The SPARC-null Mouse Model of Low Back Pain:

Mechanism, Treatment, and Translation to Humans

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ABSTRACT

Persistent low back pain (LBP) is the most common form of chronic illness in Canadians age 60 and under, affecting 15% of the population. In addition to being a major health problem, it also has serious economic consequences. Current diagnostic and therapeutic approaches to chronic back pain are limited by our narrow understanding of the underlying biological mechanisms. As a consequence, treatment is not always effective and individuals may suffer for years without relief. LBP is often associated with signs of degeneration in the intervertebral discs. LBP encompasses a wide spectrum of pain disorders including axial pain in the back region and radiating pain down one or both legs.

This thesis investigates the mechanisms and treatment of axial and radiating pain associated with disc degeneration (DD) in a transgenic mouse model and in human subjects. SPARC-null mice show anatomical signs of disc degeneration and behavioral signs of pain and are thus a valid model of LBP due to DD.

Our results show that loss of lumbar disc height correlates with radiating pain in mice. In addition, we show that axial pain and radiating pain have different pharmacological profiles, suggesting the involvement of different mechanisms. Furthermore, we explore the use of opioid-adrenergic combination therapy to improve therapeutic outcome in our model. Finally, we propose an epigenetic mechanism governing SPARC expression, disc degeneration, and back pain in aging wild-type mice and in human subjects with LBP.

The results shown in this thesis will serve to increase our understanding of the mechanisms of pain due to DD. This mechanism-based approach is key in improving therapeutic options for the millions of patients suffering from chronic low back pain.

RESUMÉ

La douleur persistante du bas du dos (lombalgie) est la forme la plus commune des maladies chroniques chez les Canadiens de 60 ans et moins, touchant 15% de la population. En plus d'être un problème majeur de santé, la lombalgie a également de graves conséquences économiques. Les approches actuelles de diagnostic et de traitement de la lombalgie chronique sont limitées par notre compréhension limitée des mécanismes biologiques sous-jacents et la prise en charge n'est pas toujours efficace. Ainsi, les patients peuvent souffrir pendant de très longues périodes de temps sans soulagement. La lombalgie est souvent associée avec des signes de dégénérescence des disques intervertébraux. Elle englobe un large éventail de désordres douloureux et peut être sub-divisée en douleur axiale (dans la région du dos) et/ou douleur radiculaire (le long d'une jambe ou deux).

Cette thèse étudie les mécanismes et le traitement des douleurs axiales et radiculaires associées à la dégénérescence des disques (DD) dans un modèle murin transgénique et chez des sujets humains. Les souris SPARC-nul montrent simultanément des signes anatomiques de dégénérescence des disques intervertébraux et des signes comportementaux de douleur. Ces souris représentent donc un modèle valide de lombalgie due à la dégénérescence des disques.

Nos résultats prouvent que la perte de hauteur des disques lombaires corrèle avec le développement de douleur radiculaire chez la souris. En outre, les douleurs

axiales et radiculaires présentent des profils pharmacologiques différents, suggèrant l'implication de mécanismes différents. Dans notre modèle, nous explorons également l'utilisation d'une thérapie combinant des traitements opiacés et adrénergiques pour améliorer des résultats thérapeutiques. Finalement, nous proposons un mécanisme épigénétique régissant l'expression de SPARC, la dégénérescence des disques, et la lombalgie chez des souris sauvages agées et des sujets humains.

Nous croyons que les résultats présentés dans cette thèse serviront à améliorer notre connaissance des mécanismes de la douleur liée à la dégénérescence des disques intervertébraux. Une telle approche mécanistique permettra d'augmenter les options thérapeutiques pour des millions de patients souffrant de douleur lombo-sacrée chronique.

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LIST OF ABBREVIATIONS

AF: Annulus Fibrosus

DD: Disc Degeneration

DRG: Dorsal Root Ganglion

H&E: Hematoxylin and Eosin

IL: Interleukin

IVD: Intervertebral Disc

LBP: Low Back Pain

MRI: Magnetic Resonance Imaging

NGF: Nerve Growth Factor

NP: Nucleus Pulposus

PG: Prostaglandin

SP: Substance P

SPARC: Secreted Protein, Acidic, Rich in Cysteine

TNFα: Tumor Necrosis Factor Alpha

1 INTRODUCTION

1.1 Statement of the problem

Persistent back pain is one of the most common forms of chronic illness in Canadians age 60 and under affecting 15% of the population (Rapoport et al., 2004). It is also the most common reason for disability in people younger than 45 years of age and the second most frequent reason for visits to the physician. Because pain impairs one's ability to carry out a productive life, it is a serious economic problem (Paul et al., 2012) in addition to a major health problem, costing an estimated $10 billion/year in Canada (Rapoport et al., 2004). Current diagnostic and therapeutic approaches to chronic back pain are limited by our narrow understanding of the underlying biological mechanisms.

There are many potential causes of chronic **low back pain (LBP**). One of the most common causes is **disc degeneration (DD)**. Both age-related degeneration of **intervertebral discs (IVDs)** (Boden et al., 1990, Jensen et al., 1994) and pathological, accelerated degeneration can occur, resulting in pain, instability, and biomechanical movement disadvantages. Depending on the underlying mechanisms, responses to different treatments may vary widely. Unfortunately, therapeutic decisions are based on trial and error, a situation frustrating to physician and patient. In order to improve therapeutic outcome and quality of life for people with chronic pain, we need an advanced understanding of the mechanisms underlying LBP in general and DD in particular.

Despite the significant impairment associated with this disease, there is a lack of animal models incorporating both anatomical and functional components of

human DD (Singh et al., 2005). To study the mechanisms of pain due to pathological disc degeneration, we used a mouse model that presents with accelerated, progressive disc degeneration and signs of LBP to identify the mechanisms underlying DD-associated LBP.

The studies described in this thesis will:

1. Use the SPARC-null mouse model to examine the relationship between DD and LBP.

2. Investigate the pharmacological profile of axial and radiating pain in SPARC-null mice.

3. Study analgesic drug interactions in SPARC-null mice.

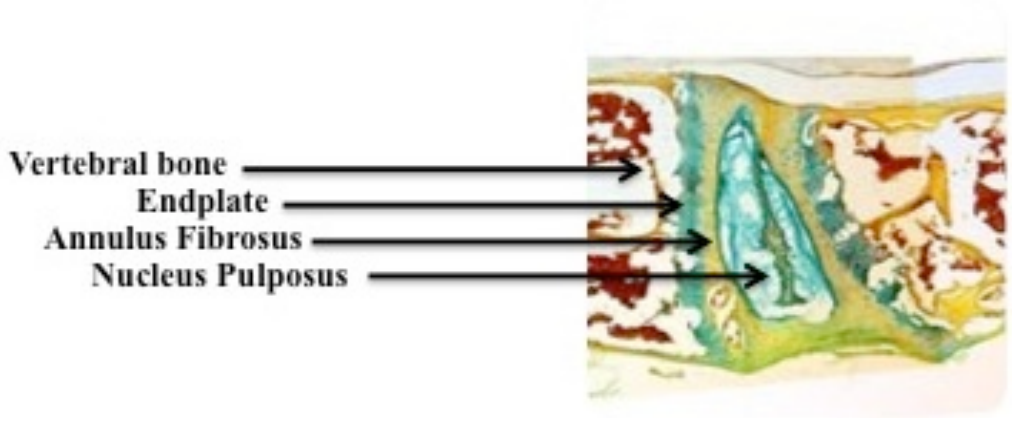
4. Identify the role of SPARC in disc degeneration in normal aging mice and in humans with DD-related LBP.

The resulting improvement in our understanding of DD will ultimately contribute to the development of novel diagnostic and therapeutic approaches for the treatment of chronic LBP.

1.2 Background

Intervertebral Discs

IVDs account for nearly a quarter of the height of the complete spinal column. They are articular joints sandwiched between vertebral end plates and act to distribute loads placed on the spine through hydrostatic pressures. They also separate vertebral bone structures so that nerve roots may exit the spinal column. IVDs have a unique composition especially rich in glycoproteins that bind water molecules and give the IVD its biomechanical properties by acting as a hydrostatic cushion between adjacent vertebrae. Their organization is different from other joints: they are composed of a fibrous outer layer, the **annulus fibrosus (AF)**, and an inner layer, the **nucleus pulposus (NP)**. These layers are compromised of cells that appear to be morphologically differentiated: cells in the outer AF have a fusiform structure, while inner AF cells appear to be more rounded. NP cells change their morphology during development. They start off as large vacuolated cells but soon transition to smaller, less active cells (Choi et al., 2008).



The lumbar IVD is the largest avascular structure in the human body (Nerlich et al., 2007) and resident cells survive in a very restricted environment in terms of oxygen and nutrients (Urban et al., 2004). In healthy discs, hydrostatic pressures force nutrients and water in and out of the disc like an expanding and contracting sponge. Under normal conditions, sensory and sympathetic innervation of IVDs is limited to the outer layers of the annulus fibrosus and the posterior and anterior ligaments that surround the disc (for review see (Edgar, 2007)). Pro-nociceptive neuropeptides have been described in the outer annulus, suggesting that some of these fibers are nociceptive (McCarthy et al., 1991a, Freemont et al., 1997).

Intervertebral Disc Degeneration

In a healthy disc the nucleus pulposus is a soft, amorphous structure that acts like a ball of fluid. In the early stages of DD, the NP starts losing its proteoglycan content, which is believed to initiate dehydration that is associated with degeneration (Raj, 2008). As degeneration progresses, discs become increasingly fibrotic, dehydrated, and narrow. Furthermore, tears begin to appear in the annulus. In the late stages of DD, radial fissures extend throughout the entire annulus, and eventually the annular wall fails, allowing the disc contents to escape (i.e. herniate) into the surrounding tissue (Fardon and Milette, 2001). At the biochemical level, minor damage due to repeated stress initiates local inflammation (Ulrich et al., 2007). As a result, biochemical mediators implicated in the breakdown of articular cartilage are up-regulated and released including interleukins, proteases, **prostaglandin E2** (**PGE2**) and tumor necrosis factor alpha (**TNFα**) (Sobajima et al., 2005, Podichetty, 2007).

There is a general lack of consensus concerning the exact definition of disc degeneration. For the purposes of our work, we will utilize the following definition proposed by Adams and Roughley in 2006:

"*The process of disc degeneration is an aberrant, cell-mediated response to progressive structural failure. A degenerate disc is one with structural failure combined with accelerated or advanced signs of aging. Early degenerative changes should refer to accelerated, age-related changes in a structurally intact disc. Degenerative disc disease should be applied to a degenerate disc that is also painful*" (Adams and Roughley, 2006).

One of the factors commonly associated with chronic LBP is DD (Schwarzer et al., 1994). It is unclear when degeneration will lead to pain, since MRI studies show a low correlation between the extent of degenerative changes and pain found upon physical evaluation (Boden et al., 1990, Jensen et al., 1994, Antoniou et al., 1998). This may be because imaging techniques are not yet sophisticated enough for detecting subtle signs of degeneration. Another factor could be the sample size, since the correlation between DD and LBP is much clearer in larger sample sizes (de Schepper et al.). Finally, it is possible that biochemical factors, undetectable by imaging, play an important role in the development and perpetuation of the chronic pain state.

Disc degeneration-related Low Back Pain

LBP is defined by a continuum of painful conditions. The first condition is axial LBP and is characterized by spontaneous or movement-evoked pain or soreness

confined to the spine and low back region. The second condition is non-axial pain in which the pain radiates from the back down one or both legs. This condition is often referred to as radiating pain or sciatica, because the pain usually follows the course of the sciatic nerve.

Theories of DD-Induced Low Back Pain

Axial Pain: Pathological Disc Innervation

Under normal conditions, nerve endings penetrate only the outer layers of the disc (McCarthy et al., 1991a). The depth and density of nerve fibers is increased in lumbar IVDs obtained from chronic LBP patients compared to discs harvested post-mortem from subjects without back pain (Freemont et al., 1997). Furthermore, studies in disc puncture models of DD show that it is accompanied by increased innervation in the scar tissue (Aoki et al., 2006). A proportion of abnormally deep fibers are immunopositive for substance P (SP), a neuropeptide expressed by some nociceptive nerve fibers. The increased innervation may be due to increased levels of **nerve growth factor (NGF)** within the disc. NGF is implicated in nerve ingrowth into painful IVDs (Freemont et al., 2002) and is upregulated in disc cells by the proinflammatory cytokines **interleukin-1β (IL-1β)** and **tumor necrosis factor alpha (TNFα)** (Abe et al., 2007). Cell culture studies have shown that NGF-sensitive neurons, cultured with TNFα, have a greater potential to spread their axons (Aoki et al., 2007). The presence of nociceptive nerve fibers deep within degenerating IVDs could provide an

explanation for the phenomena of 'discogenic' pain, in which the disc itself generates pain.

Radiating Pain: Degenerating IVDs Release Pro-Nociceptive Substances:

The contents of the NP may be involved in the etiology of non-axial LBP by irritation of nerve roots even in the absence of mechanical compression (Kawakami et al., 1997a, Olmarker et al., 1998, Mulleman et al., 2006). Application of NP or some of its constituents to the epidural space produces increased spinal nerve root excitability and hypersensitivity to painful stimuli (Aoki et al., 2002a, Obata et al., 2002, Chen et al., 2004a). Moreover, numerous inflammatory mediators known to produce pain are upregulated in DD. In degenerating discs, normal diffusion of NP contents may be accelerated by (1) increasing osmolarity of the NP resulting from loss of water and negatively charged glycoproteins and (2) the appearance of annular tears. Therefore, as discs break down, the contents of the NP become both increasingly noxious and more likely to escape into the surrounding area, where contact with nerve fibers will result in the experience of pain (Mulleman et al., 2006).

Radiating pain: Nerve Root Compression

Disc narrowing, bulging, and herniation can result in compression of spinal nerve roots or dorsal root ganglia (DRG) neurons (Cavanaugh, 1995a), producing activity in the peripheral sensory nerve fibers (Howe et al., 1977). In DD, pain due to mechanical compression of nerve roots develops secondary to disc rupture

and/or severe loss of disc height and can result in nerve damage and neuropathic pain.

Current Animal Models of Disc Degeneration

There exist numerous animal models of DD. In these models the degeneration may be naturally occurring such as in the Desert Sand Rat (Gruber et al., 2002), induced by acute injury to the disc such as disc puncture (Masuda et al., 2005, Rousseau et al., 2007), initiated by chemical mediators that result in inflammation (Aoki et al., 2004a, Aoki et al., 2004b) or result from genetic deletion of a protein important in disc integrity such as the SPARC-null mouse (Gruber et al., 2005). Each model is accompanied with advantages and disadvantages. In the naturally occurring models, the underlying cause of DD is unknown. In the induced models, the mechanistic relevance is questionable (Singh et al., 2005).

The SPARC-null Mouse as a Model of DD-related LBP

SPARC (secreted protein, acidic, rich in cysteine; aka osteonectin or BM-40) is an evolutionarily conserved collagen-binding protein present in IVDs. SPARC is known to influence bone remodeling, collagen fibrillogenesis, and wound repair. Decreased expression of SPARC has been associated with aging and degeneration in human IVDs (Gruber et al., 2004) and the targeted deletion of the SPARC gene results in accelerated disc degeneration in the aging mouse (Gruber et al., 2005). SPARC-null mice demonstrate early signs of DD at 2 months of age and extensive disc degeneration and herniation by 14 months of age (Gruber et al.,

2005). Prior to our studies, the behavioral impact of DD in these mice had not been explored.

We have identified a series of behavioral assays to measure axial and non-axial hypersensitivity in SPARC-null mice, and our initial studies validating this model have been appended to this thesis (Millecamps et al., 2011b, Millecamps et al., 2012). In brief, we show that these mice exhibit anatomical and histological signs of DD, which gets progressively worse with age. Furthermore, they show signs of axial pain detectable by the tail suspension and grip assays and signs of radiating pain in the hindpaw in the form of cold sensitivity, detectable by the acetone test.

We believe that the behavioral differences observed between SPARC-null and WT mice are indices of disc degeneration-related pain as opposed to other, non-specific etiologies for the following reasons:

- ***First,*** the behavioral changes are not due to overall increases in reactivity since they are modality specific; hypersensitivity is observed to cold but not to mechanical or heat**.**

- ***Second,*** the behavioral changes are not due to overall motor impairment or lethargy because motor function is not impaired.

- ***Third,*** the behavioral changes are not due to abnormal skin, peripheral nerve or CNS dysfunction because they are region-specific. For example, hypersensitivity to intra-dermal capsaicin develops with age in the hindpaw but not in the upper lip (See appendix 2).

- ***Fourth,*** the behavioral changes observed in SPARC-null mice cannot be attributed to prenatal or early developmental effects of the gene deletion because many of the phenotypes do not fully develop until the animals are 2-6 months old (Millecamps et al., 2012). Interestingly, it is in this time period that signs of disc degeneration begin to appear (Gruber et al., 2005).

- **Finally,** the behavioral indices of axial and/or non-axial LBP observed in SPARC-null mice are sensitive to one or more therapeutic agents used clinically to treat LBP.

The existence of well characterized disc degeneration in SPARC-null mice by previous studies, together with our demonstration of behavioral indices of axial and non-axial low back pain, make the SPARC-null mouse an excellent tool for studying the underlying mechanisms of DD-induced low back pain.

Age-related Disc Degeneration

Our SPARC-null mice exhibit signs of accelerated disc degeneration, which is ideal to mimic pathological DD in humans. However, "natural" or age-related DD is an important type of DD that can be studied in aging, WT mice. Signs of DD have been shown to increase with age in animals (Leung et al., 2008, Nuckley et al., 2008, Cho et al., 2011) and in both symptomatic and asymptomatic human subjects (Hicks et al., 2009). The mechanisms by which disc degeneration progresses with age are diverse. They include, but are not limited to, cellular senescence (Gruber et al., 2007), changes in proteoglycan synthesis (Johnstone

and Bayliss, 1995), changes in the collagen content of the nucleus pulposus (Naylor et al., 1954), and decrease in nutrient supply to the IVD (Grunhagen et al., 2011). Another factor that is involved in aged-related DD is decreased SPARC expression within degenerating IVDs (Gruber et al., 2004, Tajerian et al., 2011b), However, the mechanisms responsible for decreased SPARC expression are not clear.

In addition to studies using the SPARC-null model of accelerated IVD degeneration to understand DD-induced LBP, this thesis also explores the role of SPARC in age-related disc degeneration in WT mice and in humans. Specifically, we examine the regulation of SPARC as a function of age in mice and as a function of disc degeneration in human subjects.

Epigenetic Regulation of Gene Expression

In the last few decades, comprehensive sequencing of human and animal genomes has generated great promise in understanding the basis of phenotypic differences in general and disease states in particular. However, it is now clear that many conditions involve chronic changes in gene expression caused by mechanisms outside of changes in DNA sequence.

The epigenome consists of the chromatin and its modifications, noncoding RNA such as microRNA (Bergmann and Lane, 2003) as well as a covalent modification by methylation of cytosine rings found at the dinucleotide sequence CG in DNA (Razin, 1998). DNA methylation is the most widely studied epigenetic mechanism. In eukaryotes, this consists of the covalent addition of a methyl group

at 5'-position of cytosines, usually at CpG dinucleotides and is usually associated with gene silencing.

What distinguishes DNA methylation in vertebrate genomes is the fact that not all CpGs are methylated in any given cell type, generating cell type specific patterns of methylation (Razin and Szyf, 1984). The DNA methylation pattern confers upon a genome its cell-type specific identity. Active regulatory regions of the chromatin, which enable gene expression, are associated with hypo-methylated DNA whereas hyper-methylated DNA is packaged in inactive chromatin resulting in gene silencing (Razin and Cedar, 1977, Razin, 1998). DNA methylation silences gene expression by two principal mechanisms: direct interference of a methyl residue in a recognition element for a transcription factor (Comb and Goodman, 1990, Inamdar et al., 1991) and through attracting the binding of methylated-DNA binding proteins such as methyl CpG binding protein 2 (MeCP2) (Nan et al., 1997). MeCP2 recruits other proteins such as SIN3A and histone-modifying enzymes that lead to a formation of "closed" chromatin configuration and silencing of gene expression (Nan et al., 1997). Recent data suggest that the chromatin state could dictate DNA methylation states. Thus, there is a bilateral relationship between DNA methylation and chromatin modification (D'Alessio and Szyf, 2006).

Epigenetics could provide us with an explanation regarding how the environment relates to chronic pain conditions. For instance, epidemiological evidence indicates that external stressors, history of previous pain, abuse or other adverse events that precede the onset of pain are predictors for the development and

maintenance of chronic pain (Marchand, 2008, Young Casey et al., 2008). Furthermore, while a genetic basis for individual variations in the development of chronic pain is well established (Diatchenko et al., 2005b), genetic factors only account for approximately half of the inter-individual variability in chronic low back pain (MacGregor et al., 2004a).

Since DNA methylation is a well-validated epigenetic mechanism, we decided to make it our focus. Changes in DNA methylation have been associated with aging (Rodriguez-Rodero et al., 2010b) and with various pathological states (Issa et al., 1994, Ahuja et al., 1998b, Landmark-Hoyvik et al., 2010), but its role in pain had not been explored when we initiated the studies described in Chapter 5. Indeed, throughout the course of last few years, great advances have marked the start of the field of pain epigenetics (Tajerian et al., 2011b, Denk and McMahon, 2012b). This is an interesting area of research since epigenetic mechanisms act as the interface between genes and the environment and might help explain how environmental exposures can affect disease later in life. Furthermore, interventions targeting these epigenetic changes can be a possible therapeutic option for patients.

1.3 Rationale

Persistent back pain is a debilitating condition effecting millions of people worldwide. Unfortunately, therapeutic outcome for the back pain patient is less than satisfactory since treatment is not mechanism-based. Increasing understanding of the mechanisms of pain due to DD will lead to better treatment outcome for the millions suffering from LBP.

1.4 Hypothesis

The central hypothesis of the thesis is that the lack of SPARC is involved in the progression of DD, which leads to chronic axial and radiating LBP both in murine and human subjects.

Specifically, we demonstrate that:

1. The SPARC-null mouse is a useful model of pain due to disc degeneration, where pain is correlated with anatomical signs of disc degeneration.

2. SPARC-null mice show signs of axial and radiating pain with distinct pharmacological profiles.

3. Combination therapy might be useful in treating axial and radiating LBP in SPARC-null mice.

4. Epigenetic changes in SPARC may be responsible for LBP in aging WT mice and in human subjects with LBP.

1.5 Contribution of authors

Chapter II-Research Manuscript (in preparation):

Design of the study: M. Tajerian, M. Millecamps, A. Danco, and L.S. Stone

Execution and analysis of the study: M. Tajerian and A. Danco

Writing of the manuscript: M. Tajerian and L.S. Stone

Supervision: L.S. Stone

Chapter III-Research Manuscript (Submitted):

Design of the study: M. Tajerian, M. Millecamps, and L.S. Stone

Execution and analysis of the study: M. Tajerian and M. Millecamps

Writing of the manuscript: M. Tajerian and L.S. Stone

Supervision: L.S. Stone

Chapter IV-Research Manuscript (Pain research and treatment, Published):

Design of the study: M. Tajerian, M. Millecamps, and L.S. Stone

Execution and analysis of experiments: M. Tajerian

Writing of the manuscript: M. Tajerian and L.S. Stone

Supervision: L.S. Stone

Chapter V-Research Manuscript (Molecular pain, Published):

Design of the study: M. Tajerian, S. Alvarado, M. Millecamps, M. Szyf, and L.S. Stone

Execution and analysis of experiments: M. Tajerian, S. Alvarado, and T. Dashwood (epigenetic analysis); K.M. Anderson (functional assays in human subjects).

Human tissue collection: K.M. Anderson, L. Haglund, and J. Ouellet

Writing of the manuscript: M. Tajerian, S. Alvarado and L.S. Stone

Supervision: L.S. Stone and M. Szyf

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2 The Relationship between Disc Degeneration and Low Back Pain

Preface

The work shown in the following manuscript addressed the question: Is intervertebral disc height a predictor of pain? In other words, does the severity of disc degeneration predict the extent of axial and/or radiating pain? This question has been the subject of debate for many decades and studies in humans have reached conflicting conclusions.

Upon close examination, it is apparent that SPARC-null animals show anatomical and histological signs of disc degeneration. Furthermore, these signs are accompanied by behavioral changes indicative of axial and radiating pain. Although DD and LBP seem to occur concurrently in SPARC-null animals, there are no studies directly linking anatomical signs of disc height to behavioral signs of pain. We therefore decided to use our mouse model to answer the question posed earlier.

The following manuscript explores the relationship between lumbar disc height and shape and behavioral signs of axial and radiating pain and overall motor function.

Manuscript: Loss of Disc Height Correlates with Radiating Pain in a Mouse Model of Low Back Pain and Disc Degeneration

2.1 Abstract

Chronic low back pain is a serious health and economic problem affecting millions of patients worldwide. It can be divided into 2 main components: axial pain in the low back region, and non-axial pain radiating down one or both legs. One of the factors commonly associated with back pain is the degeneration of intervertebral discs. However, the relationship between measures of axial and radiating pain and anatomical signs of disc degeneration remains unclear.

We used the SPARC-null model of disc degeneration and low back pain to explore the relationship between lumbar disc height, disc wedging, and behavioral indices of axial pain, radiating pain, and motor function.

Our results show that by 6 months of age, SPARC-null animals show reduced lumbar disc height and increased disc wedging compared to WT controls. Furthermore, we show that radiating pain correlates negatively with disc height and positively with wedging in SPARC-null mice only.

Our data supports the hypothesis that there is a relationship between pain and disc anatomy and emphasizes the need to distinguish between the different pain components involved in the broad spectrum of back pain.

2.2 Introduction

Persistent **low back pain (LBP)** is one of the most common forms of chronic illness in Canadians age 60 and under affecting 15% of the population (Rapoport et al., 2004). LBP can include axial and non-axial elements. Axial LBP is characterized by spontaneous or movement-evoked pain or soreness confined to the spine and low back region. Non-axial pain is pain that radiates from the back down one or both legs. It is often referred to as radiating pain or sciatica, because the pain usually follows the course of the sciatic nerve.

Despite the fact that many individuals with **disc degeneration (DD)** do not suffer from LBP, studies show that **intervertebral disc (IVD)** degeneration is associated with an increased risk of chronic LBP (Boden et al., 1990, Jensen et al., 1994, Luoma et al., 2000b, Takatalo et al., 2011), and that restoring disc height can reverse back pain (Apfel et al., 2010). However, research examining the exact relationship between axial and radiating pain and anatomical signs of disc degeneration (i.e. disc height and shape) is scarce (Frymoyer et al., 1984).

In this study we used a previously established mouse model of LBP due to DD to investigate the relationship between disc degeneration and axial and radiating LBP. SPARC-null mice exhibit accelerated, age-dependent disc degeneration (Gruber et al., 2005), which is accompanied by signs of axial and radiating pain (Millecamps et al., 2011b, Tajerian et al., 2011b, Millecamps et al., 2012). In the current study, we studied 6 month old animals for the following reasons: 1) both the DD and the LBP phenotypes are observed this age 2) high variability exists in

both the number of effected discs and in the severity of DD and 3) animals do not yet exhibit signs of disc herniation or motor impairment at this age. Thus, in the current study we are using it as a model of DD-related LBP in the absence of herniation.

The aim of this study is to determine whether radiographic measurements of disc height and shape correlate with axial and radiating components of pain in the SPARC-null model of DD. This will help us elucidate the link between pain and disc anatomy in the SPARC-null model of disc degeneration.

2.3 Materials and Methods

Mice

SPARC-null mice were developed on a mixed C57BL/6 x129 SVJ background (Norose et al., 1998). Since inbred mouse strains can have different pain behavioral responses, SPARC-null mice were backcrossed onto a standard C57BL/6 background for > 12 generations and are considered to be fully congenic. We therefore used commercially available C57BL/6 mice (Charles River, Quebec, Canada) as wild-type (WT) controls.

6 month-old male SPARC-null and WT control mice, both bred in-house, were used. Sample size ranged between 34-55 mice/group. Animals were housed in groups of 2-5, had unrestricted access to food and water and were on a 12-hr light-dark cycle. All experiments were performed blind to genotype. All experiments were approved by the Animal Care Committee at McGill University, and conformed to the ethical guidelines of the Canadian Council on Animal Care and the guidelines of the Committee for Research and Ethical Issues of IASP published in PAIN, 16 (1983) 109-110.

Radiological Assessment of Disc degeneration

Animals were deeply anesthetized and perfused through the left cardiac ventricle with PBS. The lumbar spine (L1-S1) was fixed overnight in 4% PFA solution following the gross removal of the lumbar spinal muscles. Lateral x-ray images of

the intact lumbar spine were then taken at 4x using a Faxitron ® MX-20 (Faxitron X-Ray LLC, Lincolnshire, IL).

Calculation of Disc Height

Disc Height Index (DHI) was determined according to the following equation: Disc Height Index (DHI) = 2 x (DH1 + DH2 + DH3) / (A1 + A2 + A3 + B1 + B2 + B3), where A and B represent the length of the vertebral bone immediately rostral and caudal to the IVD, respectively; and DH represents the disc height between adjacent vertebrae (**Figure 1a**) (Lu et al., 1997).

Calculation of Disc Wedging

To calculate the wedging angle, a blind observer traced a vector from the dorsal to the ventral end of each vertebral growth plate using ImagePro© software. The two vectors adjacent to each IVD were then extended to their intersection point, and the resulting angle was recorded (**Figure 1b**).

Behavioral Analysis

Behavioral Measures of Radiating Hypersensitivity

Sensitivity to Cold Stimuli: We used a modified version of the acetone drop test (Choi et al., 1994), where the total duration of acetone-evoked behaviors (AEBs, flinching, licking or biting) were measured in seconds for 1 minute after a drop of acetone (~25 µl) was applied to the dorsal surface of the hindpaw. Increased behavioral response to acetone suggests the development of cold hypersensitivity and decreased reactivity is suggestive of anti-hyperalgesic activity.

Sensitivity to Mechanical Stimuli: A series of calibrated monofilaments (Stoelting Co., Wood Dale, IL) were applied with increasing force to the plantar surface of the hindpaw until the animal responded by withdrawing. The 50% threshold to withdraw (grams) was calculated as previously described (Chaplan et al., 1994). The stimulus intensity ranged from 0.008g to 4g. A decrease in threshold suggests the development of mechanical hypersensitivity and increased threshold is suggestive of anti-nociceptive or anti-hyperalgesic activity.

Behavioral Measures of Axial Discomfort

Grip Force Assay: This assay is adapted from a protocol previously developed to assess chronic deep tissue pain evoked by movement in rodents subjected to muscle inflammation or tumor implantation (Kehl et al., 2000, Kehl et al., 2003, Wacnik et al., 2003). Mice grip a metal bar attached to a Grip Strength Meter (Stoelting Co., Wood Dale, IL) and are gently pulled back by the tail, exerting a stretching force. The peak force in grams at the point of release is recorded twice at a 10-minute interval, and the average measurement was calculated. A decrease in grip force is interpreted as a measure of stretch-induced discomfort (Millecamps et al., 2011b).

Tail Suspension Assay: This test is adapted from a traditional assay used to measure depression (Steru et al., 1985), and we have shown that it reliably measures signs of axial pain in mice (Millecamps et al., 2011a, Millecamps et al., 2012). The duration of immobility reflects the animal's willingness to stretch its main body axis and decreased immobility is indicative of axial discomfort. Mice

were suspended individually underneath a platform by the tail with adhesive tape attached 0.5 to 1 cm from the base of the tail and were videotaped for 180 sec. The duration of time spent in a) immobility (not moving but stretched out), b) rearing (trying to reach the underside of the platform), c) full extension (actively reaching for the floor), and d) self-supported (holding either the base of its tail or the tape) are determined. This test is adapted from a traditional assay used to measure depression (Steru et al., 1985).

Behavioral Measures of Motor Capacity

Rotorod Assay: We used the accelerating rotorod assay to monitor animals for decreased motor function (Jones and Roberts, 1968) (IITC Life Science Inc., Woodland Hills, CA) with the mouse adapter (rod diameter, 3.2 cm). The task includes a speed ramp from 0 to 30 rotations per minute over 60 seconds, followed by an additional 240 seconds at the maximal speed. Mice were not trained prior to testing sessions.

Open Field Assay: A transparent open field apparatus (24 x 24 cm) was placed in a quiet room illuminated with white light. The floor of the apparatus was equally divided into nine squares (8 x 8 cm^2). Mice were individually placed into the open field on the central square, and their spontaneous behavior was videotaped for 5 min. Subsequent analysis of the total number of squares visited was used to assess general motor activity (Millecamps et al., 2007).

Data Analysis

All data are expressed and plotted as mean ± S.E.M. $P<0.05$ was considered to be statistically significant.

IVD anatomy

IVD height and wedging angle were quantified and comparisons between SPARC-null and WT mice were performed using the 1-tailed student's t-test.

Behavioral measures

Comparisons between SPARC-null and WT mice were performed for each behavioral measure using the 2-tailed student's t-test. Welch's correction was used when the condition of equal variances was not met.

Correlation analysis

Correlations between behavioral and radiographic measures were quantified by calculating Pearson's coefficient for data that followed a Gaussian distribution, and by calculating Spearman's coefficient when data did not follow a Gaussian distribution.

All statistical analyses were performed using the Graphpad prism software©.

2.4 Results

SPARC-null mice show radiographic signs of disc degeneration at 6 months of age

Disc Height

To determine if SPARC-null mice show loss of IVD height, we performed radiographic measurements of all 5 lumbar IVDs per mouse normalized to the length of adjacent vertebrae (**Figure 1a**). At the age of 6 months, SPARC-null mice have significantly narrower average lumbar discs than WT mice (mean =0.11±0.0 in WT vs. 0.08±0.002 in SPARC-null mice, $p<0.0001$, n=26-48/group, data not shown**)**. At the individual IVD level, differences were observed at the L2-L3 ($p=0.03$) and L3-L4 ($p=0.04$) levels (**Figure 1c**).

Disc Wedging

To determine changes in the shape of the IVD, the angle of disc wedging was measured for individual lumbar vertebra (**Figure 1b**). At the age of 6 months, SPARC-null mice show an average increase in lumbar disc wedging (mean=16.5±0.9° in SPARC-null vs. 12.9±0.5° in WT mice, $p=0.001$, n=34-39/group, data not shown**)**. Furthermore, they show greater wedging at each of the lumbar levels (n=34-39/group, **Figure 1d**)

SPARC-null mice show behavioral signs of radiating and axial low back pain at 6 months of age in the absence of motor impairment

Radiating LBP

SPARC-null mice show cold allodynia on the hindpaw in the acetone assay (2.32±0.38s vs. 0.7±0.2s for SPARC-null and WT mice, respectively, p=0.0003, **Figure 2a**). However, they do not differ from WT mice in their thresholds for mechanical sensitivity measured by the von Frey test (0.75±0.05g for WT vs. 0.72±0.07g for SPARC-null, p=0.8, **Figure 3a, first row)**.

Axial LBP

In order to assess axial discomfort, the grip force and the tail suspension assays were used. The first assay measures the willingness of the animal to stretch its body axis by using applied force. SPARC-null animals exhibit a significantly lower grip force (113.1±3.8g) than their WT conspecifics (126.9±3.4g, p=0.008, **Figure 3a, second row). In** the second assay, the duration of immobility in the tail suspension task is used as a measure of axial discomfort. In WT mice, this task is relatively well tolerated, since WT mice spend most of the time in immobility. This is not the case in SPARC-null mice that are more reluctant to remain immobile and allow their body axes to be stretched by gravity (110.9±7.4s vs. 73.7±6.8s in WT and SPARC-null mice, respectively, p=0.0004, **Figure 3a, third row).**

Motor function: SPARC-null mice do not show signs of motor impairment, since they cover more distance in the open field assay compared to WT animals (37.2±3.2squares vs. 57.8±3.7squares in WT and SPARC-null mice, respectively, $p<0.0001$, **Figure 3a, fourth row).** Furthermore, the two groups perform equally well in the rotorod assay of motor coordination 37.2±3.2s vs. 57.8±3.7s in WT and SPARC-null mice, respectively, $p<0.0001$, **Figure 3a, fifth row).**

Lumbar IVD height and wedging correlate with cold allodynia in SPARC-null mice only

Cold allodynia, the only manifestation of radiating pain in the SPARC-null model, showed a negative correlation with disc height at the L3-L4 level (Spearman correlation, $r=-0.4$, $p=0.008$ $n=44$). Disc wedging showed a positive correlation with cold sensitivity at the L1-L2 (Spearman correlation, $r=0.3$, $p=0.02$ $n=54$) and L2-L3 levels (Spearman correlation, $r=0.3$, $p=0.02$ $n=54$). No correlations between cold sensitivity and lumbar IVD anatomy were observed in WT mice (**Figure 2c, Tables 1 and 2**).

Mechanical sensitivity, measured by the von Frey filament test, did not correlate with any of the anatomical measures in both strains of mice. The correlation coefficients for disc height and wedging are shown in **Tables 1** and **2**, respectively.

Radiographical measures of IVD height and shape do not correlate with axial discomfort or motor function

Measures of axial pain, represented by scores on the grip and tail suspension assays, did not show a significant correlation with either disc height or wedging (**Figure 3b,c**). This was true for both SPARC-null and WT strains. Furthermore, signs of overall activity and motor function also did not show a significant correlation with IVD height or wedging in both strains (**Figure 3b,c**).

The correlation coefficients for disc height and wedging are shown in **Tables 1** and **2**, respectively.

Summary

At 6 months of age, SPARC-null mice show signs of disc degeneration that are detectable by radiography. This is accompanied by behavioral indices of axial pain along the low back region and radiating pain in the form of cold sensitivity on the hindpaw. Disc height and disc wedging both show significant correlations with the degree of cold sensitivity in this model of LBP.

2.5 Discussion

Chronic LBP is often ascribed to intervertebral disc degeneration (Allan and Waddell, 1989, Adams et al., 2000). In contrast, several studies have shown changes indicative of disc degeneration in subjects without overt signs of back pain (Boden et al., 1990, Jensen et al., 1994, Carragee et al., 2000), casting doubt on the association between DD and LBP. Understanding the relationship between signs of DD and LBP is crucial for the following reasons: First, it paves the way for further exploration into the mechanisms and treatment of back pain. For instance, therapies targeting the repair and regeneration of the IVD (Sobajima et al., 2004, Leung et al., 2006, Masuda, 2008) would be beneficial in cases where DD is known to be the cause of LBP. Second, it can improve diagnostic measures since diagnosing patients with LBP due to DD has always been a challenge for the clinician (Waddell, 1987) and a "non-specific LBP" verdict is commonly given. This is partly because there are many anatomical changes accompanying LBP, and it is not clear which of these changes could be considered pathological. Furthermore, mechanism-based treatment is only useful when the physician can make the proper diagnosis.

We report a significant correlation between radiographic signs of disc narrowing and behavioral signs of radiating pain in a mouse model of chronic LBP. This is consistent with the proposed mechanism of radiating pain, which is thought to be mainly neuropathic: decreases in disc height could lead to nerve root compression, thus leading to pain. These results are also consistent with clinical

findings where the anterior disc height at the L3-L4 level correlates with signs of sciatica (Hurme et al., 1989). It is important to note that, in our study, this correlation was observed in the absence of disc herniation. Finally, our data shows that radiographic measurements of disc height and disc shape did not reflect the severity of axial pain. This is consistent with reports of lack of correlation between axial low back pain and DD in humans (Borenstein et al., 2001).

The observed discrepancy between axial pain and DD implies that other factors, undetectable by imaging, play a major role in the development of axial LBP. Within the disc, changes in disc biochemistry such as decreased pH (Kitano et al., 1993), decreased proteoglycan content in the NP (Livshits et al., 2011), and increased levels of inflammatory cytokines (Le Maitre et al., 2007) are all well-recognized signs of DD which could be observed in the absence of loss of disc height or change in disc shape. Furthermore, increased innervation into the inner layer of the annulus could be a potential cause of nociception (Freemont et al., 1997) without directly affecting disc height or shape.

Other factors contributing to chronic LBP include anatomical changes outside of the intervertebral disc itself. “Modic changes”, defined as changes in the vertebral body marrow adjacent to the end plate (Modic et al., 1988), show a strong association with LBP (Kjaer et al., 2005). Also, changes in the endplate (Grant et al., 2002) are often concurrent with LBP in the late stages of DD (Harrington et al., 2001) and other tissues such as facet joints, muscles and ligaments could be involved (Kuslich et al., 1991, Schwarzer et al., 1994, 1995). Finally, psychosocial factors such as catastrophizing, poor coping skills, lack of social

support, and anxiety could play an important role in the severity of LBP in humans.

