 1972). Accordingly, the sexual strategies of high-investing females largely rely on mate choice based on traits that confer information about mate value (e.g., intersexual selection), whereas sexual strategies of low-investing males focus on male-male competition for access to fertile females (e.g., intrasexual selection). Sex differences in reproductive investment are also thought to underlie psychological mechanisms of human sexual desire and resultant sexual behavior (Symons, 1979).

Sexual motivation manifests in distinct, sex-specific patterns of behavior when animals are allowed to select their sexual environment. Male rodents develop conditioned place preferences (CPP) for compartments paired with copulation and post-ejaculatory satiety, whereas female rodents only develop CPP in specific contexts, when they are allowed to effectively "pace" (i.e., control the timing and rate of) sexual activity (Pfaus, Kippin, & Coria-Avila, 2003).

Parental investment theory assumes genetic variation in animals' abilities to pursue and secure potential mates. Yet even genetically "fit" animals may

face circumstances that result in injury or illness, which can compromise reproductive success. Injury can disrupt the physical act of copulation, as well as the motivational states that drive animals to seek out and mate with desirable and available partners. Pain, which often accompanies physical injury and disease, modulates diverse motivational states (fear, pleasure, reward; see Leknes, 2008), and thus pain may diminish the rewarding properties of sexual activity.

Different sexual selection strategies might predict a sex-specific effect of pain on sexual activity. Across numerous species, for instance, physical and environmental stress can diminish female sexual motivation (Cotton, 2006), suggesting that female sexual behavior is context-dependent. In contrast, environmental novelty, sensory disruption, and even castration do not affect the sexual behavior of sexually experienced male rats (Pfaus, Kippin, & Coria-Avila, 2003). In classical rat studies, only intense electrical shock inhibited male sexual performance (Beach, 1956). We are unaware of any study that has evaluated the libido-reducing effects of any stressor on males and females simultaneously.

The link between pain and sexual motivation is evident in human sexual relations. The widespread adage, “Not tonight dear, I have a headache” refers to a lack of female sexual motivation due to pain, or the use of pain as an excuse for a lack of desire. No human data exist on the direct impact of pain on sexual

motivation, yet the high prevalence of reduced sexual desire in chronic pain populations suggests that pain may adversely impact sexual motivation, especially in women with chronic pain (Basson, 2005). Animal and human literatures indicate that females are more susceptible to developing chronic pain and have higher pain sensitivity than males (Greenspan, 2007). It remains unclear to what extent these sex differences are mediated by biological, psychological or sociocultural factors.

We demonstrate here that females with inflammatory pain show inhibited sexual solicitation behavior and reduced frequency of copulatory behavior, whereas male sexual behavior is virtually unaffected by pain. The interaction between pain and sexual motivation conforms to the patterns of sexual behavior predicted by parental investment theory, and suggests that the context-dependence of human female sexuality (Meana, 2010) has a biological rather than sociocultural basis.

## Materials and Methods

### *Animals*

Mice were sexually experienced (see below) outbred CD-1® (ICR:Crl, Charles River, Boucherville, QC) males (ages 10–25 weeks) and ovariectomized (by supplier, at 5 weeks of age) females (ages 7–15 weeks). Mice were

maintained on a 12:12 h light/dark cycle (lights off at 19:00) in a vivarium maintained at ≈21oC, with access to food and water ad libitum. All testing was conducted during the dark cycle, between 20:00–24:00 h. All procedures were approved by the McGill University Animal Care Committee.

### *Apparatus*

For the male open field (M-OF) paradigm, mating occurred in clear Plexiglas® chambers (Fig. 1, dimensions: 8” × 8.5” × 14”), in which the male had unrestricted access to a sexually receptive female. The female paced mating (F-PM) paradigm utilized identical chambers except for a clear Plexiglas partition bisecting the chamber into two halves, a “male” side (8” × 8.5” × 7”) and an “escape” side (8” × 8.5” × 7”). Four semi-circular openings approximately 1” in diameter (with 0.75” space separating each opening) were made in the bottom of the partition that were large enough for females (<25 g) to freely transverse between the male and escape sides, yet were too small to permit the larger (>45 g) male mice to cross. The partition thus allowed the female to enter and leave the male side of the chamber, allowing her to control the timing or “pace” of mating, while the male was confined to one side. Red lights installed above each mating chamber enabled clear video capture of sexual behavior with cameras mounted 12” from the “male” side, allowing visualization of the male side during

F-PM testing, and of the entire cage during M-OF testing. The video feeds were recorded live using Virtual Dub v1.6.15® software, and Lab Spy v.1.5.2® software was used by blinded observers to later code the behaviors.

*Inflammatory Pain*

A number of different combinations of inflammatory mediators (zymosan, a mixture of fungal antigens, and carrageenan, which consists of red algae mucopolysaccharides) and injection sites (both genital and non-genital) were used. As shown in Fig. 2, experimental pain groups received: a) s.c. injections of either zymosan (0.5 mg/ml in 10 μl physiological saline, Sigma) into the genitalia (center-posterior vulva or center-dorsal penile shaft) or mid-plantar right hind paw, b) 2% carrageenan (Sigma) dissolved in 10 μl physiological saline into the ventral tail (halfway from base to tip) or right cheek (whisker pad), or c) saline only into these same locations.

In a separate group of male and female mice not used for mating testing, mechanical sensitivity was quantified immediately prior to and 4 h post-zymosan injection using von Frey monofilaments as previously described (Mogil et al., 2006).

### *Sexual Vigour Training*

Sexual receptivity was induced in ovariectomized females with subcutaneous (s.c.) injections of estradiol benzoate (5 μg/0.1 ml in sesame oil; Sigma) given 48 h pre-test and progesterone (500 μg/0.1 ml in sesame oil) 5.5 h pre-test. These parameters are known to induce a state of optimal sexual receptivity, comparable to the estrus phase (Blaustein & Erskine, 2002). Hormonally primed ovariectomized females were paired with novel sexually experienced males for a "sexual vigour" training session (1–2 times/week) in the same mating chambers used for testing. Each vigour session lasted 30 min, and only male-female pairs that exhibited ≥10 intromissions (50-60% of tested pairs) were randomly assigned to F-PM or M-OF testing. This procedure ensured comparable baselines of sexual behavior across groups and ensured that differences in mate preference would not confound results. Vigour testing also served as habituation to the testing environment.

### *Sexual Behavior Testing (F-PM and M-OF)*

F-PM and M-OF testing lasted 60 min, based on pilot experiments showing that this duration captured the vast majority of mouse mating behavior, with most mice initiating sexual contact 10-20 min into testing. The following groups were tested in males and hormonally primed females (n=7-12 per group): 1) vulva (F-

PM) or penis (M-OF) zymosan, 2) vulva or penis saline control, 3) hind paw zymosan, 4) hind paw saline control, 5) tail carrageenan, 6) tail saline control, 7) cheek carrageenan, 8) cheek saline control, and 9) no treatment control. Two different inflammatory stimuli were used to establish generalizability, and various non-genital body parts were tested to address the possibility that tenderness in the hind paw might physically impede sexual behavior. Injections were given 3 or 4 h pre-test (for carrageenan and zymosan, respectively, based on pilot data measuring peak mechanical allodynia of the hind paw produced by these inflammatory agents) to mice briefly anaesthetized with isoflurane/oxygen.

The male (for M-OF) or female (for F-PM) "test" mouse was placed into the mating chamber 15 min prior to the introduction of the opposite-sex, untreated "stimulus" mouse. For F-PM testing, males were placed on the side of the partition nearest to the cameras (i.e., the "male" side). Immediately following testing, mice in all pain groups were euthanized.

### *Behavioral Measures*

Observers were blinded to experimental conditions. Extensive pilot work revealed differences between mouse sexual behavior and published descriptions of rat sexual behavior, both in the open field and paced mating paradigms, which have not been previously noted (Johansen et al., 2008). Notably, very little

hopping and darting was observed by females, and sexually receptive female mice showed variable intensities of lordosis posture during mating. The following behaviors were observed and analyzed: 1) frequency of mount episodes (defined as direct genital contact by the male onto flanks of the female without achieving penetration, often accompanied by female lordosis); 2) frequency of intromission episodes (defined as two or more distinct thrusts with penetration); 3) ejaculation frequency; 4) latency to first mount (with or without intromission); 5) inter-intromission interval; 6) average intromission duration; 7) ratio of mounts to intromissions; and, 8) ratio of male versus female termination of intromissions. For F-PM testing, additional variables included: 9) number of exits/entrances to male side; 10) total time spent on male side (overall and following first intromission); and, 11) average contact return latency following each intromission.

### *Drug Administration*

In one experiment, mice received drugs known to affect libido (Pfaus, 2009). Some mice received a single intraperitoneal (i.p.) injection of the non-selective dopamine D1 and D2 receptor agonist, apomorphine (100 mg/kg in 10 ml/kg 50% polyethylene glycol vehicle, Sigma), or vehicle control, 45 min prior to mating.

### *Data Analysis*

Data were analyzed by ANOVA and/or Student's t-test as appropriate, using Systat v. 13 (SPSS Inc.). Following ANOVA, group differences were assessed by Tukey's posthoc test. A criterion $\alpha = 0.05$ was adopted in all cases. In two cases, statistical outliers (Studentized residual >3) were omitted from the analyses.

## Results

### *Reduced sexual activity and solicitation behavior in female mice experiencing inflammatory pain*

In the F-PM paradigm, vehicle treatment showed no effect on total mount frequency compared to no treatment ($F_{1,41} = 0.32$, $p = 0.58$). Because each inflammatory/site condition was tested concurrently with its own vehicle controls, we then compared each pain group to its vehicle control group by Student's t-test. Pain in females produced a reduction in total mount frequency across body sites, including the vulva ($t_{1,14} = 2.46$, $p = 0.03$), hind paw ($t_{1,15} = 2.22$, $p = 0.04$), tail ($t_{1,11} = 3.86$, $p < 0.01$, and cheek ($t_{1,12} = 6.04$, $p < 0.01$) (Fig. 3). Thus the presence of female pain, genital or non-genital, resulted in an overall reduction in sexual behavior in the paced mating paradigm. The fact that the reductions were

as large (or larger) in the tail pain and cheek pain groups compared to the genital and hind paw groups suggests that physical inability to engage in sexual behavior and/or support the male's weight does not account for our observations.

Given that total mount frequency is a behavioral measure that involves male participation, we sought to determine whether these robust differences were indeed the result of reduced female sexual motivation. Females in genital or non-genital pain spent significantly less time on the male side ($F_{2,69}$ = 8.7, $p<0.001$) (Fig, 4), suggesting that the overall reduction in sexual behavior was indeed a product of reduced female sexual motivation. Furthermore, this lowered solicitational behavior was not due to pain-related impairments in motor functioning, given that the total number of barrier crossings did not differ between mice with and without pain ($t_{1,70}$ = 0.59, p = 0.56).

*No change in sexual activity in male mice experiencing inflammatory pain*

In the M-OF paradigm, vehicle treatment showed no effect on total mount frequency compared to no treatment ($F_{1,34}$ = 0.55, p = 0.46). Pain in male mice was found to have no effect on total mount frequency in any body site (Fig. 5), including the penis ($t_{1,15}$ = 1.66, p = 0.12), hind paw ($t_{1,11}$ = 0.52, p = 0.61), tail ($t_{1,10}$ = 0.6, p = 0.56), and cheek ($t_{1,13}$ = -0.19, p = 0.85).

*Females and males show equivalent mechanical sensitivity to inflammatory pain*

To address the possibility that the sex differences in sexual behavior noted above derived from sex-specific pain intensity, a separate of group of mice were tested for mechanical allodynia following hind paw zymosan injections using parameters identical to those employed in the mating experiment. Male and female mice displayed equivalent baseline mechanical sensitivity ($t_{10}$ = 0.4, p=0.70). Repeated measures ANOVA revealed significant mechanical allodynia (defined as the decrease in withdrawal thresholds after injection) ($F_{1,10}$ = 14.7, p<0.001) but no sex x repeated measures interaction ($F_{1,10}$ = 0.01, p=0.92). As seen in Fig. 6, male and female mice displayed equivalent mechanical allodynia, and thus the female-specific inhibition in mating behavior cannot be due to increased inflammatory pain levels in this sex.

*Pro-sexual drug apomorphine reverses the reductions in sexual behavior caused by female inflammatory pain*

The administration of apomorphine, a dopamine D1/D2 receptor agonist, reversed the pain-induced reduction in paced (F-PM) sexual behavior (Fig. 3a). A two-way ANOVA revealed a significant main effect of pain on total mounting frequency ($F_{1,29}$ = 6.87, p = 0.01), no main effect of apomorphine ($F_{1,29}$ = 0.88, p = 0.36), and a significant pain x apomorphine interaction ($F_{1,29}$ = 5.34, p = 0.03).

Given that the groups did not differ in frequency of barrier crossings ($F_{3,28}$ = 1.01, p = 0.4), it is unlikely that the restoration of mating behavior is due to apomorphine-induced hyperlocomotion. Rather, a one-way ANOVA indicated that the groups differed in time spent on the male side following the first intromission ($F_{3,28}$ = 3.81, p = 0.02), with the vehicle + carrageenan group spending the least amount of time on the male side (Fig. 7). Thus, the administration of apomorphine restored female solicitational behavior despite the concurrent presence of hind paw pain.

## Discussion

We have observed, for the first time, sex differences in the causal interaction between pain and sexual behavior. Sexually receptive female mice in pain reduce the amount of time voluntarily spent in proximity to male mice (their previously successful sexual partners), leading to reduced copulatory behavior. In contrast, male sexual behavior under equivalent circumstances is wholly unaffected by pain. This sex difference is independent of the site or type of pain, and as there is no evidence for sex differences in pain levels in this study, we conclude that the sex differences are driven by altered sexual motivation secondary to pain. The reduced sexual motivation displayed by female mice can be rescued by pre-treatment with the libido-enhancing drug apomorphine,

indicating that the observed changes are indeed due to alterations in sexual motivation *per se*.

