Diet-induced Obesity Leads to Behavioral Indicators of Pain Preceding Structural Joint Damage in Wild-Type Mice.

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Abstract

Introduction: Obesity is one of the largest modifiable risk factors for the development of musculoskeletal diseases, including intervertebral disc (IVD) degeneration and back pain. Despite the clinical association, no studies have directly assessed whether diet-induced obesity accelerates IVD degeneration, back pain, or investigated the biological mediators underlying this association. In this study we examine the effects of chronic consumption of a high-fat or high-fat/high-sugar (western) diet on the IVD and pain-associated outcomes.

Methods: Male C57BL/6N mice were randomized into one of three diet groups (control chow; high-fat; high-fat, high-sugar western diet) at 10-weeks of age and remained on the diet for 12, 24 or 40 weeks. At endpoint, animals were assessed for behavioral indicators of pain, joint tissues were collected for histological and molecular analysis, and IBA-1, GFAP and CGRP were measured in spinal cords by immunohistochemistry .

Results: Animals fed obesogenic (high-fat or western) diets showed behavioral indicators of pain beginning at 12 weeks and persisting up to 40 weeks of diet consumption. Histological indicators of joint degeneration were not detected in the IVD or knee until 40 weeks on the experimental diets. Mice fed the obesogenic diets showed increased intradiscal expression of inflammatory cytokines and circulating levels of MCP-1 compared to control. Linear regression modeling demonstrated that age and diet were both significant predictors of most pain-related behavioral outcomes, but not histopathological joint degeneration.

Conclusion: Diet-induced obesity accelerates IVD degeneration and knee OA in mice; however, pain-related behaviors precede and are independent of histopathological structural damage. These findings contribute to understanding the source of obesity-related back pain and the contribution of structural IVD degeneration.

Introduction

Obesity – traditionally defined as a body mass index over 30 – is a worldwide epidemic. Obesity substantially increases the risk of developing metabolic, cardiovascular, neurological and musculoskeletal diseases (1), and with the prevalence nearly tripling over the last 30 years (1), it poses a large public health concern. Obesity decreases both life expectancy (2) and quality of life, and is associated with increased disability, mental illness and unemployment (1, 3). A significant contributor to obesity-induced disability is low back pain (LBP) (4, 5), which is the single most common cause of long-term pain and disability worldwide (6). Despite efforts to improve the clinical management of LBP, treatments are largely limited to symptomatic relief, without treating the underlying cause of the pain (7). This is largely due to an incomplete understanding of the tissues and pathways involved in the initiation and progression of LBP. While several tissues appear to be involved in LBP, including the paraspinal

muscles, ligaments, and facet joints (8–10), degeneration of the fibrocartilaginous intervertebral disc (IVD) is believed to be the major contributor to pain in approximately 40% of cases (8).

Despite the clinical associations between LBP, IVD degeneration, and obesity, the underlying mechanisms and biological pathways responsible remain unknown. One contributing factor appears to be increased mechanical loading. Excess weight alters the mechanical load experienced by the IVD (11), a known regulator of IVD cellular function (12, 13). Increased body weight is associated with indices of lumbar disc degeneration including disc space narrowing and decreased lumbar disc signal intensity detected by MRI (14, 15). In articular cartilage, excess weight and altered mechanical loading has also been suggested to contribute to osteoarthritis (OA) (16), a degenerative musculoskeletal disease with many similarities to IVD degeneration (17). Of note, increased mechanical load alone does not account for the association between obesity and OA, as obese individuals also present more frequently with OA in non-weight bearing joints, such as the hand (18).