The absence of evidence linking disc degeneration to axial pain is by no means evidence of absence of such a link. Additional studies are needed to clarify the relationship between the two.

Limitations and future directions

The use of transgenic animals and animal models, in general, has inherent limitations. For example, measuring spontaneous pain behavior in mice poses a challenge, since we infer the existence of pain from mainly reflexive measures. However, the SPARC-null model is well validated (Millecamps et al., 2011b, Millecamps et al., 2012). It is also difficult to incorporate or account for psychosocial factors in animal models that are well established to play a role in clinical LBP.

The current study focused on radiological measures obtained only at the lumbar level. It is possible that including additional spinal segments or using more sensitive, histological based measurements of disc degeneration would yield additional information regarding the etiology of axial and/or radiating low back pain.

Conclusions

We show that loss of disc height and disc wedging are correlated to radiating but not axial pain in the SPARC-null mouse model of LBP, suggesting that the two

conditions have different underlying mechanisms. Our findings support the link between DD and LBP and emphasize the need to distinguish between axial and radiating pain in the study of and in the treatment of chronic low back pain.

2.6 Figures

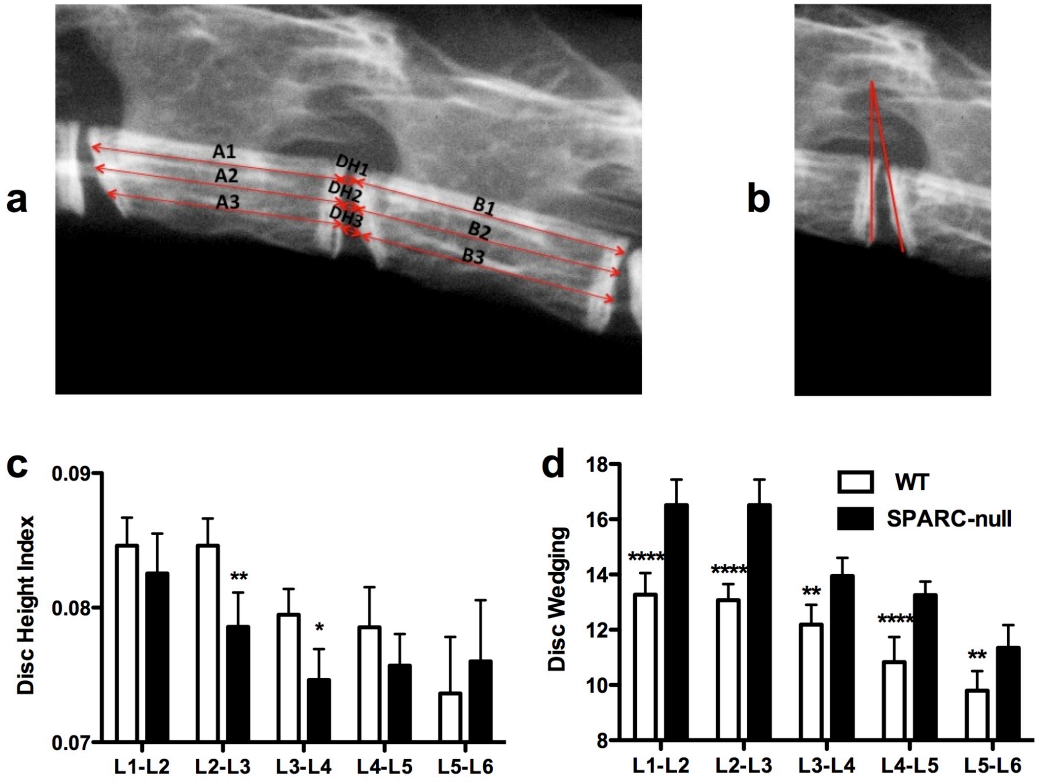


Figure 1 Lumbar disc height index (DHI) was calculated according to the formula: DHI = 2X(DH1 + DH2 + DH3) / (A1 + A2 + A3 + B1 + B2 + B3) as shown in **(a)**. Wedging was measured by calculating the angle between adjacent endplates as shown in (**b**). SPARC-null mice show decreases in lumbar disc height at 6 months of age **(c)** and increased signs of disc wedging at multiple spinal levels (**d**). *=p<0.05, **=p<0.001, ***=p<0.0001, ****=p<0.00001. n=34-40/group. Error bars indicate S.E.M.

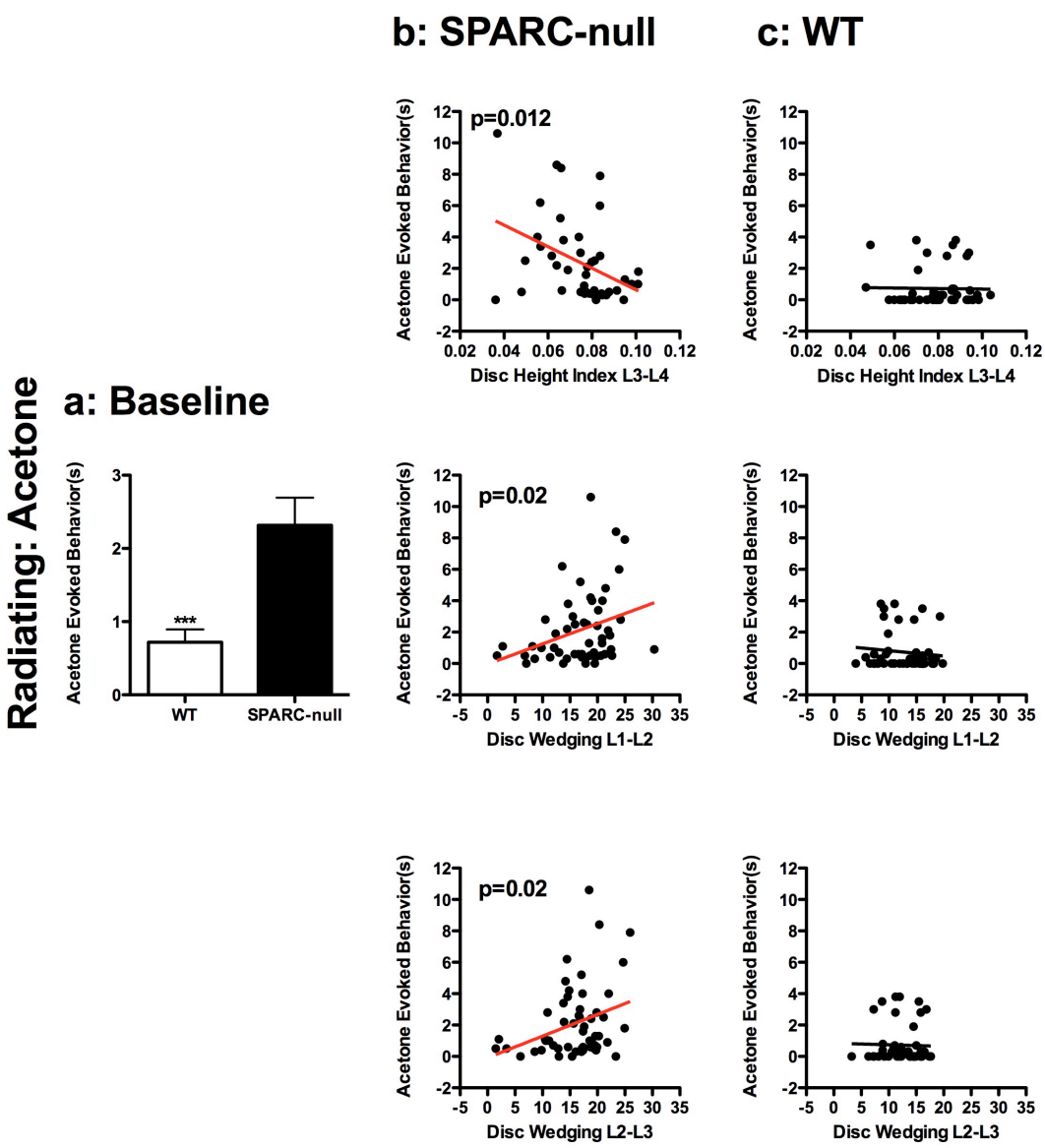
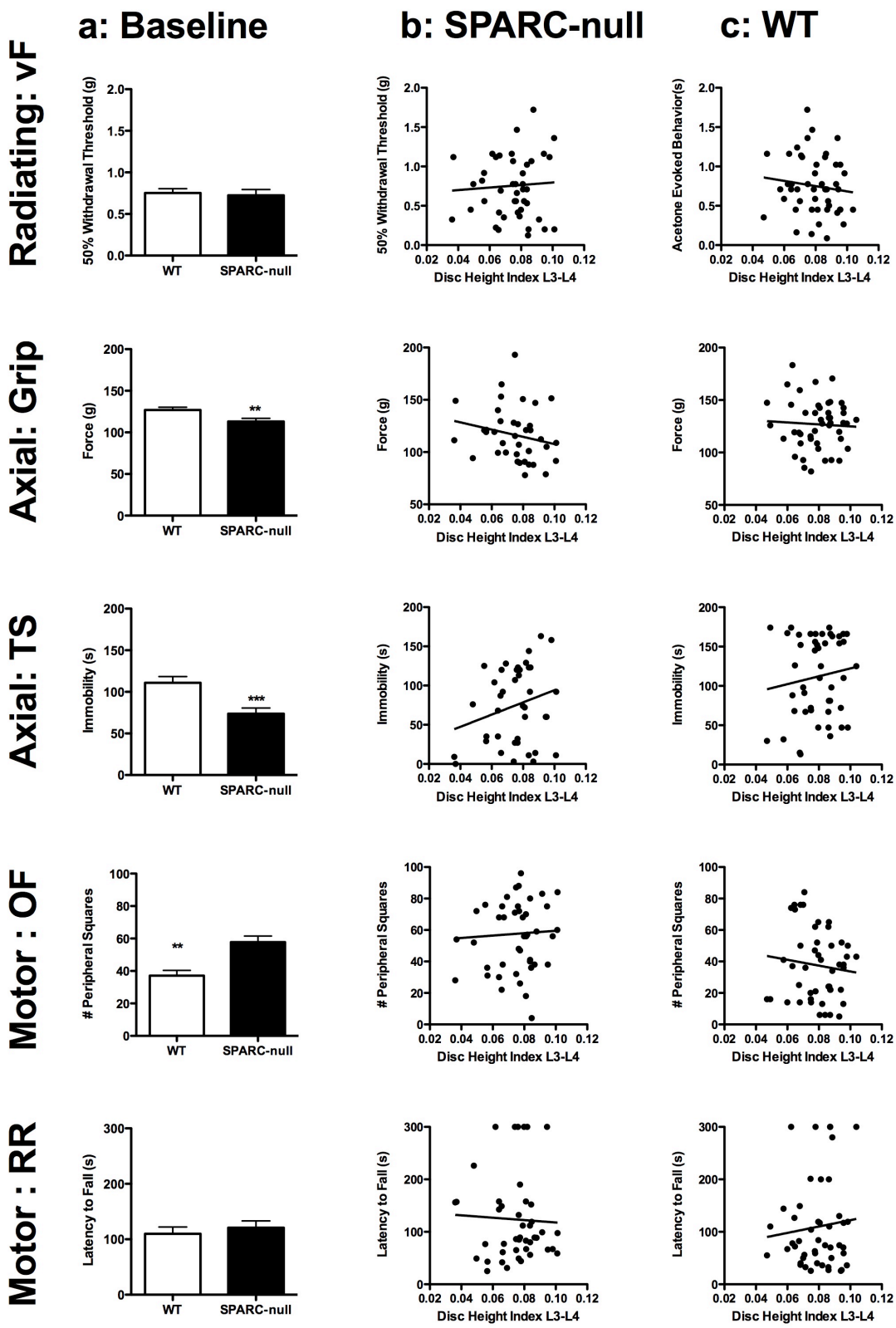


Figure 2 SPARC-null mice show signs of cold allodynia measured by increased duration of acetone-evoked behaviors (**a**). Cold sensitivity in these animals correlates inversely with disc height at the L3-L4 level and directly with disc wedging at both L1-L2 and L2-L3 levels (Pearson correlation) (**b**). Such correlations are absent in WT mice (**c**). ***=p<0.0001. n=34-54/group. Error bars indicate S.E.M.

Figure on Next Page

Figure 3 Baseline measurements show that SPARC null animals show signs of axial discomfort (rows 2&3), but no signs of mechanical hypersensitivity (row 1) or motor impairment (rows 4&5) compared to WT mice (**a**). Furthermore, mechanical sensitivity, axial pain, and motor function all fail to show a correlation (Pearson correlation) with disc height or wedging. While the data for L3/4 are depicted in this figures (**b,c**), the data were similar at all lumbar levels (see Tables 1,2). **=$p<0.001$. n=34-54/group. Error bars indicate S.E.M.


a: Baseline
b: SPARC-null
c: WT
Radiating: vF
Axial: Grip
Axial: TS
Motor : OF
Motor : RR
50% Withdrawal Threshold (g)
Acetone Evoked Behavior(s)
Force (g)
Immobility (s)
Peripheral Squares
Latency to Fall (s)
Disc Height Index L3-L4
WT
SPARC-null
**
***

2.7 Tables

Table 1: Summary of correlation coefficients between behavior and lumbar disc height index.

Assay	Strain	L1/2	L2/3	L3/4	L4/5	L5/6	Average
Acetone (Cold allodynia)	**SPARC-null**	-0.04 (-0.3-0.25)	0.02 (-0.3-0.3)	**-0.4 ** (-0.6-0)**	0.05 (-0.3-0.4)	0.15 (-0.2-0.5)	-0.09 (-0.5-0.3)
	WT	0.3 (0-0.5)	0.2 (0-0.5)	-0.02 (-0.3-0.3)	0.1 (-0.2-0.4)	0.3 (0-0.5)	0.2 (-0.1-0.5)
Von Frey (Mechanical sensitivity)	**SPARC-null**	-0.3 (-0.6-0)	0 (-0.3-0.3)	0.06 (-0.2-0.3)	0.2 (0.1-0.5)	0 (-0.4-0.4)	-0.1 (-0.4-0.2)
	WT	0.1 (-0.2-0.4)	0.02 (-0.3-0.3)	-0.1 (-0.4-0.2)	-0.1 (-0.4-0.2)	0.04 (-0.3-0.3)	-0.06 (-0.4-0.3)
Grip (Axial pain)	**SPARC-null**	-0.09 (-0.4-0.2)	-0.1 (-0.4-0.2)	-0.1 (-0.4-0.2)	0.1 (-0.2-0.4)	-0.03 (-0.4-0.3)	-0.02 (-0.3-0.3)
	WT	-0.1 (-0.4-0.2)	-0.02 (-0.3-0.3)	0.1 (-0.4-0.2)	-0.1 (-0.4-0.2)	-0.2 (-0.5-0.2)	0.1 (-0.4-0.2)
Tail Suspension (Axial pain)	**SPARC-null**	0.1 (-0.2-0.4)	-0.03 (-0.3-0.3)	0.2 (-0.1-0.4)	-0.2 (-0.5-0.09)	-0.3 (-0.7-0)	-0.2 (-0.4-0.1)
	WT	0.03 (-0.3-0.3)	-0.01 (-0.3-0.3)	0.1 (-0.2-0.4)	0.03 (-0.3-0.3)	0.1 (-0.2-0.4)	0.08 (-0.2-0.4)
Open field (Overall activity)	**SPARC-null**	0.01 (-0.3-0.3)	0.02 (-0.3-0.3)	-0.3 (-0.5-0.03)	-0.2 (-0.4-0.2)	-0.2 (-0.5-0.2)	-0.2 (-0.6-0.1)
	WT	0.03 (-0.3-0.3)	-0.3 (-0.6-0)	-0.1 (-0.4-0.2)	-0.07 (-0.4-0.2)	-0.01 (-0.3-0.3)	-0.08 (-0.4-0.2)
Rotorod (Motor incoordination)	**SPARC-null**	0.03 (-0.3-0.3)	0.1 (-0.2-0.4)	-0.2 (-0.4-0.1)	-0.05 (-0.4-0.3)	-0.1 (-0.5-0.2)	-0.07 (-0.4-0.3)
	WT	0.1 (-0.2-0.4)	0.1 (-0.2-0.4)	0.02 (-0.3-0.3)	0.02 (-0.3-0.3)	-0.08 (-0.4-0.2)	0.06 (-0.2-0.4)

Table 2: Summary of correlation coefficients between behavior and angle of lumbar disc wedging.

Assay	Strain	L1/2	L2/3	L3/4	L4/5	L5/6	Average
Acetone (Cold allodynia)	**SPARC-null**	**0.3* (0.05-0.5)**	**0.3* (0.05-0.5)**	0.2 (-0.02-0.5)	0.09 (-0.2-0.3)	0.1 (-0.2-0.4)	0.3 (-0.03-0.6)
	WT	-0.1 (-0.4-0.2)	-0.03 (-0.3-0.3)	0.1 (-0.2-0.4)	0.1 (-0.2-0.4)	0.3 (-0.1-0.6)	0.06 (-0.3-0.4)
Von Frey (Mechanical sensitivity)	**SPARC-null**	-0.07 (-0.3-0.3)	0.03 (-0.3-0.3)	-0.04 (-0.3-0.3)	0.2 (-0.1-0.4)	0.1 (-0.2-0.4)	0.04 (-0.3-0.4)
	WT	-0.1 (-0.4-0.2)	-0.05 (-0.3-0.2)	-0.2 (-0.4-0.1)	-0.08 (-0.4-0.2)	-0.2 (-0.5-0.1)	-0.3 (-0.5-0.1)
Grip (Axial pain)	**SPARC-null**	0.1 (-0.2-0.4)	0.06 (-0.3-0.3)	0.02 (-0.3-0.3)	0.1 (-0.2-0.4)	-0.1 (-0.4-0.1)	0.04 (-0.3-0.3)
	WT	0.07 (-0.2-0.3)	0.01 (-0.3-0.3)	-0.1 (-0.4-0.2)	-0.2 (-0.5-0.1)	-0.1 (-0.4-0.2)	-0.01 (-0.3-0.3)
Tail Suspension (Axial pain)	**SPARC-null**	0.04 (-0.3-0.4)	-0.06 (-0.4-0.3)	0.01 (-0.3-0.3)	-0.02 (-0.3-0.3)	-0.04 (-0.4-0.3)	-0.02 (-0.3-0.3)
	WT	0.01 (-0.3-0.3)	-0.02 (-0.3-0.3)	-0.03 (-0.3-0.3)	-0.06 (-0.4-0.2)	0.1 (-0.2-0.4)	0.03 (-0.3-0.3)
Open field (Overall activity)	**SPARC-null**	-0.2 (-0.5-0.1)	-0.03 (-0.3-0.2)	-0.02 (-0.3-0.3)	0 (-0.3-0.3)	0.05 (-0.3-0.3)	-0.05 (-0.4-0.2)
	WT	-0.2 (-0.5-0.1)	0.1 (-0.2-0.4)	-0.2 (-0.5-0.1)	0.08 (-0.2-0.4)	-0.1 (-0.5-0.2)	-0.2 (-0.5-0.2)
Rotorod (Motor incoordination)	**SPARC-null**	0.03 (-0.3-0.3)	-0.04 (-0.3-0.3)	0.02 (-0.3-0.3)	-0.03 (-0.4-0.3)	0.09 (-0.2-0.3)	0.02 (-0.3-0.3)
	WT	0.3 (-0.1-0.6)	0.3 (-0.1-0.6)	0.09 (-0.2-0.4)	0.02 (-0.3-0.3)	0.05 (-0.3-0.4)	0.3 (-0.04-0.6)

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3 Pharmacological Profile of Chronic Low Back Pain in SPARC-null Mice

Preface

While the previous chapter focused on understanding the link between disc radiography and pain, the current chapter will focus on the mechanisms of axial and radiating pain in the SPARC-null mouse using pharmacological tools.

Understanding pain mechanisms is crucial in choosing an efficient treatment route in patients suffering from chronic LBP. Prescribing pharmacological agents that do not target the mechanism causing pain will not only result in lack of analgesic efficacy, but can also be accompanied by unnecessary side effects.

SPARC-null mice exhibit signs of both axial and radiating pain. We therefore tested the analgesic efficacy of various pharmacological agents in behavioral assays measuring these pain components. The choice of drugs tested was based on many factors such as: guidelines for treating patients with chronic LBP, the tolerability of the drug in mice, and the route of administration. Since the object of the study was to explore the mechanisms of axial vs. radiating pain, we selected drugs with well-characterized mechanisms of action. In addition to morphine, the "gold standard" in analgesia, we examined the efficacy of an alpha-adrenergic agonist (clonidine), anti-inflammatory agents (dexamethasone and ibuprofen) and anti-neuropathic agents (pregabalin and amitriptyline). A full dose-response curve was generated for most of the drugs. In cases where only a single dose was tested, the choice of dose was based on published literature.

Manuscript: Axial Pain and Radiating Pain have Distinct Pharmacological Profiles in the SPARC-Null Mouse Model Of Low Back Pain

3.1 Abstract

Chronic low back pain is a devastating and common condition that comprises of axial pain in the low back region and radiating pain in one or both legs. Many structures of the lumbar spine play a role in low back pain, including intervertebral discs. Depending on the pathology, it might involve nociceptive, inflammatory, or neuropathic components. The mechanisms of axial and radiating low back pain are not well understood, leading to the adoption of ineffective treatment strategies.

The SPARC-null mouse is a model of chronic low back pain associated with intervertebral disc degeneration that incorporates behavioral signs of both axial and radiating pain. We have used six-month old SPARC-null and WT mice to test the effect of intraperitoneally administered pharmacological compounds belonging to different classes: morphine, clonidine, pregabalin, amitriptyline, dexamethasone, and ibuprofen, compared to a saline control. The following behavioral assays were used: tail suspension for axial pain, von Frey filament test

and the acetone test for radicular pain, and the rotorod and open field assays for overall motor function.

Our results show that axial pain was reversed by morphine, clonidine, pregabalin, amitriptyline, and ibuprofen, while radiating pain was only sensitive to clonidine and pregabalin. These distinct pharmacological profiles suggest mechanistic differences: while axial pain has a mixed nociceptive/inflammatory/neuropathic profile, radiating pain is primarily neuropathic.

This study draws attention to the need for mechanism-based treatment of axial and radiating low back pain as independent phenomena, using agents targeting their respective underlying mechanisms.

3.2 Introduction

Chronic low back pain (LBP) is a multifactorial condition with serious health and economic consequences (Kent and Keating, 2005). It is estimated that roughly half of all adults suffer from LBP during any given year (Reisbord and Greenland, 1985, Deyo et al., 2006), with ~10% of cases leading to chronic LBP (Quittan, 2002). LBP can be differentiated into axial vs. radiating pain. Axial LBP is characterized by spontaneous or movement-evoked pain or soreness confined to the spine and low back region. Radiating pain radiates down one or both legs. One factor associated with an increased risk of chronic LBP is degeneration of the intervertebral discs (IVDs) (Luoma et al., 2000b, Raj, 2008, Samartzis et al., 2011).

Despite the development of many treatment guidelines for chronic LBP (Chou et al., 2007, Savigny et al., 2009), adequate pain relief remains lacking (Morlion, 2011). Depending on the underlying mechanisms, responses to treatments vary widely. Unfortunately, therapeutic decisions are based on trial and error, a situation frustrating to physician and patient. In order to improve therapeutic outcome and quality of life for people with chronic pain, we need an improved understanding of the underlying mechanisms. Given the complexity of LBP, an animal model that incorporates clinically relevant signs of axial and radiating LBP is needed to accelerate discovery in this area.

We have recently introduced the SPARC-null mouse model of chronic LBP (Millecamps et al., 2011b). SPARC (secreted protein, acidic, rich in cysteine; aka

osteonectin or BM-40) is an evolutionarily conserved collagen-binding protein present in IVDs. Decreased expression of SPARC is associated with aging and degeneration in human IVDs and targeted deletion of the SPARC gene results in accelerated disc degeneration in mice (Gruber et al., 2004, Gruber et al., 2005). We have shown that SPARC-null mice exhibit behavioral signs of both axial and radiating pain that increase with age and severity of disc degeneration (Millecamps et al., 2011b). SPARC-null mice resist gravity induced stretching of the spine in the tail suspension and grip force assays, which serve as measures of axial discomfort (Millecamps et al., 2011b). SPARC-null mice also develop cold allodynia on the hindpaw by 6 months of age, which we interpret as a sign of radiating pain. Cold allodynia is consistent with patient reports of cold sensations in their feet (Ljunggren, 1983).

The goal of the current study is to use the SPARC-null mouse model of chronic LBP to evaluate the pharmacological profiles of axial and radiating components of LBP associated with disc degeneration. We therefore assessed the efficacy of clinically-used compounds with diverse mechanisms of action: morphine - the "gold standard" in analgesic treatment, clonidine- a broad spectrum analgesic effective in inflammatory and neuropathic pain, pregabalin - the "gold standard" for nerve-injury induced pain, amitriptyline - a tricyclic anti-depressant (TCA) commonly used in the treatment of chronic LBP, dexamethasone - a steroidal anti-inflammatory agent, and the non-steroidal anti-inflammatory agent, ibuprofen, which is often the first line of defense against LBP. These drugs were selected

because of their different mechanisms of action, thus enabling us to explore the mechanisms of axial and radiating pain in our model.

This study supports the hypothesis that axial and radiating components of chronic LBP have different mechanisms that respond differentially to pharmacological treatment. These results could be useful in inspiring mechanism-based treatment of pain in the clinic.

3.3 Materials and Methods

Mice

SPARC-null mice were developed on a mixed C57BL/6 x129 SVJ background and backcrossed onto a standard C57BL/6 background for > 12 generations to be considered fully congenic (Norose et al., 1998). We therefore used commercially available C57BL/6 mice (Charles River, Quebec, Canada) as wild-type (WT) controls as has been done in previous studies (Brekken et al., 2003, Millecamps et al., 2011b, Tajerian et al., 2011a).

4-6 month old male SPARC-null (n=210) and WT control mice (n=185), both bred in-house, were used in this study. Animals were housed in groups of 2-5 at ~24°C, had unrestricted access to food and water, and were on a 12-hr light-dark cycle. All experiments were performed blind to genotype and treatment, using a randomized block design. All experiments were approved by the Animal Care Committee at McGill University, and conformed to the ethical guidelines of the Canadian Council on Animal Care and the guidelines of the Committee for Research and Ethical Issues of IASP published in PAIN, 16 (1983) 109-110.

Assessment of Disc Degeneration

Histological Analysis

Animals were deeply anesthetized and perfused through the left cardiac ventricle with PBS followed by 200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer,

pH 7.4, at room temperature. The T1 to S4 spinal segment was collected and post-fixed in the same fixative overnight at 4°C. Spinal columns were dissected and decalcified by immersion in 4% EDTA at 4°C for 14 days. The EDTA was changed twice a week. Samples were then cryo-protected in 30% sucrose in phosphate buffer for 4 days at 4°C, and embedded in OCT (Tissue-Tek). 16-micron sections were cut with a cryostat (Leica CM3050S) in the sagittal plane and thaw-mounted onto gelatin-coated slides for subsequent staining. Staining was performed using the FAST protocol developed by Leung et al., 2009 for intervertebral discs(Leung et al., 2009). In brief, staining consisted of consecutive baths in (1) acidic Alcian Blue, 10 min, (2) Safranin-O, 2:30 min, (3) 50% Ethanol, 1 min, (4) Tartrazine, 10 sec, (5) Fast-green, 5 min. After drying, slides were mounted with DPX (all from Sigma-Aldrich, USA). This method clearly distinguishes intervertebral disc compartments and shows matrix remodeling within the disc (Leung et al., 2009).

Evans blue

This assay was used to measure the degree of vascular permeability to the dye in lumbar IVDs extracted from SPARC-null and WT mice. 300µl of 2% Evans blue solution was injected intravenously through the tail vein. 4 hours after injection, mice were deeply anesthetized and perfused through the left cardiac ventricle with PBS for 20 minutes. The 5 lumbar IVD were then carefully dissected and allowed to dry at 60°C overnight. The desiccated IVDs were kept in 1ml formamide for 48 hours to extract the Evans blue. Reading of the Evans blue concentration was done at λ=620 using a SpectraMax M2E plate reader (Molecular Devices, USA).

A standard curve was constructed with Evans blue diluted in formamide.

Behavioral Analysis

Tail Suspension Assay

Mice were suspended individually underneath a platform by the tail with adhesive tape attached 0.5 to 1 cm from the base of the tail and were videotaped for 180 sec. The duration of time spent in a) immobility (not moving but stretched out), b) rearing (trying to reach the underside of the platform), c) full extension (actively reaching for the floor), and d) self-supported (holding either the base of its tail or the tape) were determined. This test is adapted from a traditional assay used to measure depression (Steru et al., 1985).

Sensitivity to Cold Stimuli

Animals were habituated to the testing apparatus for 1 hour before the start of the experiment. We used a modified version of the acetone drop test(Choi et al., 1994), where the total duration of acetone-evoked behaviors (AEBs, flinching, licking or biting) were measured in seconds for 1 minute after a drop of acetone (~25 µl) was applied to the plantar surface of the hindpaw. Increased behavioral response to acetone suggests the development of cold hypersensitivity and decreased reactivity is suggestive of anti-hyperalgesic activity. A cutoff of 4 seconds was applied when interpreting the data.

Sensitivity to Mechanical Stimuli

Animals were habituated to the testing apparatus for 1 hour before the start of the experiment. A series of calibrated monofilaments (Stoelting Co., Wood Dale, IL) was applied with increasing force to the plantar surface of the hindpaw until the animal responded by withdrawing. The 50% threshold to withdraw (grams) was calculated as previously described (Chaplan et al., 1994). The stimulus intensity ranged from 0.008g to 4g. A decrease in threshold suggests the development of mechanical hypersensitivity and increased threshold is suggestive of anti-nociceptive or anti-hyperalgesic activity. A cutoff of 2g was applied when interpreting the data.

Rotorod Assay

We used the accelerating rotorod assay to monitor animals for motor function (IITC Life Science Inc., Woodland Hills, CA) with the mouse adapter (rod diameter, 3.2 cm) (Jones and Roberts, 1968). The task includes a speed ramp from 0 to 30 rotations per minute over 60 seconds, followed by an additional 240 seconds at the maximal speed. Mice were not trained prior to testing sessions. A cutoff of 200s was used when interpreting the data.

Open Field Assay

A transparent open field apparatus (24 x 24 cm^2) was placed in a quiet room illuminated with white light. The floor of the apparatus was equally divided into nine squares (8 x 8 cm^2). Mice were individually placed into the open field on the

central square, and their spontaneous behavior was videotaped for 5 min. Subsequent analysis of the total number of squares visited was used to assess general motor activity (Millecamps et al., 2007). Following drug administration, animals underwent tail suspension just before being placed in the open field.

Timeline

The schedule of testing was as follows: 16 weeks of age: habituation to tail suspension; 20 weeks: baseline open field and tail suspension assays; 24 weeks: baseline and post-drug administration in the von Frey, acetone, and rotorod assays; 26 weeks: tail suspension and open field after drug administration; 30 weeks: Euthanasia and histology. A washout period of 2 weeks was included between drug exposures to ensure that only the acute effects of each drug were studied.

Pharmacological Treatment

Analgesic agents or saline control were administered to SPARC-null and WT mice by i.p. injection (5ml/kg injected directly in the intra-peritoneal cavity). The following drugs were dispensed: Morphine 0.1, 1, 3, 6, 10, and 30 mg/kg (Medisca Inc., Montreal, Quebec); Clonidine 0.001, 0.01, 1, and 10mg/kg (Sigma-Aldrich Canada Ltd., Oakville, Ontario); Pregabalin 1, 10, and 100mg/kg (Pfizer Inc., Groton, Connecticut); Amitriptyline 10 mg/kg (Sigma-Aldrich Canada Ltd., Oakville, Ontario); Dexamethasone 10mg/kg (Sigma-Aldrich Canada Ltd., Oakville, Ontario); and Ibuprofen 200mg/kg (Sigma-Aldrich Canada Ltd., Oakville, Ontario). All drugs were dissolved in 0.9% saline. Animals were tested

60 minutes after drug administration, except for dexamethasone (45 minutes) and ibuprofen (120 minutes). Since data from animals receiving saline did not differ at 45, 60 or 120 minutes, all saline groups were pooled together.

Data Analysis

IVD Permeability

IVD permeability by Evans blue was quantified and comparisons between the SPARC-null and WT groups were performed by 1-tailed, unpaired t-test. Sample size = 6 mice/group.

Baseline Behavioral Measurements

Data was tested for normality (D'Agostino-Pearson test) and, having passed the normality test, a 2-tailed, unpaired t-test was then used for comparisons between baseline SPARC-null and WT mice. This included all animals used in this study and tests were performed for each assay. Welch's correction was used when the condition of equal variances was not met. Sample size ranged between 35-96 mice/group.

Post-treatment Behavioral Measurements

Single-dose drug treatment groups were tested for normality (D'Agostino-Pearson test) and, having passed the normality test, were then compared to the saline – treated group by 2-tailed, unpaired t-test. Welch's correction was used when the condition of equal variances was not met. Sample size ranged between 7-15 mice/group.

All statistical analysis described above were performed using the Graphpad prism software©. All data are expressed and plotted as mean ± S.E.M. $P<0.05$ was considered to be statistically significant.

Dose-Response Analysis

Dose-response analysis was performed to evaluate the efficacy of drugs where 3 or more doses were tested. Individual dose points were converted to % Maximum Possible Effect according to the following equations: Tail suspension: % MPE = ((drug-saline)/(180-saline)) x 100; Mechanical threshold: % MPE = ((drug-saline)/(2-saline)) x 100; Acetone, Rotorod and Open Field: % MPE = ((saline-drug)/(saline)) x 100.

ED_{50} values and confidence limits were calculated according to the graded dose-response method of Tallarida and Murrayon the linear portion of each dose-response curve(Tallarida and Murray, 1981, Tallarida and Murray, 1987). A minimum of three doses was required for dose-response analysis. ED_{50} values were calculated if the slope of the curve was different from 0. All dose-response analyses were performed with the FlashCalc pharmacological statistics software package generously supplied by Dr. Michael Ossipov.

3.4 Results

Disc Degeneration in six month-old SPARC-null mice

Deletion of the SPARC gene has been previously reported by us and others to accelerate age-dependent disc degeneration (Gruber et al., 2005, Tajerian et al., 2011a). Since the current study was performed in 6 month-old mice, we evaluated six month-old SPARC-null and WT mice for disc degeneration.

Histology

Histological evaluation of SPARC-null lumbar spinal columns at 6 months of age revealed signs of disc degeneration, including loss of clear compartmentalization in the IVD and changes in proteoglycan content. Similar to what is observed in humans, where not all lumbar discs are equally degenerated, the degree of degeneration is not uniform across lumbar IVDs at this age. In the example shown in **Figure 1a,** the same animal has both healthy **(Figure 1b)** and degenerating **(Figure 1c)** discs. No signs of degeneration were observed in over >50 age-matched WT spines examined.

Permeability to Evans Blue

Vascular permeability of lumbar IVDs was measured in 6 month-old SPARC-null and WT mice following intravenous administration of Evans blue. SPARC-null IVDs were found to be more permeable to Evans blue 4 hours following injection compared to WT controls (WT = 0.29±0.03μg/mg, SPARC-null = 0.45±0.07μg/mg, **Figure 1c**).

Our results show that lumbar IVDs extracted from SPARC-null mice at 6 months of age show changes in proteoglycan composition, degradation of overall disc integrity, and increased vascular permeability compared to WT mice. In addition, reductions in disc height index are observed in SPARC-null mice by x-ray image at this age (data not shown, WT = 0.11±0.005, SPARC-null = 0.08±0.002, p=0.0001, also see Tajerian et. al., 2011)(Tajerian et al., 2011a).

Pharmacological Profile of Axial Low Back Pain

We have previously reported the use of the tail suspension assay as a measure of sensitivity to gravity-induced stretching of the spine(Millecamps et al., 2011a, Tajerian et al., 2011a). In this assay, animals can adopt one of four postures: immobility (no movement), full extension (reaching for the floor), rearing, and self-supported (holding on to either the tape or the tail). In WT mice, this task is relatively well tolerated and WT mice spend most of the time in immobility. In contrast, SPARC-null mice are more reluctant allow their body axes to be stretched by gravity in the immobile position; they therefore spend less time in this posture (Baseline values: WT = 113±11s, SPARC-null = 68±8s, p=0.0014, **Figure 2a**)**.** The duration of time spent in immobility was therefore used as an experimental endpoint for axial pain.

In the tail suspension assay, morphine (ED_{50}=10.1mg/kg, 95%CI=6.6-15.6**)**, clonidine (ED_{50}=0.05mg/kg, 95%CI=0.02-0.012), pregabalin (ED_{50}=5.3mg/kg, 95%CI=2.4-11.7), amitriptyline (10mg/kg), and ibuprofen (200mg/kg) all significantly increased the time spent in immobility in SPARC-null mice **(Figure**

2). In WT mice, efficacy was also observed with morphine (ED_{50}=17.5mg/kg, 95%CI=12.7-24.2), clonidine (ED_{50}=8.3mg/kg, 95%CI=0.6-116), amitriptyline (10mg/kg), and ibuprofen (200mg/kg). However, both morphine and clonidine were significantly less potent in WT mice. Saline treated animals did not differ from baseline measurement in this assay in either strain **(Figure 2a, table 1)**.

Pharmacological Profile of Radiating Pain

Radiating pain was assessed by measuring sensitivity to mechanical and cold stimuli on the hindpaw using the von Frey and acetone tests, respectively. At 6 months of age, SPARC-null mice exhibited cold allodynia measured as the total duration of acetone-evoked behaviors (AEBs), (Baseline values: WT=0.59±0.1s, SPARC-null=1.8±0.3s, $p<0.0001$, **Figure 3a**).

Morphine lacked efficacy in reversing acetone-induced behaviors both in SPARC-null and WT mice (**Figure 3b)**, while clonidine inhibited cold sensitivity in both SPARC-null (ED_{50}=0.08mg/kg, 95%CI=0.03-0.23) and WT (ED_{50}= 0.13mg/kg, 95%CI=0.04-0.44) strains with similar potency (**Figure 3c)**. Pregabalin produced dose-dependent analgesia in SPARC-null (ED_{50}=30.1mg/kg, 95%CI=14.4-63.0) but lacked efficacy in WT mice (**Figure 3d)**. Both SPARC-null and WT Saline treated animals showed increased AEBs compared to baseline measurements (WT baseline=0.59±0.1s vs. saline=1.01±0.1s, p=0.02; SPARC-null baseline=1.8±0.3s vs. saline=2.5±0.2s, p=0.004) **(Figure 3a, table 1)**.

In contrast to sensitivity to cold, SPARC-null mice do not differ from WT mice in mechanical sensitivity (Baseline values: WT=0.72±0.034g, SPARC-

null=0.76±0.003g, p=0.412, **Figure 4a)**. Since SPARC-null animals do not show signs of mechanical hypersensitivity on the hindpaw, only the full dose-response curve was explored for morphine. Morphine produced dose-dependent analgesia in both SPARC-null (ED_{50}=66mg/kg, 95%CI=44-97) and WT mice (ED_{50}=132mg/kg, 95%CI=82-213, **Figure 4b**). Clonidine (10 mg/kg) and pregabalin (100 mg/kg) also produced analgesia in both strains (**Figure 4c,d)**. Saline treated animals did not differ from baseline measurement in this assay in either strain **(Figure 4a, table 1)**.

Pharmacological Profile of Motor Impairment and Sedation

The behavioral assays used in this study rely on motor function and could be influenced by motor impairment, hyperactivity, or sedation. Any intervention that can affect motor ability can potentially mask or exaggerate a presumed analgesic effects of a drug. We therefore measured the efficacy of each drug in the rotorod and open field assays.

Six month-old SPARC-null mice show no signs of motor impairment in the rotorod assay measured as the latency to fall in seconds (Baseline values: WT=102±10s, SPARC-null=113±9s, p=0.39, **Figure 5a**). A small but significant improvement in the assay was observed in saline-treated SPARC-null mice (136±6s) compared to baseline (p=0.04, **Figure 5a**).