*Sex differences in sexual motivation or pain sensitivity?*

In the paced mating (F-PM) paradigm, sexual motivation is inferred from the amount of time spent on the male side, and the female regulates the timing and frequency of sexual activity. When pain-free females are allowed to pace sexual activity, the preferred frequency of mounting is reduced compared to the non-paced (M-OF) situation wherein the male has unrestricted access to a receptive female (compare No Treatment groups in Fig. 3 and 5). This baseline sex difference in preferred rates of (female) paced and non-paced mating is described in the rat paced mating literature (Pfaus, Kippin, & Coria-Avila, 2003). We find here that the presence of pain—regardless of its location (genital vs. non-genital)—dramatically reduces the time a female spends with a previously preferred sexually vigorous male, and results in decreased frequency of copulation. This effect cannot be explained by sex differences in pain sensitivity, which although well-known (see Mogil & Bailey, 2010), depend on stimulus modality and interact with genotype (see Mogil, 2000), and were not seen here. Nor can the reduction in mounting behavior in females be attributed to discomfort due to weight bearing on the affected hind paw (or perhaps also on the tail) being

aggravated by the physical act of mounting, since cheek pain produced equivalent reductions in sexual activity. Although reduced cutaneous sensitivity on the rump or genitals may adversely impact lordosis and copulation rate (Pfaff, Montgomery, & Lewis, 1977), non-genital pain was equally effective as genital pain at impairing behavioral indices of female sexual motivation. Furthermore, if these effects were due to the continued physical discomfort caused by pain, one would expect mounting behavior to decrease over time; in contrast, females with and without pain showed consistent rates of copulation throughout testing (data not shown). Mating-induced analgesia has been reported for male as well as female rodents (i.e., vaginocervical stimulation-evoked analgesia), but vaginocervical analgesia, if present, has only been shown to reduce pain sensitivity for brief periods of time (Komisaruk & Whipple, 2000), and obviously failed to alleviate the tonic inflammatory and neuropathic pain in the current study. Finally, it is unlikely that the hormone priming regimen itself enhanced pain sensitivity in females, given the repeated demonstrations of decreased pain following supraphysiological doses of estradiol such as those used here (Aloisi, 2010; Craft, 2008; Sanoja, 2008). Therefore, it is highly unlikely that purely sensory mechanisms can account for the present findings.

*Pain-induced reductions in sexual motivation and libido-enhancing drugs*

Dopamine and melanocortin modulation with apomorphine reversed the pain-induced sexual behavior deficit in female mice, suggesting either that either this drug directly augmented sexual motivation, or that is has analgesic properties. The apomorphine doses we administered have been previously shown to produce no thermal or mechanical sensory changes (Pelissier et al., 2006), and thus it is unlikely that they produced analgesia here. Pro-sexual effects in rodents and humans have been reported with dopamine agonists, showing enhanced female solicitations in the rat (Pfaus, 2009). In the male rat, apomorphine enhances penile reflex through a spinal 5-HT2C-dependent mechanism (Kimura, 2008). Apomorphine may also modulate dopamine transmission in the nucleus accumbens during sexual behaviour (Olivier et al., 2007), part of the neural circuitry implicated in reward and motivation. Indeed, exposure to, pacing of, and copulation with males are associated with dopamine release in the nucleus accumbens and striatum of female rats (Pfaus, Damsma, Wenkstern, & Fibiger, 1995).

The possibility remains that the decreased sexual activity observed in the paced (F-PM) paradigm could have, in part, reflected reduced motivation of the male. Exposure to (and subsequent pursuit of) a sexually receptive female is reinforcing for male rats (Pfaus, Kippin, & Coria-Avila, 2003). Given that female

mice in pain showed reduced solicitation compared to pain-free females, their behavior may have been less reinforcing to males. Similarly, male mice may find the scent of a female in pain less appealing and reduce their own approach behavior. However, in rats even aversive scents such as cadaverine on a receptive female fails to inhibit male sexual behavior (Pfaus, Kippin, Coria-Avila, 2003), suggesting that aversive scent does not impact male sexual motivation. In the present experiment, there were no group differences in latency to mount the female once the female appeared in the male side (data not shown), suggesting that male motivation was unaffected by female pain.

Finally, we note that in our hands apomorphine produced no increases in mounting behavior when given to pain-free female mice, but only when given to female mice whose sexual motivation was inhibited by pain. This suggests that the use of pain to reduce sexual motivation might be a superior (i.e., more sensitive) screening tool for libido-enhancing drugs. It can be argued that demonstrating efficacy of such drugs in the context of reduced libido is more clinically relevant than current paradigms in which increases from a “normal” level of sexual behavior are screened.

*Biological basis of context-dependent sexuality in females*

Although sexuality relies on biological mechanisms to facilitate reproduction, it is widely believed that the expression of human sexuality is

dictated by culturally-constructed expectations of sexual motivation and behavior, and the tremendous cross-cultural variation in sexual mores and practices reinforces this assumption (Foucault, 1978; Gagnon & Simon, 1973). Western culture assumes that women's sexual behavior is highly context-dependent, and this bias is evident in popular accounts of sexual desire and arousal (e.g., "Not tonight dear, I have a headache"; (Basson, 2007; Meana, 2010). Clinical data supports these assumptions, indicating that female sexual desire and arousal is strongly regulated by inhibitory factors that restrict sexual motivation and behavior (e.g., negative mood, distraction, fear of pregnancy or disease), compared to men (Bancroft & Graham, 2001). In contrast, our results suggest that female sexual motivation can be modulated by strictly biological factors that modulate the sensitivity to sexual context, given that rodents do not develop within the context of culture or social constructions of sexuality.

## Conclusions

In addition to baseline sex differences in patterns of sexual behavior, male and female mice exhibit sexually dimorphic behavioral responses to the presence of pain during sexual activity. Whereas males show robust motivation for sexual activity that is not affected by genital or non-genital pain, female sexual motivation is blunted by the presence of any type of physical discomfort. This

biologically-based sensitivity to sexual context may reflect fundamental differences in sexual selection strategies that characterize the female sex, for which pregnancy and child rearing demand enormous energy and resources. Pain states thus have the ability to powerfully shape basic motivational drives in a sex-specific manner, lending credence to the popular belief that female sex drive can be stifled with a headache, or for that matter any ache at all.

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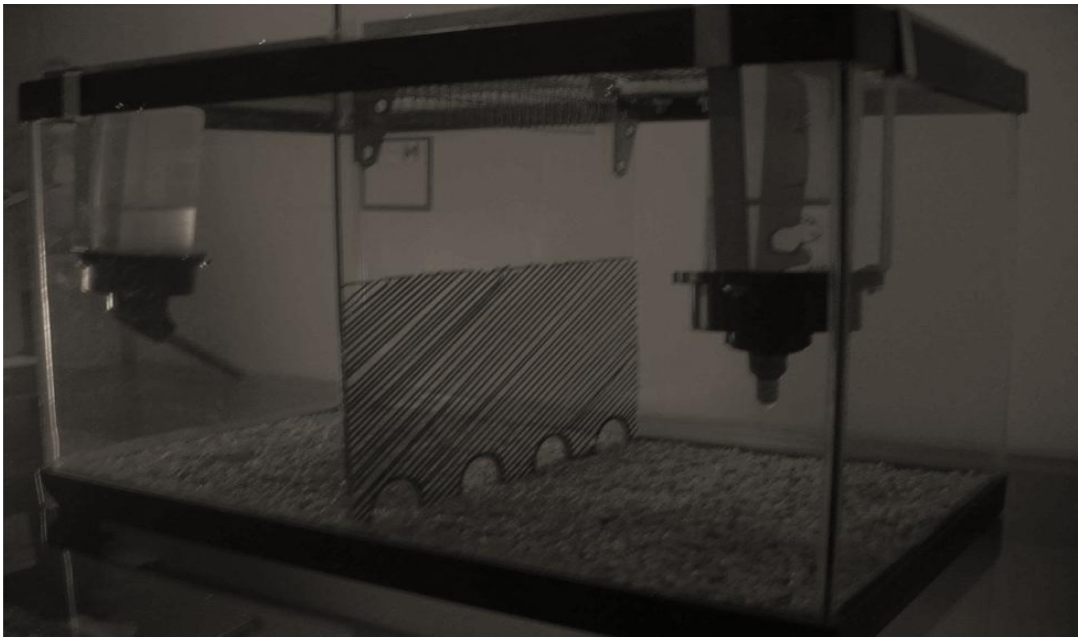

Figure 1. Paced mating chamber, with a dividing partition featuring four holes to allow females to freely traverse between both sides of the chamber.

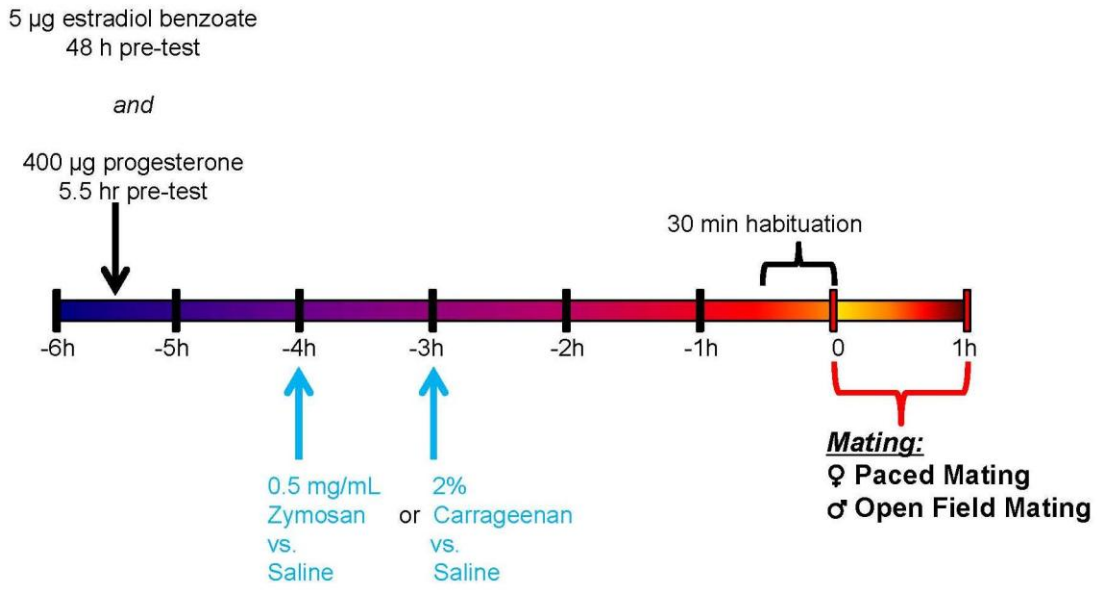


Figure 2. Experimental design for female paced mating (F-PM) and male open field (M-OF) paradigms.

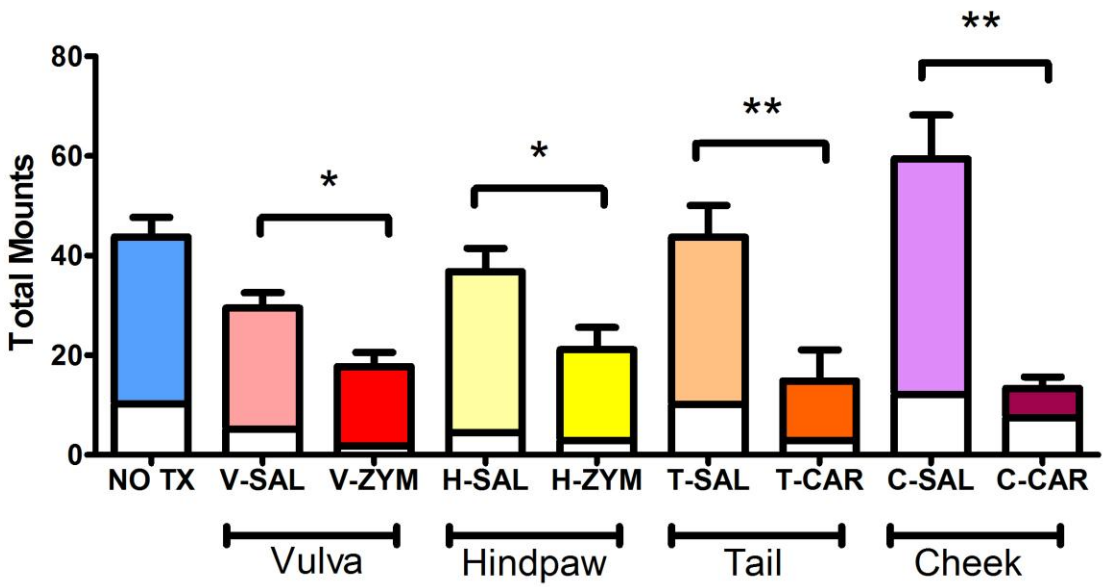


Figure 3. Frequencies of female sexual behavior in the paced mating paradigm, by group. No significant differences were found between saline (n = 7-9 per group) and no treatment (n=13) groups; however significant differences were found between each inflammatory pain- (zymosan or carrageenan) and saline-treated body sites (n=7-8 per group). White areas depict frequency of mounts without intromissions, colored areas depict frequency of mounts with intromissions, and standard error bars reflect variance in the total mounts (with and without intromissions) for each group.

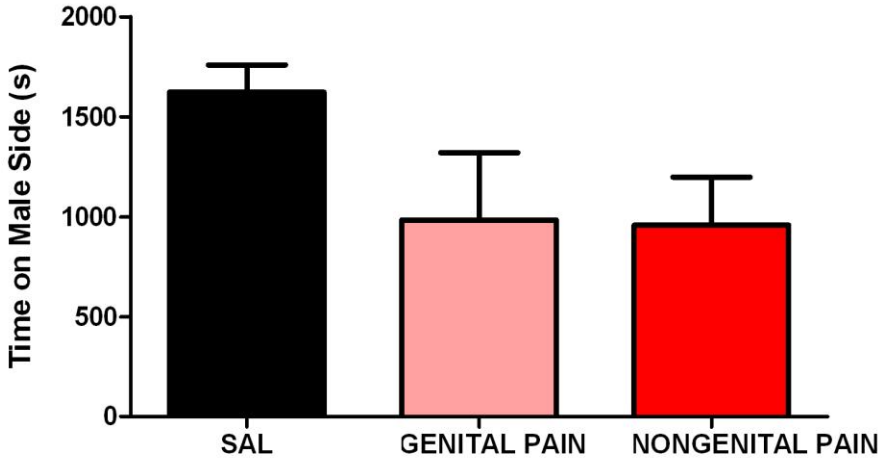


Figure 4. Time on male side (s) in pooled saline-treated control group (n=45), compared to genital (n=8) and non-genital pain groups (n=23).

Note: Genital pain group = vulvar zymosan; non-genital pain group= pooled data from hind paw zymosan, tail carrageenan, and cheek carrageenan groups.