In addition to increased mechanical load, metabolic abnormalities associated with obesity impact musculoskeletal health (19, 20). Obesity is associated with chronic metabolic disorders including hypertension, diabetes mellitus and dyslipidemia, collectively known as metabolic syndrome (21). In the context of OA, it is postulated that each component of metabolic syndrome may independently contribute to disease progression, as comprehensively reviewed by Zhuo et al. (22) Specifically, alterations in the release of systemic factors (inflammatory cytokines, adipokines), nutrient exchange, advanced glycation end-products (AGEs) levels and glucose/lipid metabolism are believed to be major contributors to OA progression (19, 22). Using mouse models, multiple groups have demonstrated that obesity induced by a high-fat diet accelerates the progression of both age- and surgically-induced knee OA (23–27), accompanied by behavioral indicators of pain (23). Aside from its role in energy storage, adipose tissue is also a major endocrine organ and has been shown to secrete hormones termed adipokines (e.g. leptin, adiponectin, visfatin, resistin) and inflammatory cytokines (e.g. TNF-α, IL-6, TGF-β) (28). Studies investigating the role of adipokines have highlighted their importance in obesity-associated pathologies. For example, leptin-deficient mice become obese yet they do not develop knee OA, suggesting leptin may play a key role in obesity-induced OA (29). In the IVD, exposure of NP cells to adipokines, such as leptin and resistin, promotes catabolic metabolism associated with increased expression of matrix remodeling enzymes such as MMP and ADAMTS genes (30, 31). Adipokines also appear to play a role systemically as modulators of pain sensitivity (32). In addition to back pain, obese individuals are more likely to develop chronic pain conditions such as fibromyalgia, headaches and abdominal pain (33). While the underlying mechanisms linking obesity and chronic pain remains unknown, it has been suggested that systemic immune and endocrine alterations play a role in the altered pain response (34). This systemic modulation of pain may contribute to LBP in addition to structural alterations and local inflammation within the IVD itself.

While there is extensive clinical evidence supporting the association between obesity, LBP and IVD degeneration (35, 36), no studies have directly assessed whether diet-induced obesity accelerates IVD degeneration, back pain, or investigated biological mediators underlying this association. The current

study was designed to investigate whether chronic consumption of a high-fat or high-fat, high-sugar western diet alters the progression of age-related IVD degeneration or back pain using the mouse as a model.

Materials And Methods

Mice and Diets

Wild-type, male, C57BL/6N (Charles River: Wilmington, MA, USA) mice were used. Mice were fed standard chow (Envigo 2018) after weaning and randomized at 10-weeks of age into one of three diet groups (n=9-16 mice/group; **Supplementary Table 1**) based on previous reports of obesity and metabolic derangement in mice (26,37): high-fat diet (60% kcal fat, 21% kcal carbohydrate; Envigo TD.06414), western diet (45% kcal fat, 41% kcal carbohydrate; Envigo TD.10885), or standard chow (18% kcal fat, 58% kcal carbohydrate). Mice remained on the experimental diets until sacrifice at 5, 8 or 11.5 months-of-age (12-, 24-, 40-weeks on diet, respectively). Mice were housed in standard cages and maintained on a 12 hr light/dark cycle, with food and water consumed *ad libitum*; food consumption and body weight were measured weekly. All aspects of this study were conducted in accordance with the policies and guidelines set forth by the Canadian Council on Animal Care and were approved by the Animal Use Subcommittee of the University of Western Ontario (protocol 2017-154).

Characterization of pain-associated behaviors

Behavioral analysis was conducted on mice following 12-, 24-, or 40-weeks on experimental or control diets. Behavioral studies were preceded by a two-week habituation to the neurobehavioral testing facility, and mice were habituated to all tests one week prior to data collection. On data collection days, animals were habituated to the testing room for 1 h before test start. To avoid confounding variables associated with the diurnal cycle, all behavioral assessments were conducted between 8 and 11 AM.

Stretch-induced axial discomfort: Stretch-induced axial discomfort was measured using the tail suspension test and grip force during axial stretch, as described previously(38–40). For the tail suspension test, spontaneous reaction to gravity-induced stretch was assessed in mice suspended by the base of their tails for 180 s. Two observers blinded to the experimental groups independently scored video recordings of the duration of time spent by mice in immobility, full extension, rearing, or self-supporting using ANY-maze software (Stoelting Co.: Wood Dale, IL). Voluntary activity was quantified for 5 min immediately before (pre) and after (post) tail suspension using open field activity monitors (AccuScan Instruments, Omnitech Electronic: Columbus, OH), to quantify movement-evoked discomfort. The difference in total distance between the two open-field sessions (post - pre) was calculated for each mouse.