In SPARC-null mice, morphine (ED_{50}=8.0mg/kg, 95%CI=3.7-17, **Figure 5b**) and clonidine (ED_{50}=0.34mg/kg, 95%CI=0.13-0.88, **Figure 5c**) caused significant reductions in rotorod performance. Similar impairment was observed for

morphine (ED_{50}=16.6mg/kg, 95% CI=7.2-38.7 **Figure 5b**) and clonidine (ED_{50}=0.13mg/kg, 95% CI=0.03-0.62 **Figure 5c**) in WT mice. Pregabalin was not effective in either strain (**Figure 5d**), while Amitriptyline (10mg/kg) also significantly inhibited performance in SPARC-null animals only (**Figure 5e).**

The open field assay was performed twice in each animal, once before tail suspension and once immediately after tail suspension. In the initial 5-minute baseline testing period prior to tail suspension, SPARC-null mice covered more distance (measured by the number of peripheral squares crossed) compared to WT (WT=37.9±3.8squares, SPARC-null=58.9±3.4squares, $p<0.0001$, **Figure 6a**).

The second open field was done immediately after tail suspension in either saline or drug-treated animals. Saline-treated SPARC-null covered less distance than they did at baseline prior to tail suspension (Baseline=58.9±3.4squares, Saline-treated= 43.4±3.9squares, p=0.003, **Figure 6a**).

Morphine treatment resulted in a strong, dose-dependent increase in *activity* in both SPARC-null (ED_{50}=0.20mg/kg, 95%CI=0.13-0.30) and WT (ED_{50}=0.57mg/kg, 95%CI=0.35-0.93) mice (**Figure 6b**). Morphine is therefore not a sedative agent in this assay. In contrast, clonidine treatment resulted in a dose-dependent *decrease* in activity in both SPARC-null (ED_{50}=0.17mg/kg, 95%CI=0.05-0.59) and WT (ED_{50}=0.14mg/kg, 95%CI=0.05-0.44) mice (**Figure 6c)**. Sedation was also observed in WT mice following amitriptyline (10 mg/kg, **Figure 6e**) and ibuprofen (200 mg/kg, **Figure 6g**).

Summary of Results

At 6 months of age, SPARC null animals show signs of disc degeneration (**Figure 1**) accompanied by axial and radiating pain in the absence of motor dysfunction (**Figure 2a-6a**).

In SPARC-null animals, axial discomfort was improved by morphine, clonidine, pregabalin, amitriptyline, and ibuprofen (**Figure 2**). Cold allodynia, the only indication of radiating sensitivity in our model, was reduced by clonidine and pregabalin (**Figure 3)**. The rotorod test showed morphine, clonidine, pregabalin, and amitriptyline to cause motor impairment (**Figure 5)**, while the open field test showed morphine to cause severe hyper-activity (**Figure 6**). A summary of the applicable ED_{50} values is shown is **Table 1.**

3.5 Discussion

The SPARC-null model of LBP

The SPARC-null model is associated with progressive, chronic disc degeneration and the development of behavioral signs of chronic LBP by six months of age (Gruber et al., 2005, Millecamps et al., 2011b). The presence of disc degeneration was confirmed by histological analysis and increased extravasation, and by previously reported reductions in disc height (Tajerian et al., 2011a). Furthermore, at six months of age, these mice show signs that may be considered to be indicative of axial pain and radiating hypersensitivity (cold allodynia).

We have shown that axial and radiating pain have different pharmacological profiles in the SPARC-null model of LBP associated with disc degeneration. These findings suggest that axial and radiating pain have different underlying mechanisms and encourage increased use of mechanism-based treatment paradigms.

Mechanisms of Axial LBP

Our data shows that anti-nociceptive, anti-inflammatory, and anti-neuropathic agents can ameliorate axial pain in SPARC-null mice, suggesting a mixed inflammatory/neuropathic profile. Several theories regarding the origin of disc degeneration-related axial pain, including aberrant innervation of the IVD and changes in disc biochemistry, are consistent with these observations.

Under normal conditions, nerve endings penetrate only the outer layers of the disc (McCarthy et al., 1991b). However, the depth and density of nerve fibers increases in lumbar IVDs obtained from chronic LBP patients compared to discs harvested from subjects without LBP (Coppes et al., 1997, Freemont et al., 1997). These nerve fibers will then be exposed to mechanical forces and chemical stimuli (e.g. low pH) that activate nociceptors. Conditions within the disc that are not normally painful would therefore result in morphine- and clonidine-sensitive nociceptive pain.

Our data shows that Ibuprofen can ameliorate signs of axial pain only in SPARC-null mice, suggesting that axial pain has an inflammatory component. Furthermore, we have observed increased plasma extravasation in SPARC-null IVDs, indicative of potential angiogenesis and inflammation, contributing to the inflammatory component of pain (Tolonen et al., 2001, Lee et al., 2011). Another factor is increased expression of inflammatory mediators (e.g. TNFa, IL-1b) within degenerating discs (Ohtori et al., 2011). In addition to activating nerve fibers directly, exposure to these mediators can result in peripheral sensitization.

IVDs are composed of a fibrous outer layer, the annulus fibrosus, and an inner gelatinous core, the nucleus pulposus (NP). Under normal conditions the NP contains pro-nociceptive and pro-inflammatory mediators, many of which are upregulated in degenerated discs (Richardson et al., 2009). Application of the NP or some of its constituents to the epidural space produces increased spinal nerve root excitability and hypersensitivity (Aoki et al., 2002b, Obata et al., 2002, Chen et al., 2004b). Therefore, as discs break down, the contents of the NP become both

increasingly noxious and more likely to escape into the surrounding area, where contact with nerve fibers will result in the experience of pain (Mulleman et al., 2006). Furthermore, inflammation of structures around the IVD can be caused by spinal instability, thereby promoting axial pain (Panjabi, 2006). Finally, contact with the NP is sufficient to induce nerve damage (Kawakami et al., 1996, Kawakami et al., 2003a, Inoue et al., 2006b, Murata et al., 2006), resulting in a neuropathic component to axial pain.

Mechanisms of radiating LBP

SPARC-null mice suffer from cold allodynia as a manifestation of radiating pain. The anti-epileptic agent pregabalin, known to be effective in treating neuropathic pain (Tassone et al., 2007), was the only compound that could reverse cold sensitivity in the absence of significant motor impairment and sedation. This suggests the presence of a strong neuropathic component in radiating pain associated with disc degeneration.

Disc degeneration could result in neuropathic pain via several mechanisms. First, the narrowing of lumbar IVDs in the SPARC-null mice could result in nerve compression upon exiting the spinal column. Second, disc narrowing, bulging, and herniation can result in compression of spinal nerve roots, producing spontaneous activity in the peripheral sensory nerve fibers (Howe et al., 1977, Cavanaugh, 1995b), or compression of dorsal root ganglia, which results in cold allodynia (Lee et al., 2011). Finally irritation of nerve roots due to pro-nociceptive substances released from the IVDs could result in radiating pain even in the

absence of mechanical compression (Kawakami et al., 1997b, Olmarker et al., 1998, Mulleman et al., 2006).

Clinical Correlates

Despite the existence of many guidelines for LBP treatment (Chou and Huffman, 2007, Savigny et al., 2009, Pillastrini et al., 2011), sufficient pain relief remains rare (Morlion). While there are many non-pharmacological strategies to minimize LBP, pharmacotherapy remains the most prevalent treatment, aiming to reduce pain and dysfunction while limiting adverse drug reactions (Dworkin et al., 2007).

In patients with axial LBP, NSAIDs, opioids, anticonvulsants, and acetaminophen are all indicated in treatment guidelines (Chou and Huffman, 2007, Savigny et al., 2009). Guidelines for the treatment of radiating pain are less clear, and often strategies for treating general neuropathic pain are used. In the current study, we assessed the effects of different classes of drugs in the SPARC-null mouse model. It is important to consider how the pharmacological profile in the preclinical model relates to clinical observations in patients suffering from LBP.

1. Opioids

Opioids can be effective in the treatment of chronic pain, but are associated with intolerable side-effects that can limit its clinical utility (Fine et al., 2009). Opioids are particularly effective in treating nociceptive and inflammatory pain (Collett, 2001), but only partially effective in reversing neuropathic pain (Furlan et al., 2006), Our data shows that morphine is effective in improving axial, but not

radiating pain in the SPARC-null mouse. In addition, morphine administration was accompanied by motor dysfunction and severe hyperactivity. Overall, morphine was efficacious in axial pain, but was accompanied with undesired side effects, much like the case in a clinical setting (Walder et al., 2001).

2. Clonidine

Alpha-2 adrenergic agonists such as clonidine are effective in acute, inflammatory, and chronic neuropathic pain (Rauck et al., 1993, Cheng et al., 1999, Cohen et al., 2002). Despite its analgesic efficacy, the clinical use of systemic clonidine is limited by its sedative and hypotensive effects. In the SPARC-null mouse, clonidine was effective against axial and radiating pain, but only in dose ranges that also induced sedation. The use of clonidine in combination with other agents, such as morphine, or by intrathecal administration, might improve analgesia with reduced side effects (Eisenach et al., 1994b).

3. Anticonvulsants

Pregabalin and gabapentin are the main anticonvulsant agents indicated in the treatment of neuropathic pain. Although pregabalin is efficient in treating pain caused by a broad range of neuropathic conditions (Gilron et al., 2011), it is not efficient in the treatment of LBP (Gatti et al., 2009). This is perhaps due to the significant inflammatory component in axial LBP. In our study, pregabalin was effective in axial pain. This could suggest that pregabalin targets neuropathic components of axial pain and/or that pregabalin has a broader mechanism of action, and should be considered in the treatment of LBP with or without radiating

pain. Importantly, pregabalin was the only drug in the current study that improved radiating pain in he absence of severe sedation.

4. Antidepressants

Despite the lack of clear evidence showing efficacy of tricyclic antidepressants (Urquhart et al., 2008), they are nonetheless recommended for the treatment of chronic LBP in patients where acetaminophen, NSAIDs, and opioids did not provide sufficient pain relief (Savigny et al., 2009). In the SPARC-null mouse, amitriptyline improved axial but not radiating pain. This could be because of newly-described anti-inflammatory effects of amitriptyline (Sadeghi et al., 2011).

5. Anti-inflammatory agents

A meta-analysis of the effectiveness of NSAIDs in acute and chronic LBP shows that their use is a feasible option in the absence of radiating pain (Roelofs et al., 2008). Our data is consistent with clinical findings in that ibuprofen was effective in relieving axial but not radiating pain. While steroidal anti-inflammatory agents are effective in post-operative and inflammatory pain (Aasboe et al., 1998), there is no clear indication in LBP. While a chronic treatment regimen might be needed to better evaluate the utility of dexamethasone in our model, it improved neither the axial nor radiating components of LBP.

Limitations and Future Directions

The current study used six month-old mice. At this age, SPARC-null mice have mild to moderate disc degeneration associated with axial and radiating pain but no

motor impairment. We believe this represents the stage at which patients might seek medical intervention. With increasing age, disc degeneration becomes increasingly severe in humans and SPARC-null mice. Future studies will examine the effect of pharmacological agents at these more severe stages of disc degeneration.

Several of the drugs tested in this study were only administered at a single dose and each drug was only tested following a single, acute administration. Full exploration of the dose-response curve and the incorporation of chronic dosing schedules are required to make conclusive statement about efficacy. While outside the scope of the current study, these possibilities require further investigation.

Conclusions

Despite the availability of treatment guidelines for LBP, insufficient relief is common. This is partly due to the failure to implement mechanism-based treatment strategies. For instance, despite the fact that radiating pain often accompanies axial pain, and has a mainly neuropathic mechanism, steroids and NSAIDS are still commonly used in the absence of additional drugs targeting the neuropathic component. Furthermore, while axial pain has a mixed inflammatory/neuropathic profile, pharmacotherapy usually targets the inflammatory component. Targeting both the inflammatory and neuropathic mechanisms of pain promises better pain management for chronic LBP patients (Rainov et al., 2001).

In this study, we make use of a transgenic model where disc degeneration is progressive and is accompanied by behavioral signs of pain. SPARC-null mice, much like patients suffering from LBP, exhibit signs of axial and radiating pain. This study also draws attention to the need for more mechanism-based clinical studies. Since pharmacological responsiveness varies between the axial and radiating components of LBP, clinical studies that do not distinguish between these components may produce false negative results. Separating axial from radiating LBP in the clinic will not only result in better outcomes, but will also prevent the administration of drugs that are unlikely to be effective.

3.6 Figures

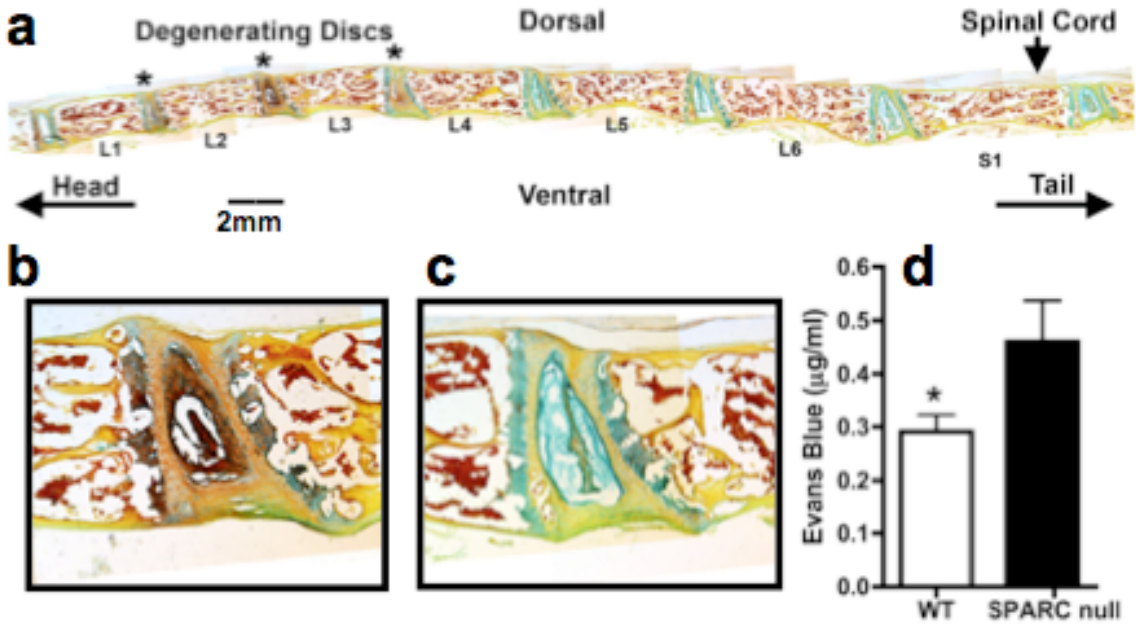

Figure 1: Evidence of Disc Degeneration in 6 month-old SPARC-null mice

a: Representative image using the multichromatic FAST staining protocol in a lumbar spinal column from a 6 month-old SPARC-null mouse. Note the signs of degeneration in several discs (*) including compromised IVD integrity and altered proteoglycan content (indicated by the appearance of orange within the disc). **b,c:** Higher magnification of a degenerating **(b)** and healthy **(c)** disc from **a**. Over 50 spines have been evaluated from each strain and no histological signs of degeneration are observed in WT mice at this age (data not shown). **d:** Vascularization of the lumbar IVDs was measured by the uptake of Evans blue in lumbar IVDs 4 hours following intravenous injection. At 6 months of age, SPARC-null IVDs are more permeable than WT discs. *=$p<0.05$, unpaired, 1-tailed t-test, n=6/group. Error bars indicate S.E.M.

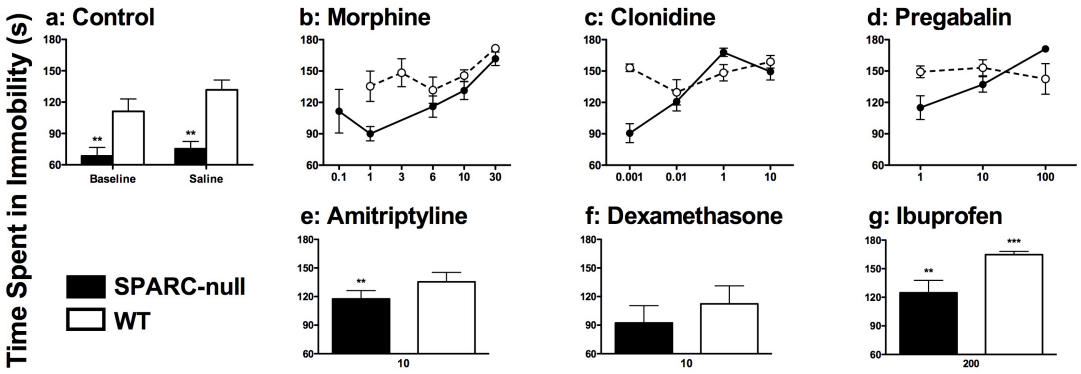

Figure 2: Behavioral Signs of Axial Low Back Pain in the Tail Suspension Assay have a Mixed Pharmacological Profile. a: Baseline measurements show that 6-month old SPARC-null mice avoid gravity-induced stretching along the axis of the spine by reducing the time spent in the immobile position during the tail suspension assay compared to age-matched WT mice. **b-g:** Effect of intraperitoneal morphine **(b)**, clonidine **(c)**, pregabalin **(d)**, amitriptyline **(E)**, dexamethasone **(f)** and ibuprofen **(g)** on the duration of immobility in SPARC-null and WT mice. In SPARC-null mice, all drugs showed efficacy except dexamethasone. In WT mice, efficacy was observed with morphine, clonidine and ibuprofen. Significance was determined compared to saline by 2-tailed, unpaired t-test for single-dose treatments, and by calculating the ED_{50} values for full dose-response curves. See Table 1 for ED_{50} values for morphine, clonidine and pregabalin. **=p<0.01, ***=p<0.0001, n=7-15/treatment group, error bars indicate S.E.M.

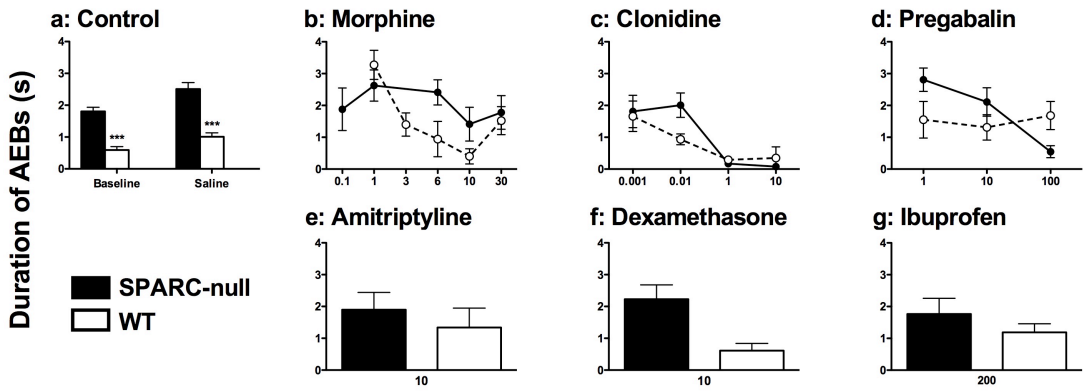

Figure 3: Behavioral Signs of Radiating Pain in the Acetone Test Have a Neuropathic Pharmacological Profile.

a: Baseline measurements show that 6-month old SPARC-null mice spend greater duration exhibiting acetone-evoked behaviors (AEBs) compared to age-matched WT mice. **b-g:** Effect of intraperitoneal morphine **(g)**, clonidine **(c)**, pregabalin **(d)**, amitriptyline **(e)**, dexamethasone **(f)** and ibuprofen **(g)** on the duration of AEBs in SPARC-null and WT mice. Low dose of morphine caused an increase in AEBs in WT mice, clonidine dose-dependently inhibited it in both strains and pregabalin was effective in SPARC-null mice only. Significance was determined compared to saline by 2-tailed, unpaired t-test for single-dose treatments, and by calculating the ED_{50} values for full dose-response curves. See Table 1 for ED_{50} values for morphine, clonidine and pregabalin. ***=$p<0.0001$, n=7-15/treatment group, error bars indicate S.E.M.

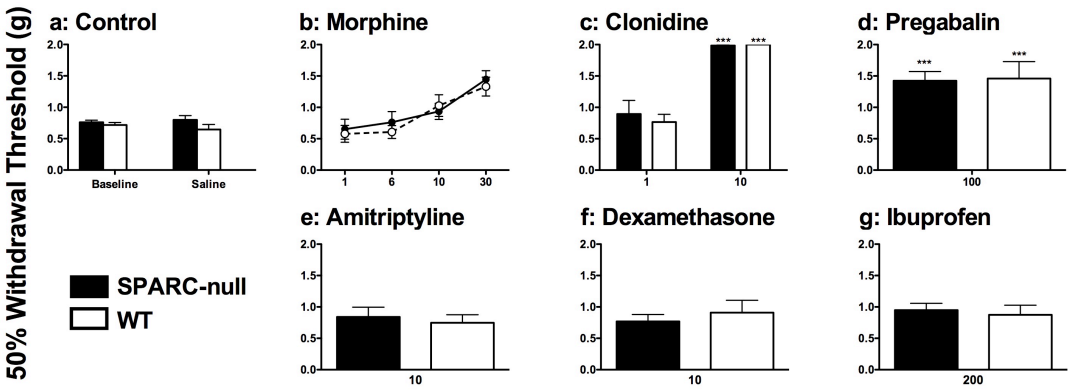

Figure 4: Similar Mechanical Withdrawal Thresholds and Pharmacological Profile in WT and SPARC-null Mice.

a: Baseline measurements show that 6-month old SPARC-null mice do not differ from age-matched WT mice in the von Frey assay of mechanical sensitivity. **b-g:** Effect of intraperitoneal morphine **(b)**, clonidine **(c)**, pregabalin **(d)**, amitriptyline **(e)**, dexamethasone **(f)** and ibuprofen **(g)** on the 50% withdrawal threshold (g). Morphine, clonidine, and pregabalin were similarly effective in increasing the thresholds in both strains. Significance was determined compared to saline by 2-tailed, unpaired t-test for single-dose treatments, and by calculating the ED_{50} values for full dose-response curves. See Table 1 for ED_{50} values for morphine, clonidine and pregabalin. ***=$p<0.0001$, n=7-11/treatment group, error bars indicate S.E.M.

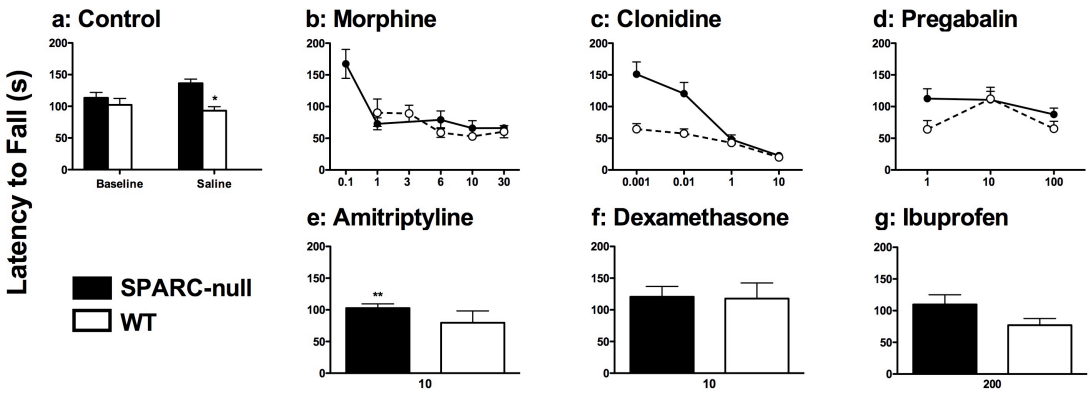

Figure 5: Similar Motor Impairment and Pharmacological Profile in WT and SPARC-null Mice in the Rotorod Assay.

a: Baseline measurements show that 6-month old SPARC-null mice do not differ from age-matched WT mice in the latency to fall from an accelerating rotorod. **b-g:** Effect of intraperitoneal morphine **(b)**, clonidine **(c)**, pregabalin **(d)**, amitriptyline **(e)**, dexamethasone **(f)** and ibuprofen **(g)** in the rotorod assay. Morphine and clonidine significantly decreased the time on the rotorod in both SPARC-null and WT mice while amitriptyline decreased the latency in SPARC-null mice only. Significance was determined compared to saline by 2-tailed, unpaired t-test for single-dose treatments, and by calculating the ED_{50} values for full dose-response curves. See Table 1 for ED_{50} values for morphine, clonidine and pregabalin. *=$p<0.05$, **=$p<0.01$, n=7-11/treatment group, error bars indicate S.E.M.

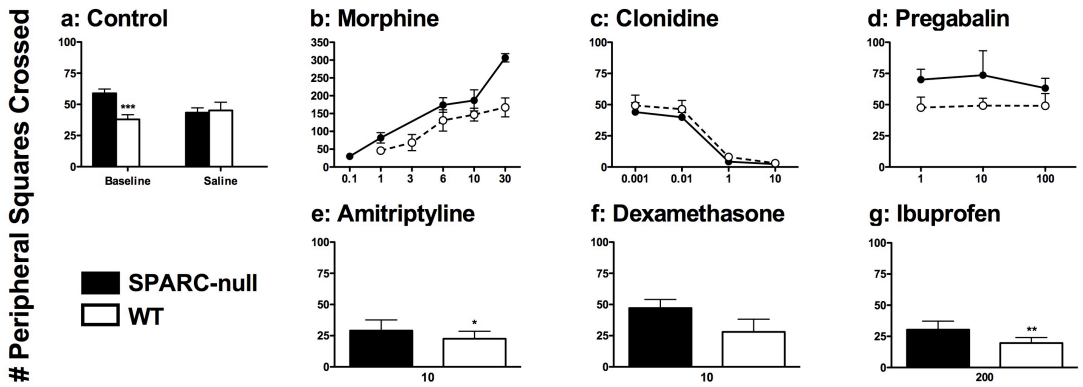

Figure 6: Pharmacological Effects on Voluntary Activity in the Open Field Assay in both SPARC-null and WT mice.

a: Baseline measurements show that 6-month old SPARC-null mice cover more distance in the open field compared to age-matched WT mice. **b-g:** Effect of intraperitoneal morphine **(b)**, clonidine **(c)**, pregabalin **(d)**, amitriptyline **(e)**, dexamethasone **(f)** and ibuprofen **(g)** on total activity in the open field assay. Activity was measured as the number of peripheral squares crossed in an open field that is divided by a 3x3 grid. Morphine significantly increased and clonidine significantly decreased total activity in both strains. Sedation was also observed in WT mice only after amitriptyline and ibuprofen administration. Significance was determined compared to saline by 2-tailed, unpaired t-test for single-dose treatments, and by calculating the ED_{50} values for full dose-response curves. See Table 1 for ED_{50} values for morphine, clonidine and pregabalin. *=$p<0.05$, **=$p<0.01$, ***=$p<0.0001$, n=7-11/treatment group, error bars indicate S.E.M.

3.7 Tables

Summary of ED_{50} Values for Morphine, Clonidine and Pregabalin in SPARC-null and WT mice. # = The values for morphine in the Open Field Assay reflect % increase in activity while the values for clonidine reflect % reduction in activity. *=$p<0.05$ between ED_{50} values for SPARC-null vs. WT strains.

Drug	Strain	Tail Suspension	Acetone	Von Frey	Rotorod	Open Field
Morphine ED_{50} (95% CI)	**SPARC-null**	10.1 (6.6-15.6)	*No efficacy*	66 (44-97)	8.0 (3.7-17)	*0.20# (0.13-0.30)*
	WT	17.5 (12.7-24.2)	*No efficacy*	132 (82-213)	16.6 (7.2-38.7)	*0.57# (0.35-0.93)*
	Potency Ratio SPARC-null:WT	**1:2***	-	1:2	1:2	**1:2.8***
Clonidine ED_{50} (95% CI)	**SPARC-null**	0.05 (0.02-0.12)	0.08 (0.03-0.23)	-	0.34 (0.13-0.88)	0.17 (0.05-0.59)
	WT	8.3 0.6-116	0.13 (0.04-0.44)	-	0.13 (0.03-0.62)	0.14 (0.05-0.44)
	Potency Ratio SPARC-null:WT	**1:166***	1:1.5	-	2.5:1	1:1
Pregabalin ED_{50} (95% CI)	**SPARC-null**	5.3 (2.4-11.7)	30.1 (14.4-63.0)	-	*Insufficient efficacy*	*No efficacy*
	WT	*No efficacy*	*No efficacy*	-	*No efficacy*	*No efficacy*
	Potency Ratio SPARC-null:WT	-	-	-	-	-

3.8 References

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4 Combination Therapy in Low Back Pain

Preface

One of the main advantages of having a valid animal model of pathology is the ability to test the outcome of various treatments. While in chapter 3 we examined the effects of acute administration of individual drugs, this chapter will focus on the co-administration of morphine and clonidine and its effect of axial and radiating pain in addition to motor function in the SPARC-null model of LBP due to DD.

Prior to our studies, there were no pharmacological studies carried out in SPARC-null animals. Therefore, we had to explore a wide range of doses of drugs in various assays before deciding on a fixed ratio for our drug co-administration studies. Furthermore, we tried two different drug combinations: morphine + clonidine and morphine + pregabalin. Morphine was chosen because it is the "gold standard" in analgesia and is highly prescribed to pain patients, clonidine was chosen because of its high efficacy, and pregabalin was chosen since it targets the radiating pain aspect of LBP in our model.

Since the efficacy of drugs varied depending on the behavioral assay, we had to test various drug ratios. We tested 300:1 and 100:1 morphine:clonidine ratios and 10:1 and 1:1 pregabalin:morphine ratios. In this thesis, we only show the effects of morphine + clonidine co-administration in a 100:1 fixed ratio since it was the one where significant analgesic synergy was observed.

Manuscript: Morphine and Clonidine Synergize to Ameliorate Low Back Pain in Mice

4.1 Abstract

Chronic low back pain (LBP) is a debilitating condition associated with signs of axial and radiating pain. In humans with chronic LBP, opioids are often prescribed with varying outcomes and a multitude of side effects. Combination therapies, in which multiple pharmacological agents synergize to ameliorate pain without exasperating adverse reactions, may be key in improving therapeutic outcome in these patients.

We have used the SPARC-null mouse model of low back pain due to disc degeneration to assess the effects of opioid (morphine) and a_2-adrenergic agonist (clonidine) co-administration on measures of axial and radiating pain. Our results indicate that systemic morphine and clonidine, co-administered at a fixed dose of 100:1 (morphine:clonidine), show a synergistic interaction in reversing signs of axial pain, in addition to improving the therapeutic index for radiating pain. Furthermore, these improvements were observed in the absence of synergy in assays of motor function, indicative of side effects such as sedation and motor incoordination.

Our data shows that the addition of low dose systemic clonidine can improve therapeutic outcome both in axial and radiating pain measures, which could be of enormous benefit to patients suffering from chronic LBP.

4.2 Introduction

Low back pain (LBP) is a common condition associated with disability, decrease in quality of life, and significant economic burden (Stewart et al., 2003, Luo et al., 2004, Grabois, 2005). Chronic LBP can include both axial and non-axial symptoms (Devereaux, 2009). While axial LBP is characterized by spontaneous or movement-evoked pain or soreness confined to the spine and low back region, non-axial pain is pain that radiates from the back down one or both legs. This condition is often referred to as radicular pain or sciatica, because the pain usually follows the course of the sciatic nerve (Lindholm et al., 1981, Nygaard and Mellgren, 1998, Mosek et al., 2001, Raj, 2008). Although the exact mechanisms of LBP remain unclear, evidence suggests that the degeneration of intervertebral discs (IVDs) is associated with an increased risk of chronic LBP (Boden et al., 1990, Jensen et al., 1994, Luoma et al., 2000b, Takatalo et al., 2011).

Pharmacotherapy is the most common treatment option for patients suffering from LBP with or without radiating pain (Koes et al., 2010). Although non-steroidal anti-inflammatory drugs are the first line of defense against LBP, they do not sufficiently treat chronic and severe LBP. Opioids are often prescribed with varying therapeutic outcome (Bartleson, 2002, Grabois, 2005, White et al., 2011) and are associated with side effects that limit their use, such as constipation, nausea, somnolence and fatigue (Brown et al., 2011). Since opioids such as morphine remain the gold standard of chronic pain treatment, it is vital to investigate strategies that would decrease required doses without diminishing the

therapeutic effects. One such strategy is the addition of a non-opioid analgesic that will potentiate the analgesic effects of morphine without potentiating the undesirable adverse reactions.

The addition of **a_2-adrenergic agonists (a_2ARs)** improves opioid-induced antinociception in rodents following both systemic and spinal administration (Wilcox et al., 1987, Drasner and Fields, 1988, Ossipov et al., 1990b, Ossipov et al., 1990c, Ossipov et al., 1990d, Meert and De Kock, 1994, Ossipov et al., 1997, Przesmycki et al., 1997, Fairbanks and Wilcox, 1999). Evidence from human studies suggests that the use of opioid-a_2AR agonist combinations in clinical pain management could minimize the side effects associated with both a_2AR and opioid therapeutics (Eisenach et al., 1994a, Paech et al., 2004). Furthermore, combination therapy may be effective in the treatment of chronic, opioid-insensitive pain states (Coombs et al., 1986). To date, the therapeutic benefit of opioid-a_2AR agonist co-administration in chronic axial and non-axial LBP has not been systematically explored in either humans or in animal models.

In this study, we used the SPARC-null mouse model of LBP due to disc degeneration (DD) to examine opioid-a_2AR agonist combinations. SPARC (secreted protein, acidic, rich in cysteine; aka osteonectin or BM-40) is an evolutionarily conserved collagen-binding protein present in IVDs. SPARC is known to influence bone remodeling, collagen fibrillogenesis, and wound repair. Decreased expression of SPARC has been associated with aging and DD in human IVDs (Gruber et al., 2004) and targeted deletion of the SPARC gene results in accelerated disc degeneration in the aging mouse (Gruber et al., 2005).

DD in these mice is also associated with behavioral signs of axial and radiating LBP (Millecamps et al., 2011a, Tajerian et al., 2011a).

The aim of the current study is to use the SPARC-null mouse model of low back pain to study the interaction between an opioid (morphine) and an alpha-2 adrenergic agonist (clonidine) in treating signs of chronic axial and radiating pain. Our results support the hypothesis that combination therapy using morphine and clonidine has the potential to improve therapeutic outcome for the chronic back pain patient.

4.3 Materials and Methods

Mice

SPARC-null mice (backcrossed to the C57BL/6 background) and wild-type (WT) controls (C57BL/6, Charles River, Quebec, Canada) were used as in previous studies (Norose et al., 1998, Brekken et al., 2003, Millecamps et al., 2011a, Tajerian et al., 2011a).

4-6 month old male SPARC-null and WT control mice were bred in-house. Animals were housed in groups of 2-5, had unrestricted access to food and water, and were on a 12-hr light-dark cycle. All experiments were performed blind to genotype and treatment, using a randomized block design. All experiments were approved by the Animal Care Committee at McGill University, and conformed to the ethical guidelines of the Canadian Council on Animal Care and the guidelines of the Committee for Research and Ethical Issues of IASP (Zimmermann, 1983).

Behavioral Analysis

Tail Suspension Assay

Mice were suspended individually underneath a platform by the tail with adhesive tape attached 0.5 to 1 cm from the base of the tail and were videotaped for 180s. The duration of time spent in a) immobility (not moving but stretched out) and b) escape behaviors (rearing to reach the underside of the platform, extending to reach the floor, or self-supported at the base of the tail or the suspension tape) were determined. The duration of immobility reflects the animal's willingness to

stretch its main body axis. Decreased immobility is indicative of axial discomfort. This test is adapted from a traditional assay used to measure depression (Steru et al., 1985), and we have shown that it reliably measures signs of axial pain in mice (Millecamps et al., 2011a). A cutoff of 180s seconds was applied when interpreting the data.

Sensitivity to Cold Stimuli

A modified version of the acetone drop test was used (Choi et al., 1994), where the total duration of acetone-evoked behaviors (AEBs: flinching, licking, or biting) were measured in seconds for 1 minute after a drop of acetone (~25 μl) was applied to the plantar surface of the hindpaw. Increased behavioral response to acetone suggests the development of cold hypersensitivity and decreased reactivity is suggestive of anti-hyperalgesic activity. A cutoff of 4s was applied when interpreting the data.

Rotarod Assay

The accelerating rotarod assay was used to monitor animals for motor function (IITC Life Science Inc., Woodland Hills, CA) with the mouse adapter (rod diameter, 3.2 cm) (Jones and Roberts, 1968). The task includes a speed ramp from 0 to 30 rotations per minute over 60s, followed by an additional 240s at the maximal speed. A decline in the latency to fall off the rotarod reflects motor incoordination. Mice were not trained prior to testing sessions. A cutoff of 200s was used when interpreting the data.

Open Field Assay

A transparent open field apparatus (24 x 24 cm^2) was placed in a quiet room illuminated with white light. The floor of the apparatus was equally divided into nine squares (8 x 8 cm^2). Mice were individually placed into the open field on the central square, and their spontaneous behavior was videotaped for 5 min. Subsequent analysis of the total number of squares visited was used to assess general motor activity (Millecamps et al., 2007). An increase in the number of peripheral squares covered reflects hyperactivity, while a decrease is indicative of sedation. Following drug administration, animals underwent tail suspension just before being placed in the open field.

Timeline

The schedule of testing was as follows: 16 weeks of age: habituation to tail suspension; 20 weeks: baseline open field and tail suspension assays; 22 and 26 weeks: baseline and post-drug administration for acetone and rotarod assays; 24 and 28 weeks: tail suspension and open field after drug administration. A washout period of 2 weeks was included between drug exposures to ensure that only the acute effects of each drug were studied.

Pharmacological Treatment

Analgesic agents or saline control were administered to SPARC-null and WT mice by i.p. injection (5ml/kg injected directly in the intra-peritoneal cavity). Morphine (Medisca Inc., Montreal, Quebec) and clonidine (Sigma-Aldrich

Canada Ltd., Oakville, Ontario) were dissolved in 0.9% saline either alone or in combination at a constant dose ratio of 100:1 (morphine:clonidine). Animals were tested 60 minutes after drug administration.

Data Analysis

Behavioral Phenotype of LBP

Comparisons between saline-treated SPARC-null and WT mice were performed for each assay by 2-tailed, unpaired t-test. Welch's correction was used when the condition of equal variances was not met. Sample size ranged between 35-48 mice/group of saline-treated mice.

Dose-Response Analysis (Table 1)

Individual dose points are reported as raw data for both strains and all pharmacological treatments as means with standard error of the mean (SEM). In order to calculate ED_{50} values, individual dose points were first converted to % Maximum Possible Effect **(%MPE)** according to the following equations:

Tail suspension: % MPE = (drug-saline)/(maximum-saline) x 100; maximum effect = 180 seconds in immobility.

Acetone: % MPE = (saline-drug)/(saline-maximum) x 100; maximum effect = 0 seconds of AEB-induced behavior.

Rotarod: % MPE = (saline-drug)/(saline- maximum) x 100; maximum effect = 0 seconds latency to fall.

Open Field: % MPE = (saline-drug)/(saline- maximum) x 100; maximum effect = 0 number of peripheral squares.

ED_{50} values and confidence limits were calculated according to the graded dose-response method of Tallarida and Murray (Tallarida and Murray, 1987) on the linear portion of each dose-response curve. ED_{50} values were determined by extrapolation in cases where maximum efficacy was between 30 and 50%. If 30% efficacy was not reached, ED_{50} values were not calculated and the drug was considered to lack efficacy. A minimum of three doses was used for each drug or combination of drugs.

Isobolographic Analysis (Table 1)

Isobolographic analysis is the 'gold standard' for evaluating drug interactions (Tallarida and Murray, 1987, Tallarida, 1992). Dose-response curves were constructed for each agonist administered alone and the ED_{50} values were calculated. The two drugs were then co-administered at a constant dose-ratio approximately equal to their potency ratio, a third dose-response curve was constructed, and an experimentally derived combination ED_{50} was calculated.