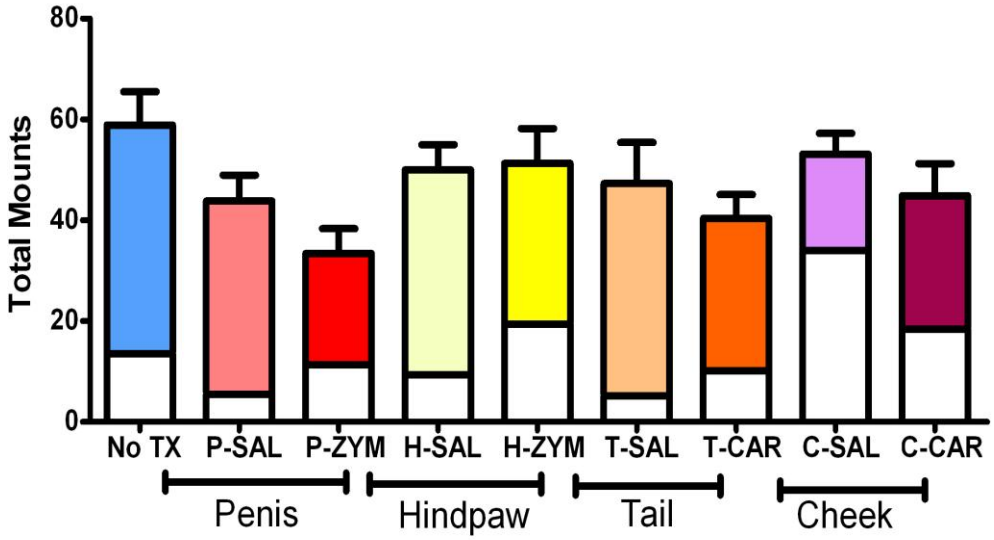


Figure 5. Frequencies of male sexual behavior in the open field mating paradigm, by group. No significant differences were found between saline and no treatment groups (n=6-8 per group), or between inflammatory pain- (zymosan or carrageenan) and saline-treated body sites (n=7-10). White areas depict frequency of mounts without intromissions, colored areas depict frequency of mounts with intromissions, and standard error bars reflect variance in the total mounts (with and without intromissions) for each group.

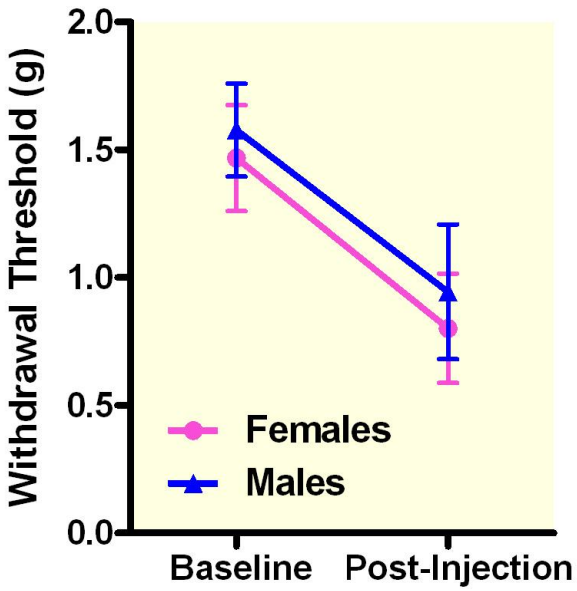


Figure 6. Lack of a sex difference in hind paw mechanical sensitivity (n=12) 4 h post-zymosan injection (corresponding to the commencement of mating testing).

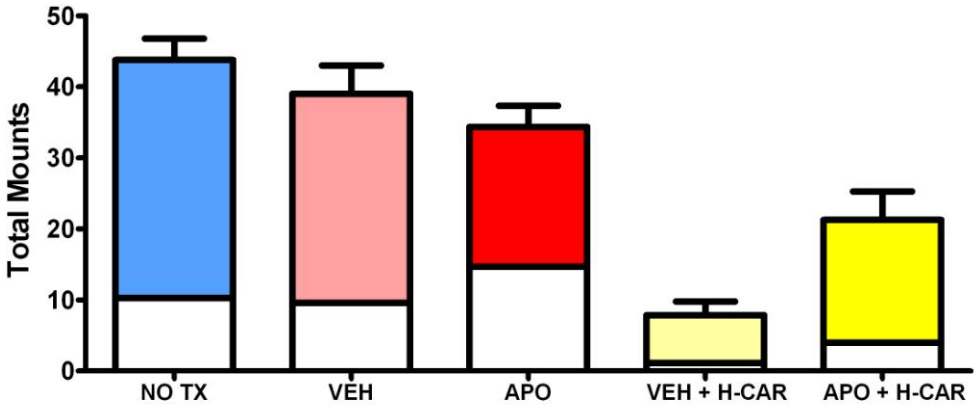


Figure 7. Apomorphine restores carrageenan-induced reductions in female paced sexual activity (n=9-17 per group).

# GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The review and two empirical investigations contained in this dissertation examine novel animal models of urogenital pain that provide insight into vulvar pain etiology and sexual behavioural consequences of genital and non-genital pain. These models were developed in reference to clinical reports and observations of women with PVD, and methodological design decisions were made in accordance with clinical definitions and practices (Haefner et al., 2005).

The finding that repeated and prolonged bouts of infection and inflammation can produce long-lasting vulvar hypersensitivity constitutes the first empirical evidence of an etiology for provoked vestibulodynia. A large percentage of women with PVD have reported repeated yeast infections (Bohm-Starke, Hilliges, Blomgren, Falconer, & Rylander, 2001; Witkin, Gerber, & Ledger, 2002), and this evidence in mice suggests that a subset of women with PVD may indeed have persistent vulvar hypersensitivity *due to previous infection(s)*. Interestingly, frank signs of vulvar inflammation were absent following multiple *Candida* infections in allodynic mice. This observation is consistent with the human data showing no difference in inflammatory infiltrate between women with and without PVD (Halperin et al., 2005; Lundqvist et al., 1997; Slone et al., 1999; but see Chadha et al., 1998; Foster & Hasday, 1997).

This finding of no frank inflammation has two important clinical ramifications: 1) presence of inflammatory infiltrate in women with PVD may reflect factors that cause or maintain vulvar pain, as well as more recent, unrelated immune threats; and therefore 2) previous human data showing equivocal findings of inflammatory infiltrate may not be adequate evidence for or against a particular inflammatory etiology of PVD given that previous inflammation cannot be evaluated after its resolution. A careful study of the transition from acute to chronic pain using animal models may provide insight into the mechanisms underlying the maintenance of vulvar pain, and these maintaining factors are likely related to the vestigial “clues” of previous inflammation that characterize the chronic vulvar pain symptoms seen by gynaecologists.

Alternately, these findings may also suggest the existence of inflammatory and non-inflammatory PVD subtypes, which have not been previously detected in mixed clinical samples. Such subtypes would explain the contradicting pattern of inflammatory findings that characterize some PVD samples, and not others (see Gerber, Witkin, & Stucki, 2008 for an overview). Recent conceptualizations of the different mechanisms underlying subtypes of pelvic pain have suggested that pelvic pain may initially develop due to local pathology (e.g., inflammation), and later develop into systemic pain conditions if pain persists despite treatment attempts (e.g., Rodriguez, Afar, Buchwald, &

NIDDK Working Group, 2009). Evidence for such subsets of women with PVD who have underlying systemic abnormalities in pain processing includes extra-dermatomal (i.e., non-genital) altered pain sensitivity (e.g., Pukall et al., 2002) and symptom overlap with fibromyalgia (Pukall, Baron, Amsel, Khalife, & Binik, 2006). Such hypotheses could be tested with this mouse model of PVD, combined with cystitis or colitis models.

Both empirical investigations described in this dissertation indicate the great potential for clinically relevant animal models to explain a diverse array of physiological and behavioural processes that impact the experience of pain. The validity of the repeated infection mouse model of PVD is supported by the replication of the primary clinical symptom of the condition—localized, provoked vulvar mechanical hypersensitivity—and the finding of altered vulvar innervation in allodynic vulvar tissue, which closely parallels findings of altered vulvar innervation in women with PVD (Bohm-Starke, 2010). Similarly, the demonstration that female mice show reduced sexual motivation and behaviour following genital and non-genital pain closely mirrors the clinical reality of comorbid sexual pain and desire dysfunction in women (Farmer & Meston, 2007; Meana, Binik, Khalife, & Cohen, 1999). These mouse models suggest that pain can reorganize physiology, behaviour, and even motivational processes, thereby fundamentally altering the way an organism interacts with its environment.

Sex differences in experimental pain sensitivity have been previously described (Greenspan et al., 2007), yet the paced mating experiment demonstrates a sex difference in how pain affects basic motivational drives. Evolutionarily, this sex difference may be linked to differential parental investment (Trivers, 1972), such that high-investing females show optimal sexual motivation when they perceive that they are capable of sustaining the physical demands of pregnancy. A variety of physical stressors can impair female sexual motivation and behaviour in a number of species (Cotton et al., 2007), suggesting a biologically ingrained sensitivity to specific contextual factors that could compromise her ability to bear offspring, thereby modulating her motivation to pursue sexual activity. Recent models of sexual motivation in women have emphasized biological, psychological, and sociocultural context specificity in enhancing and reducing a woman's attention to, emotions about, and behaviour facilitating sexual activity (Basson, 2005). Whereas sociocultural factors are thought to play a dominant role in the way in which a woman responds to her sexual context (e.g., Lewis, 2004; Tiefer, Hall, & Tarvis, 2002; Tolman & Diamond, 2001), mice have no such culture, and thus the paced mating evidence suggests a biological basis for this female sensitivity to contextual factors that cannot be explained by hormonal status.

While these animal models show great promise in the study of pain and sexual dysfunction, there are methodological concerns that need to be addressed before these models are applied to clinical research. The primary concern is the time commitment required to execute these studies. For example, a repeated *Candida* infection experiment lasts a minimum of 5-6 months and requires a large number of mice given that approximately only 50% at most end up developing vulvar allodynia. The repeated vulvar zymosan injection experiment may serve as a proxy for the repeated infection scenario, yet the cost of this convenience is a loss of the ecological validity gained from repeatedly inducing and resolving infections with a common pathogen seen in the PVD clinical population. Similarly, female mice require a minimum of one month for hormonal priming to correct the post-ovariectomy loss of sex steroid hormones and to gain adequate sexual experience within the paced mating context. Furthermore, approximately 20-30% of male mice show unreliable sexual performance, and using sexually sluggish males can confound results that are aimed to potentially reduce sexual behaviour. However, the scientific value of the data yielded from these animal models relies on a small time investment, compared to years of inconclusive clinical studies and effort. Development of animal models that allow us to ask the critical clinical questions about PVD are invaluable if they improve the understanding and treatment of the condition.

Numerous methodological questions remain about the generalizability of the post-infection PVD mouse model, including whether the vulvar allodynia can be produced with other *Candida* strains or even bacteria (to model bacterial vaginosis pain). Given evidence of genetic polymorphisms in some women with PVD that may predispose them to altered inflammatory responses (Gerber, Witkin, & Stucki, 2008), follow-up studies with knockout mice (genetically engineered mice with strategically inactivated genes that result in altered appearance, behaviour, or biochemistry) would be needed to evaluate the mechanistic impact of these genetic risk factors. Additionally, an examination of more inflammatory markers (cytokines, mast cells, macrophages, etc.) in vaginal lavage fluid and vulvar tissue during acute and chronic phases of the inflammatory process may provide additional mechanistic insight, but was not pursued in the described study due to time/effort constraints. There is conflicting evidence as to whether the mouse has vulvar tissue that is embryologically analogous to the vulvar vestibule in the human. Whereas the perinatal mouse vagina is characterized by urogenital sinus tissue in the lower 2/5 of the vagina and Müllerian tissue in the upper 3/5, the epithelia of these two types of tissues becomes homogeneous neonatally and it is no longer possible to definitively track their respective growth (Boutin & Cunha, 1996), thereby making a morphological comparison difficult. Finally, the “ceiling” values (4+ g of force)

used for von Frey tests of vulvar mechanical sensitivity do not reflect an actual baseline, but a rather a baseline range—this indicates that baseline vulvar sensitivity measurements cannot be obtained using these procedures.

Despite these limitations, these models provide fruitful paths for future vulvodynia research. The repeated infection/inflammation PVD model can be adapted to test other suspected vulvar pain etiologies (e.g., pain secondary to oral contraceptive use, bacterial vaginosis, chronic bladder inflammation, or even psychological stress), including additional investigations into the role of acute inflammatory factors that may facilitate the development of persistent pain. This model can also be used to assess the effectiveness of novel pharmacologic treatments of vulvar pain, given that so few effective treatments currently exist. Similarly, the paced mating paradigm can be used to examine the effect of repeated exposures to pain during sexual activity to better understand the effect of learning on sexual motivation in the presence of pain. An exploration of the impact of other aversive experimental manipulations that model acute illness or visceral pain, such as a lithium chloride model, may expand our understanding of the generalizability of our findings in acute, somatic pain. Furthermore, this model shows promise in evaluating libido-enhancing drugs that require baseline depressions of sexual motivation to demonstrate their therapeutic effects, as seen with apomorphine. Strain comparisons may also reveal genetic differences

in female susceptibility to pain-induced reductions in sexual desire, with the potential of testing knockout mice to directly examine the role of genes in the pain-sexual motivation interaction.

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# Appendix

(Reprint of Publications)

30

# CHAPTER 30
# Animal Models of Dyspareunia

*Melissa A. Farmer, Yitzchak M. Binik, Jeffrey S. Mogil*
McGill University Health Centre, Montréal, Québec, Canada

## Introduction

In recent years, the development and application of new animal models of disease processes has been a popular scientific trend [1]. However, few of these models have focused on sexuality, and fewer still have modeled pain conditions that impact sexuality. Urogenital and abdominal pain conditions associated with dyspareunia impact a staggering percentage of women, yet very few of these conditions are well understood. Although imaging studies have greatly advanced human research in this area [2], experimental options using human subjects are still limited. Animal models allow experimental manipulations to evaluate the causal relationships between pathological causes and physiological effects. These models are convenient and cost-effective, and they permit the testing of hypotheses that are otherwise ethically implausible in humans. The development of viable animal models for conditions that are associated with painful intercourse, such as endometriosis, interstitial cystitis (IC), irritable bowel syndrome (IBS), and provoked vestibulodynia (PVD) might have profound implications for our understanding of the etiology, maintenance, and treatment of these debilitating conditions.