For the grip force assay, mice were positioned to grab onto a metal bar attached to a grip force meter (Stoelting Co.: Wood Dale, IL), and then gently pulled back by their tails to exert axial stretch. Tolerance

was assessed by measuring the grip strength, in grams, for each mouse at the point of release averaged over 3 trials.

Hind limb sensitivity to mechanical and cold stimuli: Mechanical sensitivity was measured through application of calibrated Von Frey filaments (Stoelting Co.: Wood Dale, IL) to the plantar surface of the hind paw for 3 s or withdrawal. 50% withdrawal threshold was calculated using the Chaplan up-down method (41).The stimulus intensity ranged from 0.07-6.0 g, beginning with a stimulus intensity of 1.4 g. Cold sensitivity was assessed by measuring the total time spent by mice in behavior evoked by evaporative cooling of acetone (flicking, stamping or licking of ventral surface of the paw) during the first 40 s following application of 50 µL acetone to the ventral surface of the hind paw. The test was carried out twice for each paw, with at least 5 min recovery between each test. Times were then averaged between paws.

Spontaneous activity: Voluntary locomotor activity was assessed using open field activity monitors (AccuScan Instruments, Omnitech Electronic: Columbus, OH). Mice were placed into individual boxes and their activity was monitored over 2 h. This was repeated for 3 consecutive days and values were averaged for each mouse.

Micro-computed tomography (micro-CT)

Forty-eight hours before sacrifice, µCT imaging was performed using a cone-beam imaging system (eXplore SpeCZT scanner, GE Healthcare Biosciences: London, CAN). For imaging, mice were anesthetized using 2-3% inhaled isoflurane (CA2L9100, Baxter: Mississauga, CAN) infused with oxygen at a flow rate of 1.0 mL/min. To maintain sedation, a nose cone apparatus was used to administer 1.75% inhaled isoflurane for 20 min while scanning was performed. During a single 5 min rotation of the gantry, 900 X-ray projections were acquired (peak voltage of 90 kVp, peak tube current of 40 mA, and integration time of 16 ms). A calibrating phantom composed of air, water, and cortical bone-mimicking epoxy (SB3; Gammex, Middleton WI, USA) was included in each scan. Data were reconstructed into 3D volumes with an isotropic voxel spacing of 50 µm and scaled into Hounsfield units (HU). Using MicroView software (GE Healthcare Biosciences) three signal-intensity thresholds (-200, -30, and 190 HU) were used to classify each voxel as adipose, lean, or skeletal tissue, respectively. Custom software was used to calculate tissue masses from assumed densities of 0.95 (adipose), 1.05 (lean), and 1.92 (skeletal) g/cm^3, as previously reported (42).

Histological analysis

Intact lumbar spine segments (L1-S1) and knees were isolated, fixed, decalcified and paraffin embedded, as previously described(43). Spines were sectioned sagittally, and knees were sectioned coronally at a thickness of 5 µm using a microtome (Leica Microsystems: Wetzlar, DEU). Lumbar spines were stained using a 0.1% Safranin-O/0.05% Fast Green. Knees were stained with either 0.1% Safranin-O/0.05% Fast Green or 0.04% Toluidine Blue. Sections were imaged on a Leica DM1000 microscope, with Leica Application Suite (Leica Microsystems: Wetzlar, DEU).

To evaluate IVD degeneration, spine sections were scored by 2 independent scorers using the modified Boos system (44). Knee joint health was assessed using the murine Osteoarthritis Research Society International (OARSI) histopathological scale (45). Articular surfaces of the medial femoral condyle (MFC), medial tibial plateau (MTP), lateral femoral condyle (LFC) and lateral tibial plateau (LTP), were scored by two blinded observers and averaged. For each knee joint surface, scores from 10 serial sections spanning 500 μM of the joint were summed to represent OARSI score for each quadrant. Total scores from each of the four quadrants were then added together to generate whole joint OARSI score. Thickness of articular cartilage of the MTP and LTP was quantified using the OsteoMeasure7 Program (v.4.2.0.1, OsteoMetrics Inc., Decatur, GA, USA). Articular cartilage thickness within each quadrant was averaged using three serial sections spanning 150 μM of the weight-bearing region of the knee. Using the same histomorphometry system, trabecular bone area was calculated by measuring the total surface area of the bone between the articular cartilage and growth plate and subtracting the area of bone marrow. Measurements were taken for both medial and lateral compartments of the joint and averaged from 3 serial sections.