To test for interactions between agonists, the ED_{50} values and standard error of all dose-response curves were arithmetically arranged around the ED_{50} value using the following equation**:** (ln(10) x ED_{50}) X (S.E.M. of log ED_{50}) (Tallarida, 1992). Isobolographic analysis necessitates this manipulation. When testing an interaction between two drugs, a theoretical additive ED_{50} value is calculated for the combination based on the dose-response curves of each drug administered separately. This theoretical value is then compared by a *t*-test with the observed experimental ED_{50} value of the combination. An interaction is considered

synergistic if the experimental ED_{50} is significantly less ($p<0.05$) than the calculated theoretical additive ED_{50}.

Visualization of drug interactions can be facilitated and enhanced by graphical representation of isobolographic analysis (**Figures 1-3, C-C'**). This representation depicts the ED_{50} of each agent on the x- or y-axes. For example, **Figure 1C** presents the ED_{50} of morphine on the y-axis and the ED_{50} of clonidine on the x-axis. The line connecting these two points depicts the dose combinations expected to yield 50% efficacy if the interaction is purely additive and is called the theoretical additive line. The theoretical additive ED_{50} and its confidence interval are determined mathematically and plotted spanning this line. The observed ED_{50} for the combination is plotted at the corresponding x,y co-ordinates along with its 95% confidence interval for comparison to the theoretical additive ED_{50}. Isobolographs were plotted only when both drugs alone and the combination showed efficacy.

All dose-response and isobolographic analyses were performed with the FlashCalc pharmacological statistics software package generously supplied by Dr. Michael Ossipov.

Therapeutic Index (Table 2)

Therapeutic Index (**TI**) is a measure of the amount of an agent required to produce the desired effect (i.e. analgesia) compared to the amount that produces the undesired effect (i.e. motor impairment). Specifically, the TI is defined as the ED_{50} (undesired effect)/ED_{50} (desired effect). A TI <1 indicates the drug is more

potent in the production of the undesired effect than the desired effect. A TI > 1 indicates that the desired effect can be achieved in the absence of the side effect. Higher indices are more advantageous therapeutically.

4.4 Results

Morphine and Clonidine Synergize to Improve Axial Pain in the Tail Suspension Assay

SPARC-null mice show signs of axial pain compared to WT mice as shown in the tail suspension assay (135.4±5.2s in WT vs. 86.8±5.7s in SPARC-null, $p<0.0001$, 2-tailed t-test, **Figure 1A**). Both in SPARC-null and WT mice, systemic administration of either morphine or clonidine produced dose-dependent increases in immobility, indicative of reduced axial discomfort, 60 minutes post-injection (**Figure 1B, B'**).

The dose-response data from **Figures 1B** is represented graphically as an isobologram in **Figure 1C**. As shown in **Figure 1C**, the ED_{50} of the combination (closed circle) in SPARC-null mice is lower than the theoretical additive ED_{50} (open circle), indicating that this interaction is synergistic. This synergistic interaction was confirmed by statistical comparison between the observed combined ED_{50} value and the theoretical additive ED_{50} value.

In WT mice, no efficacy was observed with morphine+clonidine co-administration (**Figure 1B'**), and thus isobolographic analysis could not be performed (**Table 1**).

Morphine and Clonidine are Additive in the Acetone Test of Cold Allodynia

SPARC-null mice show signs of cold allodynia on the hindpaw compared to WT mice, as shown in the acetone assay (1.2±0.1s in WT vs. 2.6±0.2s in SPARC-null,

$p<0.0001$, 2-tailed t-test, **Figure 2A**). In SPARC-null mice, systemic administration of clonidine produced dose-dependent analgesia in the acetone assay at 60 minutes post-injection; while morphine failed to reach 50% MPE but was of sufficient maximum efficacy (45%) to extrapolate an ED_{50} value (**Figure 2B**).

In WT mice, the administration of either morphine or clonidine alone produced dose-dependent antinociception in the acetone assay (**Figure 2B'**). This interaction was tested statistically by comparing the observed combined ED_{50} value and the theoretical additive ED_{50} value, and was shown to be additive. The dose-response data from **Figure 2B,B'** are represented graphically as isobolograms in **Figure 2C,C'**. As shown in **Figures 2C,C'**, the ED_{50} of the combination (closed circle) in both strains is not significantly different from the theoretical additive ED_{50} (open circle), indicating that this interaction is additive (**Table 1**).

Morphine and Clonidine are Additive in the Rotarod Test of Motor Impairment

SPARC-null mice do not show signs of motor impairment at 6 months of age. Rather, they perform better than WT mice in the rotarod assay at this age (92.1±5.9s in WT vs. 136.2±6.2s in SPARC-null, $p<0.0001$, 2-tailed t-test, **Figure 3A**). In SPARC-null mice, systemic administration of either morphine or clonidine produced dose-dependent motor impairment in the rotarod assay at 60

minutes post-injection (**Figure 3B**). Only clonidine produced a dose-dependent effect in WTs (**Figure 3B'**).

The SPARC-null dose-response data from **Figure 3B** is represented graphically as an isobologram in **Figure 3C**. As shown in **Figure 3C**, the ED_{50} of the combination (closed circle) is not significantly different from the theoretical additive ED_{50} (open circle), indicating that this interaction is additive. In WT mice, morphine+clonidine co-administration lacked efficacy, and thus it was not possible to perform isobolographic analysis in this assay (**Table 1**).

Opposing Effects of Morphine and Clonidine in the Open Field Test of Voluntary Activity

SPARC-null mice do not differ from WTs in the number of peripheral squares covered in the open field, indicative of no overall change motor activity (49.1±5.9 squares in WT vs. 45.7±3.8 squares in SPARC-null, p=0.6, 2-tailed t-test, **Figure 4A**). In both SPARC-null and WT mice, systemic administration of morphine produced dose-dependent hyperactivity, while clonidine produced dose-dependent sedation in the open field assay at 60 minutes post-injection in (**Figure 4B, 4B'**). Since the two agonists exert opposite effects on overall activity, isobolographic analysis was not performed for the open field test.

Effect of Morphine and Clonidine Co-Administration on Therapeutic Index

The data presented above demonstrate that co-administration of morphine and clonidine produces antinociceptive but not sedative synergy following i.p.

administration. We therefore examined the impact of co-administration on the therapeutic index **(TI)** between sedation and antinociception. In **Table 2**, the TI has been calculated for morphine and clonidine alone and in combination following systemic administration for both axial pain and cold allodynia. In SPARC-null mice, the index for each drug given alone ranged from 0.2-6.8, indicating little separation between the antinociceptive and sedative effective dose ranges. In contrast, the addition of a small amount of clonidine to morphine increased these values to 700 for axial LBP and 16 for non-axial LBP. These changes reflect the fact that analgesia is reached before sedation when the drugs are co-administered. These increases in therapeutic index are the result of potentiation in the antinociceptive assays in parallel with an additive interaction in the undesired side effect (motor impairment).

4.5 Discussion

Morphine and Clonidine Synergy Improves Therapeutic Outcome for Axial Pain

SPARC-null mice have been previously reported to develop behavioral signs of axial pain by 4-6 months of age (Millecamps et al., 2011a, Tajerian et al., 2011a). In the current study, we show that while morphine and clonidine both dose-dependently attenuate axial pain, the side effects of motor impairment, sedation (clonidine) and hyperactivity (morphine) develop in a similar dose range. Systemic co-administration of morphine and clonidine not only resulted in synergy in SPARC-null; the therapeutic index of the combination was greater than for either drug administered alone. The pharmacological effects observed in SPARC-null animals are not likely due to motor impairment or sedation, since the morphine+clonidine combination lacked efficacy in our tests of motor function. Furthermore, while morphine produced increases in overall activity, morphine-treated animals spent more time in immobility in the tail suspension assay, indicative of antinociception.

The majority of pre-clinical studies examining opioid-a_2AR interactions to date have been carried out in naïve rodents, where the measured endpoint is antinociception to cutaneous noxious stimuli (Drasner and Fields, 1988, Ossipov et al., 1990b, Ossipov et al., 1990c, Ossipov et al., 1990d, Meert and De Kock, 1994, Fairbanks and Wilcox, 1999) or inhibition of chemically-evoked behaviors (Stone et al., 1997, Overland et al., 2009). In contrast, the current study focused

on pharmacological reversal of pathological signs of axial LBP in a pre-clinical model of intervertebral disc degeneration-related pain. To our knowledge this is the first demonstration of an opioid-adrenergic antinociceptive synergy in axial LBP in either pre-clinical or human studies.

In patients suffering from axial LBP, pain management remains inadequate. Patients with mild or severe LBP are often prescribed two or more medications in addition to opioids, reflecting the challenging nature of LBP (Taylor-Stokes et al., 2011). Currently the primary use of clonidine as a pain management tool is as a spinal adjuvant for opioids in intractable cancer pain (Eisenach et al., 1995, Ghafoor et al., 2007). Although not currently indicated for patients with chronic axial LBP, our results suggest that low doses of systemic clonidine may be a useful addition to opioid therapy.

Co-administration of Morphine and Clonidine Increases the Therapeutic Index for Radiating Pain

Cold allodynia in the hindpaw of SPARC-null mice is a behavioral measure of non-axial, radiating pain. While cold allodynia is reversed by systemic clonidine, that efficacy is associated with side effects including motor impairment and sedation. Although the co-administration of morphine and clonidine was additive in our model, we did, nonetheless, observe an improvement in the therapeutic index, such that therapeutic effects were observed at doses associated with minimal side effects.

Radiating pain, which may accompany axial pain in patients suffering from LBP (Lindholm et al., 1981, Nygaard and Mellgren, 1998, Mosek et al., 2001, Raj, 2008), is thought to have a mainly neuropathic mechanism (Morlion, 2011). As a result, anti-neuropathic agents and not opioids are the treatment of choice in these patients. Consistent with the reduced opioid efficacy commonly associated with neuropathic pain conditions, morphine failed to reach 50% efficacy in cold hypersensitivity in SPARC-null mice in the current study. Furthermore, while the ED_{50} values for morphine were between 8-10 mg/kg in the tail suspension and rotarod assays, the extrapolated ED_{50} value for morphine in non-axial pain was >30 mg/kg. These observations are supportive of the predictive validity of the current model.

Studies evaluating opioid-a_2AR agonist interactions in rodent models of neuropathic pain have demonstrated synergistic interactions between morphine and the a_2AR agonists clonidine and moxonidine (Ossipov et al., 1997, Fairbanks et al., 2000). While morphine and clonidine co-administration did not result in synergy in radiating pain in the current study, it did improve the therapeutic index in this modality.

These results, together with the synergy observed in axial analgesia, show that combining morphine and clonidine targets both the axial and radiating pain aspects observed in humans with chronic LBP. The ability to obtain sufficient relief of both axial and radiating pain with the combination of morphine and a low dose of clonidine could result in less adverse drug reactions, fewer undesired or

unanticipated drug interactions, increased patient compliance, and improved quality of life.

Opioid-a_2AR Agonist Interactions

It is well established that the nature of opioid/a_2AR agonist interactions depends on both the route of administration and the test used. For example, the interaction between the a_2AR agonist medetomide and the opioids morphine, fentanyl and meperidine is additive in both the hot plate and tail flick tests following intravenous administration, whereas they are synergistic in the tail flick test but not the hot plate test following intrathecal administration (Ossipov et al., 1990c). Similarly, intravenous clonidine combined with morphine, fentanyl or meperidine is additive in the tail flick test whereas these same combinations delivered intrathecally interact in a synergistic manner in the same test (Ossipov et al., 1990a). These observations have led to the suggestion that opioid/a_2AR agonist interactions are mediated primarily within the spinal cord.

In humans, only a few studies have examined the interaction between opioid/a_2AR agonists in chronic pain conditions. In one study, the addition of epidural clonidine benefited patients with intractable cancer pain, particularly those with a significant neuropathic component (Eisenach et al., 1995), and the combination of intrathecal morphine + clonidine is useful for the management of chronic pain after spinal cord injury (Siddall et al., 1994, Siddall et al., 2000). In order to maximize the clinical relevance of the current study, systemic administration was selected; spinal delivery requires invasive procedures that add

additional risks. A variety of systemically delivered adrenergic agonists (i.e. clonidine, dexmedetomidine, moxonidine, tizanidine) are currently available for use in humans and could be utilized as adjuvants in patients not receiving sufficient efficacy from opioids.

Although there are many studies reporting functional interactions between opioids and a_2AR agonists (for review see (Fairbanks et al., 2009)), the molecular mechanisms underlying these interactions are not clear. Evidence from immunohistochemical studies suggests that opioid receptors are co-expressed in the same population of sensory neurons as a_2ARs, and that antinociceptive synergy requires activation of protein kinase C (Wei and Roerig, 1998, Overland et al., 2009, Riedl et al., 2009). It has been proposed that the physical association between these two receptors may account for the synergistic effects observed, at least at the spinal level (Jordan et al., 2003, Rios et al., 2004, Riedl et al., 2009). In addition, interactions at other sites in the CNS, such as the locus coeruleus (Illes and Norenberg, 1990), as well as in the periphery, are possible.

Future Directions

We have studied the acute effects of morphine, clonidine, and their combination 60 minutes after systemic administration. However, in clinical situations most patients undergo chronic pharmacotherapy. It would therefore be informative to study these interactions using a chronic dosing paradigm. The use of multimodal therapy may be of even greater therapeutic benefit if chronic studies reveal protective effects of the combination against the development of tolerance or

opioid-induced hyperalgesia. Clonidine is also know to reduce opioid withdrawal symptoms, a property that may be beneficial in long term management of chronic non-cancer pain (Milne et al., 1985). Ultimately further studies in both pre-clinical models and human subjects are required to fully understand the therapeutic benefit of adrenergic adjuvant therapy.

Conclusions

We have used a mouse model of chronic LBP due to progressive disc degeneration to explore the effects of morphine and clonidine co-administration on measures of axial and radiating pain. Side effects including motor impairment and overall change in activity were also assessed. The data indicates that the addition of low dose systemic clonidine can improve therapeutic outcomes both in axial and radiating pain measures, which could be of enormous benefit to patients suffering from chronic LBP.

4.6 Figures

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Figure 1: Morphine and Clonidine synergize to attenuate axial pain in SPARC-null mice.

A. Saline-treated SPARC-null animals spend less time in immobility compared to WT mice in the tail suspension assay, indicative of axial pain. **B, B'.** In SPARC-null mice (B), morphine (●) and clonidine (■) dose-dependently inhibited axial pain when administered systemically either alone or co-administered (i.p.) at a constant dose ratio of 100:1 (morphine: clonidine). In WT mice (B'), morphine (●) and clonidine (■) dose-dependently inhibited axial pain when administered systemically, but the combination lacked efficacy. **C.** Isobolographic analysis applied to the data from Figure 1B. The y-axis represents the ED_{50} for morphine and the x-axis represents the ED_{50} for clonidine. The lines directed from each ED_{50} value toward zero are the lower 95% confidence limits of each ED_{50}. The line connecting these two points is the theoretical additive line. The open circle on the theoretical additive line represents the calculated theoretical ED_{50} value of the combination if the interaction is additive. The observed combination ED_{50} (●) was significantly ($p<0.05$; t-test) lower than the theoretical additive ED_{50} (○), indicating that the interaction is synergistic. An isobolograph was not plotted for WT mice, since the combination lacked efficacy in this assay. Error bars represent ±SEM for each dose point (*n* = 5-11 animals/dose). See Table 1 for ED_{50} values. ***=$p<0.0001$.

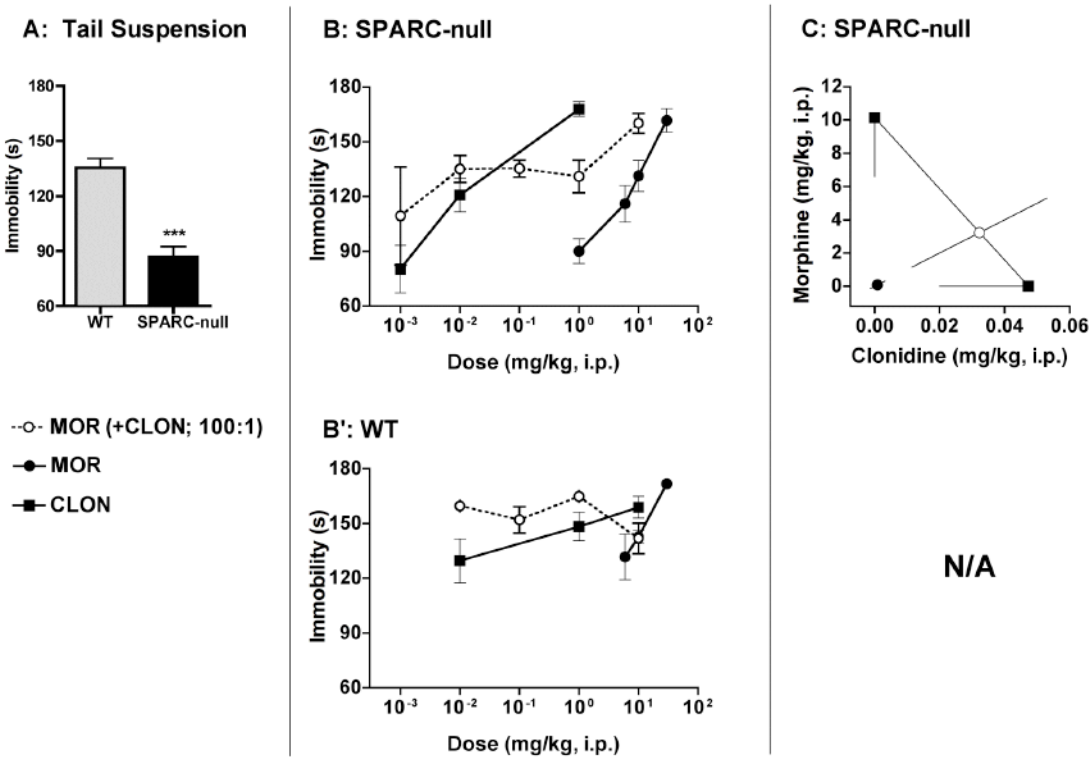
A: Tail Suspension
Immobility (s)
180
150
120
90
60
***
WT
SPARC-null
MOR (+CLON; 100:1)
MOR
CLON
B: SPARC-null
Immobility (s)
Dose (mg/kg, i.p.)
B': WT
Immobility (s)
Dose (mg/kg, i.p.)
C: SPARC-null
Morphine (mg/kg, i.p.)
Clonidine (mg/kg, i.p.)
N/A

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Figure 2: Effect of co-administration of morphine and clonidine on cold allodynia

A. Saline-treated SPARC-null animals exhibit more acetone-evoked behaviors compared to WT mice in the acetone assay, indicative of cold hypersensitivity on the hindpaw. **B, B'.** In both SPARC-null (B) and WT (B') mice, morphine (●) and clonidine (■) dose-dependently inhibited cold allodynia when administered systemically either alone or co-administered (i.p.) at a constant dose ratio of 100:1 (morphine:clonidine). **C, C'.** Isobolographic analysis applied to the data from Figures 2B,B'. The y-axis represents the ED_{50} for morphine and the x-axis represents the ED_{50} for clonidine. The lines directed from each ED_{50} value toward zero represent the respective lower 95% confidence limits of each ED_{50}. The line connecting these two points is the theoretical additive line. The open circle on the theoretical additive line represents the calculated theoretical ED_{50} value of the combination if the interaction is additive. The observed combination ED_{50} (●) was not significantly different (t-test) from the theoretical additive ED_{50} (○) in either strain, indicating that the interaction is additive in both cases. Error bars represent ±SEM for each dose point (n = 5-11 animals/dose). See Table 1 for ED_{50} values. ***=$p<0.0001$.

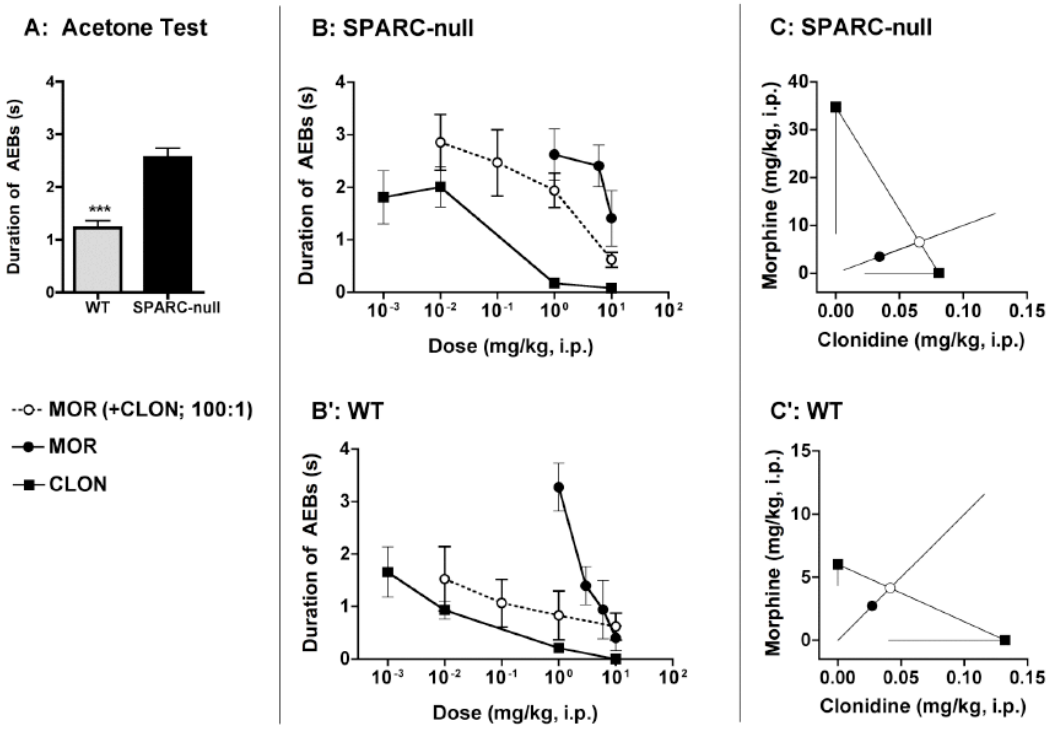
A: Acetone Test
Duration of AEBs (s)
***
WT
SPARC-null
B: SPARC-null
Duration of AEBs (s)
Dose (mg/kg, i.p.)
C: SPARC-null
Morphine (mg/kg, i.p.)
Clonidine (mg/kg, i.p.)
MOR (+CLON; 100:1)
MOR
CLON
B': WT
Duration of AEBs (s)
Dose (mg/kg, i.p.)
C': WT
Morphine (mg/kg, i.p.)
Clonidine (mg/kg, i.p.)

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Figure 3: Effect of co-administration of morphine and clonidine on motor function

A. Saline-treated SPARC-null animals perform better on the rotarod assay compared to WT mice, indicative of an absence of motor impairment in SPARC-null mice. **B, B'.** In SPARC-null mice (B), morphine (●) and clonidine (■) dose-dependently caused motor impairment when administered systemically either alone or co-administered (i.p.) at a constant dose ratio of 100:1 (morphine:clonidine). In WT mice (B'), morphine (●) and clonidine (■) dose-dependently caused motor incoordination when administered systemically, but the combination lacked efficacy. **C.** Isobolographic analysis applied to the data from Figure 1B. The y-axis represents the ED_{50} for morphine and the x-axis represents the ED_{50} for clonidine. The lines directed from each ED_{50} value toward zero represent the respective lower 95% confidence limits of each ED_{50}. The line connecting these two points is the theoretical additive line. The open circle on the theoretical additive line represents the calculated theoretical ED_{50} value of the combination if the interaction is additive. The observed combination ED_{50} (●) was not significantly different (t-test) from the theoretical additive ED_{50} (○), indicating that the interaction is additive. Isobolographic analysis was not performed in WT mice since the combination lacked efficacy in this assay. Error bars represent ±SEM for each dose point (*n* = 5-11 animals/dose). See Table 1 for ED_{50} values. ***=$p<0.0001$.

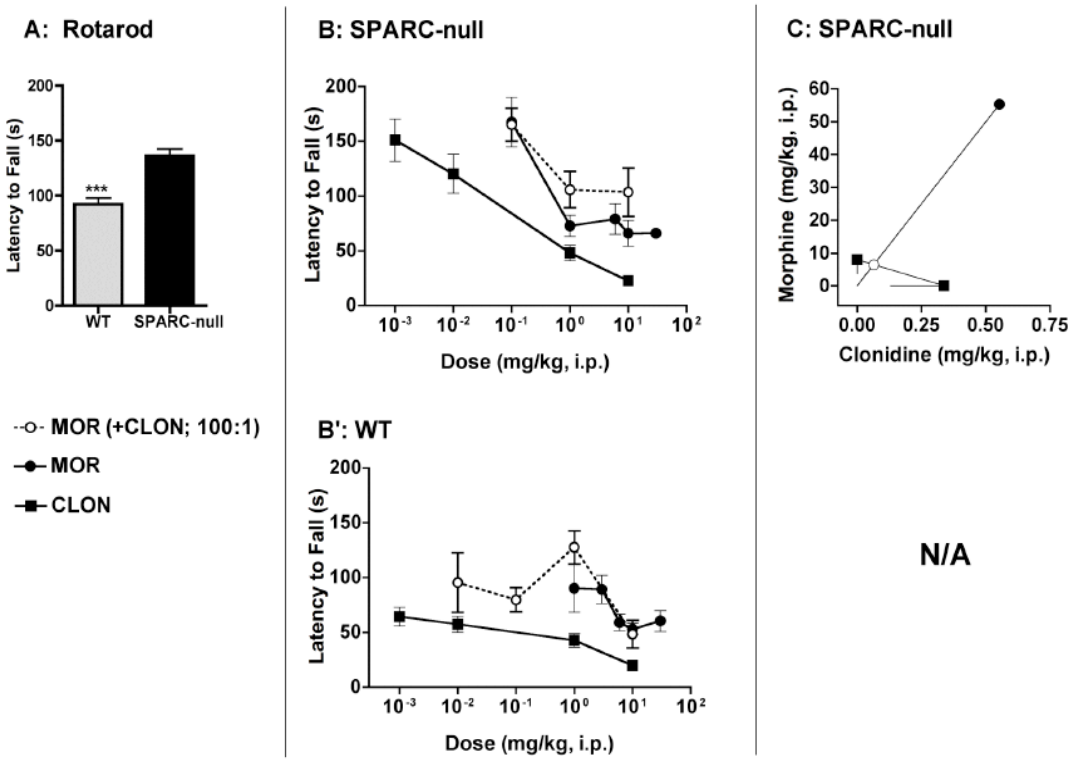
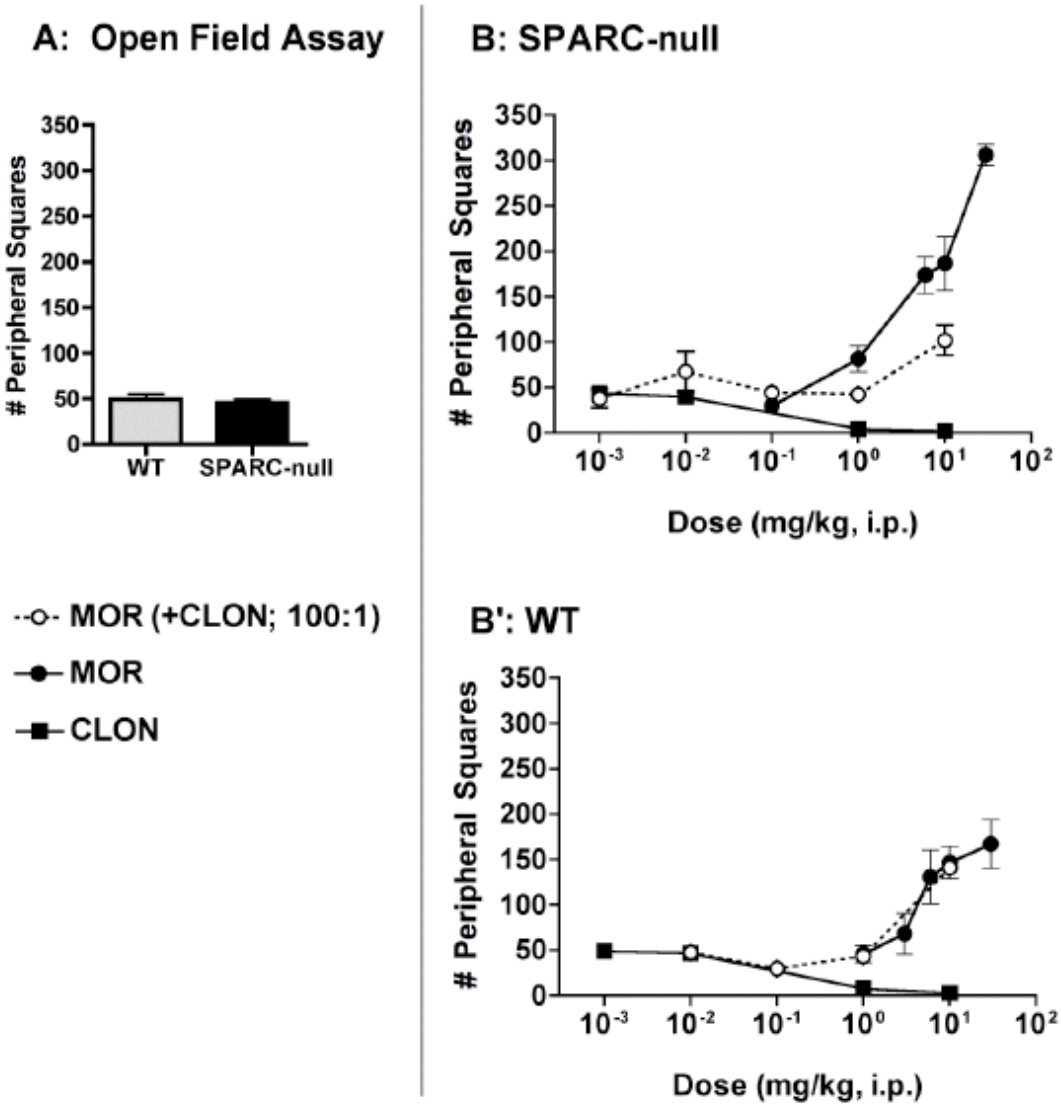

A: Rotarod
Latency to Fall (s)
200
150
100
50
0
***
WT
SPARC-null
B: SPARC-null
Latency to Fall (s)
Dose (mg/kg, i.p.)
C: SPARC-null
Morphine (mg/kg, i.p.)
Clonidine (mg/kg, i.p.)
MOR (+CLON; 100:1)
MOR
CLON
B': WT
Latency to Fall (s)
Dose (mg/kg, i.p.)
N/A

Figure on next page

Figure 4: Effect of co-administration of morphine and clonidine on overall activity

A. Saline-treated SPARC-null animals do not differ from WT mice in the number of peripheral squares covered in the open field, indicative of comparable overall activity between the two strains. **B, B'.** Both in SPARC-null (B) and WT (B') mice, morphine (●) caused an increase in activity, while clonidine (■) dose-dependently caused sedation. When co-administered (i.p.) at a constant dose ratio of 100:1 (morphine:clonidine), the combination showed no efficacy in SPARC-null mice and produced hyperactivity in WT mice. No isobolographs were plotted for either strain as the drugs had opposing effects. Error bars represent ±SEM for each dose point (n = 5-11animals/dose). See Table 1 for ED_{50} values.


A: Open Field Assay
Peripheral Squares
350
300
250
200
150
100
50
0
WT
SPARC-null
B: SPARC-null
Peripheral Squares
Dose (mg/kg, i.p.)
MOR (+CLON; 100:1)
MOR
CLON
B': WT
Peripheral Squares
Dose (mg/kg, i.p.)

4.7 Tables

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Table 1: Effect of Combination Therapy on Drug Potency

Morphine and Clonidine ED_{50} values (mg/kg, i.p.) either alone or in combination at a dose-ratio of 100:1 (Observed Combination ED_{50}). The Theoretical Combination ED_{50} is the predicted ED_{50} for the combination in the absence of any interaction. The Interaction indicates if the observed combination ED_{50} was statistically different from the theoretical combination ED_{50}. ~ indicates that the ED_{50} value was determined by extrapolation if maximum efficacy was less than 50%. *In the open field assay, morphine had no potency as a sedative but caused hyperactivity. A drug or drug combination was considered to exhibit no efficacy if maximum efficacy was under 30%. NA = not available (it is not possible to calculate these values when one drug lacks efficacy).

Assay	Strain	Morphine ED_{50}	Clonidine ED_{50}	Observed Combination ED_{50}	Theoretical Combination ED_{50}	Interaction
Tail Suspension (Axial pain)	**SPARC-null**	10 (±4.0)	0.05 (±0.04)	0.08 (±0.23)	3.3 (±2.1)	**Synergistic**
	WT	18 (±6.0)	8.2 (±21)	No efficacy	17 (±5.7)	NA
Acetone (Cold allodynia)	**SPARC-null**	*~35 (±50)*	0.08 (±0.09)	3.5 (±6.3)	6.6 (±6.0)	**Additive**
	WT	6 (±2.0)	0.1 (±0.2)	2.7 (±8.9)	4.2 (±1.8)	**Additive**
Rotorod (Motor incoordination)	**SPARC-null**	8 (±6.1)	0.3 (±0.3)	*~56(±85)*	6.5 (±4.5)	**Additive**
	WT	*~17 (±14)*	0.1(±0.2)	No efficacy	7.3 (±7.4)	NA
Open field (Overall activity)	**SPARC-null**	*0.2 (±0.1)	0.2 (±0.2)	No efficacy	NA	NA
	WT	*0.6 (±0.3)	0.14 (± 0.16)	*0.13 (±0.08)	NA	NA

Table 2: Combination Therapy Improves Therapeutic Index

Strain	Drug(s)	ED_{50} Value (± SE; mg/kg, i.p.)			Therapeutic Index	
		Motor	Axial	Non-Axial	Motor/ Axial	Motor/ Non-Axial
SPARC-null	Morphine	8 (±6.1)	10 (±4.0)	*~35 (±50)*	0.8	0.2
	Clonidine	0.3 (±0.3)	0.05 (±0.04)	0.08 (±0.09)	6.8	4.3
	Morphine (+Clon; 100:1))	*~56 (±85)*	0.08 (±0.23)	3.5 (±6.3)	**700**	**16**
WT	Morphine	*~17 (±14)*	18 (±6.0)	6.0 (±2.0)	0.9	2.8
	Clonidine	0.1 (±0.2)	8.2 (±21)	0.1 (±0.2)	0.01	1.0
	Morphine (+Clon; 100:1))	*No efficacy*	*No efficacy*	2.7 (±8.9)	N/A	N/A

The **Therapeutic Index** is the ratio of the ED_{50} value (mg/kg, i.p.) of the undesired effect (motor impairment) to the desired effect (inhibition of axial or non-axial pain). A larger Therapeutic Index suggests the drug or drug combination will be analgesic at doses that do not produce motor impairment. ~ indicates that the ED_{50} value was determined by extrapolation if maximum efficacy was less than 50%. NA = not available (the combination lacked efficacy in the rotorod assay in WT mice). Note the much-larger Therapeutic Index achieved with the addition of clonidine to morphine.

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5 Epigenetic Studies of Intervertebral Disc Degeneration

Preface

The use of transgenic animals as model organisms for various pathological states has been beneficial in advancing our understanding of biological processes. The use of SPARC-null mice as a model of chronic LBP due to DD has helped us to understand the role of SPARC in the IVD, to study the progression of LBP when the gene is inactivated, and to test different pharmacological agents to ameliorate the condition. Furthermore, the SPARC model is meant to elucidate the mechanisms of accelerated IVD degeneration as opposed to naturally occurring age-related degeneration. It was for these reasons that we decided to study the link between SPARC and LBP in aging WT mice. Furthermore, we decided to take it a step closer to the clinic by studying lumbar IVD samples from humans with or without DD and LBP.

In this study, we focused on understanding the underlying mechanisms that may control SPARC expression. We were fortunate to collaborate with the laboratory of Dr. Moshe Szyf, a specialist in the field of epigenetics. The resulting manuscript was the first to report methylation changes associated with any painful condition. Since then many papers have been published linking various epigenetic changes (including methylation) to pain.

Manuscript: DNA methylation of SPARC and chronic low back pain

5.1 Abstract

The extracellular matrix protein SPARC (Secreted Protein, Acidic, Rich in Cysteine) has been linked to degeneration of the intervertebral discs and chronic low back pain (LBP). In humans, SPARC protein expression is decreased as a function of age and disc degeneration. In mice, inactivation of the SPARC gene results in the development of accelerated age-dependent disc degeneration concurrent with age-dependent behavioral signs of chronic LBP.

DNA methylation is the covalent modification of DNA by addition of methyl moieties to cytosines in DNA. DNA methylation plays an important role in programming of gene expression, including in the dynamic regulation of changes in gene expression in response to aging and environmental signals.

We tested the hypothesis that DNA methylation down-regulates SPARC expression in chronic LBP in pre-clinical models and in patients with chronic LBP.

Our data shows that aging mice develop anatomical and behavioral signs of disc degeneration and back pain, decreased SPARC expression and increased methylation of the SPARC promoter. In parallel, we show that human subjects with back pain exhibit signs of disc degeneration and increased methylation of the

SPARC promoter. Methylation of either the human or mouse SPARC promoter silences its activity in transient transfection assays.

This study provides the first evidence that DNA methylation of a single gene plays a role in chronic pain in humans and animal models. This has important implications for understanding the mechanisms involved in chronic pain and for pain therapy.

5.2 Introduction

Chronic low back pain (LBP) is a complex continuum of painful conditions that includes both axial and radicular pain (Devereaux, 2009): Axial LBP is defined as spontaneous or movement-evoked pain or discomfort localized to the spine and low back region. Non-axial, radiating LBP is pain in one or both legs. Often referred to as radicular pain or sciatica, it usually follows the course of the sciatic nerve. Current diagnostic and therapeutic approaches to chronic back pain are limited by our narrow understanding of the underlying biological mechanisms. There are many potential causes of chronic LBP including degenerative disc disease (DDD). While natural age-related degeneration of intervertebral discs (IVDs) is common (Boden et al., 1990, Jensen et al., 1994), chronic LBP is associated with increased signs of disc degeneration (Luoma et al., 2000b, Samartzis et al., 2011). Like most other conditions, back pain is the product of genetic (Costigan et al., 2010, Nyman et al., 2010) and environmental (Bovenzi, 2010, Chou and Shekelle, 2010) influences.

SPARC (secreted protein, acidic, rich in cysteine; aka osteonectin or BM-40) is an evolutionarily conserved collagen-binding protein present in IVDs. SPARC is known to influence bone remodeling, collagen fibrillogenesis, and wound repair (Brekken and Sage, 2001). Decreased expression of SPARC has been associated with aging and degeneration in human IVDs (Gruber et al., 2004). Furthermore, targeted deletion of the SPARC gene results in accelerated disc degeneration in the aging mouse and a behavioral phenotype resembling chronic LBP in humans

(Gruber et al., 2005, Millecamps et al., 2011a). The genetic evidence from mice and the clinical observation that SPARC is down-regulated in humans with disc degeneration suggests that long-term down-regulation of SPARC expression may play a critical role in chronic LBP. What are the mechanisms that could lead to lasting down-regulation of genes such as SPARC?

One mechanism that is now well established for stable, long-term programming of gene expression is DNA methylation. The DNA is covalently modified by the addition of methyl moieties by an enzymatic DNA methyltransferase reaction that catalyzes the transfer of a methyl group from the methyl donor S-adenosyl methionine. What distinguishes DNA methylation in vertebrate genomes is the fact that not all CpGs are methylated in any given cell type, generating cell type-specific patterns of methylation (Razin and Szyf, 1984), which confer upon a genome its cell type-specific identity. Active regulatory regions of the chromatin, which enable gene expression, are associated with hypo methylated DNA, whereas hyper methylated DNA is packaged in inactive chromatin resulting in gene silencing (Razin and Cedar, 1977, Razin, 1998). Patterns of DNA methylation are generated during gestation and until recently were believed to be restricted to life-long programming of cell type-specific gene expression (Razin and Riggs, 1980). However, recent data suggests that DNA methylation is dynamic in adult non-dividing cells and is responsive to environmental signals (Weaver et al., 2004). It might therefore play a role in the modulation of gene function in response to a plethora of environmental signals after birth and throughout life (Szyf, 2009a).

We tested the hypothesis that DNA methylation occurring later in life or in pathological conditions might play a role in chronic LBP. The possibility that DNA methylation might precipitate chronic pain through down-regulation of expression of critical genes such as SPARC has not been previously addressed.

Our results are consistent with the hypothesis that alterations in DNA methylation associate with chronic LBP and IVD degeneration in mice and in humans with chronic LBP. This data provides the first line of evidence that supports the hypothesis that DNA methylation is involved in chronic pain.