## Evaluation of Animal Models of Pain

Pain in animals is defined as "an aversive sensory experience caused by actual or potential injury that elicits progressive motor and vegetative reactions, results in learned avoidance behavior, and may modify species-specific behavior, including social behavior" [3]. Animals cannot verbally rate their pain intensity, quality, or location, nor can they communicate the impact of emotion on pain. Instead, researchers infer the presence of pain from abnormal behaviors that are (hopefully) unique to the experimentally induced nociceptive state. The difficulty in measuring an animal's emotional or cognitive responses to pain suggests that we are largely using *nociceptive models*, rather than true pain models [4]. However, just because we can't measure something doesn't mean it isn't there. Nevertheless, the word *pain* will be used throughout this chapter.

Pain can be typified as spontaneous or provoked, depending on whether or not it is elicited by exogenous stimulation. Many existing animal models of pain are limited in their duration; chronic, spontaneous pain—the most clinically relevant form—has proved particularly difficult to model in animals [5]. Pain conditions can also be visceral or somatic in nature. Visceral pain originates from the internal organs contained within the chest and abdomen, and it is characterized by increased autonomic reactivity, emotional salience, and diffuse pain that may be referred to other visceral or somatic tissue that shares common innervation at the level of the spinal cord [6]. Referred pain is perceived in areas distal from the site of injury that receives common spinal input as the region where pain originates [7, 8]. The majority of animal models of pain conditions associated with dyspareunia are visceral in nature, including uterine inflammation, vaginal and uterine distension, endometriosis, and abdominal pain (including cystitis and colitis).

In animal models, behavioral responses may reflect the location of the pain in the case of somatic tissue (e.g., withdrawal of a heated hind paw), whereas visceral pain

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may be manifested as referred somatic pain [7]. Patterns of pain behavior can increase in frequency or magnitude with higher levels of noxious stimulation. Abnormal behaviors that show temporal correspondence with tissue injury or inflammation are thought to reflect injury-specific pain, although the correlation may not be strong. For visceral pain in particular, the *absence* of behavior may be indicative of pain, as evidenced by reduced mobility or motivation to engage in normal activity [9]. In addition, estrous cyclicity may significantly impact some behaviors [10], but many indices of behavioral pain show equal variability when male versus female animals are used [11]. Empirical validation that a behavior is specific to pain is often achieved via the administration of known analgesics, such as nonsteroidal anti-inflammatory drugs, lidocaine, or morphine.

Ultimately, many behaviors have been associated with pain in rodents. Table 30.1 lists behaviors that have been linked to animal models of dyspareunia. Notably, the criteria for dyspareunia vary between models. Whereas some models directly measure vaginal sensitivity to noxious stimuli, other models induce pain conditions associated with dyspareunia. Ideally, pain behaviors are unique to an experimental manipulation, easily quantifiable with minimal need for interpretation, frequent enough to allow for statistical comparisons between groups, and reliably observed in afflicted animals (and rare in healthy animals). Such behavior should coincide with the duration and severity of injury and be mitigated by analgesics in a dose-dependent manner. Most importantly, the validity of an animal model of pain relies on whether the researchers have accurately identified a pain behavior that closely parallels the clinical characteristics of the condition it is intended to model. This chapter will be limited to reviewing rodent models of female urogenital and abdominal pain that include the measurement of pain *behavior*, not electrophysiological or electromyographic proxies, as a primary outcome measure [4].

## Animal Models of Dyspareunia

### Ureteral Calculosis

Women with dysmenorrhea, or painful menstruation, often report dyspareunia and are more likely to experience urinary calculosis (kidney stones). Based on this comorbidity, animal models of ureteral calculosis (UC) may indirectly induce dyspareunia, although this link has never been formally tested.

The first detailed behavioral characterization of UC-induced visceral pain was conducted by Giamberardino's laboratory [12]. Within a day of implantation of an artificial stone into the left ureter, rats displayed a variety of spontaneous pain behaviors including stretching, hunched back, abdominal/flank licking, flank muscle contractions accompanied by ipsilateral inward hindlimb motions, lower abdominal squashing (against the floor), and the adoption of a supine position with the left hindlimb retracted into the abdomen. These behaviors slowly decreased in frequency and duration over four days postimplantation. Rats with frequent visceral pain behaviors were more likely to vocalize to electrical stimulation of the ipsilateral oblique muscles, indicative of referred pain. These behaviors are similar to the protracted abdominal stretching observed in early visceral pain models [13, 14]. Pain behaviors were reduced with intraperitoneal 5 mg/kg/day morphine. Giamberardino's model established a typology for abnormal pain behaviors associated with visceral pain that would be replicated or modified by the majority of subsequent abdominal visceral pain models.

Based on preliminary human evidence linking dysmenorrhea, endometriosis, and UC [15], Giamberardino and colleagues [16] developed a dual rat model of endometriosis with UC to investigate whether abdominal pain behaviors found in either condition are enhanced by the comorbidity. Animals with endometrial autografts plus stone implantations showed significantly longer bouts of pain behavior compared to stone implantation only or sham groups. Although all animals developed some referred hyperalgesia caused by the presence of a ureteral stone, the endometriosis + UC group displayed the greatest magnitude of referred pain as indicated by reduced vocalization threshold in response to electrical stimulation of the left oblique muscles.

### Uterine Inflammation

Wesselmann and colleagues [17] characterized pain behavior associated with uterine inflammation in the rat. The pain behaviors they examined were based on the Giamberardino model of UC [12]. To induce inflammation, 10% mustard oil and a mineral oil vehicle were injected into the uterine lumen, and pain behaviors were videotaped for seven days postsurgery. Of animals receiving

**Table 30.1** Common pain behaviors used in animal models of dyspareunia.

| Pain Behaviors | Type of Pain | Condition Modeled | References |
|---|---|---|---|
| Pushing abdomen against floor ("stretch-flat" position) | Spontaneous, visceral | Ureteral calculosis, endometriosis, uterine inflammation, colitis, parturition | 12, 16, 17, 41, 49 |
| Lifting abdomen off floor | Spontaneous, visceral | Colitis | 42–43 |
| Sharp back hunch ("lambda" position) | Spontaneous, visceral | Ureteral calculosis, endometriosis, uterine inflammation | 12, 16–17 |
| Abdomen pressed against floor with nose facing toward tail of afflicted side ("alpha" position) | Spontaneous, visceral | Ureteral calculosis, endometriosis, uterine inflammation | 12, 16–17 |
| Lower abdomen pressed against floor while standing/sitting ("squash-pelvic" position) | Spontaneous, visceral | Ureteral calculosis, endometriosis, uterine inflammation, parturition | 12, 16–17, 49 |
| Stretching (back arched) | Spontaneous, visceral, mechanical distension | Ureteral calculosis, uterine inflammation, uterine distension, cystitis, colitis | 12, 17–18, 32, 41-43 |
| Experimenter observed abdominal contractions | Spontaneous, visceral | Cystitis, colitis | 32–33, 41–44 |
| Hunched posture | Spontaneous, visceral, mechanical distension | Ureteral calculosis, uterine inflammation, uterine distension, cystitis, parturition | 12, 17–18, 31, 33, 35–36, 49 |
| Inward turning of hindlimb | Spontaneous, visceral | Ureteral calculosis, uterine inflammation, parturition | 12, 17, 49 |
| Jumping or retreating from palpation/pressure | Provoked, mechanical or thermal or electrical, referred | Ureteral calculosis, referred hypersensitivity from: uterine inflammation, cystitis, colitis, ovariectomy, YIST model | 12, 17, 34–35, 37, 41, 43–44, 46–47 |
| Operant response | Provoked mechanical distension, spontaneous, referred | Vaginal and uterine distension, endometriosis, ovariectomy | 18, 20–21, 24–25, 29 |
| Licking afflicted area | Spontaneous or provoked | Ureteral calculosis, uterine inflammation, cystitis, colitis, parturition | 12, 17, 31–34, 41, 49 |
| Writhing | Spontaneous, visceral, tonic | | 13–14 |
| Reduced physical activity | Spontaneous | Cystitis, uterine inflammation, colitis | 7, 17, 32, 41–42 |
| Vocalization | Spontaneous or provoked | Ureteral calculosis + endometriosis, uterine inflammation, uterine distension | 12, 16–18 |
| Piloerection | Spontaneous | Cystitis (rat model only) | 31–35 |
| Abnormal defecation/urination | Spontaneous or provoked | Colitis | 43 |
| Facial expression (eye squint, blink) | Spontaneous | Cystitis | 36 |

uterine inflammation, 79% displayed prolonged periods of spontaneous pain behavior, with behavior frequency peaking two days after surgery. Dramatic individual differences were found in the frequency and duration of pain behaviors, and animals with uterine inflammation showed reductions in overall mobility. Of animals displaying spontaneous pain behavior, 66% also showed referred muscle hypersensitivity in the lower back and flanks that actually outlasted the occurrence of spontaneous pain behaviors.

Wesselmann's study was especially significant in that it established that pain from distinct viscera—the ureter and the uterus—resulted in very similar behaviors, including behavioral evidence of referred pain. Although this behavioral similarity may support the validity of these behaviors as being specific to pain, it also indicates that the behaviors are not specific enough to distinguish between visceral pains of different origins. The poor localization of visceral pain, however, makes it very unlikely that different visceral pains would be manifested in unique behavioral patterns.

## Vaginal and Uterine Distension

Berkley and colleagues [18] established one of the earliest rat models of reproductive tract pain using vaginal and uterine distension. The elegance of this model relies on the novel operant task devised by the authors, which required rats to learn that a discrete behavioral response (extending the nose to interrupt a photocell circuit) would terminate an aversive stimulus (vaginal or uterine mechanical distension with a latex balloon). The authors argued that the rats' motivation to perform the escape behavior in response to high levels of distension indicated that intense mechanical distension constituted an aversive stimulus to the rats. The intense level of stimulation employed by this model is in contrast to innocuous levels of vaginal stimulation, which have positively reinforcing and analgesic properties in rodents [19].

Berkley et al. [18] validated this behavioral pain model in adult virgin female rats with low levels of ovarian hormones (i.e., Metestrus), to control for the potentially confounding effects of estrous cycle hormone fluctuations. Rats reliably escaped distension with increasing speed and frequency as the vaginal distension volume increased, and this response pattern held throughout the estrous cycle [20]. The rats' ability to detect and escape from uterine distension, however, was less predictable—many rats produced operant responses during control trials when distension volumes were minimal, and a large minority of animals did not show behavioral discomfort with maximum levels of uterine distension. The authors noted that rats often responded to uterine distension with stretching behavior.

Interestingly, escape behaviors increased in response to higher vaginal and uterine pressures when estrogen levels were low during Metestrus and Diestrus [20]. Similarly, ovariectomy (OVX) also induced moderate to high levels of vaginal hyperalgesia that were promptly reversed with 17β-estradiol replacement [21]. This pattern of estrogen-dependent vaginal sensitivity has adaptive reproductive significance. The increased tolerance to vaginal pressure, such as that induced by penile penetration, would be functionally important during the height of sexual activity in late proestrus, after estrogen and progesterone levels have peaked.

The development of this model exemplifies the successes and hazards of validating reliable behavioral correlates of pain. The authors succeeded in identifying a reliable pattern of behaviors for vaginal distension, yet uterine distension pain proved more difficult to characterize. Escape responding during uterine distension correlated with a prominent visceral pain behavior, which lends support to the aversive quality of the distension stimulus. One strength of this model is that it relies on an organized motor response that requires cerebral processing, which is thought to more accurately reflect the sensory perception of pain compared to simple reflex responses [4].

## Endometriosis

Endometriosis is a painful condition defined by dysmenorrhea, dyspareunia, infertility, and chronic abdominal and low back pain [22]. To induce endometriosis, a segment of uterine horn is removed (i.e., hysterectomy), and pieces of endometrial tissue from the uterine horn (or fat for sham-operated controls) are autotransplanted onto blood vessels in the left ovary, the internal lower abdominal wall, or the cascade mesenteric arteries. Cysts rapidly develop at uterine transplant sites. The endometriosis rat model shares important similarities with endometriosis in women, including pelvic pain, infertility, in vitro and in vivo tissue and cell properties, and treatment responses [23].

Berkley and colleagues [24] combined the distension-induced pain model with the endometriosis rat model.

Animals subjected to the endometriosis surgery showed a significant increase in escape behavior in response to vaginal distension compared to baseline, whereas sham-operated animals without cysts showed no change in behavior. The findings of increased hypersensitivity to vaginal distension in rats with endometriosis have immense clinical relevance given the comorbidity of endometriosis and dyspareunia [22].

In a follow-up study, Cason and colleagues [25] found time- and estrous cycle–dependent changes in distension-induced vaginal hypersensitivity following endometriosis surgery. When postsurgical data from all stages of the estrous cycle were pooled together, the rate of escape responding to vaginal distension steadily increased for eight weeks in proportion to the growth of endometrial cysts. When specific stages of the estrous cycle were examined, rats with endometriosis increased escape responding from vaginal distension during Metestrus, Diestrus, and Proestrus (but not Estrus).

This finding is interesting for two reasons: First, the robust impact of endometriosis on vaginal sensitivity is fully reversed for about a day during the estrous cycle; second, this effect appears to be independent of normal patterns of vaginal hypersensitivity wherein moderate and high levels of estrogen during estrus and proestrus enhance tolerance to vaginal pressure [20]. The difference may be that nonpathological fluctuations in vaginal sensitivity are due to the direct effects of estrogen on vaginal tissue [26–27], whereas the pathological mechanisms underlying endometriosis-induced vaginal hyperalgesia may become centrally mediated [28]. Even a profound drop in ovarian hormones due to OVX does not change endometriosis-induced vaginal hyperalgesia [29], suggesting that either a reduction in estrogen levels does not alter the mechanisms underlying the hyperalgesia or that the capacity for both endometriosis plus OVX to produce hyperalgesia is not additive. Estrogen replacement following endometriosis plus OVX reverses the vaginal hypersensitivity, and this reversal may in part be due to central effects of estrogen [29].

## Interstitial Cystitis

Interstitial cystitis (IC) is highly comorbid with dyspareunia and may be accompanied by a burning or aching vaginal pain [30]. Animal models of cystitis use a variety of irritants to induce bladder inflammation, including cyclophosphamide (an antitumor agent), turpentine, and even bacteria.

The cystitis-induced visceral pain model was first developed in the rat [31] and then in the mouse [9, 32]. Following cystitis induction, spontaneous pain behaviors progressively increased in frequency and were correlated with increasing severity of bladder inflammation [31–33]. Cystitis pain behaviors may be more pronounced in the rat compared to the mouse, with the former exhibiting spontaneous abnormal behaviors (i.e., hunched posture, abdominal licking and contractions, reduced locomotion) and the latter exhibiting a general reduction in physical activity [9, 31, 32], although one study found comparable hunching behaviors in the mouse [34]. Rat and mouse models show that cystitis produces referred pain to other areas receiving common innervation, such as the tail, hindpaw, and abdomen [9, 34–36]. In both species, cystitis-induced referred mechanical and thermal hypersensitivity were dose-dependently reduced with morphine [9, 34, 35]. The development of cystitis-induced behaviors does not vary across estrous stages, but interestingly, the onset of pain behaviors progresses more rapidly in female compared to male rats [33].

A model of bacteria-induced cystitis demonstrated that mice showed reduced hindpaw-withdrawal latencies to noxious radiant heat for 14 days following *Escherichia coli* administration, whereas otherwise genetically similar mice but with deficient Toll-like receptor 4 (TLR-4) function failed to show this thermal hypersensitivity [37]. Toll-like receptors are part of the innate immune defence against foreign pathogens, and TLR-4 recognizes bacterial wall components, contributing to nuclear factor-kappa B (NF-$\kappa$B) activation and subsequent increases in proinflammatory cytokine expression [38]. Central TLR-4 has also been implicated in behavioral hypersensitivity to neuropathic pain [39, 40]. These findings indicate that a bacterium is a sufficient inflammatory irritant to induce experimental, TLR-4-dependent cystitis.

## Colitis

In order to model functional abdominal pain like IBS, an animal model of visceral pain from colitis was developed by the Cervero laboratory which measured behavioral responses to colonic irritation from capsaicin and mustard oil [41]. Colonic irritation rapidly and dose-dependently produced abdominal pain behaviors

(i.e., abdominal licking and hunching postures), as well as increased mechanical sensitivity on abdominal, tail, and hindpaw tissues indicative of referred pain. Abdominal pain behaviors were dose-dependently reduced by morphine. Similar models that correlated colonic irritation with increased acute and chronic abdominal pain behaviors showed no apparent structural damage to colonic mucosa [42, 43]. Furthermore, a minority of animals (about one-quarter) may develop chronic mechanical and thermal hypersensitivity lasting up to 16 weeks after severe colitis, indicating the presence of referred pain long after colitis-associated inflammation has resolved [44]. Due to the production of abdominal pain without detectable colonic pathology, these animal models are thought to mimic the clinically important characteristics of IBS, including visceral hypersensitivity and referred somatic pain [45].

Estrogen levels may play an important role in visceral pain. Sanoja and Cervero [46, 47] demonstrated that OVX mice developed robust mechanical, thermal, and visceral allodynia and hyperalgesia in abdominal, hindpaw, and proximal tail skin within a month of OVX surgery. Compared to control groups, the OVX group showed significantly greater numbers of referred visceral pain behaviors following intracolonic capsaicin (including abdominal licking, stretching, squashing, and retractions). This shift in pain sensitivity was reversed with 17β-estradiol replacement. A potential mechanism for this model involves serotonin, which is implicated in the descending inhibitory modulation of pain [48].

## Parturition

One of the most commonly encountered forms of visceral pain occurs during labor, when the lower uterus and cervix are stretched and sometimes even torn to permit passage of the offspring. A rat model of parturition pain found that pain behaviors observed in the 1.5 hr preceding birth are similar to behaviors outlined in other animal models of visceral pain [49]. Rats in labor displayed frequent abdominal straining and squashing and an inward turning of the hindpaw, and the rate of these behaviors increased proportionately with labor duration. Systemic oxytocin (10 μg/kg) reduced the labor duration and increased the rate of pain behaviors, which were reduced with epidural morphine (30 ug/10 μL).

## Yeast-Induced Sensitization to Touch (YIST) Model

Provoked vulvar pain—involving somatic tissue—is the most common cause of dyspareunia, and yet the majority of existing animal models of pain conditions that cause dyspareunia in women are visceral. We have developed a method of testing vulvar mechanical sensitivity in order to evaluate a mouse model of PVD. The testing method is an adaptation of the classic von Frey [50] psychophysical test and involves stimulation of mouse posterior vulvar tissue, located ventrally from the anogenital ridge, with calibrated nylon monofilaments (0.009–2.0 g). Mice display varying intensities of behavior in response to vulvar stimulation, including sniffing or licking of the vulva, body repositioning, or jumps. Because a rapid, full jump (all four paws off the ground) was the behavior most reliably elicited (albeit at high levels of applied force), we adopted this behavior as the criterion for an aversive response to mechanical stimulation.

Based on multiple reports that women with PVD are significantly more likely to have experienced recurrent vulvovaginal candidiasis (RVVC) [51–53], we developed a mouse model of provoked vulvar pain following three successive vulvovaginal infections with *Candida albicans.* For each infection, mice were vaginally inoculated with yeast, and four days following inoculation the infections were verified and eliminated with seven days of oral fluconazole. Following three weeks of consistently negative cultures from vaginal lavage fluid, mechanical sensitivity measurements were taken and compared to baseline measurements. After three yeast infections, significant differences in vulvar mechanical sensitivity were found between RVVC mice exposed to vulvovaginal yeast compared to fluconazole and saline controls. No changes in hindpaw mechanical sensitivity were found, indicating that increases in sensitivity were specific to the vulvar tissue exposed to yeast. We are hopeful that our model may allow an improved understanding of the mechanisms underlying provoked vulvar pain, as well as the development of novel treatments for clinical use.

## Validity of Animal Models of Dyspareunia

As outlined in Table 30.2, the animal models we have reviewed do not meet the proposed criteria for

**Table 30.2** Evaluation of the validity of behavioral outcome measures presented by model.

| Pain Conditions | Behavior Specific to Condition? | Reliable | Frequently Observed? | Behavior Time Course | Related to Pathology? | Reversible with Analgesics? | Human Condition Modeled? |
|---|---|---|---|---|---|---|---|
| Ureteral calculosis | No | Yes | Varies between individuals | Onset in 1st day, reduces within 4 days | Unknown | Yes | Urinary calculosis (kidney stones) |
| Uterine inflammation | No | Yes | Varies between individuals | Onset in 2–4 days of mustard oil | No | N/A | Various uterine pathologies |
| Vaginal distension | Yes | Yes | Yes | Within sec of noxious distension | Yes, in case of endometriosis | N/A | Vaginal dyspareunia |
| Uterine distension | Sometimes | No | Varies | Within sec of noxious distension | Unknown | N/A | Unknown |
| Endometriosis | No | Yes | Yes | Abnormal sensitivity by 1–2 mo | Yes, behavior correlates with cyst growth | Yes | Endometriosis |
| Cystitis | No | Yes | Yes | Gradually increases in 1–4 hrs | Yes, behavior correlates with bladder inflammation | Yes | Cyclophosphamide-induced cystitis |
| Colitis | No | Yes | Yes | Onset within 1 hr, several days referred pain | No | Yes | Irritable bowel syndrome |
| Parturition | No | Yes | Yes | Onset 1.5 hr before birth | N/A | Yes | Labor pain |
| YIST model | Yes | Yes | Yes | Following three infections | Unknown | N/A | Yeast infection–induced provoked vulvar pain |

accurately modeling clinical symptoms of dyspareunia and its associated disorders, as the visceral pain behaviors used in most models are not specific to any particular pain stimulus. Only vaginal distension and vulvar mechanical sensitivity behaviors are unique to a stimulus. Most models produce reliable and frequent pain behaviors, although much individual variation may exist [12, 17, 18].

Pain models vary from acute onset [18] to tonic inflammatory [17, 32] and chronic referred pain [44]. A correlation between behavior and physical pathology is largely absent, with the exception of endometriosis and cystitis models [24, 25, 31–33], and most models are reversible with known analgesics. Each of these models requires substantial development, including a refinement of pain behavior patterns, improved understanding of corresponding physiological pathology, and identification of clinically relevant symptoms specific to dyspareunia.

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PAIN

# Repeated Vulvovaginal Fungal Infections Cause Persistent Pain in a Mouse Model of Vulvodynia

Melissa A. Farmer,[1,2] Anna M. Taylor,[2,3] Andrea L. Bailey,[1,2] Alexander H. Tuttle,[1,2] Leigh C. MacIntyre,[1] Zarah E. Milagrosa,[1] Halley P. Crissman,[1] Gary J. Bennett,[2,4] Alfredo Ribeiro-da-Silva,[2,3] Yitzchak M. Binik,[1,2] Jeffrey S. Mogil[1,2]*

**Provoked vestibulodynia, the most common form of vulvodynia (unexplained pain of the vulva), is a prevalent, idiopathic pain disorder associated with a history of recurrent candidiasis (yeast infections). It is characterized by vulvar allodynia (painful hypersensitivity to touch) and hyperinnervation. We tested whether repeated, localized exposure of the vulva to a common fungal pathogen can lead to the development of chronic pain. A subset of female mice subjected to recurrent *Candida albicans* infection developed mechanical allodynia localized to the vulva. The mice with allodynia also exhibited hyperinnervation with peptidergic nociceptor and sympathetic fibers (as indicated by increased protein gene product 9.5, calcitonin gene–related peptide, and vesicular monoamine transporter 2 immunoreactivity in the vaginal epithelium). Long-lasting behavioral allodynia in a subset of mice was also observed after a single, extended *Candida* infection, as well as after repeated vulvar (but not hind paw) inflammation induced with zymosan, a mixture of fungal antigens. The hypersensitivity and hyperinnervation were both present at least 3 weeks after the resolution of infection and inflammation. Our data show that infection can cause persistent pain long after its resolution and that recurrent yeast infection replicates important features of human provoked vulvodynia in the mouse.**

## INTRODUCTION

Pain is a cardinal feature of the inflammatory response to fungal, bacterial, and viral infections. In most cases, pain rapidly disappears with the resolution of the infection. Although acute pain is effectively managed with currently available analgesic strategies, chronic pain remains poorly treated. Pain secondary to previous and resolved infection is suspected to underlie numerous idiopathic chronic pain conditions, including urogenital pain (vulvodynia, endometriosis, and prostatitis), interstitial cystitis, and inflammatory bowel syndrome [for example, (*1*)], but a causal relationship between infection and persistent pain has not been demonstrated. To test whether such a causal relationship can exist, we developed a mouse model of provoked vestibulodynia (PVD), a chronic urogenital pain condition suspected to result from repeated infection by a common pathogen, the yeast *Candida albicans*.

Of the idiopathic pain conditions associated with a history of previous infection, vulvodynia (vulvar pain) is the most prevalent, affecting 9 to 12% of women of childbearing age (*2*). The predominant form of vulvodynia, PVD (previously known as vulvar vestibulitis), is characterized by burning and cutting pain localized to the vulvar vestibule in response to light touch (vulvar mechanical allodynia), with physical findings limited to occasional erythema (*3*). Chronic vulvar pain is associated with significant psychological distress because of its interference with sexual intercourse and nonsexual activities (bike riding, walking, and even standing); as a result, mood disturbances and reduced quality of life are often reported in this population (*4*). Reduced vulvar tactile and pain thresholds in PVD have been experimentally confirmed with standardized mechanical stimuli (*5*). Histological changes in vulvar vestibule tissue, including increased density of free nerve endings (*6*, *7*), suggest that neural mechanisms may underlie these clinical symptoms. Brain imaging of patients with PVD reveals patterns of activity similar to those observed in experimental and clinical pain, as well as neuroanatomical abnormalities suggestive of compensatory central reorganization secondary to chronic pain (*8*).

No definitive causes of PVD have yet been identified. However, women with PVD have a high prevalence of recurrent vulvovaginal candidiasis (RVVC; or recurrent yeast infections), defined as three or more yeast infections annually, compared to healthy women: 42 to 60% (*9*, *10*) versus 5 to 8%, respectively (*11*). The commensal yeast, *C. albicans*, is thought to cause 85 to 90% of all yeast infections in women (*11*). The comorbidity between RVVC and chronic vulvar pain has led to the hypothesis that vulvar hypersensitivity in PVD results from abnormal sensory processing secondary to past inflammation from prolonged and/or repeated vaginal yeast colonization. This correlational hypothesis remains untested. Here, we assess whether persistent vulvar mechanical hypersensitivity can develop in mice after multiple rounds of vulvovaginal infection or after a single, long-lasting infection with *C. albicans*.