5.3 Materials and Methods

Animals

All procedures were approved by the Animal Care Committee at McGill University, and conformed to ethical guidelines of the Canadian Council on Animal Care. Female C57BL/6 mice were used in this study. The SPARC-null mouse (Norose et al., 1998) was backcrossed onto a standard C57BL/6 background for >12 generations (Brekken et al., 2003). All animals were bred and aged in-house and experienced identical environments. Experiments were performed blind to genotype and age.

Mouse Behavioral Assays

Axial Pain: Tail Suspension Assay

Mice were suspended underneath a platform by the tail with adhesive tape attached 0.5-1.0 cm from the base of the tail and videotaped for 3 minutes. The duration of time spent in a) immobility (not moving but stretched out), b) rearing (trying to reach the underside of the platform), c) full extension (actively reaching for the floor), and d) self-supported (holding either the base of its tail or the tape) were determined (Steru et al., 1985).

Radicular Pain: Sensitivity to Cold Stimuli

Spontaneous nociceptive behaviors (flinching, licking or biting) were measured for 1 minute after a drop of acetone (~25 µl) was applied to the hindpaw as previously described (Millecamps et al., 2011a).

Radicular Pain: Sensitivity to Mechanical Stimuli

A series of calibrated monofilaments (von Frey filaments) were applied with increasing force to the plantar surface of the hindpaw until the animal responded by withdrawing. A decrease in threshold suggests the development of mechanical hypersensitivity. Results are expressed as the threshold (in grams) to withdraw 50% of the time as previously described (Millecamps et al., 2011a).

Rotarod Assay

We used the accelerating rotarod assay to monitor animals for decreased motor function (Jones and Roberts, 1968). The latency to fall was recorded in seconds. The maximum duration of the test was 5 minutes.

Radiographic assessment of IVD degeneration in Mice

Aging WT mice were anaesthetized and x-rays were obtained using a Faxitron 3000. For SPARC-null and WT control mice, radiography was performed *ex vivo*. Disc height was calculated for all lumbar IVDs (Figure 5). The observer was blind to age and genotype.

Human Subjects

Procedures involving human subjects were approved, as applicable, by the following Institutional Review Boards: McGill University Faculty of Medicine, McGill University Health Centre, The University of Minnesota and Allina Health Care (Minneapolis, MN).

Three groups of human subjects were used in this study. Additional information on human subjects can be found in table 1.

Experimental Subjects

The experimental group was recruited from a pool of patients scheduled for spinal fusion to treat severe cLBP associated with lumbar disc degeneration (mean age = 45.6 ± 2.8, n=10, 3 males, 5 females, 2 unknown; see table 1). Following surgical removal, discs were rinsed briefly in PBS, sectioned into quadrants, flash frozen in liquid nitrogen and stored at -80C.

Healthy Pain-Free Controls

A healthy control group consisting of individuals free of back pain was used as a comparator for the pain and disability assessments and disc degeneration scores in the surgical samples. (Mean age = 41.2 ± 2.3, n=23, 14 males and 9 females). No tissue was available from these subjects.

Non-Degenerated Control Discs

Non-degenerated control IVDs were acquired with the cooperation of Transplant Quebec from tissue donors. Lumbar spinal columns were extracted intact and placed on ice for transport. While age, sex and cause of death is recorded, no information is available regarding the presence of back pain or physical disability before death. X-rays were obtained of lumbar spines prior to IVD dissection and discs with obvious signs of degeneration (reduced height, calcification, bony spurs) were excluded from the study. Following x-ray of the entire column, the IVDs were dissected and stored at -80 until use. (Mean age = 58.2 ± 4.4, n=5 males, see Table 1).

Pain and disability assessment in human subjects

Pain was assessed using the numeric rating scale in which subjects rate pain intensity on a scale of 0-100, where 100 is the worst pain imaginable. Pain-related disability was determined using the Oswestry Disability Index (ODI) questionnaire (Fairbank and Pynsent, 2000).

Radiographic assessment of IVD degeneration in human subjects

Lumbar MRIs were obtained for all surgical and healthy, pain-free control subjects. Each lumbar disc was scored using the 5-point Pfirrmann scale (Pfirrmann et al., 2001) by a radiologist blind to diagnosis.

Bisulfite mapping and expression analyses

DNA was treated with sodium bisulfite and primers were designed for converted products of both mouse [GenBank:AL596207.10] and human [GenBank: AC011374.6] SPARC promoters. PCR products were sequenced using the Biotage Pyrosequencer according to the manufacturer's protocol (See Table 2 for primer sequences) (Colella et al., 2003). Expression of *SPARC* [GenBank: AK003162.1] was quantified using quantitative RT-PCR on the Lightcycler 480 using *GAPDH* [GenBank: AK002273.1] for normalization.

RNA extraction was carried out using Trizol (Invitrogen) and followed by Dnase I treatment and cDNA conversion using random hexamers (Roche Molecular Biochemicals) according to manufacturer's instructions. Expression of SPARC was then quantified using quantitative RT-PCR on the Lightcycler 480 using GAPDH for normalization. *SPARC* primers for human and mouse are listed in Table 2.

Bisulfite PCRs were amplified using two rounds of PCR using outer and nested primers (see Table 2.). Cycling conditions involved an initial step of 5 minutes at 95^{o}C followed by 35 cycles of [95 ^{o}C for 1 minute, T_m for 2.5 minutes, 72^{o}C 1 minute] Followed by 5 minutes of 72^{o}C. Luciferase construct PCRs were made with primers (See Table 2.) using the same cycling conditions only with one round of PCR. Quantitative PCR was amplified with a pre-incubation at 95 ^{o}C for 10 minutes followed by 45 cycles of [95 ^{o}C for 10 seconds, 60 ^{o}C for 10 seconds, 72 ^{o}C for 10 sec] followed by 10 minutes of 72 ^{o}C.

Luciferase reporter assay

Human and mouse SPARC promoters were subcloned into the CpG-less pCpGL luciferase reporter plasmid (Klug and Rehli, 2006) in both 5' to 3' (sense) or 3' to 5' (antisense) orientation, respectively.

The constructs were methylated in vitro with SssI CpG DNA methyltransferases (New England Biolabs, Inc.) as recommended by the manufacturers.

Transfections were performed using calcium phosphate precipitation as described previously (Rouleau et al., 1992). Cells were harvested 48 h after transfection and luciferase activity was assayed using the Luciferase Assay System (Promega).

Statistical analysis

All data are plotted as mean ± SEM. The student t-test (2-tailed, unpaired) was used in data comparing two groups. One-way ANOVA followed by Bonferroni's test was used when comparing 3 groups. For the luciferase assay, data is shown in the percentile form. All data was analyzed and graphed using Prism 4.0 (GraphPad Software, Inc., La Jolla, CA).

5.4 Results

Aging and SPARC-null mice exhibit signs of disc degeneration and back pain

Disc degeneration is known to increase as a function of aging in both humans and rats (Boden et al., 1990, Zhang et al., 2009) and is associated with an increased risk of chronic LBP (Luoma et al., 2000b). In order to determine the effect of aging on disc degeneration in mice, disc height was determined from x-rays of the lumbar spine in young (3 month), middle-aged (7 month), and old (15 month) C57B wild-type (WT) mice. Our data shows reduction in lumbar disc height as a function of aging (**Figure 1a**). A similar decrease in disc height was observed in young 4-month old SPARC-null mice compared to their age-matched WT controls, such that a 4-month old SPARC-null mouse is roughly equivalent to a normal 15-month old animal in terms of disc anatomy (**Figure 1f**). Thus, both normal aging and the deletion of the SPARC gene results in disc degeneration in mice, supporting a role for SPARC in maintaining disc integrity.

We then tested whether age-dependent disc degeneration in mice is associated with behavioral signs of axial and radicular pain and motor impairment, and whether it is impacted by loss of function of SPARC.

Axial pain was assessed using a modified version of the tail suspension assay in which the spontaneous reaction to gravity-induced strain along the axis of the spine was measured (Steru et al., 1985). Young animals (3 months) spent significantly more time in the immobility posture than did older animals (**Figure**

1b). This implies a reluctance to stretch the body axis, which is suggestive of axial pain. A similar decrease in immobility was observed in 4-month old SPARC-null mice compared to their age-matched WT controls (**Figure 1g**).

Sensitivity to mechanical and cold stimuli in the hindpaw was used as behavioral indices of radicular pain. While 7- and 15-month old WT mice developed hypersensitivity to cold as a function of age (**Figure 1c**), mechanical paw withdrawal thresholds were not significantly altered (**Figure 1d**). Similarly, 4-month old SPARC-null mice presented with cold but not mechanical hypersensitivity compared to their WT controls (**Figure 1h,i**).

Physical ability was assessed using the accelerating rotarod assay, in which the latency to fall from a rotating treadmill is measured. Aging WT mice showed impaired performance on this task at 7- and 15- months of age, suggesting the presence of movement-evoked pain and/or motor dysfunction (**Figure 1e**). SPARC-null animals showed a trend towards impaired motor function compared to WT mice at 4-months of age (**Figure 1j**).

Together, these data indicate that naive WT mice develop age-dependent disc degeneration along with behavioral signs of axial and radicular low back pain and motor impairment.

Aging mice demonstrate changes in expression and DNA methylation of SPARC

The aging phenotype in WT mice is remarkably similar to 4-month old SPARC-null mice, suggesting that the absence of the SPARC gene accelerates the normal aging process in the IVDs, resulting in low back pain. We therefore tested whether aging is accompanied by reduced SPARC expression. A significant decrease in SPARC mRNA expression was observed in lumbar IVDs taken from 15-month-old mice compared to 3-month-old mice (**Figure 2b**). This is consistent with the hypothesis that age-dependent down-regulation of SPARC might be associated with chronic LBP and that mechanisms that stably down-regulate SPARC expression are involved in precipitating chronic pain.

We therefore addressed the question of what is the mechanism that down-regulates SPARC during aging. The SPARC promoter has been shown previously to be modulated by DNA methylation in cancer. Specifically, it is hyper methylated and silenced in the majority of invasive cervical cancer cases (Sova et al., 2006). Cancer epigenetics has taught us that tumor suppressor genes that are suppressed in cancer because of genetic mutations are silenced in many other cases by DNA methylation, leading to similar phenotypes. Interestingly, genes that are frequently hyper methylated in cancer are also hyper methylated with age (Ahuja et al., 1998a). We therefore hypothesized that a similar mechanism operates in chronic LBP, whereby the age-dependent decrease in SPARC mRNA expression is due to increased methylation of the SPARC promoter. DNA was prepared from mouse lumbar discs and the state of methylation of the SPARC

promoter region was mapped in 3-, 7-, and 15-month old mice. A pyrosequencing analysis of the methylation state of 6 CpG sites residing in the SPARC gene promoter region (**Figure 2a**) showed increased methylation in IVDs with increasing age at several sites located in the promoter (**Figure 2c**).

The DNA demethylating agent 5-azacytidine silences SPARC gene expression in the mouse IVD *in vivo*

To test whether DNA methylation is involved in silencing SPARC gene expression in IVD *in vivo,* we treated 1-year old mice with the DNA demethylating agent 5-azacytidine (5AC) (30 mg/kg, i.v. and 250 fmol i.t.). We observed a significant > 4-fold increase in SPARC mRNA in the 5AC-treated mice compared to the vehicle-treated controls (**Figure 2d**). A pyrosequencing analysis revealed demethylation of the SPARC promoter in animals treated with 5-AC (**Figure 2e**). To further test the involvement of SPARC methylation in chronic pain we determined the relationship between state of methylation of the SPARC promoter and cold allodynia as a measure of pain. Our data indicates that the state of DNA methylation was significantly correlated with the magnitude of cold allodynia *in vivo* (**Figure 2e, inset**).

The mouse and human SPARC promoters are silenced by DNA methylation *in vitro*

Our results with 5AC presented in Figure 2 show that non-specific demethylation can activate the SPARC gene *in vivo*. In order to directly determine whether methylation of the SPARC gene promoter region silences either mouse or human

SPARC promoters we subcloned the promoter region into a CpG-deficient luciferase reporter plasmid pCpGL (Klug and Rehli, 2006). The same region was subcloned in the antisense direction as a negative control. We methylated exclusively the sites that were shown to be methylated *in vivo* using Sss1 DNA methyltransferase (**Figure 3a,b**), and compared luciferase activity after transient transfection into HEK293 cells with the same plasmid that was mock-methylated. The luciferase expression observed in the control condition was completely silenced by methylation of both the mouse (**Figure 3c**) and human SPARC promoters (**Figure 3d**), demonstrating that methylation silences SPARC promoter activity.

SPARC promoter is hyper methylated in LBP patients with disc degeneration

Given the potential clinical significance of these findings, we examined whether these results could be translated to humans. Patients with severe chronic LBP were recruited from a pool of individuals scheduled for spinal fusion surgery due to severe disc degeneration. Pain free controls were recruited from the general population. As anticipated, the surgical group had higher pain and disability levels than control subjects (**Figure 4a,b**). The degree of degeneration of the lumbar spine was determined by lumbar MRI and was also significantly increased in patients vs. controls (**Figure 4c**). These data indicate that our patient population had significant increases in disc degeneration, pain, and disability compared to the general population.

Decreases in SPARC expression with age and degeneration have been reported elsewhere (Gruber et al., 2004). Unfortunately, the quality of the RNA derived from the human IVD didn't allow us to accurately measure SPARC mRNA. However, the stability of DNA allowed us to perform bisulfite mapping of the promoter region. Increased methylation of the SPARC promoter (**Figure 4d**) was observed in surgical samples compared to controls. Five out of the thirteen individual sites examined had significantly increased methylation in the surgical samples (**Figure 4e**). Our data supports the hypothesis that increased DNA methylation of promoters such as SPARC gene promoter is associated with pain in humans.

5.5 Discussion

The prevalence of chronic pain in the general population is estimated at 15-40%. In addition to being a major health care problem, chronic pain has serious economic consequences, costing billions of dollars per year in lost productivity and medical expenses. Chronic pain is often resistant to therapeutic intervention, and individuals suffer for years without relief. New therapeutic strategies are desperately needed. Despite enormous efforts, significant advances in pain management continue to be elusive.

While a genetic basis for individual variations in the development of chronic pain is well established (Diatchenko et al., 2005a), genetics only accounts for approximately half of the inter-individual variability in chronic low back pain (MacGregor et al., 2004b). One concept that is beginning to receive attention in pain research is the fact that gene function can be altered not only by differences in gene sequence but also by differences in epigenetic modification. Epigenetic modulation refers to chemical modifications of DNA including DNA methylation that produce long-term changes in gene expression. These changes can have long-lasting biological consequences and could become maladaptive, leading to chronic diseases such as obesity (Vucetic et al., 2010), fatigue (Landmark-Hoyvik et al., 2010) or neurological and mental disorders (Szyf, 2009a, Sweatt, 2010).

In humans, decreased expression of SPARC is observed in painful, degenerating discs (Gruber et al., 2004) and deletion of the SPARC gene triggers accelerated age-dependent disc degeneration (Gruber et al., 2005) and chronic pain in mice

(Millecamps et al., 2011a). We therefore hypothesized that the SPARC gene may be silenced by DNA methylation as a function of aging in degenerating discs.

In the current study we show that disc degeneration is accompanied by signs of axial and radicular pain and physical impairment in mice (Figure 1) and with pain and physical disability in humans (Figure 4). Targeted inactivation of the SPARC gene results in early onset of both disc degeneration and behavioral indices of LBP in mice (Gruber et al., 2005, Millecamps et al., 2011a). Having established that deletion of SPARC increases chronic pain and disc generation, we determined whether this gene is commonly silenced by DNA methylation during normal aging. Increased disc degeneration and chronic pain are associated with age in rodents and humans.

There are multiple mechanisms by which IVD degeneration can result in chronic axial and/or radicular LBP. In the periphery, spinal instability due to disc degeneration could result in irritation of other structures such as the facet joints, muscles and ligaments. Increased innervation of degenerating discs by sensory neurons (Freemont et al., 1997) is also thought to contribute to discogenic pain (Garcia-Cosamalon et al., 2010) and contact with the contents of the disc results in increased neuronal excitability and sensitization (Kawakami et al., 2003b, Inoue et al., 2006a). In addition, disc degeneration may result in radicular pain following nerve compression due to disc bulging or herniation (Song et al., 1999, Lacroix-Fralish et al., 2006).

Within the central nervous system, ongoing nociceptive input from peripheral structures may result in sensitization within the spinal cord or supraspinal structures, resulting in an exaggerated response to subsequent peripherally applied stimuli (for reviews see (Zhuo, 2007, Ruscheweyh et al., 2011)). Furthermore, chronic LBP results in changes in brain structure and function (Apkarian et al., 2004, Seminowicz et al., 2011). Interestingly, therapeutic interventions that target the spinal column such as facet joint blocks or spinal surgery can reverse pain-related changes in the brain, suggesting that ongoing input from the periphery actively maintains pain-related CNS plasticity (Seminowicz et al., 2011). While the current study is focused on the epigenetic modulation of the degeneration of a peripheral structure, studies examining the role of epigenetics in pain-related CNS plasticity are needed.

We provide several lines of evidence that support the hypothesis that DNA methylation occurs during aging and that it results in silencing of SPARC. First, the state of methylation of several CG sites in the promoter is increased with aging. Second, DNA methylation inhibitors resulted in demethylation of the SPARC promoter and increased expression of SPARC *in vivo*. Third, methylation of CG sites in the promoter lead to silencing of promoter activity. The combination of the genetic evidence for the role of SPARC in chronic pain and the DNA methylation analysis provide strong support for the idea that DNA methylation occurs during aging and that it is involved in disc degeneration and chronic LBP. Although it was not possible to provide direct evidence of SPARC mRNA expression in clinical samples, we tested whether the clinical situation is

consistent with our hypothesis. We show that discs removed from patients that suffered from chronic LBP also exhibited increased methylation of the SPARC gene (Figure 4). The human promoter, like the mouse promoter, is silenced by DNA methylation (Figure 3).

Unlike the static genome, the epigenome is in dynamic equilibrium throughout our lifespan and DNA methylation is a bidirectional process that can be altered by pharmacological agents (Szyf, 2009b). Changes in methylation appear to be a common feature in aging cells and tissues. Epigenetics also plays a key role in the development of diseases associated with aging including cancer (Issa et al., 1994, Ahuja et al., 1998b), atherosclerosis, and neurodegenerative and autoimmune disorders (Rodriguez-Rodero et al., 2010a). Interestingly, age itself is a risk factor for chronic pain in humans (Thomas et al., 1999). Using the candidate gene approach, we demonstrate that aging is one physiological process that could lead to hyper methylation and silencing of SPARC. It is likely that aging results in silencing of additional genes that are involved in chronic pain either in the periphery or the central nervous system. Moreover, it stands to reason that other transient environmental exposures such as tissue injury could result in DNA methylation of many other genes, which could serve as a long-term memory of such exposures in the genome, resulting in chronic pain.

Our study provides the first line of evidence that DNA methylation is involved in chronic pain. Specifically, we present evidence from both mouse and human studies supporting the hypothesis that DNA methylation of the SPARC promoter is increased with age and intervertebral disc degeneration, resulting in the

silencing of a gene that is protective against accelerated disc degeneration. The SPARC gene is likely to be just one example of many pain-relevant genes that are similarly regulated by DNA methylation in both peripheral tissues and in the central nervous system.

Epigenetic modifications are at the interface between environment and genetics, creating a mechanism by which life experience can lead to long-lasting changes in gene expression. If DNA methylation is implicated in chronic pain, it will provide not only new understanding of the underlying mechanisms involved in generation and maintenance of chronic pain, but also new therapeutic possibilities.

5.6 Figures

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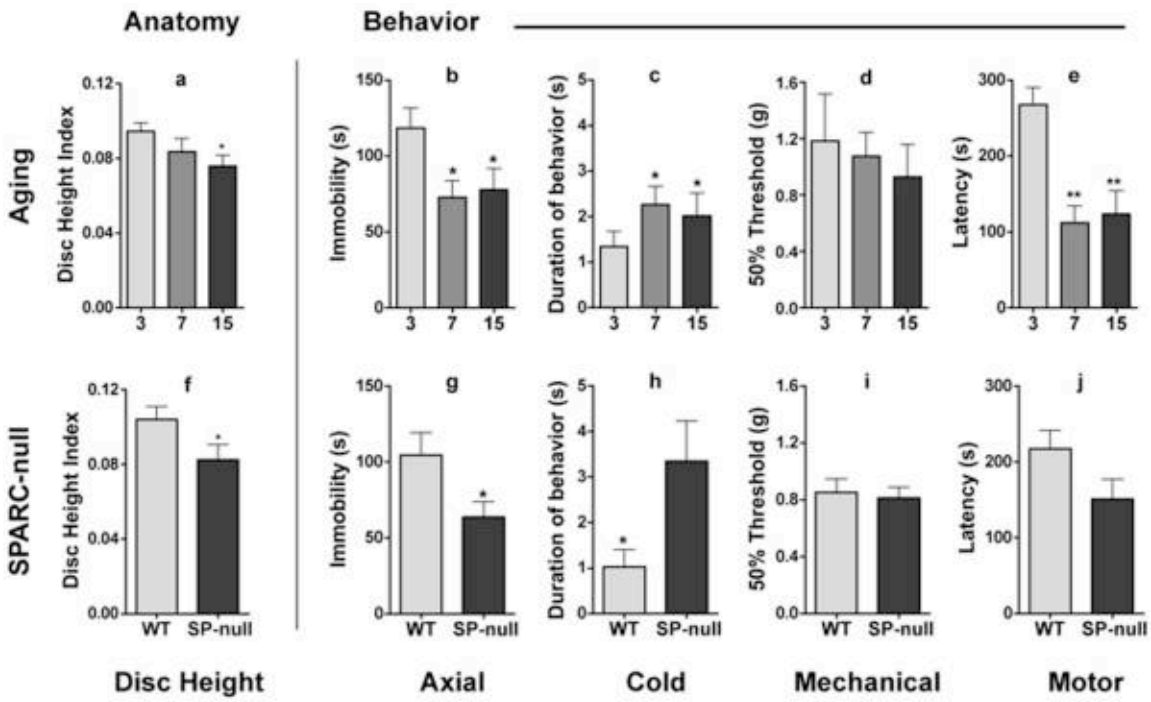
Figure 1. Disc degeneration and behavioral signs of low back pain in aging and SPARC-null mice.

Anatomy: The disc height index measured in 15-month old mice is smaller than that in 3-month old mice (a). 4-month old SPARC-null mice show smaller average disc height compared to age-matched WT controls (f). *Pain Behavior:* Aging mice exhibit signs of axial discomfort (b), cold sensitivity (c), but not mechanical sensitivity (d) in the hindpaw, in addition to overall motor impairment (e) when compared to 3-month old mice. 4-month old SPARC-null animals show a behavioral profile similar to older WT mice (g-j). *=$p<0.05$, **=$p<0.01$, One-way ANOVA followed by Bonferroni's test (aging cohorts), two-tailed student t-test (SPARC-null vs. WT). n=8-15/group. Error bars=S.E.M.


Anatomy
Behavior
Aging
SPARC-null
Disc Height Index
Immobility (s)
Duration of behavior (s)
50% Threshold (g)
Latency (s)
a
b
c
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e
f
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h
i
j
3
7
15
WT
SP-null
Disc Height
Axial
Cold
Mechanical
Motor

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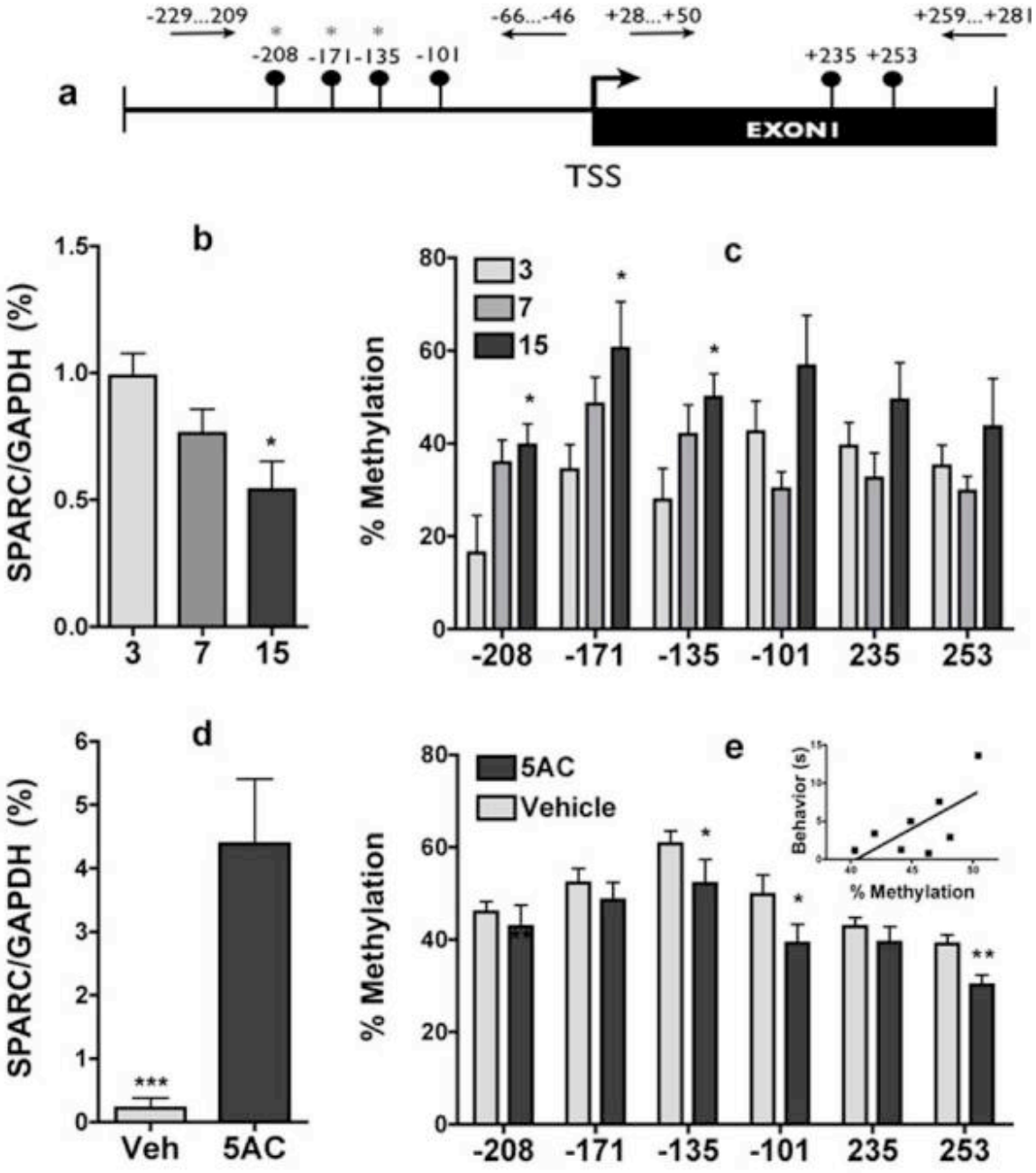
Figure 2. Changes in expression and DNA methylation of SPARC in aging mice. SPARC mRNA expression (relative to *GAPDH*) at different time points in life (b). Age-dependent changes in methylation of CG sites in the SPARC promoter (a) in IVDs as quantified by pyrosequencing (c). Treatment of 1-year old mice with the demethylating drug 5AC or vehicle (30 mg/kg, i.v. and 250 fmol i.t.) resulted in a 4-fold increase in SPARC mRNA expression and (d) decreased methylation of CG sites in the SPARC promoter as quantified by pyrosequencing in IVDs. Inset: Increased cold sensitivity following 5AC injection was significantly correlated with total SPARC methylation. *=$p<0.05$, ***=$p<0.001$. One-way ANOVA followed by Bonferroni's test (aging cohorts) and two-tailed student t-test (5AC vs. vehicle treated). Pearson's correlation ($p=0.03$, $r^2=0.46$). n=3-15/group. Error bars=S.E.M.


a
-229...209
-66...-46
+28...+50
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-171
-135
-101
+235
+253
EXON I
TSS
b
SPARC/GAPDH (%)
1.5
1.0
0.5
0.0
3
7
15
c
% Methylation
80
60
40
20
0
3
7
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-208
-171
-135
-101
235
253
d
SPARC/GAPDH (%)
6
5
4
3
2
1
0
***
Veh
5AC
e
% Methylation
5AC
Vehicle
Behavior (s)
% Methylation
**

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Figure 3. DNA methylation silences murine and human SPARC promoter activity. The mouse (a) and human promoter regions (b) were cloned into a pCPGL-basic plasmid (the CpG positions are indicated as balloons). There were no CpGs in the vector. The plasmids were either methylated or mock methylated *in vitro* and then transfected into HEK293 cells in the sense or antisense (1st column) configuration for 48 h (c, d). Relative luciferase activity (percentage) in the extracts is shown as average per group (triplicate transfection). The decrease in expression in the methylated (3rd column) vs. unmethylated (2nd column) treatment groups indicates gene silencing. One-way ANOVA followed by Bonferroni's test. **=$p<0.01$ Error bars=S.E.M.

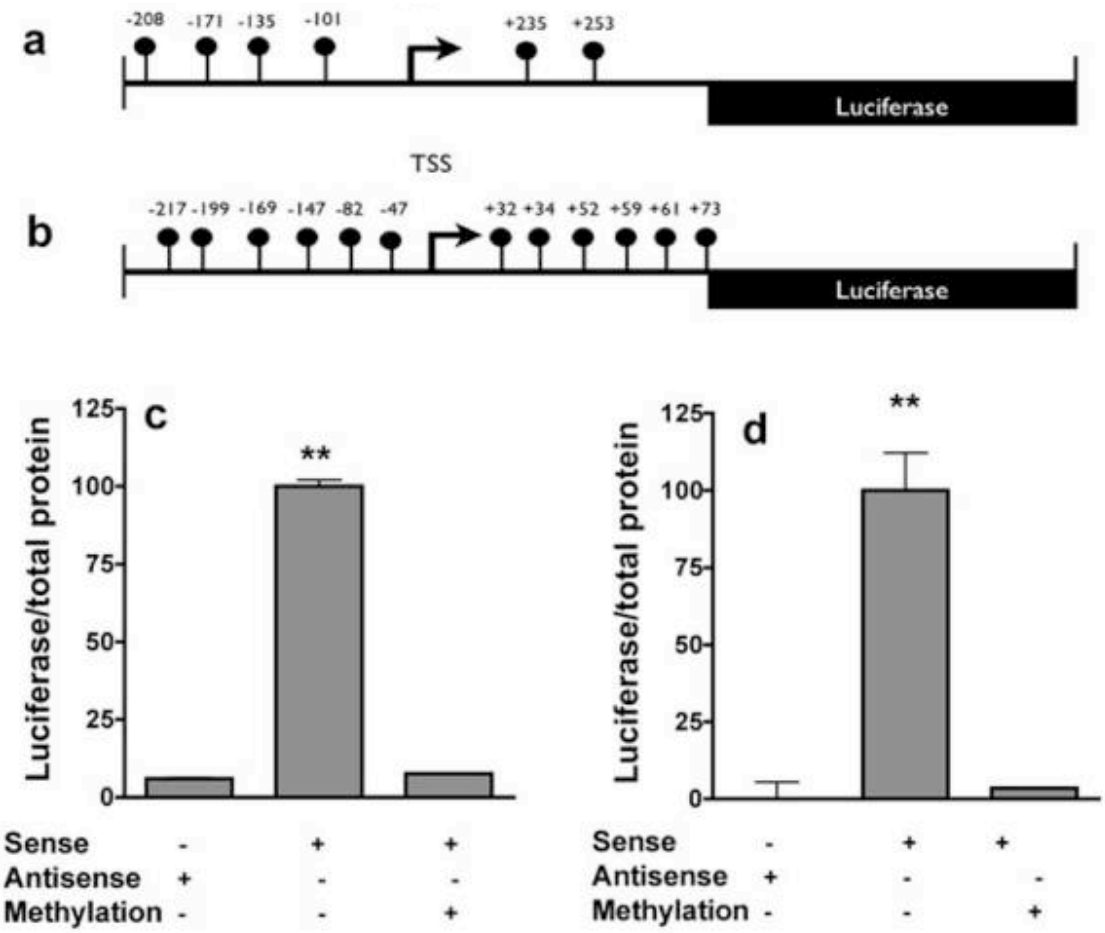
a
-208
-171
-135
-101
+235
+253
Luciferase
TSS
b
-217
-199
-169
-147
-82
-47
+32
+34
+52
+59
+61
+73
Luciferase
c
Luciferase/total protein
125
100
75
50
25
0
**
Sense - + +
Antisense + - -
Methylation - - +
d
Luciferase/total protein
125
100
75
50
25
0
**
Sense - + +
Antisense + - -
Methylation - - +

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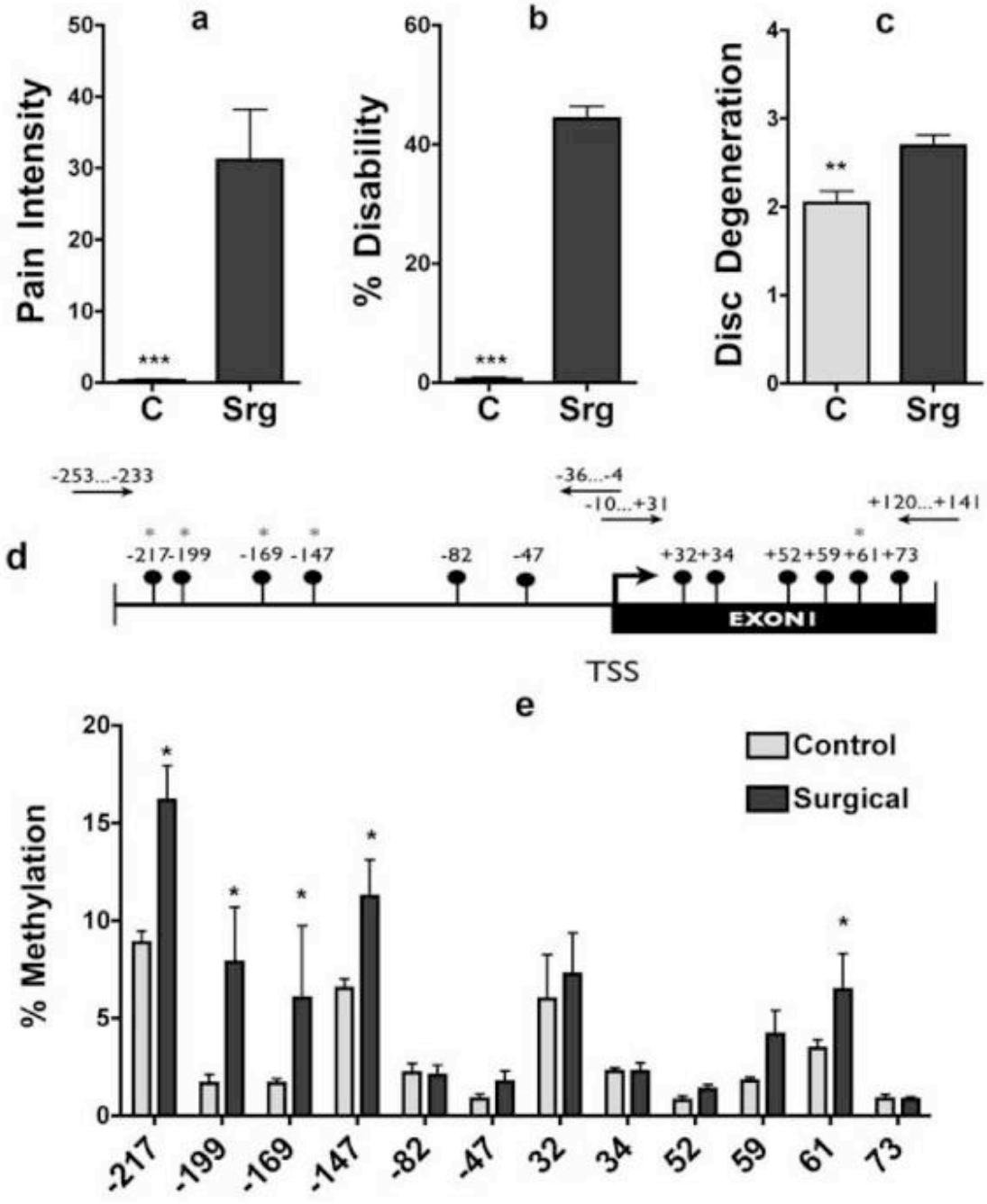
Figure 4. SPARC mRNA expression and DNA methylation in IVDs from chronic LBP patients with disc degeneration.

Pain intensity measured with the numeric rating scale (a) and physical disability as determined by the Oswestry Disability Index (b) were increased in surgical patients, as was lumbar disc degeneration based on scoring of MRI images (c). The state of methylation of CG sites in the human SPARC promoter (d) in L4-L5 IVDs was increased as quantified by pyrosequencing (e) *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$. Two-tailed student t-test. n=5-8/group. Error bars=S.E.M.


a
Pain Intensity
50
40
30
20
10
0
***
C
Srg
b
% Disability
60
40
20
0
***
C
Srg
c
Disc Degeneration
4
3
2
1
0
**
C
Srg
d
-253...-233
-36...-4
-10...+31
+120...+141
-217 -199
-169 -147
-82
-47
+32+34
+52+59+61+73
EXON I
TSS
e
% Methylation
20
15
10
5
0
Control
Surgical
-217
-199
-169
-147
-82
-47
32
34
52
59
61
73

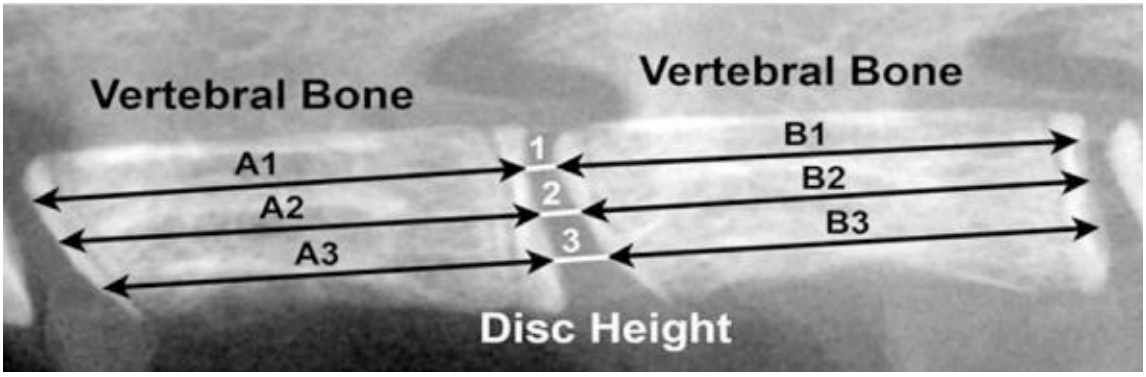


Figure 5. Calculation of Disc Height Index from Mouse Lumbar Spinal X-ray.

Lateral x-ray images of the intact lumbar spine were taken at 4x using a Faxitron ® MX-20 (Faxitron X-Ray LLC, Lincolnshire, IL). Disc Height Index (DHI) was determined according to the following equation: Disc Height Index (DHI) = 2 x (DH1 + DH2 + DH3) / (A1 + A2 + A3 + B1 + B2 + B3) where A and B represent the length of the vertebral bone immediately rostral and caudal to the IVD, respectively; and DH represents the disc height between adjacent vertebrae.

5.7 Tables

Table 1. Subject information from cadaveric and surgical human IVD samples

Subject ID	Gender	Age	Cause of Death
tq8	Male	62	Stroke (brain)
tq10	Male	62	Myocardial infarction
tq12	Male	55	Motor Vehicle accident
tq16	Male	43	Anoxia (hanging)
tq47	Male	69	Unknown
1007	Female	43	N/A
1009	Unknown	49	N/A
1015	Female	52	N/A
1016	Female	44	N/A
1106	Female	43	N/A
1112	Male	28	N/A
1124	Female	55	N/A
1172	Male	49	N/A
2000	Male	36	N/A
9634	Unknown	57	N/A

Description: Gender, Age and Cause of Death (if applicable) is indicated for all human intervertebral disc samples used in this study.