## RESULTS

### RVVC can cause vulvar allodynia

Mice were tested for baseline mechanical sensitivity of the vulva and hind paws with von Frey filaments. For each round of infection with *C. albicans* strain SC5314 cells, mice were tested again at 4 days (active infection), 11 days (treated infection), and 32 days (3 weeks after infection resolution) after inoculation (Fig. 1A). Quantification of

[1]Department of Psychology, McGill University, 1205 Doctor Penfield Avenue, Montreal, Quebec H3A 1B1, Canada. [2]Louise and Alan Edwards Centre for Pain Research, McGill University, 740 Doctor Penfield Avenue, Montreal, Quebec H3G 1A4, Canada. [3]Department of Pharmacology and Therapeutics, McGill University, 3655 Promenade Sir-William-Osler, Montreal, Quebec H3A 1Y6, Canada. [4]Department of Anesthesia, McGill University, Montreal, Quebec H3A 1Y6, Canada.
*To whom correspondence should be addressed. E-mail: jeffrey.mogil@mcgill.ca

vaginal fungal burden confirmed infection status at each time point (fig. S1A). At baseline, most mice (79%) exhibited vulvar mechanical withdrawal thresholds exceeding 4.0 g, with no between-group differences ($\chi^2$ = 5.0, $P$ = 0.17). This was true at every testing session for mice in the fluconazole control group (Saline + FLU; Fig. 1B). By contrast, the group of mice with repeated infections (*Candida* + FLU; Fig. 1C) was allodynic during active SC5314 infection (day 4) and was still allodynic after treatment (day 11). By day 32, when the infection was long resolved, evidence of allodynia was absent after the first two rounds of infection. However, after the third round of infection, persistent allodynia was present ($\chi^2$ = 9.5, $P$ = 0.02). We obtained similar results when we applied a within-subjects analysis, defining allodynic mice as those displaying a ≥33% decrease in vulvar threshold from their own baseline (Fig. 1D). On day 32 after the third round of infection, 40% (6 of 15) of the infected subjects were allodynic by this definition compared to 5.5% (1 of 18) of the fluconazole control subjects

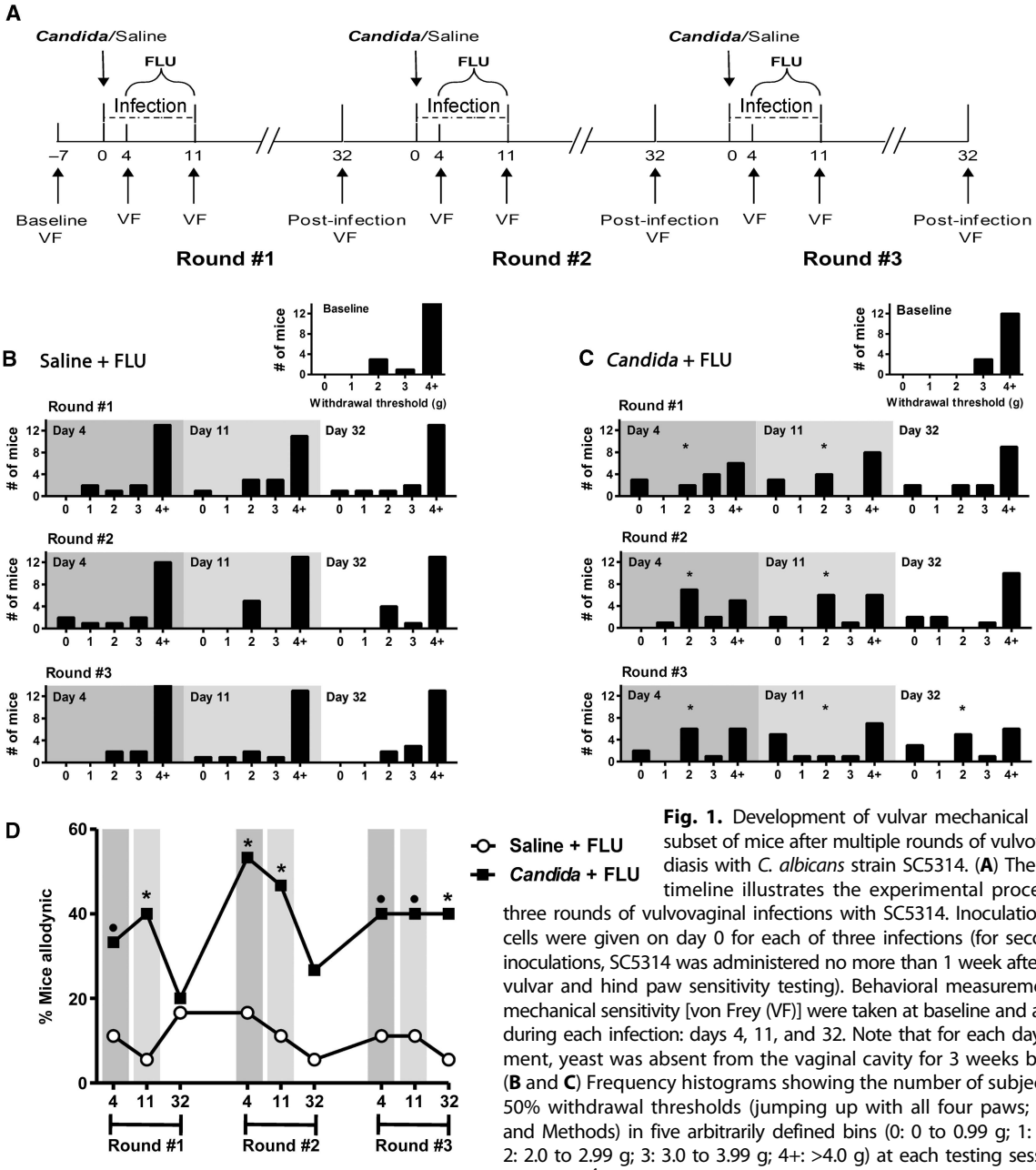


**Fig. 1.** Development of vulvar mechanical allodynia in a subset of mice after multiple rounds of vulvovaginal candidiasis with *C. albicans* strain SC5314. (**A**) The experimental timeline illustrates the experimental procedures across three rounds of vulvovaginal infections with SC5314. Inoculations of $5 \times 10^4$ cells were given on day 0 for each of three infections (for second and third inoculations, SC5314 was administered no more than 1 week after the previous vulvar and hind paw sensitivity testing). Behavioral measurements of vulvar mechanical sensitivity [von Frey (VF)] were taken at baseline and at three points during each infection: days 4, 11, and 32. Note that for each day 32 measurement, yeast was absent from the vaginal cavity for 3 weeks before testing. (**B** and **C**) Frequency histograms showing the number of subjects displaying 50% withdrawal thresholds (jumping up with all four paws; see Materials and Methods) in five arbitrarily defined bins (0: 0 to 0.99 g; 1: 1.0 to 1.99 g; 2: 2.0 to 2.99 g; 3: 3.0 to 3.99 g; 4+: >4.0 g) at each testing session. Infection status is indicated by shading. Mice were inoculated vaginally with saline (B) or $5 \times 10^4$ SC5314 cells (C) on day 0 of each infection round ($n$ = 15 to 18 per group); all mice received fluconazole (FLU; 15 mg/kg, orally, once daily) from day 4 to day 11. *$P$ < 0.05 by $\chi^2$ analysis compared to within-group baseline. (**D**) Mice displaying ≥33% decreases in withdrawal threshold compared to their own baseline at each testing session. *$P$ < 0.05 compared to Saline + FLU group by one-tailed Fisher's exact test; •$P$ < 0.10 compared to Saline + FLU group.

($P = 0.02$, one-tailed Fisher's exact test). This allowed the separation of mice in the *Candida* + FLU group into allodynic and nonallodynic subgroups, from which tissues were obtained for immunohistochemical studies (see below). There were no statistically significant alterations in mechanical sensitivity of the hind paw produced by fluconazole or strain SC5314 over the course of the three rounds of infection (fig. S2).

### RVVC does not have morphological or inflammatory effects

Visual inspection of hematoxylin and eosin (H&E)–stained sections obtained after the third infection revealed no edema and no obvious intergroup differences in inflammatory infiltrate (fig. S3). The presence of a small number of immune cells is typical of healthy vaginae, and a few basophils, macrophages, and mast cells were evident throughout the lamina propria and along blood vessel walls in all groups. Inflammatory cells did not penetrate the epithelial layer. No evidence of altered vulvar epithelial morphology was found in Saline + FLU or in *Candida* + FLU mice (allodynic or nonallodynic) after the resolution of the third infection (fig. S3). Epithelial thickness at the broad ($F_{3,19} = 1.4$, $P = 0.29$) and narrow ($F_{3,19} = 0.8$, $P = 0.50$) aspects of the epithelium did not differ between groups, and in all cases, the keratin layer was intact across the posterior surface of the vulva (table S1). Whereas fungal burden covaried with hypersensitivity during acute infection, leukocyte levels showed no such correlation (table S2).

### Only allodynic RVVC mice show increases in vulvar innervation

Immunohistochemical analyses after repeated infection in allodynic ($n = 3$ to 6) mice, after repeated infection in nonallodynic ($n = 5$) mice, and in fluconazole control ($n = 4$) mice were conducted on postmortem vulvar tissue. We observed an almost 300% increase in the density of nerve fibers [as detected by immunoreactivity (IR) for the pan-axonal marker protein gene product 9.5 (PGP 9.5)] throughout the lamina propria of vulvar tissue taken from allodynic compared to nonallodynic and control mice ($F_{2,10} = 12.8$, $P = 0.002$; Fig. 2, A to D). The increased density reflected both increased number of fibers and thicker, longer fibers. A significant, almost 400% increase in the density of peptidergic fibers, as assessed by calcitonin gene–related peptide (CGRP)–IR, was found in allodynic animals compared to the nonallodynic group ($F_{2,14} = 4.6$, $P = 0.03$; Fig. 2, E to H). In all groups, CGRP-IR fibers were observed throughout the lamina propria, but few fibers penetrated the basal cell layer of the epithelium. Allodynic mice displayed increased (more than four times higher) sympathetic innervation, as revealed by vesicular monoamine transporter 2 (VMAT2)–IR, compared to nonallodynic and control groups ($F_{2,12} = 8.0$, $P < 0.01$; Fig. 2, I to L). Sympathetic fibers in all mice were typically distributed in the deeper layers of the lamina propria; in allodynic mice, there was increased fiber density, with some thin processes seen to penetrate the lamina propria beneath the epithelium (Fig. 2K).

### Extended primary fungal infection can cause vulvar allodynia

In a new experiment, mice were tested for vulvar and hind paw mechanical sensitivity throughout a single but extended-duration (14-day) vulvar *C. albicans* strain SC5314 infection (Fig. 3A). Vaginal fungal burden throughout the extended infection is shown in fig. S1B. At baseline, 96% of this cohort exhibited vulvar mechanical withdrawal thresholds exceeding 4.0 g, with no between-group differences ($\chi^2 = 0.7$, $P = 0.41$). The fluconazole control group continued to exhibit unchanged thresholds throughout the experiment (Fig. 3B). In contrast, a significant proportion of extended-infection mice became allodynic after the acute phase of SC5314 infection (day 14; $\chi^2 = 8.1$, $P = 0.005$) (Fig. 3C). This hypersensitivity persisted after completion of antifungal treatment (day 21; $\chi^2 = 5.0$, $P < 0.05$) and 3 weeks after infection resolution (day 42, $\chi^2 = 6.9$, $P < 0.01$). A large proportion of mice (86% exhibiting a >66% reduction from baseline threshold) continued to display allodynic behavior up to day 70, which was 7 weeks after the resolution of the SC5314 infection ($\chi^2 = 14.4$, $P < 0.001$) (Fig. 3C). No alterations in hind paw mechanical sensitivity were observed throughout the experiment (fig. S4).

### Repeated vulvar exposure to zymosan produces vulvar allodynia

To determine whether the persistent vulvar allodynia observed with SC5314 infection required exposure to a live pathogen, we subjected a new cohort of mice to repeated vulvar injections of the yeast cell wall glucan zymosan (or saline, using baseline sensitivity–matched controls). Each mouse in the experimental group received two vulvar injections of zymosan, a week apart; each week thereafter, mice were reinjected only if they recovered to nonallodynic levels of mechanical sensitivity (>66% of baseline thresholds). This design allowed us to assess individual variability in the number of zymosan injections required to produce persistent vulvar allodynia. Figure 4A shows the frequency distribution of the full data set and the number of zymosan injections required to achieve chronic vulvar allodynia in the saline- versus zymosan-treated groups ($\chi^2 = 14.8$, $P < 0.001$). We found considerable individual variation in the number of zymosan injections needed to induce persistent allodynia; data from the first six mice to be tested are shown in Fig. 4B to depict the range of patterns observed. Four hours after the first injection of vulvar zymosan, all mice displayed allodynia; for most mice, this allodynia completely resolved within a week. A single mouse (#1 in Fig. 4B) remained allodynic after the single inflammatory insult. Four hours after the second injection of zymosan, all mice showed robust vulvar allodynia; a week later, all mice remained allodynic. However, with each subsequent week, some mice maintained the allodynic state (#1 and #2 in Fig. 4B), whereas other mice returned to baseline and either required additional injections to achieve persistent allodynia (#2, #3, and #5) or never became persistently allodynic (#4 and #6). A separately performed experiment in which zymosan was injected into the hind paw revealed no evidence whatsoever of persistent allodynia development in any of the mice tested (Fig. 4A, right). In all cases, hind paw hypersensitivity resolved within a week and no chronic allodynia was ever observed even after repeated zymosan injections.

## DISCUSSION

We have observed long-lasting mechanical vulvar hypersensitivity after repeated vulvovaginal infections with the yeast *C. albicans*. A single, 14- to 21-day-long, fully resolved infection with *C. albicans* strain SC5314 also induced mechanical allodynia that greatly outlasted the resolution of active inflammation. Finally, comparably long-lasting allodynia was observed in mice receiving multiple vulvar (but not hind paw) injections of zymosan, a mixture of fungal antigens. In all three

experiments, only a subset of mice developed allodynia. In the RVVC experiment, the allodynic (but not the nonallodynic) mice displayed a significant increase in the density of vulvar nerve fibers, including identified peptidergic sensory and sympathetic fibers. Allodynia was not accompanied by gross morphological changes in the vulvar mucosa (for example, no reduced epithelial thickness or keratinization). Thus, repeated or extended infection with a common pathogen can induce a pathological pain state that persists long after the resolution of the infection.