Table 2. Primer Sequences

Bisulfite PCR Primers	Sequence	Tm (°C)
Outer Human SPARC forward	GAGGGTTATGTTTTTAAGGGGAGT	60
Outer Human SPARC reverse	CTCCAAACCTCACTTACCCTCT	60
Nested Human SPARC region 1 forward	GGGGTTGGTGTAATTATAGAAGG	60
Nested Human SPARC region 1 reverse	AAACCCAACCCAAAACTCTAAA	60
Nested Human SPARC region 2 forward	GAGTTTTGGGTTGGGTTTATTTT	60
Nested Human SPARC region 2 reverse	CATCCCTCCAAACCTATCCA	60
Outer Mouse SPARC forward	GGTTGATAGGGAGGTGTATATTT	54
Outer Mouse SPARC reverse	TCCCCCACTACCATACCAAC	54
Nested Mouse SPARC region 1 forward	AGGTAGGTAGGTAGGTAGGTAGG	60
Nested Mouse SPARC region 1 reverse	TTTTTGTTTTGTGGTAGGTTGTG	60
Nested Mouse SPARC region 2 forward	GGGTTGGAATAGTTGTTGGAA	60
Nested Mouse SPARC region 2 reverse	GAGTGAATTTGTTTGAGTATTTTT	60
Expression Primers		
Mouse SPARC forward	ATGAGGGCCTGGATCTTCTTTC	60
Mouse SPARC reverse	GGAAGAGTCGAAGGTCTTGTTGTC	60
Mouse GAPDH forward	GTCGTGGAGTCTACTGGTGTC	60
Mouse GAPDH reverse	GAGCCCTTCCACAATGCCAAA	60
Luciferase Construct Primers		
Human SPARC Sense for	AAGCTTTATAGGGGGTCACACATACCTCAG	60
Human SPARC Sense rev	GGATCCTATAAAAGAGGCTGTTCTGGGTCA	60
Human SPARC Antisense for	GGATCCTATAGGGGGTCACACATACCTCAG	60
Human SPARC Antisense rev	AAGCTTTATAAAAGAGGCTGTTCTGGGTCA	60
Mouse SPARC Sense for	AAGCTTTATACTGACAGGGAGGTGCATACC	60
Mouse SPARC Sense rev	GGATCCTATATCTGCCTTGCTGTACATTGC	60
Mouse SPARC Antisense for	GGATCCTATACTGACAGGGAGGTGCATACC	60
Mouse SPARC Antisense rev	AAGCTTTATATCTGCCTTGCTGTACATTGC	60
Pyrosequencing Primers		
Mouse SPARC Region 1 Sequencing 1	CCTCCACATTCCTACAACCC	N/A
Mouse SPARC Region 2 Sequencing 1	TAGGTTAGGTTTTGTTTAGAG	N/A
Mouse SPARC Region 2 Sequencing 2	GAGAGAGAGAGAGTTATAGAGGT	N/A
Human SPARC Region 1 Sequencing 1	AATTATAGAAGGGAAAGGTT	N/A
Human SPARC Region 2 Sequencing 1	AACCCTAACACTCTATAAAT	N/A
Human SPARC Region 2 Sequencing 2	TTGGGTTTATTTTTTTTTTA	N/A

Description: Provides the sequences and Tm (°C) for the Bisulfite PCR Primers, Expression Primers, Luciferase Construct Primers and Pyrosequencing Primers used in this study.

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6 Conclusions

6.1 Summary

We have previously shown that the SPARC-null mouse model is a useful model to study back pain due to disc degeneration in humans (Millecamps et al., 2011b, Millecamps et al., 2012). In brief, SPARC-null mice show anatomical and histological signs of disc degeneration in the form of decreased lumbar disc height and change in proteoglycan content, respectively. Disc degeneration in these mice is accompanied by behavioral signs of axial and radiating pain. The phenotype is developed by 6 months of age (Millecamps et al., 2012).

In order to elucidate the relationship between disc degeneration and pain, we conducted correlation studies between lumbar disc height index and measures of axial and radiating pain. Our results in Chapter 2 show that both disc height and shape correlate with the degree of cold allodynia in SPARC-null mice.

In order to better understand the mechanisms of pain in SPARC-null mice, we studied the pharmacological profile of axial and radiating pain. Our results show that these two types of pain are reversed by different sets of pharmacological agents, indicating different pain mechanisms. While axial pain can be reversed both by anti-inflammatory and anti-neuropathic agents, radiating pain is more sensitive to anti-neuropathic agents.

Next, we used our mouse model to test the effects of co-administration of morphine and clonidine on pain. In humans with chronic LBP, opioids are often prescribed with varying outcomes and a multitude of side effects; combination

therapy may be key in improving therapeutic outcome in these patients. We show that the administration of morphine and clonidine in a fixed 100:1 ratio acts synergistically to ameliorate axial pain and improve the therapeutic index of radiating pain. These improvements were observed in the absence of enhanced side effects.

Finally, we emphasize the role of SPARC in disc degeneration and pain by studying age-related disc degeneration in WT mice and in patients with low back pain due to disc degeneration. We have shown that SPARC expression in intervertebral discs decreases with age and parallels "natural", age-related disc degeneration in WT mice. Furthermore, we explore the mechanism behind this phenomenon and observe that the promoter for the SPARC gene gets hyper-methylated in degenerating murine and human discs, thus suppressing SPARC expression and potentially contributing to DD-related LBP. This final part provides a novel link between the fields of epigenetics and pain, and offers a direct translation of our mouse studies to human patients.

6.2 General discussion

On the prevalence and causes of low back pain

Low back pain is a highly prevalent pathology effecting subjects of diverse age groups (Papageorgiou et al., 1995, Bressler et al., 1999, Turk et al., 2011, Macfarlane et al., 2012). The natural history of LBP is variable and many patients show improvement with some care. However, chronic LBP remains a serious problem since it effects 70-85% of adults at some points in their lives (Andersson, 1999) and has enormous economical consequences (Katz, 2006). At present, treatments for LBP are based on empirical observations targeting the alleviation of the symptoms instead of understanding the underlying mechanisms.

There are many causes for LBP. Multiple structures in the spine could be involved in the generation and maintenance of LBP in humans. These include ligaments, vertebral bodies, fascia, facet joints, sacroiliac joints, muscles, and intervertebral discs (Kuslich et al., 1991, Schwarzer et al., 1994, 1995). In theory, any structure that is innervated could be a potential source of pain in the spine.

One factor that is extensively studied in relation to LBP is DD. DD is associated with LBP (Luoma et al., 2000a) and is the focus of our SPARC-null mouse model. It's difficult to describe the etiology of DD since it's poorly defined. Perhaps it is best to characterize it as a condition that has its roots in changes in the cellular microenvironment of the intervertebral disc (Freemont, 2009). These could include, but are not limited to: reduced PH, decreased water content (Kitano

et al., 1993), up-regulated pro-inflammatory cytokines, an increase in associated catabolic enzymes (Le Maitre et al., 2007), and loss of proteoglycans (Lyons et al., 1981, Ohshima et al., 1995). With time, these cellular modifications could result in the breakdown of the substructures of the IVD, therefore interfering with normal load distribution and ultimately leading to physical disability and pain (Urban and Roberts, 2003).

On the diagnosis and teatment of disc degeneration

DD is primarily diagnosed using various imaging methods. Almost a century ago, the imaging method of choice was either radiography or computed tomography (for review, see (Emch and Modic, 2011)). In the 1980s, **magnetic resonance imaging (MRI)** started gaining popularity, and owing to great technological advances, has become the gold standard of diagnostic tools for DD (Haughton, 2004). Using MRI, it became possible to detect changes in disc height, calcification, osteophyte formation, herniation (Modic and Ross, 2007), and the presence of annular tears (Yu et al., 1988).

Although MRI is an excellent and minimally invasive tool to demonstrate morphological changes in the spine, it is not always clear what these changes signify. For example, changes detectable by MRI do not always correlate with the presence or the severity of chronic LBP (Boden et al., 1990), a fact that sheds some doubt on the utility of MRI for clinical diagnostic purposes.

DD is often characterized by loss of function of the intervertebral disc, with a strong emphasis on mechanical factors, such as load bearing and joint mobility. It

is therefore not surprising that DD is associated with physical disability, in addition to pain. Hence, treatment strategies should target both analgesia and the restoration of normal mechanical function. Biological treatment strategies that aim to repair (Masuda, 2008) or regenerate (Sobajima et al., 2004, Leung et al., 2006) the disc itself would be ideal, but unfortunately are not yet available in the clinic. Consequently, surgical procedures such as spinal fusion and discectomy remain the norm. These procedures often do not restore lost function in the spine, and have mixed outcome when it comes to ameliorating LBP. Moreover, despite the severity of LBP due to DD, conservative pharmacotherapy remains the most common therapy for many patients suffering from chronic LBP.

A multidisciplinary approach to understanding chronic Low Back Pain due to Disc Degeneration

Since chronic LBP is a heterogeneous and complex condition (Fourney et al., 2011), it is essential to understand its causes and underlying mechanisms before proposing any treatment options. To date, there are many *ex vivo*, *in vitro*, and *in vivo* studies that aim at a better understanding of chronic LBP due to DD.

Most *ex vivo* and *in vitro* studies are conducted using isolated intervertebral discs (Haschtmann et al., 2006, Lee et al., 2006, Korecki et al., 2007, Johnson et al., 2008, Junger et al., 2009, Bergknut et al., 2010, Paul et al., 2012) or isolated and cultured IVD cells (Ciapetti et al., 2012, Hartman et al., 2012). Such models lack the intact physiological system needed to observe the signs and understand the mechanisms of pain due to DD. However, they have the advantage of being able

to focus on a single factor, such as disc loading (Ayturk et al., 2010, Kuo and Wang, 2010, Walter et al., 2011) and disc nutrition (Grunhagen et al., 2011, Jackson et al., 2011, Malandrino et al., 2011, Stephan et al., 2011).

There exist many animal models for the *in vivo* study of LBP, as outlined in the "Introduction" chapter of this thesis. Such models are suitable for an overall assessment of back pain behavior but may be accompanied by confounding variables that could interfere with the interpretation of the observed behavioral phenotype.

Ideally, it would be best to pair *in vivo* and *ex vivo* studies for a better understanding of DD and pain. Doing so will enable us to understand the cellular/biomechanical/immunohistochemical factors involved in DD, and to link it to behavioral measures of chronic LBP. Unfortunately, this thorough approach is often lacking in pre-clinical studies, and it is rare to see *in vivo* studies paired with *ex vivo* work (Ellman et al., 2011).

On the use of the SPARC-null animal model of disc degeneration and back pain

The work shown in this thesis makes use of the SPARC-null model for an in-depth study of the mechanisms and treatment of axial low back pain and radiating pain due to disc degeneration. One of the main strengths of our research lies in our ability to study the role of SPARC using a wide range of techniques, and in both transgenic and WT mice and in human subjects. This approach has many advantages including:

1. Behavioral testing

The thorough behavioral testing was crucial in the assessment of the different pain phenotypes in our mice. Using multiple assays to measure similar outcomes has greatly increased the reliability of our methods. For instance, axial discomfort was measured by both the grip force and the tail suspension assays (although beyond the scope of this thesis, our lab has also developed a novel testing apparatus, the *flexmaze*©, which is also indicative of axial discomfort); cold sensitivity was measured by the acetone and cold paw immersion assays (in addition to a cold-plate temperature preference paradigm testing developed in our lab, the results of which are not shown in this thesis). Furthermore, assays of overall activity and motor function served to circumvent the problem of confounding factors often encountered when studying animal models of pain. Such assays were particularly useful when carrying out pharmacological studies that involve drugs with side effects that could impair motor coordination and overall motor activity.

2. Assessment of disc degeneration

The anatomical assessment of disc degeneration helps establish the link between DD and axial and radiating pain components of chronic LBP. This is crucial since many studies focus on pain without examining the mechanisms of DD and *vice-versa*. Although we've shown simple radiographic measurements in this document, we have also evaluated disc

degeneration using other techniques, such as micro computed tomography, MRI and histological techniques using classic hematoxylin and eosin (**H&E**) staining and the multichromatic FAST staining (useful to study the proteoglycan content in the IVD). Furthermore, we have conducted correlation studies for a better understanding of the relationship between disc degeneration and low back pain.

3. Pharmacology

The use of pharmacological techniques has been useful in teasing apart the inflammatory versus the neuropathic aspects of pain associated with DD. The main advantage of our studies is that testing was carried out in various assays and with a wide range of pharmacological agents. This data is useful when considering treatment options for axial and radiating pain in humans. In particular, our morphine and clonidine study seems to be very promising for patients whose pain is not adequately controlled by single drug treatment.

4. Age-related DD

The study of SPARC, DD and chronic LBP in WT mice serves to shed light on "natural", age-related DD. This is interesting since it is often difficult to distinguish between pathological, accelerated DD and normal, age-related DD; with the two falling on different ends of a continuum (Matsumoto et al., 2010). In addition, the pre-clinical literature on age-related DD and LBP is scarce. We were therefore pleased to add a study of

age-related DD to the literature, since we believe that such a study is relevant for all the human population and not just those that exhibit accelerated signs of DD.

5. Pain epigenetics

The molecular techniques shed light on the epigenetic mechanisms behind changes in SPARC expression with age, disc degeneration, and pain. The topic of epigenetics has garnered the attention of the pain research community only in the last 2 years (Denk and McMahon, 2012a). So far, most of the work has focused on histone acetylation/deacetylation (Zhang et al., 2011), mainly using pharmacological tools (Chiechio et al., 2009, Bai et al., 2010, Chiechio et al., 2010, Kiguchi et al., 2011) with only a few reports of methylation changes and chronic pain (Tajerian et al., 2011b, Viet et al., 2011, Wang et al., 2011). We are confident that the field of pain epigenetics will continue to flourish in the next few years.

6. Translation to humans

Examining SPARC in human IVDs further supports the relevance of our mouse model and emphasizes the involvement of SPARC in DD across different species. One of the main problems in using animal models of pain is the lack of translation to humans (Mogil, 2009), and we are fortunate to be working with a model that is relevant to the human pathology. The acquisition of human IVDs, both surgical and cadaveric, has enabled us to take findings from our murine model and test their

relevance in humans. Although this doesn't completely validate the SPARC-null model (if indeed, validation of animal models is even possible), it does bring SPARC a step closer to the clinic.

Limitations of our approach

As with all scientific studies, ours had inherent limitations including:

1. SPARC is expressed everywhere in the body

Being a matricellular protein, SPARC is found in tissues other than the IVDs, which could be considered problematic since the SPARC-null phenotype could potentially encompass conditions other than DD and LBP. Further complications arise from the fact that developmental factors might be involved. A more sound approach would have been to knockout the gene conditionally, and only in lumbar IVDs. It is important to note, however, that the SPARC-null phenotype in itself is not very severe. These animals appear to be "normal" in many aspects; indeed, the SPARC-null phenotype was initially thought to be non-existent. Ultimately, it will be important to conduct a more thorough examination into other tissues (skin, bone, IVDs at levels other than those in the lumbar region, etc.) in SPARC-null mice to better understand the full phenotype.

2. Pharmacology as a tool used in understanding pain mechanisms

Although pharmacotherapy is a common treatment option for patients with LBP, the analgesic outcome is far from ideal and patients often have to

deal with undesirable side effects. Understanding the mechanisms of pain and targeting them would therefore yield a better outcome for the pain patient. However, using pharmacology as a tool to explore pain mechanisms has its inherent limitations. This is partly because many drugs lack efficacy, have unknown/obscure or mixed mechanisms of action, or have an unknown site of action. It is therefore not possible to ascertain a pain mechanism simply based on the efficacy/potency of the tested drug. For instance, the fact that cold allodynia is responsive to pregabalin is only suggestive of a neuropathic mechanism being involved; it is not conclusive evidence that cold allodynia is purely neuropathic in nature. In fact, pregabalin is also effective for migraine, which is not thought to be neuropathic. Furthermore, all drugs in our studies were delivered intraperitoneally which might not be relevant if the mechanism it targets is elsewhere in the body (for instance, if there is increased inflammation in the IVD, then anti-inflammatory agents should be delivered into the disc space, a procedure that would be extremely difficult considering the small size of IVDs in the mouse and the damage it would cause to it). Finally, full dose-response curves were not explored for all drugs, risking the possibility of false negatives.

3. The unavailability of healthy control (non-cadaveric) IVDs

Unlike our work on the murine model of LBP, access to human tissues is limited. Healthy volunteers are willing to provide information regarding pain and disability, and will agree to an MRI scan. However, they do not

donate their healthy IVDs. In contrast, tissue donors from transplant Quebec provided us with healthy human discs and enabled us to study the epigenetics of SPARC, but unfortunately, we had no other information regarding their pain sensitivity (although we did get an x-ray for a basic anatomical evaluation of the lumbar region). Furthermore, the quality of RNA from cadaveric IVDs was suboptimal, which resulted in our group only reporting DNA methylation results.

Alternative interpretation of acquired results

Designing an experiment and collecting data are only part of the equation when it comes to carrying out a successful study. Equally important is the manner in which the data is analyzed and interpreted. For the purposes of this thesis, we have included some of the most likely interpretations of the data in the discussion of each manuscript chapter. However, there are always alternative explanations.

1. Cold allodynia as a manifestation of radiating pain

SPARC-null mice exhibit signs of cold allodynia that was measured reliably by multiple assays. No signs of mechanical or heat sensitivity were observed. These results were interpreted as modality-specific radiating pain, which agrees with what is seen in the clinic as described in chapter 3. However, it is possible that cold sensitivity is due to other factors, such as changes in vasculature in our mice. This possibility can be further investigated by examining the SPARC-null phenotype more carefully. Could it be that these mice show temperature differences in their

extremities? Could the involvement of SPARC in angiogenesis affect cold sensitivity in the hindlimb? Unless we carry out experiments to provide conclusive evidence supporting or refuting such claims, we have to accept the possibility that cold allodynia could possibly be due to factors separate from radiating pain and DD.

2. Aging and age-related pain

In chapter 5, we attempted the study of age-related DD and pain. With age, animals show anatomical signs of disc degeneration, behavioral signs of pain, decreased SPARC expression in IVDs, and increased SPARC promoter methylation. We concluded that these findings support the relevance of our model and further support the role of SPARC, not just in pathological DD, but also in "natural" DD. An alternate explanation would be that these changes are due to aging alone, and are independent of age-related pain. Since it is not possible to tease these two factors apart, we have to be careful in the interpretation of our results concerning back pain due to age-related DD.

6.3 Future directions

In the last few years, our team has established a novel animal model of chronic LBP due to DD using behavioral, anatomical, histological, pharmacological, and immunohistochemical techniques. This model can be used for further studies targeting different aspects of disc degeneration and pain.

It has already been shown that mimicking degenerative changes in an organ-culture setting could change the microenvironment in both the nucleus pulposus and annulus fibrosis. In particular, changes in proteoglycans, aggrecan, and matrix metalloproteinases were observed (Ponnappan et al., 2011, Illien-Junger et al., 2012). It would be interesting to study the microenvironment of SPARC-null vs. WT IVDs *ex vivo* in a cell culture setup. This may be particularly useful since degenerative changes could happen at the cellular level long before they are detectable in the overall IVD architecture.

Studying the innervation of painful discs is also of interest, since nociceptive innervation of the IVD is a major theory of discogenic pain. Furthermore, there is evidence showing altered innervation patterns in degenerated discs both in animal models and in humans (Aoki et al., 2006, Ozawa et al., 2006, Hayashi et al., 2008). Therefore, another avenue worth pursuing is the interaction between IVDs and neurons/nerve fibers. This could be done by carrying out immunohistochemical studies in order to observe innervation in both healthy and degenerated IVDs. Pilot data from our laboratory has demonstrated that the periphery of IVDs extracted from SPARC-null animals shows more staining for

the pan-neuronal marker, PGP9.5. These preliminary findings are indeed very encouraging in the pursuit of discogenic pain. Another possible method to study disc-nerve interactions could be accomplished by preparing a neuron-chondrocyte co-culture system. The latter would also provide the opportunity to test the effect of different compounds on modulating the neuron/chondrocyte interface.

Finally, it would be interesting to examine the effects of long-term environmental interventions on DD and LBP. Such studies could include environmental enrichment, physical exercise, and dietary changes, either as a preventative or palliative measure. The Stone lab has already initiated a series of studies examining the effects of running exercise and soy diet in animals with back pain with promising results. These findings could provide a mechanism that links environment and pain, as well as offer new treatment options for patients with chronic LBP.

6.4 Contributions to the field

The original contributions to the field from the work contained in this thesis can be summarized as follows:

1. The SPARC-null mouse is a useful model of chronic LBP due to disc degeneration, where radiating pain correlates with radiographical measures of disc degeneration.

The following publications are related to the work above, and are included in chapter 2 of this thesis, in addition to the manuscripts included in the appendix:

- Millecamps M, Tajerian M, Sage EH, Stone LS. Behavioral signs of chronic back pain in the SPARC-null mouse. Spine. 2011; **36**(2): 95-102.
- Millecamps M, Tajerian M, Naso L, Sage EH, Stone LS. Lumbar intervertebral disc degeneration associated with axial and radiating low back pain in ageing SPARC-null mice. Pain, 2012.
- Tajerian M., Millecamps M., Danco A., & Stone L S. Loss of Disc Height correlates with radiating pain in a mouse model of low back pain due to disc degeneration. In prep.

2. SPARC-null mice exhibit signs of axial and radicular pain components with distinct pharmacological profiles. Morphine and clonidine synergize to ameliorate signs of radiating pain and increase the therapeutic window for the treatment of axial pain.

The following publications are related to the work above, and are included in chapters 3 and 4 of this thesis:

- Tajerian M, Millecamps M, Stone LS. Axial pain and radiating pain have distinct pharmacological profiles in the SPARC-null mouse model of low back pain. Submitted.

- Tajerian M, Millecamps M, Stone LS. Morphine and clonidine synergize to ameliorate low back pain in mice. Pain Research and Treatment, 2012.

3. SPARC promoter methylation is accompanied by signs of LBP in aging WT mice and in human subjects.

The following publication is related to the work above, and is included in chapter 5 of this thesis:

- Tajerian M, Alvarado S, Millecamps M, Dashwood T, Anderson KM, Haglund L, et al. DNA methylation of SPARC and chronic low back pain. Molecular pain. 2011; 7: 65.

The main findings shown in this thesis bring us a step closer to understanding the mechanisms of disc degeneration and chronic low back pain in humans and support the implementation of mechanism-based treatment of chronic pain.

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7 Appendix

Manuscripts

Spine

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BASIC SCIENCE

Behavioral Signs of Chronic Back Pain in the SPARC-Null Mouse

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Study Design. Secreted Protein, Acidic, and Rich in Cysteine (SPARC)-null mice were examined for behavioral signs of chronic low back and/or radicular pain.

Objective. To assess SPARC-null mice as an animal model of chronic low back and/or radicular pain caused by degenerative disc disease.

Summary of Background Data. Degeneration of intervertebral discs is a major cause of chronic low back and adicular pain in humans. Inactivation of the SPARC gene in mice results in premature intervertebral disc degeneration. The effect of disc degeneration on behavioral measures of chronic pain has not been evaluated in this model.

Methods. Cohorts of young and old (3 and 6–12 months, respectively) SPARC-null and wild-type control mice were screened for behavioral indices of low back and/or radiating pain. Sensitivity to mechanical, cold and heat stimuli, locomotor impairment, and movement-evoked hypersensitivity were determined. Animals were challenged with 3 analgesic agents with different mechanisms: morphine, dexamethasone, and gabapentin.

Results. SPARC-null mice showed signs of movement-evoked discomfort as early as 3 months of age. Hypersensitivity to cold stimuli on both the lower back and hindpaws developed with increasing age. SPARC-null mice had normal sensitivity to tactile and heat stimuli, and locomotor skills were not impaired. The hypersensitivity to cold was reversed by morphine, but not by dexamethasone or gabapentin.

Conclusion. SPARC-null mice display behavioral signs consistent with chronic low back and radicular pain that we attribute to intervertebral disc degeneration. We hypothesize that the SPARC-null mouse is useful as a model of chronic back pain due to degenerative disc disease.

Key words: back pain, animal model, osteonectin, degenerative disc disease, BM-40, matricellular. **Spine 2011;36:95–102**

The American Pain Society estimates that 45% of the US population seeks medical help for chronic pain at some point in their lives.[1] Among those affected include 15% of North Americans with persistent back pain.[2,3] A common cause of chronic low back pain (LBP) is degenerative disc disease (DDD). Age-related disc degeneration is common in asymptomatic individuals; disc degeneration is also known to be associated with lower back and sciatic pain.[4–6] Despite the significant impairment associated with this disease, there is currently no animal model that incorporates both the anatomic and functional consequences that characterize human DDD.[7]

SPARC (Secreted protein, acidic, and rich in cysteine, also known as osteonectin and BM-40) is a matricellular protein important in tissue remodeling and in response to injury.[8] SPARC is detected in cells present within both the anulus fibrosus and nucleus pulposus in human intervertebral discs (IVDs), and its expression is decreased as a function of aging and disc degeneration.[9]

Targeted deletion of the SPARC gene results in accelerated disc degeneration in the aging mouse.[10] SPARC-null mice demonstrate signs of DDD as early as 2 months of age and, by the second year of life, signs of extensive disc degeneration are observed. These signs include decreased proteoglycan content, cell loss, and irregular collagen fibrils. As a consequence, the discs cannot meet the structural demands placed on them and disc herniation and spinal compression are observed.[10] Given the severe disc degeneration and herniation observed in this model, the probability of associated sensory changes is high.[6]

The aim of this study was to assess the usefulness of the SPARC-null mouse as a model of LBP due to DDD. We hypothesized that SPARC-null mice would present with

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behavioral signs of hypersensitivity indicative of chronic back pain and that this phenotype would become increasingly severe with advancing age and degeneration.

MATERIALS AND METHODS

Animals

The SPARC-null mice were developed on a mixed C57BL/6 × 129 SVJ background.[11] Because inbred mouse strains can have different pain behavioral responses, these SPARC-null mice were backcrossed onto a standard C57BL/6 background for >12 generations and are considered to be fully congenic. We therefore used commercially available C57BL/6 mice (Charles River, Quebec, Canada) as wild-type (WT) controls as has been done in previous studies.[12]

Two cohorts of male mice were used in this study. The young cohort was composed of 3-month-old SPARC-null mice (n = 10) and age-matched WT mice (n = 9), both were bred in-house. The old cohort was composed of 1 group of 9-month-old SPARC-null mice (n = 5) bred at the Benaroya Research Institute and transported to McGill University, and 2 groups of WT mice (6-month-old, n = 9; and 12-month-old retired breeders, n = 9; Charles River, Quebec). The use of the 2 WT control groups for the 9-month-old SPARC-null mice group was because of the lack of availability of age-matched animals. The SPARC-null mice (3-month: 23.7 ± 0.4 g; 9-month: 27.4_0.7 g) were slightly smaller than the WT mice (3-month: 26.0 ± 0.7 g; 6-month: 33.6 ± 0.6 g; 12-month: 34.4 ± 1.0 g).

All experiments were performed blind to genotype and treatment. All procedures were approved by the Animal Care Committee at McGill University, and conformed to ethical guidelines of the Canadian Council on Animal Care.

Behavioral Screening for Hypersensitivity to Cutaneous Mechanical, Cold, and Heat Stimuli

Animals were placed individually in the test chamber for 60 minutes before testing. Animals were tested for only 1 modality per day to avoid interference between assays. All testing was conducted between 9:00 AM and 3:00 PM. For each modality tested, 3 body sites were assessed whenever possible: hindpaw, tail, and low back. The hindpaw and tail measures have commonly been used in other animal models of chronic pain,[13] but to our knowledge we are the first to apply them to a model of disc degeneration. The measurement of cutaneous hypersensitivity on the skin of the low back was adapted from studies of referred visceral pain.[14]

Mechanical Sensitivity

Hindpaw and Back

Calibrated von Frey Filaments (Stoelting Co, Wood Dale, IL) were applied for 4 seconds or until withdrawal, and the 50% threshold to withdraw (grams) was calculated.[15] The stimulus intensity ranged from 0.6 to 4.0 g, corresponding to filament numbers (3.84, 4.08, 4.17, 4.31, 4.56). For each animal, the actual filaments used within the aforementioned series were determined based on the lowest filament to evoke a positive response followed by 5 consecutive stimulations using the up-down method. The filament range and average interval were then incorporated along with the response pattern into each individual threshold calculation.[15,16] Mechanical sensitivity was assessed on the plantar surface of the left hindpaw (response = flexion reflex) and on the bony structures of the L6–S1 lumbar spine (response = lordosis). The lower back region was shaved 1 day before testing.

Cold Sensitivity

Hindpaw and Back

Cold sensitivity was assessed by measurement of the total time spent performing acetone-evoked behaviors over 1 minute after a drop (25 μL) of acetone was applied gently to the plantar surface of the left hindpaw (behaviors = paw elevation, flinching, biting, licking, and scratching time) or the low back region (behaviors = biting, licking, scratching, and checking time).

Tail

Cold sensitivity was assessed by the cold water (2°C) tail immersion assay. Half of the length of the tail was dipped into the cold water, and the latency to tail withdrawal was measured. A maximum cut-off of 30 seconds was set to avoid tissue damage.

Heat Sensitivity

Hindpaw. Heat sensitivity was assessed by the latency to withdraw the right hind paw from a thermal stimulus.[17] Briefly, mice were placed in Plexiglas cages on top of a glass sheet. A thermal stimulus (IITC Life Science Inc, Woodland Hills, CA) was focused onto the center of the plantar surface of the hindpaw. Withdrawal latencies were measured 3 times at 10- minute intervals and the average was calculated. A cutoff of 17 seconds was set to prevent tissue damage.

Tail

Heat sensitivity was assessed by recording the latency to withdraw the tail in response to noxious heat. Briefly, tails were exposed to a focused beam of light (IITC Life Science Inc., Woodland Hills, CA). Withdrawal latencies were measured twice at intervals of 10 minute each, and the average was calculated. A cutoff latency of 17 seconds was set to prevent tissue damage.

Locomotor Capacity

Locomotor capacity was measured by the use of an accelerating rotarod (IITC Life Science Inc., Woodland Hills, CA) with the mouse adapter (rod diameter = 3.2 cm). The task includes a speed ramp from 0 to 30 rotations per minute over 60 seconds, followed by an additional 240 seconds at the maximal speed. Latency and rotation speed at fall were determined.

Movement-Evoked Hypersensitivity With Stretching

Grip Force Assay. Mice were made to grip a metal bar attached to a Grip Strength Meter (Stoelting Co., Wood Dale, IL), and

they were gently pulled back by the tail to exert a stretching force.[18] The peak force in grams at the point of release was recorded twice at 10-minute intervals, and the average measurement was calculated. Although this assay has not been used previously to measure back pain, it has been validated in models of deep muscle inflammation and cancer pain.[18,19]

Tail Suspension Assay

Mice were individually suspended by the tail underneath a platform. Adhesive tape was used to attach the tail (0.5–1 cm from the base) to the platform and the mice were videotaped for 180 seconds. The time spent in (*a*) immobility (not moving but stretched out), (*b*) rearing (trying to reach the underside of the platform), (*c*) full extension (actively reaching for the floor), and (*d*) selfsupporting (holding either the base of its tail or the tape) was analyzed by a blinded observer using digital software (Labspy, Montreal, Quebec, Canada) over the entire testing period (Figure 3C). This assay is commonly used as a measure of depression in mice.[20] To date, it has only been applied to chronic pain studies to assess depression in neuropathic mice, and no differences were reported.[21]

Pharmacological Manipulation of Cold Sensitivity

Baseline measurements of cold sensitivity were determined as aforementioned in the old cohort of SPARC-null mice (n = 5) 1 month following completion of the behavioral screening. Animals were subsequently treated with drug or vehicle, and cold sensitivity was assessed 45, 90, 135, and 180 minutes posttreatment. After a wash-out period of at least 48 hours, the procedure was repeated with alternative treatments.

Drugs

The following drugs were administered in our study: Morphine (6 mg/kg in 2 mL/kg, intraperitoneal [i.p.]; Medisca Inc., Montreal, Quebec, Canada); Dexamethasone (3 mg/kg in 2 mL/kg, i.p.; Sigma Aldrich Canada Ltd., Oakville, Ontario, Canada); or Gabapentin (300 mg/kg in 5 mL/kg, per os [p.o.]; MUHC Pharmacy, Montreal, Quebec, Canada).

Vehicle Controls

Saline solution (2 mL/kg, i.p. for morphine and dexamethasone; 5 mL/kg, p.o. for gabapentin).

Statistics

All data were plotted as mean ± SEM. For behavioral assays, measurements were analyzed by one-way analysis of variance followed by a Dunnett test (for the old cohort), or by an unpaired *t* test (for the young cohort). For the pharmacological treatments, each postdrug measure was normalized to the average predrug baseline and analyzed by paired t test (one-tailed).

RESULTS

Behavioral Indices of LBP

Cutaneous Stimulus-Evoked Hypersensitivity

Mechanical, cold, and heat sensitivity were assessed in the young and old cohorts on the hindpaw, lower back, and/or tail. In the young cohort, no significant differences were observed between SPARC-null and WT animals in any of the assays (Figure 1A). In the old cohort, no differences were observed

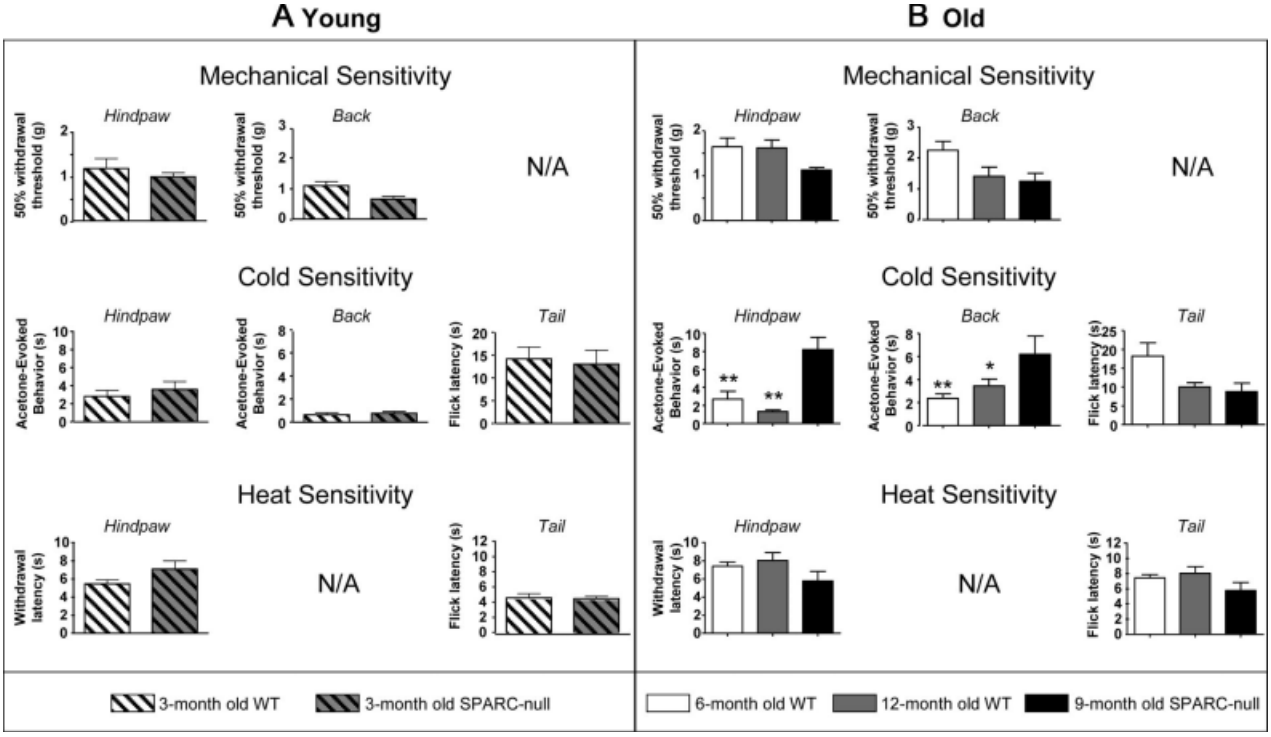

Figure 1. Behavioral screening for sensitivity to cutaneous mechanical, cold, and heat stimuli in (**A**) young (3-month-old WT [n = 10] and SPARC-null [n = 9]) and **(B)** old (6- and 12-month-old WT [n = 9/group] and 9-month-old SPARC-null [n = 5]) mice. $*P < 0.05$; $**P < 0.01$.

between SPARC-null and WT mice in terms of sensitivity to mechanical (Figure 1B, top row) or heat stimuli (Figure 1B, bottom row) or in the cold-water tail immersion assay (Figure 1B, middle row).

In contrast, old SPARC-null mice exhibited a significantly longer duration of evoked behaviors in comparison with WT control mice in response to a drop of acetone applied to the hindpaw ($F_{(79,23)} = 15.56$, $P < 0.001$) or lower back ($F_{(25,2)} = 6.087$, $P < 0.01$), a result indicative of hypersensitivity to cold.

Locomotor Capacity

Both young and old SPARC-null mice performed in the rotarod assay as well as their respective WT controls (Figure 2). Locomotor capacity was therefore intact, and locomotion did not induce discomfort in either young or aging SPARC-null animals during normal ambulation.

Movement-Evoked Hypersensitivity With Stretching

Grip Force Assay. Both young and old SPARC-null animals displayed significantly less resistive force to stretching than their WT controls (Figure 3A). In the young cohort, WT mice tolerated stretching with a greater average resistance (89.2 ± 4.3 g) on release of the grid than the SPARC-null mice (68.3 ± 3.1 g; $P < 0.0001$, unpaired t test). Similarly, the 6- and 12-month-old WT mice displayed resistive forces of 82.9 ± 5.1 g and 83.7 ± 6.2 g, respectively, whereas the 9-month-old SPARC-null mice resisted at an average of 60.5 ± 5.4 g ($F_{(999,2)} \pm 4.027$, $P < 0.05$) .

Tail Suspension Assay

Young and old SPARC-null mice presented different patterns of behavior in comparison with their respective WT controls (Figure 3B). Both 3- and 9-month-old SPARC-null animals remained immobile in a natural gravity-induced stretch for significantly less time (85.0 ± 9.8 seconds and 73.8 ± 20.5 seconds, respectively) than did 3-, 6-, and 12-month-old WT mice (121.2 ± 5.8 seconds, 149.6 ± 3.9 seconds, and 134.9 ± 5.2 seconds, respectively). In parallel, both 3- and 9-month-old SPARC-null animals spent more time rearing (57.6 ± 5.5 seconds and 56.4 ± 18.0 seconds, respectively) as compared with 3-, 6-, and 12-month-old WT mice (33.2 ± 5.1 seconds, 19.0 ± 4.0 seconds, 14.1 ± 2.6 seconds, respectively). Rearing is an attempt to catch the base of the tail to alleviate the weight-induced stretching of the spine. As a consequence, the time spent in the supported position (when the animal grabs the base of its tail or the adhesive tape) was negligible in 6- and 12-month-old WT mice (1.2 ± 1 seconds, and 2.1 ± 1.5 seconds, respectively), but was atypically elevated in 9-month-old SPARC-null mice (56.4 ± 20.2 seconds).

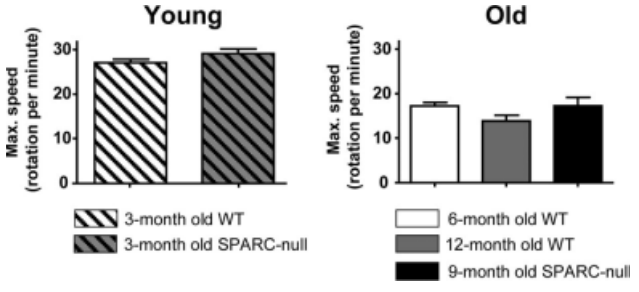

Figure 2. Behavioral screening for motor impairment in the rotarod test in young (3-month-old WT [n = 10] and SPARC-null [n = 9]) and old (6- and 12-month-old WT [n = 9/group] and 9-month-old SPARC-null [n = 5]) mice. No significant differences were observed.

Pharmacological Manipulation of Cold Sensitivity

During the aforementioned behavioral screening, SPARC-null mice displayed stretch-induced discomfort at both 3 and 9 months of age. In contrast, hypersensitivity to cold developed as a function of age. We therefore decided to use cold sensitivity in the hindpaw as our behavioral measure to test pharmacological sensitivity in aging SPARC-null mice 1 month after the behavioral screening.