As expected, we observed vulvar allodynia during and immediately after each active infection. The onset of vulvar allodynia during the acute infection corresponded with the peak of vaginal fungal burden, suggesting that acute inflammation during active infection can account for acute vulvar allodynia. Clinical reports of vulvovaginal pain during yeast infections are consistent with this finding (*11*). However, vulvar allodynia persisted despite reductions in fungal burden (day 11), indicating a dissociation between pain symptoms and fungal load. Despite the differences in vaginal and vulvar epithelium morphology (keratinization, thickness, and hormonal regulation), few differences have been identified in innate and/or adaptive immune responses throughout the lower genital tract (*12*). Innate immunity likely plays a dominant role in the acute response to *C. albicans*, given that changes associated with adaptive immunity are not observed after yeast infections in mice or women (*13*). *C. albicans* is recognized by the Toll-like receptor 2 (TLR-2) and TLR-4, which are expressed on immune and epithelial cells, and engages the innate immune response, including the up-regulation of a

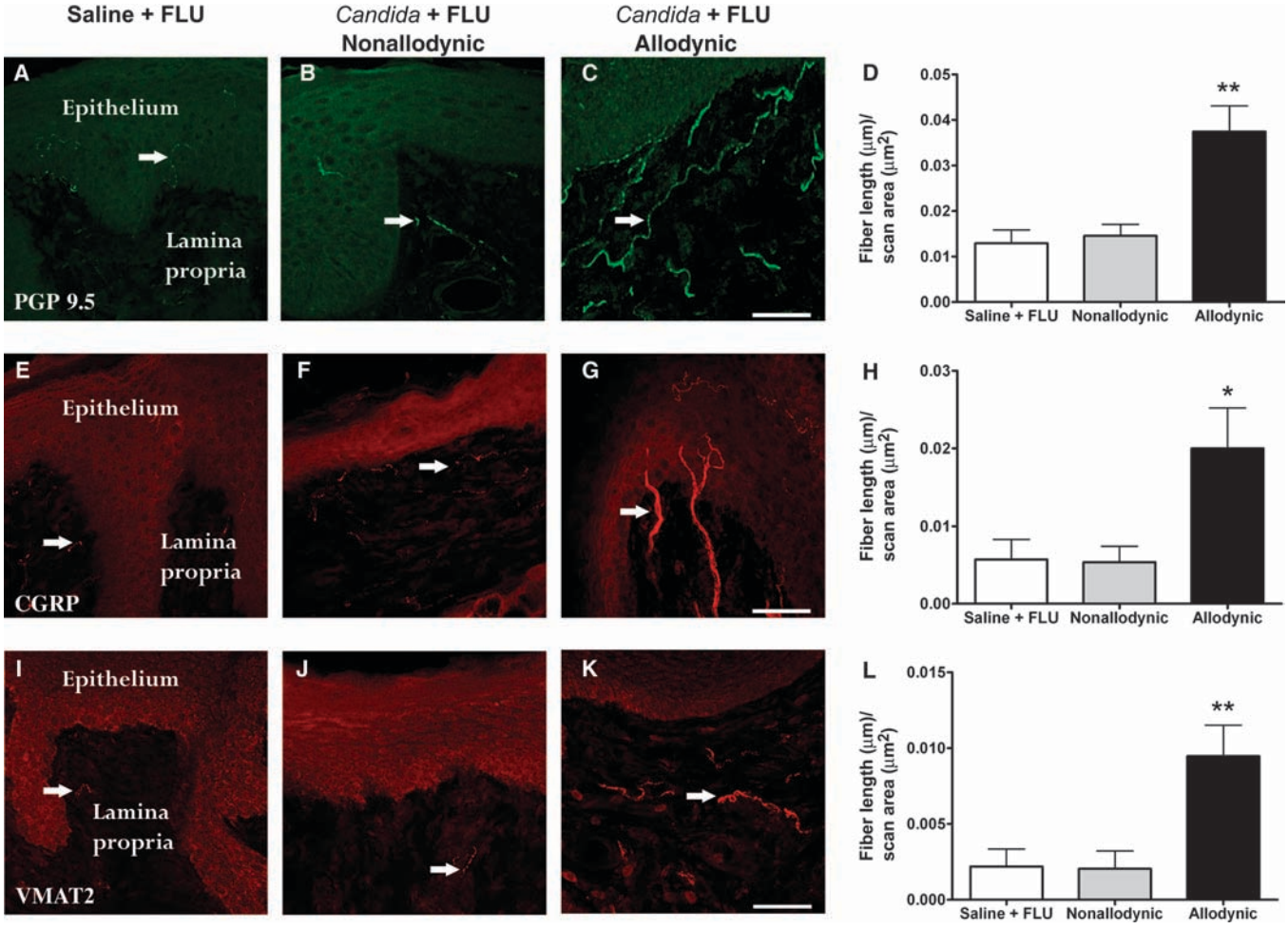


**Fig. 2.** Allodynic mice previously infected with multiple rounds of vulvovaginal candidiasis have increased expression of total, peptidergic, and sympathetic fibers compared to nonallodynic mice and controls ($n$ = 4 to 6 mice per group). (**A**, **E**, and **I**) Saline + FLU group. (**B**, **F**, and **J**) Nonallodynic *Candida* + FLU subgroup. (**C**, **G**, and **K**) Allodynic *Candida* + FLU subgroup. (**D**, **H**, and **L**) Bars represent mean ± SEM fiber length (μm) per unit area (μm$^2$). Total nerve fiber density (top row; PGP 9.5–IR) is significantly increased in the allodynic *Candida* + FLU subgroup, with long fibers lining the lamina propria beneath the epithelium (C and D). Peptidergic nerve fibers immunoreactive for CGRP, which normally consist of fine processes throughout the lamina propria that occasionally penetrate the basal cell layer of the epithelium, are significantly increased in the allodynic *Candida* + FLU group (G and H) and represent about half of the total fiber population (compare $y$ axes of D and H). Sympathetic nerve fibers immunoreactive for VMAT2 sparsely innervate the upper lamina propria in normal and nonallodynic mice, whereas a significant increase in innervation density is observed in the allodynic *Candida* + FLU group (K and L). *$P$ < 0.05; **$P$ < 0.01 compared to all other groups by one-way ANOVA followed by Tukey's post hoc test. Scale bars, 50 μm [(C), (G), and (K)]. Arrows point to fibers.

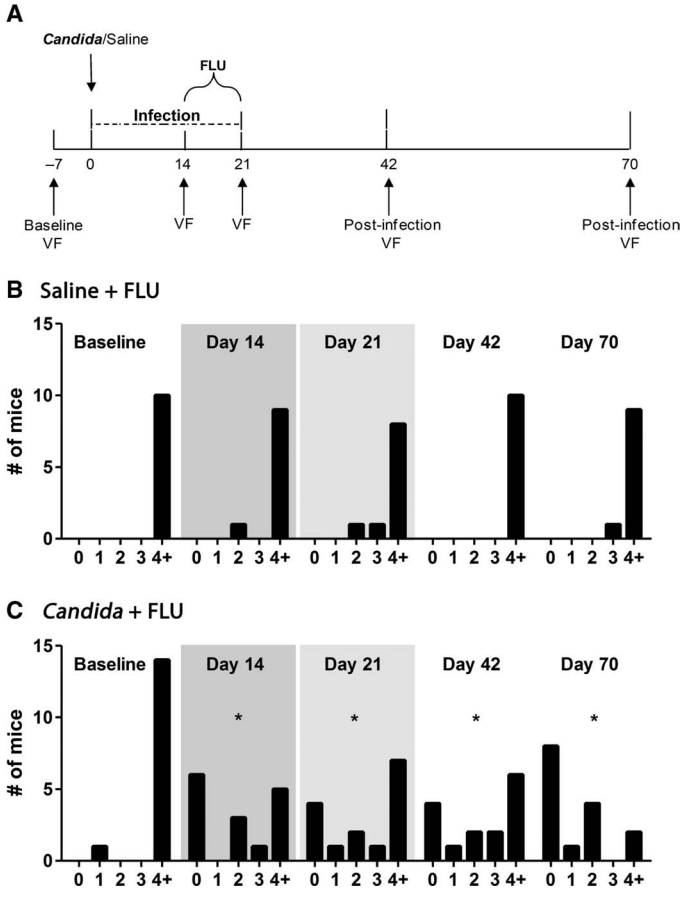


**Fig. 3.** Development of vulvar mechanical allodynia in a subset of mice after a single, extended SC5314 infection. (**A**) Experimental timeline illustrating the experimental procedures. An inoculation of $5 \times 10^4$ SC5314 cells was given on day 0; fluconazole (FLU; 15 mg/kg, orally, once daily) treatment occurred from day 14 to day 21. Behavioral measurements of vulvar mechanical sensitivity [von Frey (VF)] were taken at baseline (day –7) and at 14, 21, 42, and 70 days after inoculation. (**B** and **C**) Frequency histograms showing the number of subjects ($n$ = 10 to 15 per group) in the Saline + FLU (B) and *Candida* + FLU (C) groups displaying 50% withdrawal thresholds in five arbitrarily defined bins (0: 0 to 0.99 g; 1: 1.0 to 1.99 g; 2: 2.0 to 2.99 g; 3: 3.0 to 3.99 g; 4+: >4.0 g) at each testing session. Infection status is indicated by shading. *$P < 0.05$ by $\chi^2$ analysis compared to within-group baseline.

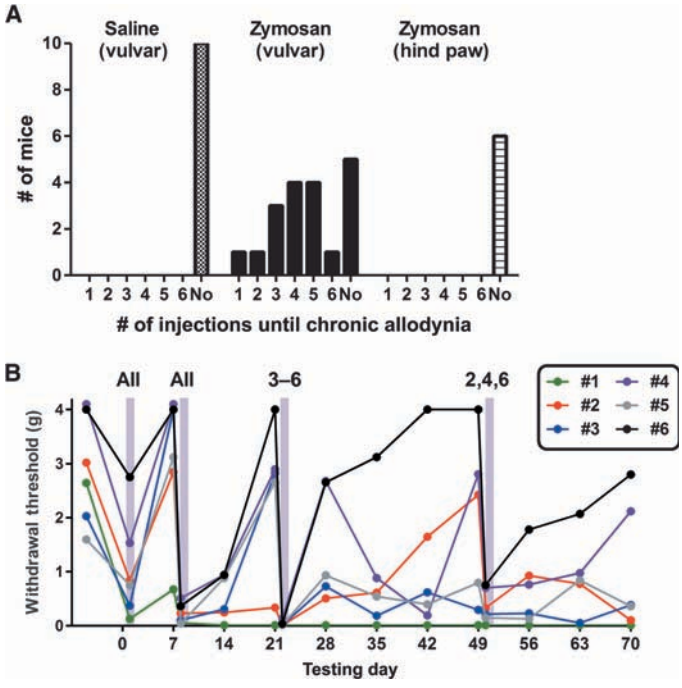


**Fig. 4.** Development of vulvar mechanical allodynia in a subset of mice after repeated vulvar injections of zymosan. (**A**) Frequency histogram showing the proportion of female mice displaying chronic allodynia after one to six weekly (or less) injections of vulvar saline (left; $n$ = 10), vulvar zymosan (middle; $n$ = 19), or hind paw zymosan (right; $n$ = 6). "No" indicates that chronic allodynia was never observed, even after six injections. (**B**) Representative patterns of vulvar mechanical sensitivity over time, using data from the first six mice to be tested (#1 to #6). Vulvar zymosan was injected into mice so indicated 4 hours before the data points highlighted in gray.

yeast-specific pattern of proinflammatory molecules through the nuclear factor κB pathway (*14*). Innate immune cells recruited during acute inflammation (macrophages, mast cells, and neutrophils) can interact directly with nerve endings to produce pain hypersensitivity and release inflammatory mediators that contribute to pain (*15*). The presence of TLR-4 on primary afferent endings (*16*) indicates another potential mechanism by which *C. albicans* activates nociceptors to induce behavioral hypersensitivity (*17*). Similarly, zymosan-induced vulvar allodynia may result from acute inflammation mediated by TLR-2 and TLR-6 and/or the direct sensitization of vulvar mechanoreceptors (*18*).

We have observed here that mechanical hypersensitivity can persist long after the resolution of the active infection. During the first and second rounds of candidiasis in the RVVC experiment, acute vulvar hypersensitivity resolved after antifungal treatment and was absent 21 days after infection resolution (day 32). By contrast, during the third round of infection, the vulvar hypersensitivity observed during the active stages of infection was maintained long after yeast were absent from the vaginal cavity. Moreover, chronic hypersensitivity was also evident after a single, extended infection with *Candida*, and the phenomenon could still be observed up to 7 weeks after infection resolution. Finally, some mice given as few as two zymosan injections exhibited allodynia lasting at least 11 weeks, indicating that the development of long-lasting hypersensitivity does not require a live pathogen and may be generalizable to fungi other than *C. albicans* (that is, *Saccharomyces cerevisiae*). The extended period of allodynia after the disappearance of detectable inflammation suggests that the chronic hypersensitivity that we observed is not inflammatory pain, at least as that term is generally understood. Given our observation of hyperinnervation, it is also problematic to characterize this phenomenon as neuropathic, which requires a neural lesion (*19*).

The fact that only a subset of infected mice developed mechanical allodynia mirrors the clinical situation, because only a minority of women with RVVC develop chronic vulvar pain. Even within the subset of (outbred) mice developing chronic allodynia after zymosan, there was considerable variability in the number of exposures required (see Fig. 4A), suggesting a classic gene-by-environment interaction

between as yet unidentified genetic susceptibility factors and inflammatory exposures. Such interactions are well known in the animal pain genetics literature (*20*). Human genes suggested to be involved in the pathogenesis of vulvodynia include those coding for mannose-binding lectin codon 54, the melanocortin-1 receptor, and the interleukin-1 receptor antagonist (*21*). Of course, an unknown environmental factor may also be responsible for the susceptibility to chronic hypersensitivity in those that develop it after repeated inflammation. Given that repeated zymosan treatment to the hind paw failed to produce chronic allodynia in any subject, these phenomena may be unique to mucosal tissue (*22*) or the genital tract (*23*).

Vulvar hypersensitivity after the third infection in the RVVC model was accompanied by increased density of sensory afferents, including increased peptide-containing nerve fibers. Previous work has shown the presence of sensory hyperinnervation during an inflammatory response, for example, in the mucosa of the urinary bladder during the inflammatory responses evoked by cyclophosphamide (*24*), in the upper dermis during the inflammatory response evoked by complete Freund's adjuvant (*25*), and in bone during the inflammatory response evoked via inoculation of prostate cancer cells (*26*). Sensory hyperinnervation in the upper dermis has also been seen during the regeneration response after a traumatic nerve injury (*27*–*29*). Notably, in the current experiment, robust sensory hyperinnervation was seen in allodynic mice 3 weeks after resolution of the infection. This suggests that the pain that persists after the resolution of infection may be due to an abnormal persistence of the hyperinnervation evoked during the acute inflammatory response. The observations in the animal studies parallel findings of greatly increased vulvar nerve density and peptidergic innervation in the allodynic vulvar vestibular tissue of women with PVD (*6*, *7*, *30*, *31*) and suggest that repeated infection is sufficient to alter innervation at the site of infection.

We also observed sympathetic (VMAT2-immunoreactive) hyperinnervation in the vulvae of allodynic mice 3 weeks after resolution of the infection. Sympathetic hyperinnervation has been observed in the skin during inflammation and after traumatic nerve injury (*25*, *27*, *28*, *32*). For example, ectopic endometrial cyst growth, which becomes sympathetically innervated, correlates with vaginal hypersensitivity in a rat model of endometriosis (*33*, *34*). Sprouting of free nerve endings (including those of peptidergic afferents) and sympathetic efferents suggest the presence of long-term physiological changes that may enhance nociceptive signaling of peripheral tactile input and promote spontaneous neuronal discharge of affected sensory fibers.

We have developed an etiologically valid and clinically relevant animal model of an idiopathic pain condition. This model will be useful in the investigation of mechanistic pathways of infection-induced pain, the evaluation of genetic and environmental risk factors, and the preclinical testing of the efficacy of new treatments for debilitating pain conditions secondary to infection. Because the most effective current treatment of PVD is surgical excision of the painful vulvar tissue (*3*), new and less invasive treatments are a clinical necessity.

## MATERIALS AND METHODS

### Subjects

Female, outbred CD-1 (ICR:Crl; Charles River) mice, 8 to 10 weeks of age, were housed in facilities equipped with Biohazard Level 2 containment. Mice were maintained on a 12:12-hour light/dark cycle (lights on at 07:00 hours) and received irradiated food (Harlan Teklad 8604) and autoclaved tap water ad libitum. All procedures, including inoculations, injections, and behavioral testing, were conducted within a class II biological safety cabinet. All procedures were approved by the McGill University animal care and use committee.

### Microorganism

A strain of *C. albicans* isolated in a clinical setting (SC5314, a gift of M. Whiteway, National Research Council of Canada) was used for vulvovaginal inoculations. See the Supplementary Material for a rationale for selection of this strain. SC5314 was grown in a phytone peptone broth for 18 hours at 25°C on an orbital shaker at 7000 rpm. Stationary-phase blastoconidia were washed twice and adjusted to $5 \times 10^4$ cells/ml. Each inoculum solution was prepared from freshly subcultured SC5314 on the day of inoculation.

### Vulvovaginal infection procedures and treatment

Mouse vaginal bacterial and fungal cultures were obtained before testing to ensure that no known pathogenic microorganisms were present. Under non–hormone-primed conditions, murine vaginal *C. albicans* infection resolves without antifungal treatment within 14 days (*13*). On day 0 of each infection, mice were lightly anesthetized with isoflurane/oxygen and inoculated vaginally with either $5 \times 10^4$ stationary-phase SC5314 blastoconidia in 20 μl of sterile phosphate-buffered saline (PBS) or saline only. The inoculum was gently pipetted into the vaginal opening, and the mouse was placed in the supine position to retain the inoculum in the vaginal cavity for 10 min. Post-inoculation vaginal lavages were collected daily until infection resolution was confirmed, and weekly thereafter. To minimize tissue irritation unrelated to infection, we took utmost care to minimize contact between the vulva and the pipette tip during lavages. Vaginal SC5314 burden and infection status were monitored with Gram- and Wright-Giemsa–stained smears prepared from vaginal lavage fluid, which were examined microscopically for the presence of polymorphonuclear leukocytes and *Candida* morphotypes (blastoconidia and pseudohyphae). Lavage fluid was serially diluted onto Sabouraud dextrose agar (Quelab) and incubated for 48 hours at 34°C, and colony-forming units (CFUs) were quantified. Loops of CFUs were submitted to two separate tests to ensure that the isolated yeast was indeed *C. albicans*: The isolated growth was submitted to a germ tube test and replated onto chromogenic agar specific to common *Candida* species (Candida CHROMagar, Hardy Diagnostics) for up to 1 week at 34°C. According to the manufacturer's guidelines, emerald green CFUs exhibiting growth characteristics consistent with *C. albicans* were considered positive.

Mice in both the SC5314-infected and the control groups were treated with fluconazole (15 mg/kg, once daily for 7 days; LKT Laboratories) administered via oral gavage. These doses are effective in eliminating *C. albicans*–induced vaginitis in mice (*35*). The treatment regimen was based on the broad use of fluconazole as a first-line treatment for RVVC in humans (*11*). The infection was considered to be resolved upon obtaining two successive negative vaginal cultures (see above), and weekly lavages were collected thereafter to ensure the absence of yeast.

### Repeated vulvovaginal infection with *C. albicans*

To simulate RVVC, mice received three separate vulvovaginal infections with $5 \times 10^4$ *C. albicans* strain SC5314. Infections were allowed to last untreated for 4 days during each round of infection, followed by 7 days of fluconazole treatment (see above). For the second and third

rounds of infection, mice were reinoculated with $5 \times 10^4$ SC5314 cells 4 weeks after clearance of the primary infection (1 week after post-infection behavior testing) to simulate RVVC. In humans, a new episode of candidiasis can begin from a few days to 3 months after a previous infection, and the 4-week interval between infection resolution and re-infection used here was deemed a valid analog of RVVC. Mice in the fluconazole control group received inoculations of saline and vaginal lavages concurrent to and in the same manner as infected mice.

Tests of baseline vulvar and hind paw thresholds were performed 1 week preceding initial SC5314 inoculation (day −7). During each round of infection, vulvar sensitivity was tested 4, 11, and 32 days after inoculation (see Fig. 1A). Hind paw sensitivity was measured after each infection, on day 33.

### Extended primary vulvovaginal infection with *C. albicans*

The extended primary infection with $5 \times 10^4$ SC5314 blastoconidia was allowed to last untreated for 14 days, followed by 7 days of fluconazole treatment (see above). Mice in the fluconazole control group received saline and vaginal lavages concurrent to and in the same manner as infected mice.

Tests of baseline vulvar and hind paw thresholds were performed 1 week preceding initial SC5314 inoculation (day −7). Vulvar sensitivity was measured at 14, 21, 42, and 70 days after inoculation (see Fig. 4A). Hind paw sensitivity was measured on days 43 and 71.

### Repeated inflammation with zymosan

A new cohort of mice was subjected to repeated subcutaneous injections of zymosan in the posterior vulva while lightly anesthetized with isoflurane/oxygen. Zymosan is prepared from *S. cerevisiae* yeast cell wall and produces sterile inflammation at the site of injection, leading to mechanical allodynia lasting up to 12 to 24 hours without the need for biohazard containment. The dose used (10 mg/ml in 10 μl of saline; 0.1 mg) was chosen on the basis of pilot experiments; lower doses produced inconsistent initial allodynia. Injections occurred no more frequently than weekly to allow acute inflammation to subside between successive injections. Mice received zymosan injections immediately after baseline behavior testing and were observed for evidence of vulvar allodynia (defined here as ≥33% reduction in mechanical threshold) 4 hours later, corresponding to the temporal peak of vulvar zymosan-induced mechanical allodynia as defined by our pilot experiments. One week later, each mouse was retested and reinjected with zymosan. Each week thereafter, vulvar von Frey measurements were obtained and additional injections were administered only if a mouse's vulvar sensitivity recovered to nonallodynic levels (defined as >66% of baseline threshold). If a mouse continued to show evidence of vulvar allodynia 1 week after zymosan injection, no injection was given, and the mouse was retested 1 week later. Mice were followed for a total of 11 weeks and received up to and including (but no more than) six zymosan injections. Mice that did not become persistently allodynic (that is, displaying a ≥33% reduction in threshold for 2 consecutive weeks) after six injections were classified as nonresponders. One control group received weekly vulvar saline injections, with timing matched to a zymosan-treated mouse with equivalent baseline vulvar sensitivity. In a separate experiment, six female mice received zymosan injections in a paradigm similar to that described above except that zymosan (0.25 mg/ml in 20 μl) was injected into the right mid-plantar hind paw. This dose was chosen because it produced equivalent levels of initial mechanical allodynia to the vulvar zymosan.

### Mechanical (von Frey) sensitivity testing

On each day of behavioral testing, animals were allowed 3 hours (11:00 to 14:00 hours) to habituate to the testing environment. Each testing session consisted of two threshold determinations separated by 1 hour; these two thresholds were averaged. An observer blinded to experimental condition applied a calibrated series of von Frey filaments (Semmes-Weinstein monofilaments; Stoelting) to the target tissue using the up-down psychophysical method of Dixon (*36*), with pressure applied to each filament until it bowed, and held for 2 s. Across species, the vulva is defined as the external female genital organs; this includes the clitoral and preputial glands in the mouse, and accordingly, we stimulated the central, hairless posterior aspect of the mouse vulva. A series of eight von Frey filaments (0.06 to 3.9 g; filaments #4 to #11) were applied to the vulva beginning with the #7 filament. Hind paw mechanical sensitivity was also monitored in all experiments, as a control. For hind paw testing, a different series of eight filaments (0.015 to 1.3 g; filaments #2 to #9) was applied to the plantar aspect of the hind paw beginning with the #5 filament. Different ranges of fibers were used for vulva and hind paw testing because different amounts of force physically lift the stimulated area off the floor (which artificially imposes a ceiling value on testing). Any mouse showing continuous positive or negative responses was assigned ceiling and floor withdrawal threshold values of 4.0 and 0.025 g, respectively, for vulvar testing and 2.0 and 0.01 g, respectively, for hind paw testing. In all other cases, the 50% withdrawal threshold was calculated as described (*36*). Aiming accuracy in mouse vulvar stimulation (a 3-mm-diameter target) was maximized by shaving anogenital hair the day before testing to improve visibility. Von Frey filaments were disinfected with 70% ethanol between each testing session, and independent filament sets were used for each experimental condition to minimize risk of cross-contamination.

The nocifensive endpoint we adopted was a clear reflexive jump (all four paws lifted) in response to vulvar stimulation, chosen because it is most similar to the clear withdrawal response used in hind paw von Frey testing. Note, however, that even the strongest usable von Frey filament (3.9 g, with larger filaments lifting the mouse off the floor without bending) rarely produced this jumping response at baseline, with >75% of mice consistently not responding to the 3.9-g fiber. It is unclear whether "positive" (<4.0 g) responses at baseline for the remaining subjects represent measurement (or testing environment) artifacts or true biological variability.

For practical reasons, it was necessary to test all mice together, regardless of their estrous stage, on each scheduled testing day. A pilot experiment confirmed that vulvar mechanical thresholds were invariant of estrous stage. In addition, vaginal lavages taken during the experiment revealed that neither SC5314 nor fluconazole treatment altered normal 4- to 5-day estrous cyclicity. Thus, it is unlikely that the changes seen in SC5314-infected mice were produced by hormonal alterations.

### Immunohistochemistry

After behavioral testing, mice were deeply anesthetized with sodium pentobarbital (≥50 mg/kg, intraperitoneally) and perfused transcardially with 5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature. The vaginal canal (from the external vulva to the cervix) was excised and postfixed in 5% paraformaldehyde in phosphate buffer for 1 hour and then cryoprotected with 30% sucrose in phosphate buffer for 24 hours. Tissue was embedded in

an optimum cutting temperature medium (Tissue Tek) and frozen at −80°C until cryosectioned. Twelve-micrometer-thick longitudinal sections were cut on a Leica CM3050 S cryostat at −25°C and placed directly on poly-L-lysine–treated slides. Slide-mounted sections were rinsed three times with 0.1 M PBS for 10 min, preincubated with 10% normal goat serum diluted with 0.3% Triton X-100 for 60 min, and then incubated for 24 hours at 4°C in one of three primary antibodies: anti–PGP 9.5 raised in rabbit (1:2000, Ultraclone), anti-CGRP (α isoform) raised in sheep (1:1000, Biomol), and anti-VMAT2 raised in rabbit (1:2000, Millipore). The next day, slides were washed three times with PBS for 10 min, incubated in Cy3 anti-rabbit and Cy2 anti-sheep secondary antibodies (1:500, Jackson ImmunoResearch Laboratories) in the dark for 2 hours, and washed three times for 10 min with PBS. For each reaction, negative controls (processed without the primary antibody) were included.

### Quantitative analysis of RVVC immunohistochemistry

Immunohistochemical analysis of a subset of post–repeated-infection allodynic ($n$ = 3 to 6, depending on the antibody), post-infection nonallodynic ($n$ = 5), and fluconazole control ($n$ = 4) animals (see below) was based on four randomly selected postmortem vulvar tissue sections per mouse, with a total of six nonconsecutive pictures per section (that is, 24 frames per mouse). Pictures were taken only of the lamina propria because very little innervation was observed in the epithelium. Images were acquired with a Zeiss Axioplan 2 imaging fluorescence microscope (lenses ranging from 40× to 60×) equipped with a Megaview II charge-coupled device (CCD) camera and processed with AnalySIS 5.0 software (Soft Imaging System). Images were saved in TIFF format and analyzed with an image analysis system (MCID Elite v.7, Imaging Research) by an observer blinded to condition. Fiber density was calculated with functions in the program configured to measure total fiber length per unit area (*25*).

### Assessment of post-infection morphology

Slide-mounted 12-μm-thick sections were processed as described above and stained with H&E to identify gross vulvar morphology, epithelial thickness, and inflammation among allodynic ($n$ = 4), nonallodynic ($n$ = 6), and fluconazole controls ($n$ = 5). Four nonconsecutive measurements were taken from the middle third of the posterior vulvar tissue, across six sections (24 measurements total). Sections were examined for signs of inflammatory infiltrate in the epithelium and lamina propria, as well as edema and plasma extravasation. Slides were digitally scanned with MIRAX Desk Scanner and visualized with MIRAX Viewer software using the 20× and 40× magnification functions for quantification (Zeiss).

### Data analysis

Normally distributed hind paw threshold data were analyzed with repeated-measures analysis of variance (ANOVA). Vulvar threshold data were analyzed with nonparametric $\chi^2$ analysis, with five arbitrarily defined threshold categories (<1.0, 1.0 to 1.99, 2.0 to 2.99, 3.0 to 3.99, and >4.0 g). Data at particular testing sessions were compared to within-group baselines, but similar results were obtained when comparing between-group at each testing session. Analysis of percentage of allodynic mice was conducted with a one-tailed Fisher's exact test on the basis of the a priori hypothesis that previously infected mice would show more allodynia. Normally distributed immunohistochemical and epithelial thickness data were analyzed by one-way ANOVA followed by Tukey's post hoc test. In all cases, a criterion level of $\alpha = 0.05$ was adopted. All statistical tests were two-tailed except as described above.

## SUPPLEMENTARY MATERIAL

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Methods

Fig. S1. Increased vaginal fungal colonization during acute infection(s) after inoculation with *C. albicans* strain SC5314, and full clearance of yeast after fluconazole treatment.

Fig. S2. No effect of repeated vulvovaginal *C. albicans* strain SC5314 infection on hind paw mechanical sensitivity.

Fig. S3. No effect of repeated vulvovaginal *C. albicans* strain SC5314 infection on gross vulvar morphology.

Fig. S4. No effect of single, extended-duration vulvovaginal *C. albicans* strain SC5314 infection on hind paw mechanical sensitivity.

Table S1. No changes in epithelium thickness produced by repeated vulvovaginal *C. albicans* strain SC5314 infection.

Table S2. Vaginal leukocyte count was not associated with the presence of infection after SC5314 inoculation.

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