First, baseline responses to acetone administered to the plantar surface of the hindpaw were reassessed in 7- and 13-month-old WT and 10-month-old SPARC-null animals; total time spent in acetone-evoked behaviors were 1.35 ± 0.25 seconds, 1.62 ± 0.38 seconds, and 5.194 ± 1.07 seconds, respectively. Because WT mice did not display a sufficient behavioral response to allow detection of pharmacological inhibition, only 10-month-old SPARC-null animals were included in the study.

Acetone-evoked behaviors were significantly attenuated relative to saline-treated controls 90 minutes after an i.p. injection of morphine (6 mg/kg, Figure 4A) (morphine = 16.2% ± 6.2% of baseline; saline = 61.2% ± 16.9% of baseline; $P = 0.0346$, paired *t* test, one-tail). After systemic treatment with dexamethasone (3 mg/kg, i.p., Figure 4B) or gabapentin (300 mg/kg, p.o., Figure 4C), acetone-evoked behaviors were not different from saline at any of the time-points tested.

DISCUSSION

One of the primary causes of chronic low back and/or radicular pain is DDD. There are several animal models of DDD. In these models the degeneration is either naturally occurring (*e.g.*, the Desert Sand Rat),[22] induced by injury to the disc (*e.g.*, disc puncture),[23,24] initiated by chemical mediators that produce inflammation,[25,26] or because of genetic inactivation of a protein important to disc integrity (*e.g.*, the SPARC-null mouse).[10,27] Despite the availability of these animal models, there is a lack of data relating disc degeneration to behavioral signs of pain and disability. An animal model that incorporates both anatomic and functional components of the disease will allow us to relate disc pathology directly to chronic low back and/or radicular pain and disability. We have confirmed the previously reported incidence of disc degeneration in SPARC-null mice by histologic and radiographic analysis. For example, signs of degeneration including wedging and loss of negatively charged proteoglycans are observed in lumbar IVDs by the age of 6 months in SPARC-null but not WT mice (data not shown).[10] The objective of the current study was to assess the usefulness of SPARC-null mice as an animal model of chronic low back and/or radicular pain caused by DDD.

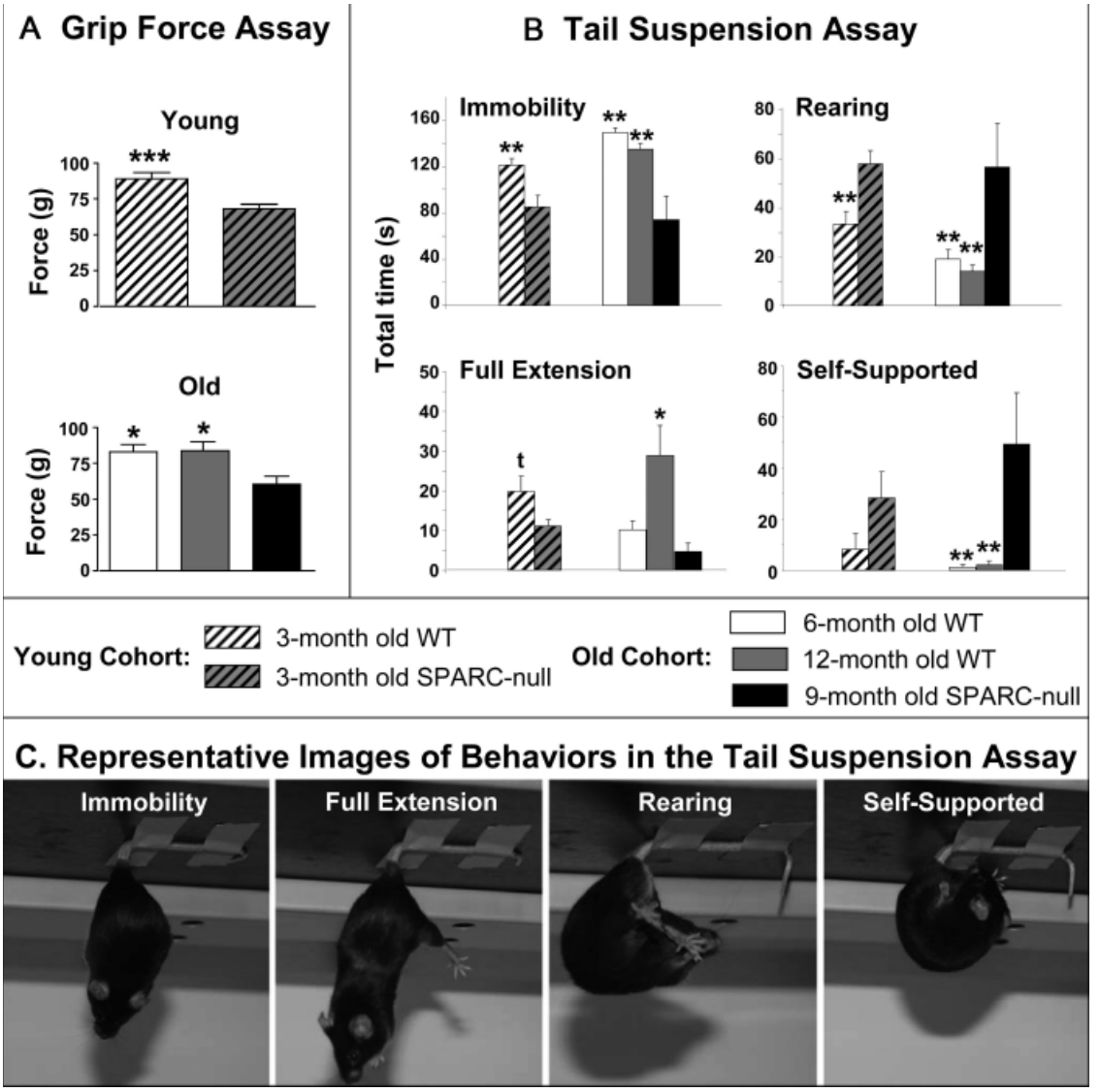

Figure 3. Movement-evoked discomfort in young and old SPARC-null and WT mice. **A**, Grip force: SPARC-null animals display a lower resistive force than WT. **B**, Tail suspension: SPARC-null mice spend less time immobile (gravity-induced stretching) and avoid this position by increased rearing and self-supported time. These differences increase with age. **C**, Representative images of the postures observed in the tail suspension assay. $t = P < 0.1$; $*P < 0.05$; $**P < 0.01$, $***P < 0.0001$.

Behavioral Phenotype of Aging SPARC-Null Mice

Although young SPARC-null animals have normal cutaneous mechanical, cold, and heat sensitivity, significant cold hypersensitivity develops with increasing age in both the hindpaw and the lower back region (Figure 1). The plantar cold hypersensitivity could be reversed by systemic treatment with morphine, but not with dexamethasone or gabapentin (Figure 4). The fact that motor ability is not impaired in SPARC-null animals supports the absence of generalized nervous system dysfunction (Figure 2). Finally, SPARC-null animals are reluctant to stretch in 2 different behavioral assays: they tolerate less stretch-induced force in the grip force assay and present with an atypical behavioral strategy in the tail suspension assay (Figure 3). Specifically, they actively avoid the natural gravity-induced stretching of the spine by increasing the time spent rearing and/or holding the base of the tail.

Cold Allodynia in SPARC-Null Mice

In the present study, SPARC-null mice developed cold allodynia on the plantar surface of the hindpaw and on the lumbar skin as a function of age, indicative of referred hypersensitivity.[28] The presence of cold but not mechanical or heat hypersensitivity in the current study differs from models of radicular pain following (i) nerve compression,[29–31] (ii) exposure of nerve to nucleus pulposus,[32] and (iii) nerve root inflammation,[33,34] in which mechanical hypersensitivity is typically observed. Our model differs from the aforementioned in that it is not initiated by acute injury or inflammation to the nerve. It was recently shown that intrathecal administration of an inflammatory mediator, complement C5, evoked cold allodynia in the absence of mechanical hypersensitivity.[35] Inflammatory mediators released as a consequence of disc degeneration could therefore theoretically produce cold allodynia in the absence of tactile changes.

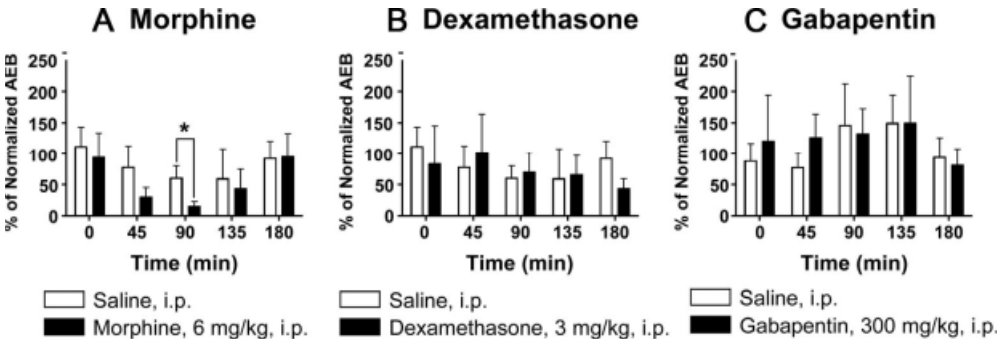

Figure 4. SPARC-null mice were treated with saline or (A) morphine (6 mg/kg, i.p.), (B) dexamethasone (3 mg/kg, i.p.), or (C) gabapentin (300 mg/kg, p.o.), and the duration of acetone-evoked behavior was assessed 45, 90, 135, and 180 minutes post-treatment. Data were normalized to the average pretreatment baseline. $*P < 0.05$.

The allodynia observed in SPARC-null mice is consistent with the human condition in which individuals experience coldness, radiating pain, and cold allodynia down one or both legs.[36–38]

Stretch-Induced Discomfort in SPARC-Null Mice

The tail suspension assay is typically used in models of depression.[20] In the current study, SPARC-null mice decreased the time spent in immobility and increased the time spent rearing and/or holding the base of the tail. This difference is not likely to be related to the slightly greater body mass of theWTmice as the increased tension on the spine of heavier animals would result in enhanced escape behaviors in WT which was not observed. Rather, we interpret this altered behavioral pattern as the avoidance of gravityinduced stretching of the spine in SPARC-null mice. To our knowledge, this study is the first to apply the tail suspension assay in the context of nociception in mice.

SPARC-null mice were impaired in the grip force assay, a phenotype observed during deep tissue pain in mice.[18] It is unlikely that this deficit is because of motor impairment as SPARC-null animals exhibited (i) normal reflexes in response to mechanical and heat stimuli (Figure 1), (ii) increased activity during the tail suspension task (Figure 3), and (iii) intact locomotor capacity (Figure 2).

The results from the grip test assay and the tail suspension task indicate that SPARC-null mice experience significant stretch-induced discomfort suggestive of axial LBP as early as 3 months of age. At this age, SPARC-null animals show signs of IVD degeneration but not herniation.[10] We propose that disc abnormalities drive the stretching-induced discomfort and hypothesize that the increased difference between aging SPARC-null and WT mice reflects the degree of degeneration. Furthermore, we propose that sensitivity to stretching will have predictive value for future drug screening as patients affected by DDD also complain of lumbar stiffness.[39] Validation of these hypotheses will require further studies, for example, extended behavioral characterization of animals (1–24 months of age), systematic assessment of the severity of DDD and its correlation to stretch-induced discomfort, and pharmacological studies.

Pharmacological Manipulation of Cold Allodynia in SPARC-Null Mice

In individual patients suffering from low back and/or radicular pain caused by DDD, morphine and gabapentin exhibit some analgesic efficacy.[40] In contrast, systemic glucocorticoids such as dexamethasone are not more effective than the placebo for the treatment of sciatica.[41]

In our study, morphine was the only treatment that reversed referred cold allodynia in 10-month-old SPARC-null mice. This reversal was not because of sedation as higher doses are typically required to induce sedation[42] and the current dose did not impair locomotor capacity in SPARC-null mice (data not shown). The ineffectiveness of gabapentin was surprising. Either the nerve injury might have been too severe at 10 months for the cold allodynia to be reversed by gabapentin, or the dose was insufficient. Future studies investigating these possibilities are required. The failure of dexamethasone to reverse referred nerve-injury induced pain is consistent with its lack of efficacy in patients41 and in animal studies of neuropathic pain.[43]

CONCLUSION

This study reports a behavioral phenotype in SPARC-null mice suggestive of chronic low back and radicular pain that is attenuated by treatment with morphine. These behavioral changes are most likely a consequence of the premature disc degeneration characteristic of these mice. The lack of mechanical hypersensitivity and motor impairment supports the absence of generalized sensory nervous system dysfunction. Furthermore, none of the other characteristics described in previously published data on these mice explains the currently reported symptoms.

The existence of a model of disc degeneration in rodents that incorporates both disc pathology and behavioral indices of chronic pain and disability will provide a platform for studies addressing the relationships between anatomic abnormalities and functional changes in vivo. Furthermore, this model will enable us to test potential therapeutic interventions in the context of both disc degeneration and chronic pain. We propose that the SPARC-null mouse is an important animal model of chronic pain due to DDD.

➢ Key Points

- ❑ Degeneration of intervertebral discs is a major cause of chronic low back and radicular pain in humans.
- ❑ Inactivation of the *SPARC* gene in mice results in premature intervertebral disc degeneration.
- ❑ Screening of SPARC-null mice in a battery of nociceptive assays revealed behavioral signs suggestive of LBP including movement-evoked discomfort and hypersensitivity to cold.
- ❑ The hypersensitivity to cold was reversed by morphine.
- ❑ The SPARC-null mouse is a useful animal model of chronic pain because of degenerative disc disease.

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Lumbar intervertebral disc degeneration associated with axial and radiating low back pain in ageing SPARC-null mice

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ABSTRACT

Chronic low back pain (LBP) is a complex, multifactorial disorder with unclear underlying mechanisms. In humans and rodents, decreased expression of secreted protein acidic rich in cysteine (SPARC) is associated with intervertebral disc (IVD) degeneration and signs of LBP. The current study investigates the hypothesis that IVD degeneration is a risk factor for chronic LBP. SPARC-null and age-matched control mice ranging from 6 to 78 weeks of age were evaluated in this study. X-ray and histologic analysis revealed reduced IVD height, increased wedging, and signs of degeneration (bulging and herniation). Cutaneous sensitivity to cold, heat, and mechanical stimuli were used as measures of referred (low back and tail) and radiating pain (hind paw). Region specificity was assessed by measuring icilin- and capsaicin-evoked behaviour after subcutaneous injection into the hind paw or upper lip. Axial discomfort was measured by the tail suspension and grip force assays. Motor impairment was determined by the accelerating rotarod. Physical function was evaluated by voluntary activity after axial strain or during ambulation with forced lateral flexion. SPARC-null mice developed (1) region-specific, age-dependent hypersensitivity to cold, icilin, and capsaicin (hind paw only), (2) axial discomfort, (3) motor impairment, and (4) reduced physical function. Morphine (6 mg/kg, i.p.) reduced cutaneous sensitivity and alleviated axial discomfort in SPARC-null mice. Ageing SPARC-null mice mirror many aspects of the complex and challenging nature of LBP in humans and incorporate both anatomic and functional components of the disease. The current study supports the hypothesis that IVD degeneration is a risk factor for chronic LBP.

1. Introduction

The Institute of Medicine estimates that chronic pain affects >100 million adults in the United States alone [40], with 10–15% of the population suffering from back pain [23,67]. The complex, multifactorial nature of low back pain (LBP) is well recognized, yet its precise cause in most individuals remains unclear [82]. Different structures can generate painful sensations in human and animals including the facet joints, intervertebral discs (IVDs), muscles, nerve roots, and dorsal root ganglia (DRG) [19,20,22,26,65,66,74]. The contribution of each structure in the global phenomenon of LBP is a matter of controversy and varies from case to case.

LBP is a complex continuum of painful conditions that can be classified as follows [14,27]: axial LBP, which is spontaneous or movement-evoked discomfort localized to the spine and low back region; and radiating LBP, which spreads into the legs and which can be either referred (perceived in regions innervated by nerves other than those that innervate the IVD) or radicular (due to injury or inflammation of the nerve root as it exits the spinal column).

Although a potential cause of chronic LBP is the degeneration of the IVDs, the relationship between LBP and disc degeneration (DD) is still controversial. One-third to two-thirds of adults without back pain have abnormalities in at least one disc, and not all

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individuals with LBP have DD. However, DD is associated with an increased risk of chronic LBP [13,41,52,78], and comparative diagnostic procedures indicate that the IVD is the most common source of chronic LBP resistant to traditional therapies [26].

To date, research into the underlying mechanisms of DD-induced LBP has been hampered by the lack of animal models that incorporate both the anatomic and functional components of the disease. Whereas preclinical models exist for the study of DD [8,15,33,35,53,69], only a few studies report an associated behavioural phenotype ([15,55,62,63]; for reviews see [51,72]), and models of radiating LBP after mechanical and/or chemical injury to the nerve root or DRG [43,47,64,74] do not incorporate the slow, progressive degeneration characteristic of discogenic LBP in humans [81].

SPARC (secreted protein, acidic and rich in cysteine) is a matricellular protein important in tissue remodeling and response to injury [17]. In humans, SPARC protein is decreased in IVD cells as a function of age and DD [34], and SPARC-null mice exhibit both accelerated age-dependent DD [36] and age-dependent behavioural signs of chronic LBP [55].

The aim of the current study was to use the SPARC-null mouse as a preclinical model to investigate the impact of progressive, age-dependent DD on the development of chronic LBP. DD, axial and radiating pain, motor impairment, and movement-evoked discomfort were observed as a function of age in SPARC-null mice. This study supports the hypothesis that DD is a risk factor for chronic LBP and describes a clinically relevant model of degenerative disc disease-induced chronic LBP.

2. Methods

2.1. Animals

The SPARC-null mice were developed on a mixed C57BL/6 x129 SVJ background [59]. Male SPARC-null and age-matched wild-type (WT) control mice, both bred in house, were used in this study. Animals were housed in groups of 2 to 5, had unrestricted access to food and water, and were on a 12-h light-dark cycle. All experiments were performed blind to genotype and treatment. All experiments were approved by the Animal Care Committee at McGill University and conformed to the ethical guidelines of the Canadian Council on Animal Care and the guidelines of the Committee for Research and Ethical Issues of IASP [85]. (See Supplementary material for additional information.)

2.2. Assessment of DD

Animals from 6 to 80 weeks of age were deeply anesthetized and perfused through the left cardiac ventricle with buffer followed by 200 mL of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at room temperature. The T1 to S4 spinal segment was collected and postfixed in the same fixative overnight at 4 °C. Samples were then cryoprotected in 30% sucrose in phosphate-buffered saline and stored at 4 °C until processing.

2.2.1. X-ray analysis

Radiographic analysis is frequently used to assess DD in humans [83]. Lateral x-ray images of the intact lumbar spine (L1 to S1) were taken at 4× with a Faxitron MX-20 (Faxitron X-Ray LLC, Lincolnshire, IL). Disc height index (DHI) and disc wedging index (DWI) were determined according to Masuda et al. [53].

2.2.2. Histologic analysis

Spinal columns were dissected and decalcified by immersion in 4% EDTA (ethylenediamine tetra-acetic acid) at 4 °C for 14 days. Samples were then cryoprotected in 30% sucrose in phosphate-buffered saline for 4 days at 4 °C and embedded in OCT cutting medium (Tissue-Tek). Sixteen-micron sections were cut with a cryostat (Leica CM3050S) in the sagittal plane and thaw-mounted onto gelatin-coated slides for subsequent staining. Staining was performed by the FAST protocol developed by Leung et al. [49] for IVDs. After drying, slides were mounted with DPX (Sigma-Aldrich, St. Louis, MO). Three sections per animal were carefully examined by a blinded observer to identify degenerating IVDs. Criteria for degeneration included the loss of clear compartmentalization between the nucleus pulposus (NP) and the annulus fibrosus (AF), annular tear, and dorsal bulging or herniation, all of which are characteristic of degenerating discs [66]. Images were obtained with a 10× objective. Representative images are shown in Fig. 1.

2.3. Longitudinal behavioural study

The longitudinal study followed a cohort of SPARC-null and WT mice (n = 9–10) aged 6 weeks to 1.5 years. All behavioural studies

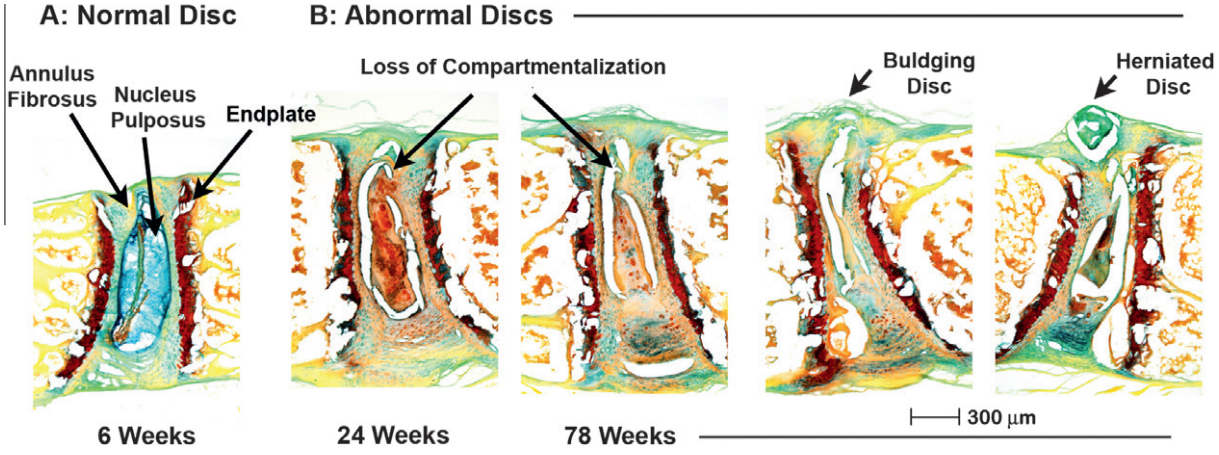

Fig. 1. FAST staining in normal and abnormal IVDs in SPARC-null mice. (A) Multichromatic FAST staining of a normal IVD from a 6-week-old SPARC-null mouse shows a clear separation between the NP and the AF. The NP is composed of a central island of red/orange cells surrounded by a blue extracellular matrix rich in negatively charged proteoglycans. The AF appears as a series of well-defined concentric blue/green collagen layers. The disc is sandwiched between anterior and posterior growth plates (GP), which appear red. Between the GP and the AF, the cartilaginous end plate appears orange matrix with large blue cells. The vertebral bones appear yellow. (B) Examples of abnormal, degenerating discs. Lumbar IVDs from 24- and 78-week-old SPARC-null mice show loss of compartmentalization between the NP and AF and altered proteoglycan content. At 78 weeks of age, bulging and/or herniation of the dorsal aspect of the disc (arrows) develops in some lumbar IVDs. This herniation resulted in compression of the spinal cord (not shown).

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were preceded by a 1-h habituation to the experimental room in the home cage. Animals were subsequently placed individually in the test chamber for an additional 60 min before testing when appropriate.

2.3.1. Sensitivity to cutaneous sensory stimuli

For each modality tested (mechanical, cold, heat), 3 body sites were assessed whenever possible: foot, tail, and low back. For the low back region, animals were shaved under brief, light gaseous anesthesia with isoflurane 48 h before testing.

2.3.1.1. Mechanical sensitivity. Mechanical sensitivity was assessed on both the plantar surface of the left foot and on the bony structures between L2 and L4 lumbar spine using the up-and-down method for von Frey filaments as previously described [21].

2.3.1.2. Cold sensitivity. Cold sensitivity for foot and back was assessed by measurement of the total time spent in acetone-evoked behaviours after acetone was gently applied to the plantar surface of the hind paw or to the low back region. For tail, cold sensitivity was assessed by the cold water (2 °C) tail immersion assay.

2.3.1.3. Heat sensitivity. Heat sensitivity was assessed for foot by the latency to withdrawal in response to exposure of the hind paw to a radiant heat stimulus as previously described [37]. For tail, heat sensitivity was assessed by recording of the latency to withdraw the tail in response to noxious heating.

2.3.2. Axial discomfort

To detect possible signs of axial discomfort in SPARC-null mice, we used 2 complementary approaches.

2.3.2.1. Grip force assay. The animal is gently stretched while gripping a bar with its forepaws until the point of release, and the force, in grams, is recorded [44,79].

2.3.2.2. Tail suspension assay. A modified version of the tail suspension assay was used to measure spontaneous reactions to natural gravity-induced stretching of the spine [55,76]. The duration of time spent in (1) immobility (not moving but stretched out), (2) rearing (trying to reach the underside of the platform), (3) full extension (trying the reach the floor), and (4) self-supported (holding either the base of its tail or the tape) was analyzed by digital software (Labspy, Montreal, QC) for the 3-min test period by an observer blinded to experimental condition.

2.3.3. Physical function

Physical function was assessed by 3 complementary approaches.

2.3.3.1. Rotarod assay. Locomotor capacity was measured with an accelerating rotarod. The experimental endpoint occurs when the animal falls off the cylinder.

2.3.3.2. Open field assay. Mice were individually placed into the open field divided equally into 9 (8×8 cm^2) squares, and the number of squares visited during a 5-min test period was used to assess general motor activity.

2.3.3.3. FlexMaze assay. This assay was developed in-house to measure lateral flexion–induced discomfort. The FlexMaze apparatus consists of a long (8×80 cm) transparent corridor with regularly spaced staggered doors and neutral (beige) 15×15 cm compartments with 6×6 cm openings on either side (Fig. 2). The natural tendency of the mouse is to explore the maze, but it is forced to undergo lateral flexion in order to progress. The FlexMaze apparatus

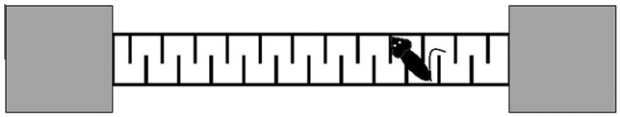

Fig. 2. FlexMaze apparatus. The FlexMaze consists of a long (8×80 cm) transparent corridor with regularly spaced staggered doors that force lateral flexion with exploration. Each end of the maze is attached to a neutral, beige compartment (15×15 cm) by a 6×6 cm opening. The 4-cm-wide doors are staggered on alternating sides and are placed every 4 cm.

was placed in a quiet room illuminated with white light. Mice were placed into one of the neutral compartments and were allowed to explore the apparatus freely for 10 min. Videotapes were analyzed for total distance covered and average velocity. The FlexMaze assay was performed by mice 6, 10, 20, 36, 61, and 78 weeks of age.

2.3.4. Testing sequence for longitudinal study

The behavioural testing sequence was as follows: Monday: Shaving of the lower back region under light isoflurane anesthesia; Tuesday: 9 to 11 AM—von Frey and acetone on left foot; 11 AM to 2 PM—home cage with food and water ad libitum; 2 to 4 pm—radiant heat paw withdrawal on right foot. Wednesday: 9 to 10 AM—von Frey and acetone on the lower back; 10 AM to 12 PM—home cage with food and water ad libitum; 12 to 1 PM—grip test; 1 to 3 PM—home cage with food and water ad libitum; 3 to 4 PM—radiant heat tail flick. Thursday: 9 to 10 AM—cold water tail flick; 10 AM to 2 PM—home cage with food and water ad libitum; 2 to 3 PM—Rotarod. Friday (when applicable): 9 AM to 12 PM—Open Field 1; 12 to 2 PM—home cage with food and water ad libitum; 2 PM to 5 PM—Tail Suspension + Open Field 2. The following Tuesday (when applicable): 9 AM to 4 PM—FlexMaze (Fig. 2).

2.4. Multiple cohort behavioural studies

A multiple-cohort, cross-sectional study design was used for the following assays to avoid the strong learning component in the cold water paw immersion assay and the possible confounding influence of long-term neurosensory changes after intraplantar capsaicin or icilin. Male SPARC-null and age-matched WT mice, bred and raised in house and in parallel, were used in these experiments.

2.4.1. Cold water paw immersion assay

The latency to hind paw withdrawal (flinching or jumping) was measured in response exposure of the ventral surface of the hind paw to a 2 °C bath.

2.4.2. Icilin-evoked behaviour

This assay was performed in a single cohort beginning at 24 weeks of age. The assay is the measurement of the total duration of evoked behaviour (biting, scratching, licking, checking) during the 5-min period after a local subcutaneous injection of the TRPM8 agonist Icilin (30 μg in 5 μL, Sigma-Aldrich) into the upper lip or the plantar surface of the hind paw. Each animal was tested at each site with both icilin and vehicle (1% dimethyl sulfoxide [DMSO] in saline), with a 4–7-day washout between treatments.

2.4.3. Capsaicin-evoked behaviour

This assay was performed on individual cohorts at 6–8, 8–16, 30, and 79 weeks of age. The assay is the measurement of the total evoked behaviour (biting, scratching, liking, and checking) during the 5 min after a local subcutaneous injection of capsaicin (2.5 μg in 5 μL, Sigma-Aldrich) or vehicle (0.25% DMSO, 0.25% ethanol, 0.125% Tween-80 in saline) in the upper lip or the plantar surface of the hind paw.

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2.5. Drug treatment

Morphine (6 mg/kg) or vehicle (0.9% saline) was delivered by intraperitoneal injection in 24–28-week-old mice, and behavioural responses were assessed in a subset of tests 60 min after injection.

2.6. Data analysis

All data are expressed and plotted as mean ± SEM. $P < .05$ was considered statistically significant.

2.6.1. X-ray image analysis

DHI and DWI were determined independently for each lumbar IVD as described above. The values for each disc were averaged to generate a single value for each animal for each of the 2 measures. To identify age or strain effects across the entire study, we analyzed data by 2-way ANOVA. Strain differences between SPARC-null and WT mice were determined by unpaired *t* test at each age.

2.6.2. Longitudinal behavioural study

Data from cutaneous sensitivity, axial discomfort, and rotarod assays were analyzed by means of a nonlinear regression curve fit program in Prism 4.0 (GraphPad, San Diego, CA), with a third-order polynomial equation curve. The best fit curves for SPARC-null and WT mice were considered to be significantly different if the sum-of-squares *F* test had a *P* value of < .05. If the data sets for the 2 strains were not distinguishable statistically, a single best fit for the combined data sets is depicted as a dotted line. If the null hypothesis is rejected and the data sets could be distinguished, then each data set is represented by its own best fit curve (SPARC-null = black line, WT = grey line). No constraints, initial values, or weighting were used for this analysis, and each replicate value was considered an individual unique value. When the 2 strains were significantly different, the data were tested for strain differences at each time point by unpaired *t* test.

To compare the distance covered in the open field assay before and after tail suspension, and the speed during the first 5 min vs the last 5 min in the FlexMaze assay, a paired *t* test was used.

2.6.3. Multiple cohort studies

Differences in the paw immersion and capsaicin-evoked behaviour assays were determined by unpaired *t* test between SPARC-null and age-matched WT animals for each cohort at each time point. In the icilin experiment, the total time spent in vehicle vs icilin was compared by paired *t* test.

2.6.4. Drug treatment

The latency to withdraw in the paw immersion assay, time spent in immobility during the tail suspension assay, latency to fall in the rotarod assay, and total distance covered during the open field after tail suspension were analyzed by unpaired *t* test between saline-treated and morphine-treated animals within the same strain, and by unpaired *t* test to compare saline-treated SPARC-null vs saline-treated WT mice.

3. Results

3.1. IVD degeneration in ageing SPARC-null and WT mice

The impact of ageing on lumbar IVD integrity was determined in young (6 weeks), middle-aged (24 weeks), and old (78 weeks) SPARC-null and WT mice by 2 complementary methods. First, x-ray analysis was performed on lumbar spinal segments, and disc height and shape were quantified (Fig. 3). Second, a multichromatic histologic approach was used to facilitate qualitative description of ageing lumbar IVDs (Fig. 4).

Analysis of DHI revealed that SPARC-null IVDs were thinner than WT IVDs throughout the life span (2-way ANOVA; age effect: $F_{(2,38)} = 40.38$, $P < .001$; strain effect: $F_{(1,38)} = 9.097$, $P = .005$; interaction: $F_{(2,38)} = 3.854$, $P = .03$), yet both SPARC-null and WT mice

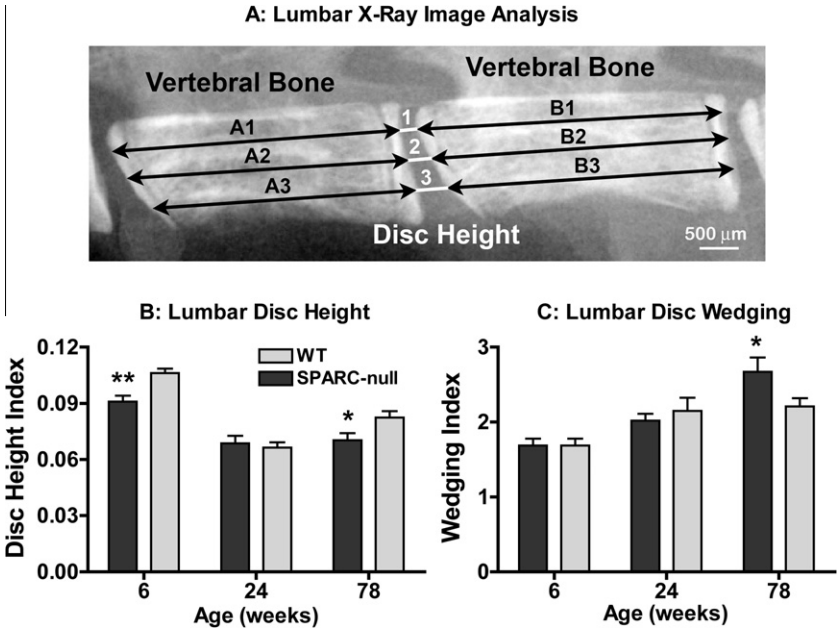

Fig. 3. Disc height and wedging in ageing SPARC-null and WT mice. (A) Analysis of lumbar spinal x-rays was used to determine the DHI and DWI. DHI is the thickness of the IVD relative to the vertebra length and is calculated as follows: DHI = [2 × (DH1 + DH2 + DH3)]/(A1 + A2 + A3 + B1 + B2 + B3). The DWI (DWI = DH3/DH1) reflects the shape of the IVD. A DWI of 1 indicates that the dorsal and ventral aspects of the disc are equivalent. If the DWI value is >1, then the ventral aspect of the disc is thicker than the dorsal aspect. Increases in DWI indicate the relative loss of the thickness of the dorsal aspect of the disc, which might result in DRG compression. Disc height (B) and wedging (C) were measured in 6-, 24-, and 78-week-old SPARC-null (dark histograms, $n = 6$, 8, and 7, respectively) and WT mice (light histograms, $n = 6$, 8, and 9, respectively). For each animal, the DHI and DWI was measured for each of the 5 lumbar IVDs and then averaged to create an overall index for each animal. Data are expressed as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, SPARC-null vs WT, 1-tailed unpaired *t* test.

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A: Disc Degeneration

Strain	WT			SPARC-null		
Age (weeks)	6	24	78	6	24	78
n	9	11	9	11	7	9
% Abnormal						
L1-L2	0	0	0	0	0	57
L2-L3	0	0	0	0	0	86
L3-L4	0	0	0	0	22	100
L4-L5	0	0	0	0	11	86
L5-L6	0	0	0	0	0	0
TOTAL (all discs)	0	0	0	0	33	100

B: Lumbar Spinal Column Histology

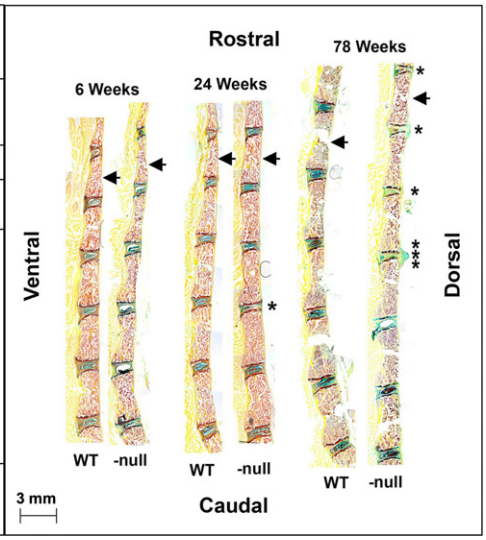

Fig. 4. Histologic analysis of DD in ageing SPARC-null and WT mice. (A) Lumbar spinal columns from 6-, 24-, and 78-week-old SPARC-null (n = 11, 7, and 9, respectively) and WT mice (n = 9, 11, and 9, respectively) were examined for signs of DD. Each lumbar IVD was independently evaluated and categorized as either normal (see Fig. 1A) or abnormal (see Fig. 1B). Values in the table indicate the percentage of animals of each strain at each age that had abnormal disc integrity at the indicated level. The total L1–L6 at the bottom of the table is the percentage of animals in each cohort in which at least 1 of the 5 lumbar discs was abnormal. (B) Representative images of FAST staining in lumbar vertebral columns of SPARC-null and WT mice at 6, 24, and 78 weeks of age. Images were obtained with a 4× objective, and reconstruction of spines was done in Photoshop. Arrows point to the L1 vertebra in each example. *Degenerated IVDs, ***herniated IVD.

developed a significant reduction in relative IVD height with age (Fig. 3).

Quantitative analysis of disc shape illustrated that the DWI increased with age in both SPARC-null and WT mice (2-way ANOVA; age effect: $F_{(2,38)} = 14.20$, $P < .0001$; strain effect: $F_{(1,38)} = 0.9571$, NS; interaction: $F_{(2,38)} = 2.759$, $P = .076$). Although SPARC-null and WT mice were similar at the earlier time points (6 and 24 weeks), SPARC-null mice exhibited increased wedging compared to WT mice at 78 weeks of age.

Complete lumbar segments in 6-, 24-, and 78-week-old SPARC-null and WT mice were examined histologically for signs of DD (ie, loss of compartmentalization, annular tears, and disc rupture/herniation) with the FAST staining protocol; each disc was scored as positive or negative for signs of degeneration. As shown in Fig. 4, neither young, 6 week-old SPARC-null, nor age-matched WT mice showed abnormal lumbar IVDs. At 24 weeks of age, abnormal lumbar IVDs, localized for the most part to the middle lumbar segments, began to appear in SPARC-null animals. Finally, by 78 weeks of age, each SPARC-null mouse presented with at least one severely degenerating IVD, and the L3–L4 IVD was abnormal in 100% of the SPARC-null mice at this age. In addition, occasional examples of severe disc bulging and/or herniation and spinal cord compression were seen at this age. In contrast, IVD structure was not affected by ageing in WT mice. Representative images of lumbar spines at 6, 24, and 78 weeks of age are shown in Fig. 4.

In summary, although decreases in disc height and increased wedging were observed in both SPARC-null and WT mice with increasing age, these changes were accelerated in SPARC-null mice (Fig. 3). In SPARC-null mice, abnormalities in disc structure could be observed as early as 24 weeks of age. By 78 weeks of age, each animal had at least one degenerating IVD (Fig. 4).

3.2. Sensitivity to cutaneous sensory stimuli in ageing SPARC-null and WT mice

Sensitivity to cutaneous mechanical, cold, and heat stimuli was assessed as a function of age on the hind paw, low back, and tail in SPARC-null and WT mice.

On the plantar surface of the hind paw, SPARC-null and WT mice were equally responsive to mechanical (Fig. 5A, first column) and heat stimuli (Fig. 5A, third column) throughout the study, and there was no significant effect of age in either strain. In contrast, SPARC-null mice exhibited signs of cold allodynia in the acetone test as evidenced by a significant increase in acetone-evoked behaviours in the hind paw compared with WT control mice (Fig. 5A, second column). In addition, ageing WT mice developed increased sensitivity to acetone, and by 1 year of age, they were as sensitive to acetone as age-matched SPARC-null mice.

The skin of the low back was slightly but consistently more sensitive to mechanical stimuli in SPARC-null mice compared to WT controls (Fig. 5B, first column). However, no differences in cold sensitivity were observed between strains (Fig. 5B, second column). Finally, hypersensitivity to neither heat nor cold developed on the tail in ageing SPARC-null mice compared to age-matched WT (Fig. 5C, second and third columns).

The hypersensitivity to cold exhibited by SPARC-null mice in the acetone test was further evaluated in a complementary study with a multicohort approach, in which the latency to withdrawal from paw immersion in noxious cold water (2 °C) was assessed (Fig. 6). In young animals (6–8 weeks old), the latency to withdrawal was similar between SPARC-null and WT mice. Starting during the third month of life (8–16 weeks old), SPARC-null mice displayed significant signs of hypersensitivity to cold that increased in severity up to 78 weeks of age. During the second year of life, ageing WT animals presented with cold hypersensitivity similar to ageing SPARC-null mice.

To determine whether the development of cold allodynia in the hind paw was be related to DD, we took advantage of the fact that the innervation of the upper lip does not pass through the vertebral column. Hypersensitivity to intradermal injection of the TRPM8 agonist icilin was measured in the upper lip and the plantar surface of the hind paw in 24-week-old SPARC-null and WT mice (Fig. 7). In WT mice, icilin-evoked behaviours were not different from vehicle in either the upper lip or the hind paw. In contrast, icilin-evoked behaviours were significantly elevated in the hind paw of

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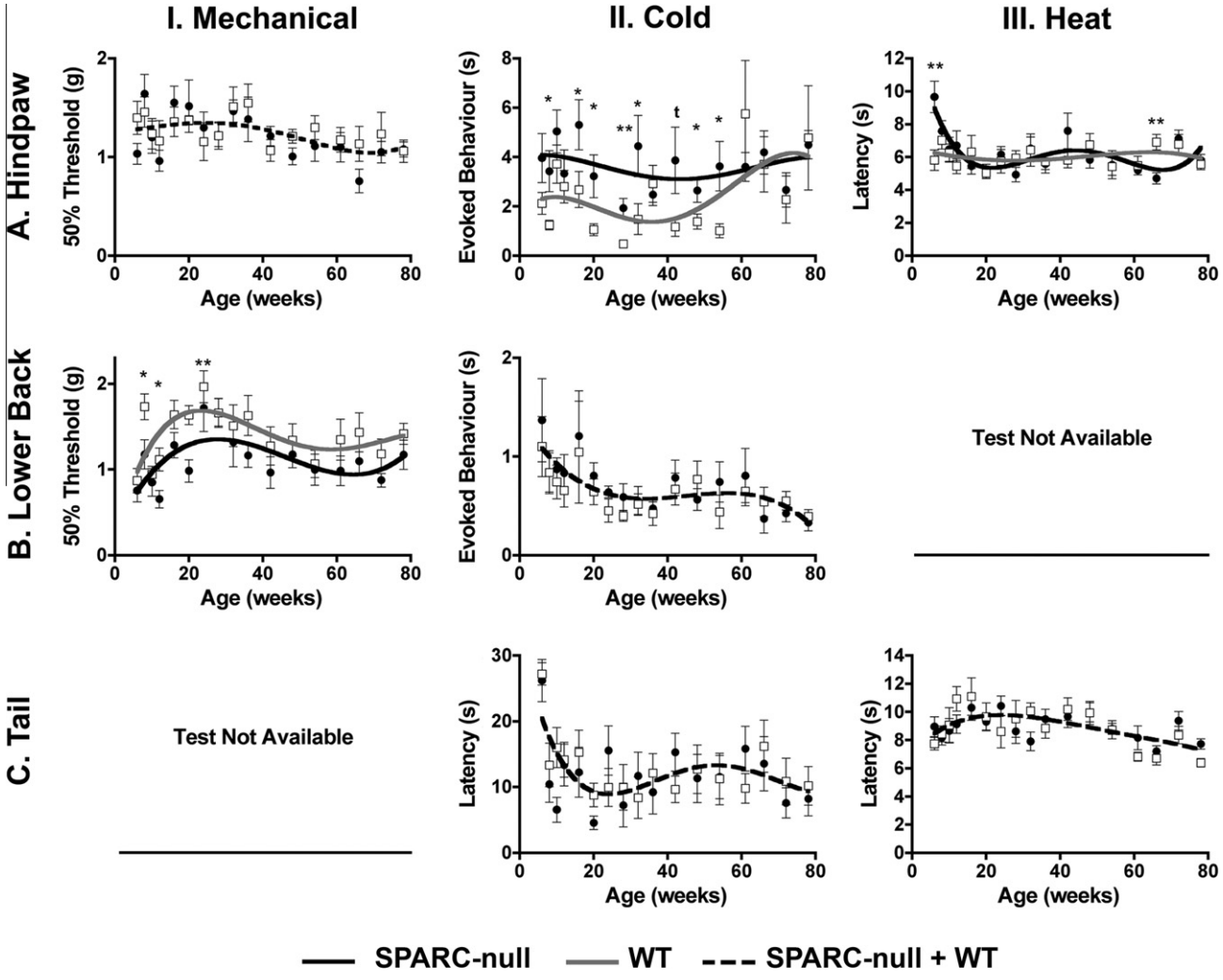

Fig. 5. Sensitivity to cutaneous sensory stimuli in ageing SPARC-null and WT mice. Sensitivity to mechanical (column I), cold (column II), and heat (column III) stimuli was assessed at 6, 8, 10, 12, 16, 20, 24 26, 32, 36, 42, 48, 54, 61, 66, 72, and 78 weeks of age in SPARC-null (n = 9, black circle, black line) and WT (n = 9, open square, grey line) mice. For each modality, 3 body sites were assessed whenever possible: hind paw (row A), low back (row B), and tail (row C). Data were analyzed by a nonlinear regression curve fit program, and the SPARC-null and WT best fit curves were compared by an F test. When $P > .05$, the 2 strains were considered not significantly different, and only 1 curve was graphed representing the data from both strains (dotted line). When $P < .05$, the 2 strains were considered significantly different, and the curve for each strain is shown separately (black line = SPARC-null, grey = WT). When the 2 strains were significantly different, data for each time point were compared by 2-tailed unpaired t test: $t = P < .1$, $^{*}P < .05$, $^{**}P < .01$, SPARC-null vs age-matched WT. Individual data points are expressed as mean ± SEM.

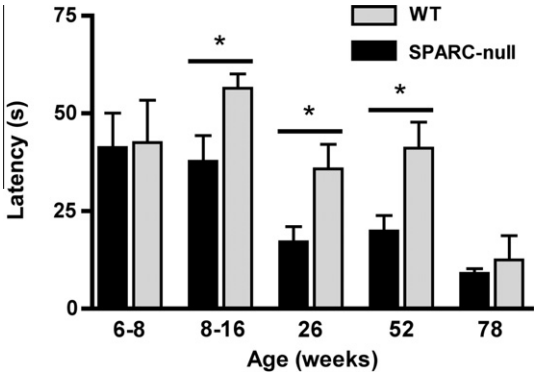

Fig. 6. Cold water paw immersion assay in ageing SPARC-null and WT mice. The latency to withdraw from cold (2 °C) water was measured in SPARC-null mice aged 6–8, 8–16, 26, 52, and 78 weeks (dark bars, n = 6, 11, 15, 9, and 8, respectively) and WT mice aged 6–8, 8–16, 26, 52, and 78 weeks (light bars, n = 5, 9, 11, 8, and 9, respectively). Data are expressed as mean ± SEM. $^{*}P < .05$, 2-tailed unpaired t test, SPARC-null vs age-matched WT.

the SPARC-null mice compared to those evoked by vehicle. This difference was not observed in the upper lip.

The region specificity observed with icilin injection was further investigated as a function of age in SPARC-null and WT mice by measurement of capsaicin-evoked behaviour in the upper lip and the plantar surface of the hind paw in different cohorts of mice of increasing ages (Fig. 8). When injected into the upper lip, age-matched SPARC-null and WT mice were not significantly different at any age. However, regardless of genetic background, older cohorts were less sensitive to capsaicin administered to the upper lip than were younger cohorts. When injected into the plantar surface of the hind paw in young animals (6–8 and 8–16 weeks old), SPARC-null and WT mice exhibited similar responses to capsaicin. In contrast, in middle-aged mice (30 weeks old), SPARC-null mice exhibited significantly more capsaicin-evoked behaviour than was observed in age-matched WT. In very old mice (78 weeks of age), regardless of the genetic background, both SPARC-null and WT animals exhibited a strong and similar increase in capsaicin-evoked behaviour.

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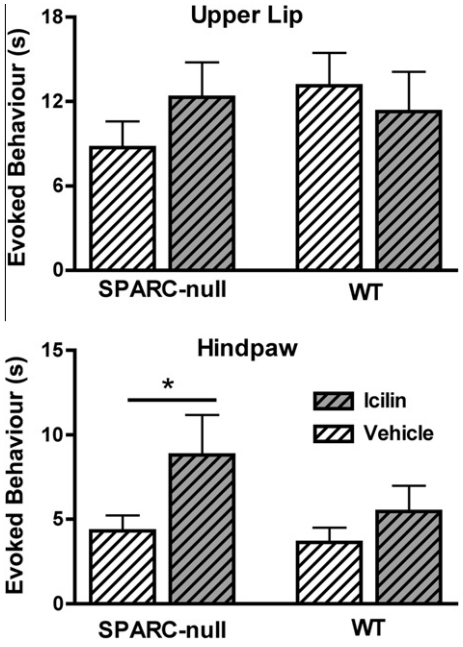

Fig. 7. Icilin-evoked behaviour in the hind paw and upper lip of ageing SPARC-null and WT mice. Twenty-four-week-old SPARC-null ($n = 10$) and WT ($n = 11$) mice received a local injection of icilin (30 μg in 5 μL, s.c.) or vehicle (1% DMSO in saline) in the plantar surface of the hind paw or in the upper lip. Each animal was used 4 times with a washout period of 3–4 days. The 4 treatments were randomly assigned between the hind paw and lip, right and left side. The total evoked behaviour (biting, scratching, liking, and checking) was measured during the 5 min after local treatment. Data are expressed as mean ± SEM. $^*P < .05$, 1-tailed, paired t test, icilin vs vehicle.

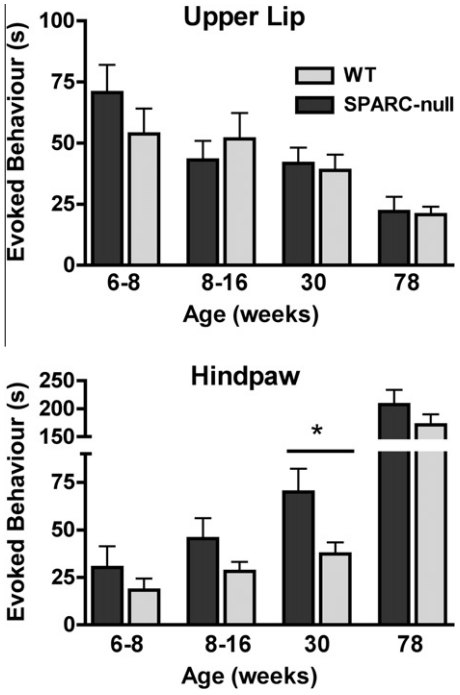

Fig. 8. Capsaicin-evoked behaviour in the hind paw and upper lip of ageing SPARC-null and WT mice. Mice received a local injection of capsaicin (2.5 μg in 5 μL, s.c.) or vehicle (0.25% DMSO, 0.25% ethanol, 0.125% Tween-80 in saline) in the plantar surface of the hind paw or in the upper lip. The total evoked behaviour (biting, scratching, liking, and checking) was measured during the 5 min after local treatment. For the upper lip, mice aged 6–8, 8–16, 30, and 78 weeks for SPARC-null (black bars, $n = 3$, 8, 15, and 5, respectively) and WT (grey bars, $n = 5$, 8, 10, and 9, respectively) were used. For the plantar site, mice aged 6–8, 8–16, 30, and 78 weeks for SPARC-null (black bars, $n = 4$, 8, 15, and 5, respectively) and WT (grey bars, $n = 5$, 8, 13, and 8, respectively) were used. Data are expressed as mean ± SEM. $^*P < .05$, 1-tailed unpaired t test, SPARC-null vs age-matched SPARC-WT.

In summary, SPARC-null mice developed hypersensitivity to cold stimuli within the first few months of age. The hypersensitivity was limited to the hind paw and became progressively more severe with age. In parallel, SPARC-null mice showed stable, slight tactile hypersensitivity localized on the lower back region, and hypersensitivity to icilin and capsaicin, localized to the hind paw, developed in middle age. Ageing WT mice developed hypersensitivity to cold and capsaicin starting in the second year of life that was indistinguishable from the SPARC-null phenotype at 78 weeks of age.

3.3. Axial LBP in ageing SPARC-null and WT mice

Behavioural signs of axial LBP were assessed in the grip force and tail suspension assays. The grip force assay has been previously used to assess use-dependent chronic deep tissue pain in rodents [44,79]. The tail suspension assay was used here to quantify the response to gravity-induced stretching of the spine [55,76].

SPARC-null animals displayed significant impairment in the grip force assay compared to WT mice at all time-points (Fig. 9A). Whereas WT mice grew progressively stronger with age, SPARC-null did not show any improvement.

In the tail suspension assay, animals can adopt 1 of 4 postures: immobility (no movement), full extension (reaching for the floor), rearing, and self-supported (holding on to either the tape or the tail) (Fig. 9B). SPARC-null and WT mice displayed significantly different patterns of behaviour in this assay throughout the entire life cycle, which we interpret as the deployment of different strategies to cope with this uncomfortable position.

Specifically, WT mice rapidly increased the time spent in immobility and reached a plateau at 18 weeks of age, where they spent more than 80% of the test period in this posture (Fig. 9C). When WT animals were not immobile, they spent very little time rearing (Fig. 9E) or self-supported (Fig. 9F). Rather, the time not in immobility was spent almost completely in full extension (Fig. 9D).

In comparison, during their first year of life, SPARC-null mice spent significantly less time in immobility (Fig. 9C), or in full-extension (Fig. 9D) than their WT counterparts. Rather, they actively avoided gravity-induced stretch and strain along the axis of the spine by increased time spent rearing (Fig. 9E) or self-supported (Fig. 9F). During the second year of life, ageing SPARC-null mice underwent changes in their pattern of behaviour: they increased their time in immobility, and decreased their time self-supported.

SPARC-null mice exhibited signs of axial LBP in both the grip force assay (reduced resistive force) and during the tail suspension assay (reduced tolerance of gravity-induced stretching and increased active escape behaviour).

3.4. Physical function in ageing SPARC-null and WT mice

To assess physical function, we used 3 complementary approaches. (i) The classical accelerating rotarod assessed motor impairment. (ii) Behaviour in an open field was measured for 5 min before and after tail suspension to determine whether strain along the axis of the spine resulted in reduced spontaneous activity. (iii) Physical activity level was assessed during ambulation in the 10-min FlexMaze assay to determine the impact of forced lateral flexion on activity.

In the accelerating rotarod assay (Fig. 10), untrained SPARC-null and WT mice performed similarly during the first 6 months of life. As they aged and their exposure to the task was increased, WT

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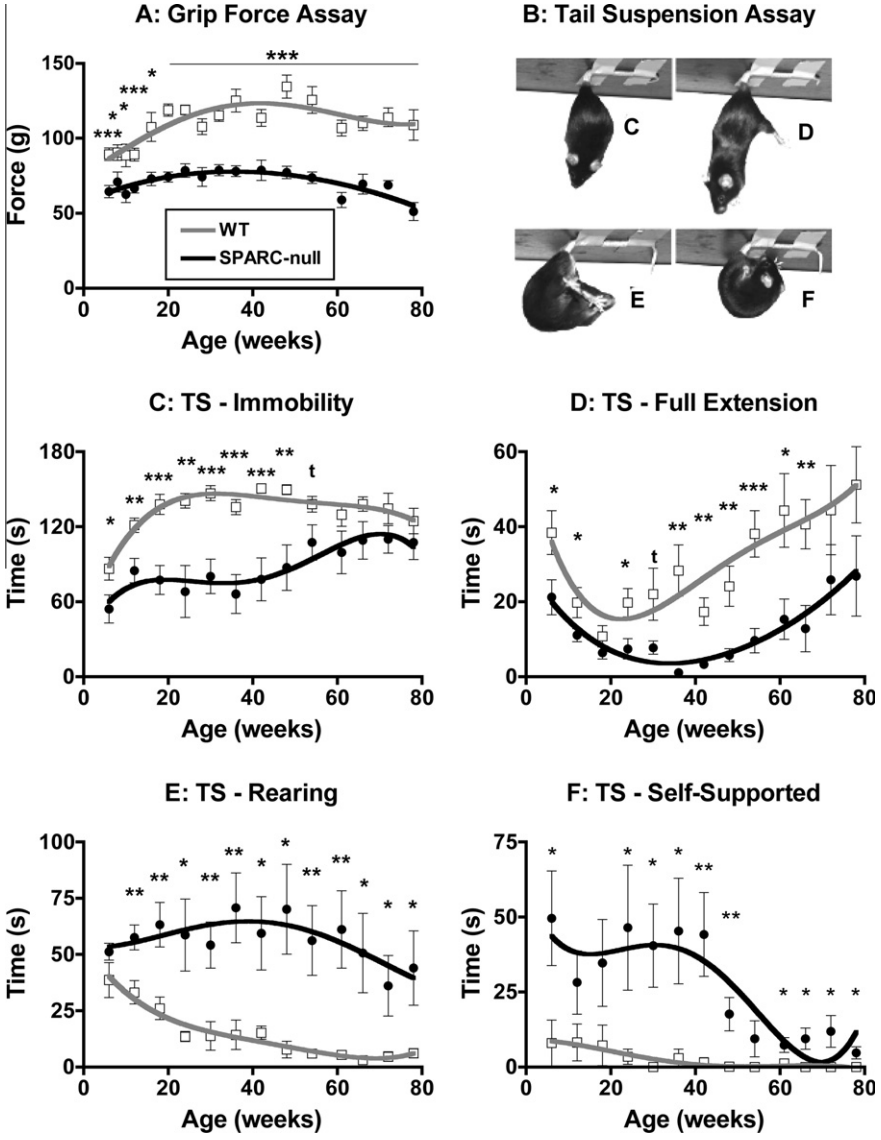

Fig. 9. Axial discomfort in ageing SPARC-null and WT mice. Axial discomfort was assessed in ageing SPARC-null mice (n = 9, black circle, black line) and WT mice (n = 9, open square, grey line). (A) The grip force assay was performed at 6, 8, 10, 12, 16, 20, 24 26, 32, 36, 42, 48, 54, 61, 66, 72, and 78 weeks of age. (B–F) The tail suspension assay was performed at 6, 12, 24, 30, 36, 42, 48, 54, 61, 66, and 78 weeks of age and time spend in immobility (C), full extension (D), rearing (E), or self-supported (F) were measured over a 3-min observation period. An illustration of the different behaviours measured in the tail suspension assay is shown in (B). Data were analyzed by a nonlinear regression curve fit program, and the SPARC-null and SPARC-WT best fit curves were compared by an F test. When $P < .05$, the 2 strains were considered significantly different, and the curve for each strain is shown separately (black line = SPARC-null, grey line = WT). When the 2 strains were significantly different, data for each time point were compared by 2-tailed unpaired t test: $t = P < .07$, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .01$, SPARC-null vs age-matched WT. Individual data points are expressed as mean ± SEM.

mice improved their performance and reached a plateau at 60–70 weeks of age. SPARC-null mice also demonstrated improved performance with age, but displayed significant motor impairment when compared to age-matched WT controls at the age of peak performance. Interestingly, all mice, regardless of their genetic background, exhibited declining motor performance starting at >70 weeks. Therefore, SPARC-null mice developed motor impairment during the second year of life.

We also tested the effect of axial strain on voluntary motor activity by comparison of total exploratory behaviour in the open field before and after tail suspension. In WT animals, no significant differences were observed in the total distance before vs after tail suspension after 6 weeks of age, and behaviour in the open field was stable over the entire life span (Fig. 11A).

Compared to WT counterparts, SPARC-null mice were significantly more active in the open field at baseline (ie, before tail suspension). However, in contrast to WT animals, total activity was significantly reduced in the open field after tail suspension. This tail suspension-induced impairment was observed at all ages above 6 weeks (Fig. 11A). (SPARC-null vs WT, 1-tailed, unpaired t test = $P < .05$, $P < .01$, $P < .001$, $P < .01$, $P < .05$, $P = .056$, and NS at 6, 12, 24, 36, 48, 61, and 78 weeks of age, respectively). Therefore, for the majority of their life span, axial strain resulted in reduced voluntary activity in SPARC-null mice.

In the FlexMaze assay, animals must undergo lateral flexion to explore the maze. After 6 weeks of age, the global exploration speed of WT mice did not change between the first 5-min period in the maze vs the period between 5–10 min. This behaviour was stable over the life span (Fig. 11B). The global speed of SPARC-null mice during the first 5 min of exploration was similar to that of WT mice at almost all ages. However, SPARC-null mice exhibited a reduction in exploration speed during the second half of the test that developed after 20 weeks of age. Therefore, beginning at 20 weeks of age, SPARC-null mice demonstrated signs of

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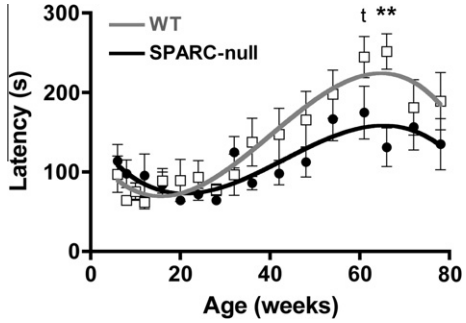

Fig. 10. Locomotor capacity in ageing SPARC-null and WT mice. Locomotor capacity was measured as the latency to fall from an accelerating rotarod in ageing SPARC-null ($n = 9$, black circle, black line) and WT ($n = 9$, open square, grey line) mice. The rotarod assay was performed at 6, 8, 10, 12, 16, 20, 24 26, 32, 36, 42, 48, 54, 61, 66, 72, and 78 weeks of age. Data were analyzed by a nonlinear regression curve fit program, and the SPARC-null and SPARC-WT best fit curves were compared by an F test. When $P < .05$, the 2 strains were considered significantly different, and the curve for each strain is shown separately (black line = SPARC-null, grey line = WT). When the 2 strains were significantly different, data for each time point were compared by 2-tailed unpaired t test: $t = P < .07$, $^{**}P < .01$, SPARC-null vs age-matched WT. Individual data points are expressed as mean ± SEM.

activity-induced decreases in physical function associated with lateral flexion and spontaneously reduce their exploration speed.

SPARC-null mice gradually developed impaired physical function that can be detected at different ages depending on the assay. The reduction in activity is first detected as early as 6 weeks of age as a decrease in exploratory activity in the open field after axial strain. In the FlexMaze assay, decreased voluntary activity with lateral flexion is detected by 20 weeks of age as a reduction in the speed of exploration in the second half of the assay. Finally, in the accelerating rotarod assay, motor impairment developed during the second year of life. These deficits in physical function model the decrease in physical function observed in humans.

3.5. Pharmacological sensitivity to morphine in SPARC-null and WT mice

In our previous study [55], plantar cold allodynia (ie, acetone test) was attenuated by morphine but not gabapentin nor dexamethasone after systemic treatment in 9 month-old SPARC-null mice. Therefore, we decided to determine the effects of systemic morphine (6 mg/kg, i.p.) in 6 month-old SPARC-null and WT mice in additional behavioural tests assessing cold allodynia, axial discomfort, physical function, and motor impairment.

The effect of morphine on cutaneous hypersensitivity to cold was measured in the cold water paw withdrawal assay. After morphine treatment, the response latency was significantly elevated in SPARC-null mice compared to saline-treated controls. Although a similar trend was observed in WT mice, it was not significant (Fig. 12A).

The effect of morphine on axial discomfort was measured in the tail suspension assay. Morphine-treated SPARC-null mice spent significantly more time in immobility in comparison to saline-treated animals (Fig. 12B). Morphine had no effect on WT mice in this assay.

Although morphine treatment did not affect motor impairment in the rotarod assay (Fig. 12C), it induced a significant increase in exploratory activity in both WT and SPARC-null mice in the post–tail suspension open field assay in comparison to saline-treated controls (Fig. 12D).

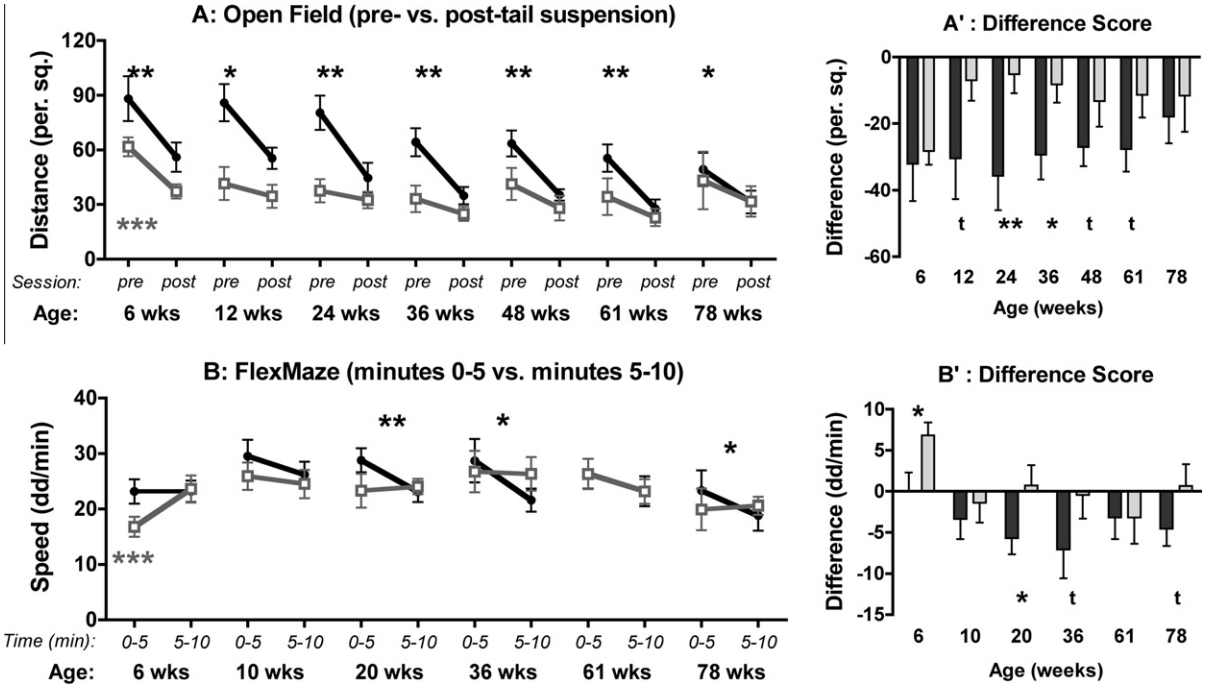

Fig. 11. Movement-evoked discomfort in ageing SPARC-null and WT mice. (A) For the open field (OF) assay, the total distance covered (number of peripheral squares explored) during a 5-min OF session before (pre) or just after (post) a 3-min tail-suspension task was assessed in SPARC-null ($n = 9$, black circle, black line, black histograms) and WT ($n = 9$, white square, grey line, grey histograms) mice at 6, 12, 24, 36, 48, 61, and 78 weeks of age. The left panel indicates the total distance before and after tail suspension for the each strain at each time point. The right panel (A′) shows the difference in total distance covered between the 2 OF sessions (Post − Pre) for each strain at each time point. (B) For the FlexMaze assay, the ambulation speed (number of double doors crossed per minute) in the FlexMaze corridor during the 5 first (0–5) or 5 last (5–10) min of the session was measured in SPARC-null ($n = 9$, black circle, black line, black histograms) and WT ($n = 9$, white square, grey line, grey histograms) mice at 6, 10, 20, 36, 61, and 78 weeks of age. The difference in speed between the 5 first and the last 5 min of the assay (last 5 min − first 5 min) for the each strain at each time point is shown in B′. A, B: 1-tailed paired t test pre vs post (A) or 0–5 vs 5–10 min (B); A′, B′: 1-tailed unpaired t test, SPARC-null vs age-matched WT. $t = P < .08$, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .0001$. Data are expressed as mean ± SEM.

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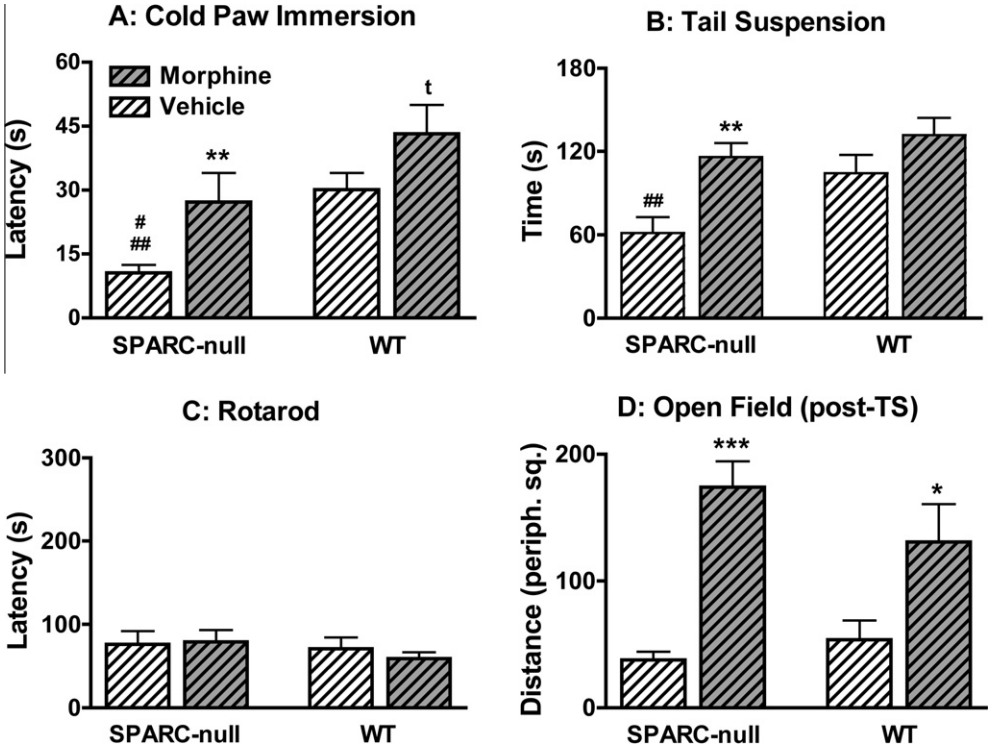

Fig. 12. Effect of morphine in SPARC-null and WT mice. The effect of morphine (6 mg/kg, i.p.) or vehicle (saline, i.p.) was determined 60 min after injection in 24–28-week-old mice. (A) For cold paw immersion, the latency to withdraw was assessed in SPARC-null (morphine $n = 11$; saline $n = 9$) and WT (morphine $n = 9$; saline $n = 24$) mice. (B) For the tail suspension assay, the time spend in immobility was assessed in SPARC-null (morphine $n = 10$; saline $n = 20$) and WT (morphine $n = 20$; saline $n = 19$) mice. (C) For the rotarod, the latency to fall was measured in SPARC-null (morphine, $n = 10$; saline $n = 20$) and WT (morphine $n = 7$; saline $n = 21$) mice. (D) For the post–tail suspension open field, the total distance covered (number of squares explored) was counted during a 5-min open-field exploration immediately after a 3-min tail suspension task in SPARC-null (morphine $n = 10$; saline $n = 22$) and WT (morphine $n = 7$; saline $n = 14$) mice. Two-tailed unpaired t test: $t = P < .1$, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .0001$, saline-treated (i.p.) vs morphine-treated (6 mg/kg, i.p.), same strain. One-tailed unpaired t test: $^{\#}P < .05$, $^{\#\#}P < .01$, $^{\#\#\#}P < .0001$, saline-treated SPARC-null vs saline-treated SPARC-WT. Data are expressed as mean ± SEM.

In summary, systemic administration of morphine reduced cutaneous sensitivity and axial discomfort in SPARC-null mice compared to saline-treated controls. At 6 mg/kg, i.p., morphine was not sedative in the rotarod assay and resulted in increased exploratory activity in both SPARC-null and WT animals. The morphine-induced increase in overall activity indicates that the increase in immobility in the tail suspension assay in SPARC-null mice might be due to a reduction in axial discomfort and not to sedation.

4. Discussion

This study describes the development of behavioural signs of axial and radiating LBP and reduced physical function with increasing age and lumbar DD in SPARC-null mice. The SPARC-null mouse models many aspects of the complex nature of LBP in humans, incorporating both anatomic and functional components. Anatomically, SPARC-null mice have reduced IVD height, increased wedging and histologic signs of degeneration. These anatomic signs of DD are associated with age-dependent cutaneous hypersensitivity (hind paw only), axial discomfort, and reduced physical function. Systemic morphine attenuates both cutaneous hypersensitivity and axial discomfort.

4.1. Radiating LBP in ageing SPARC-null and WT mice

The region-specific hypersensitivity to cold, capsaicin, and icilin observed in the hind paw of the SPARC-null mouse is consistent with the human condition in which individuals experience coldness, radiating pain, and cold allodynia down one or both legs [50,57,60,66]. It is unlikely that these phenomena are related to nonspecific changes in either the peripheral tissues or in the nervous system because they a) are region-specific, b) develop in the absence of motor impairment, and c) are modality-specific.

Ageing WT mice also developed localized hind paw hypersensitivity. Although it is not clear whether these behavioural changes are related to the signs of DD also observed at this age [76], the region-specificity is consistent with a role for the vertebral column in this phenomenon.

4.1.1. Is radiating LBP in SPARC-null mice radicular?

Radicular pain is described as a shooting or lancing pain that travels along the length of the lower limb in a 2–3-inch-wide band and is caused by ectopic discharges from a dorsal root or its ganglion [14]. These ectopic discharges may result from: (1) mechanical compression of nerves or nerve roots exiting the spinal column through narrowed spinal foramen (reduced DHI in SPARC-null mice) or by direct compression due to disc bulging or herniation [46,74], (2) exposure to pronociceptive or proinflammatory chemicals, such as escaping NP content [10,28,75,80], which result in nerve damage and neuropathic pain [39,42,43,58].

4.1.2. Is radiating LBP in SPARC-null mice referred?

Referred LBP spreads into the lower limbs and is perceived in regions innervated by nerves other than those innervating the IVD. It does not involve stimulation of nerve roots, but rather is produced by noxious stimulation of nerve endings within discs [14] and is thought to be due to converging inputs onto second-order spinal neurons. The existence of referred pain from the

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lumbar discs to the hind paw is consistent with overlapping innervation of the hind paw (primarily L3–L6 DRG) and the dorsal aspect of the L5 disc (L4–L5) [77].

4.1.3. Is radiating LBP in SPARC-null mice secondary hyperalgesia?

Converging neurons of the spinal cord can also become sensitized, resulting in an exaggerated response to subsequent peripherally applied stimuli [48]. Central sensitization can be elicited by ongoing pain [84] or muscle fatigue [73], the latter of which typically develops in the gluteal and quadriceps muscles of LBP patients [18,29]. It is therefore possible that the cutaneous hypersensitivity observed in the hind paw of SPARC-null mouse is the result of central sensitization and subsequent secondary hyperalgesia independent of peripheral nerve damage.

4.2. Axial LBP in ageing SPARC-null and WT mice

In humans, components of axial LBP include spontaneous and movement-evoked discomfort, reduced flexibility and cutaneous hypersensitivity in the low back region. The results from the grip force and tail suspension assays suggest that SPARC-null mice experience significant stretch-induced discomfort suggestive of axial LBP, the latter of which was sensitive to systemic morphine treatment. The mechanisms underlying DD-induced axial LBP remain controversial and might include multiple pathological pathways.

4.2.1. Is pathological disc innervation a source of axial LBP?

Under normal conditions, sensory and sympathetic innervation is limited to the outer layers of the AF and to the posterior and anterior ligaments that surround the disc. However, the depth and density of nerve fibers are increased in degenerating lumbar IVDs obtained from chronic LBP patients [24,30,45] or from animal models [8,9]. Moreover, increased levels of nerve growth factor (NGF) within the degenerative disc [31] are associated with increased disc innervation, suggesting that pathological innervation of degenerating IVDs contributes to discogenic pain [32].

4.2.2. Is spinal instability a source of axial LBP?

In addition to the IVDs themselves, DD influences other spinal structures, and injury or instability in any of these segments can result in pathological changes in adjacent tissues [38,65,66]. For example, DD may cause increased strain on ligaments, facet joints and muscles due to reduced spinal stability and redistribution of load. These structures, which are themselves associated with chronic LBP, may then contribute to DD-related LBP (for reviews, see [3,14].

4.2.3. Is primary afferent sensitization a source of axial LBP?

In normal conditions, sensory nerve fibers are found in the outer AF [54]. During DD, levels of the algesic chemicals NGF, TNF and IL-1β are elevated in the NP and can leak into the AF [1] where they could sensitize primary afferents, resulting in hyperalgesia and/or allodynia [10].

4.3. Physical function in ageing SPARC-null and WT mice

In people with LBP, increased movement-evoked fatigue, decreased physical activity, and reduced flexibility are commonly observed [61], and are often used as outcome measures in clinical studies. In human, some of these symptoms can result in physical disability. In SPARC-null mice, decreases in physical function developed with age and mirrored the severity of IVD degeneration from disc thinning to herniation.

Reduced physical activity after both axial strain and repeated lateral flexion are fully developed in SPARC-null mice by approximately 6 months of age, when reduced disc height and histologic signs of moderate DD (loss of compartmentalization between NP and AF) are also observed. These reductions in voluntary activity could be due to pain, muscle weakness, and/or fatigue. Although further studies are needed to distinguish between these possibilities, the phenotype is consistent with that observed in humans.

Motor impairment in the rotarod assay was observed in SPARC-null mice and corresponded temporally with the emergence of severe DD (disc bulging, herniation, and spinal cord compression). The reduced performance in this assay may therefore be due to compression of the spinal cord, nerve roots or DRG after herniation. Interestingly, the already established behavioural signs of axial and radiating LBP do not increase during this time period, further supporting the hypothesis that those changes are not attributable to disc herniation.

Physical function has been assessed in other rodent models of LBP including IVD injury [62], paraspinal muscle inflammation [56], neuropathy induced by exposure to NP [71], and transgenic animals [5]. In those studies, assessment was largely based on either rotarod assay or gait analysis. To our knowledge, the present study is the first to assess physical function based on axial discomfort in rodents. Incorporating more measures of physical function into preclinical studies, such as in the current study, will increase the clinical relevance of these models.

4.4. Advantages and limitations of the SPARC-null mouse model of LBP

The SPARC-null mouse model of LPB due to DD results from a slow, age-dependent degenerative process which progresses over the entire life of the animal. It might therefore be a more accurate representation of the natural course of the disease in humans than previously described inducible models of discogenic [53,63] or radiating pain [6,12,25,28,46], all of which involve selective, intense, physical injury of discs and/or nerves. These models are extremely useful in the study of specific aspects of LBP. For example, although IVD injury models support the study of disc innervation [7,8], repair [2], and biomechanics [11], models involving DRG compression [70] or nerve exposure to NP [39,42,43,52] study radicular pain mechanisms. Although the interpretation of results from the SPARC-null model may be more challenging, this model offers the advantage of mimicking the complexity of the human pathology.

The SPARC protein is involved in many physiological functions, and additional explanations for the observed phenotype must be considered. For example, 1-month-old SPARC-null mice have decreased collagen fibril diameter and reduced tensile strength in the skin [16,68] that could affect sensitivity to cutaneous stimuli. Although the modality and region specificity of the phenotype suggests this is unlikely, the current lack of an inducible, tissue-specific genetic model is a significant limitation.

There is a common assumption that loading of the lumbar spine in humans in greater than in quadrupeds. However, quadruped spinal columns are under constant compressive anterior–posterior forces from the paraspinal muscles and ligaments that result in intradiscal pressures similar to those observed in humans [4]. Nevertheless, it is unreasonable to expect the consequences of DD to be identical in both species, and results should be interpreted accordingly.

4.5. Conclusion

The exact relationship between DD and LBP is not clear, and the underlying mechanisms of LBP are poorly understood. The current study supports the hypothesis that DD is a risk factor for chronic LBP and describes a clinically relevant model of DD-induced

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chronic LBP. Ageing SPARC-null mice mirror many aspects of the complex and challenging nature of LBP in humans and incorporate both anatomic and functional components of the disease.

Conflict of interest statement

The authors report no conflict of interest.

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All experiments were approved by the Animal Care Committee at McGill University, and conformed to the ethical guidelines of the Canadian Council on Animal Care and the guidelines of the Committee for Research and Ethical Issues of IASP published in PAIN®, 1983;16:109–10.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pain.2012.01.027.

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