Gene expression analysis

Thoracic IVDs (4-5 per mouse; 5-8 mice per diet/per timepoint) were isolated by microdissection, placed in TRIzol reagent (Thermo Fisher Canada: Mississauga, ON, CAN) and homogenized using a PRO250 tissue homogenizer (PRO Scientific: Oxford, CT, USA). RNA was extracted according to manufacturer's instructions, quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Canada: Mississauga, ON, CAN), and 0.5 μg was reverse transcribed into complementary DNA (cDNA) (iScript; Bio-Rad Laboratories (Canada): Mississauga, ON, CAN). Gene expression was assessed by real-time PCR using a Bio-Rad CFX384 instrument. PCR analyses were run in triplicate using 120 ng of cDNA per reaction and 310 nM forward and reverse primers with 2x SsoFast EvaGreen Supermix (Bio-Rad Laboratories (Canada): Mississauga, ON, CAN) using optimized PCR parameters and primers (**Supplementary Table 2**). Primers were designed and validated to have efficiency values between 90 and 120%. Transcript levels were calculated relative to a 6-point standard curve made from pooled cDNA generated from murine IVD explants treated with lipopolysaccharide for 4 days (50 mg/mL; Thermo Fisher Canada: Mississauga, ON, CAN).

Immunohistochemistry

The intact spinal cord was removed, dissected to separate the upper (L1-L2) and lower (L3-L6) segments of the lumbar enlargement, and fixed in 4% paraformaldehyde (PFA) for 24 h at 4°. Tissues were cryoprotected for 4 days in 10% sucrose and embedded in optimal cutting temperature compound (Tissue-Tek O.C.T; Sakura Finetek US: Torrance, CA, USA) and stored at -20°C. Tissues were sectioned on a cryostat (Leica Microsystems: Wetzlar, DEU) in the transverse plane at a thickness of 14 μm, thaw mounted onto gelatin-coated slides, and stored at -80°C.

Slides were brought to room temperature, washed twice in PBS and blocked using 5% donkey serum, 0.1% Triton X-100 in PBS for 2h at room temperature. Sections were incubated overnight in a humidified

chamber at 4ºC in 5% donkey serum in PBS (with 0.1% Triton-X) containing primary antibodies directed against glial fibrillary acidic protein (GFAP) (1:500; G3893, Sigma-Aldrich: St. Louis, MO, USA), ionized calcium binding adaptor molecule 1 (IBA-1) (1:1000; AB-10341, Abcam: Cambridge, UK), or calcitonin gene-related peptide (CGRP) (1:750; BML-CA1137, Enzo Biochem: New York, NY, USA). Slides were rinsed 3 x 10 min in PBS-T (PBS + 0.01% Triton X-100) and then incubated for 45 min at room temperature with secondary antibodies diluted 1:500 in PBS: Alexa Fluor 488 conjugated donkey anti-mouse IgG for GFAP (A-21202, Thermofisher: Waltham, MA, USA); Alexa Fluor 594 conjugated donkey anti-rabbit IgG for Iba-1 (A-21207, Thermofisher: Waltham, MA, USA); or Alexa Fluor 488 donkey anti-sheep IgG for CGRP (A-11015, Thermofisher: Waltham, MA, USA). Slides were rinsed 3 x 10 min in PBS, dipped in deionized water, and cover slips mounted using Fluoroshield Mounting Medium with 4',6-diamidino-2-phenylindole to visualize nuclei (ab104139, Abcam: Cambridge, UK). Tissue sections were imaged using a Leica Microsystems DMI6000B fluorescence microscope and DFC360FX camera with Leica Advanced Application Suite software (Version 2.7.0-9329, Leica Microsystems: Wetzlar, DEU). A region of interest (ROI) was manually defined to contain lamellae 1-4 of the spinal cord dorsal horn using ImageJ software. The dorsal horn was differentiated from surrounding white mater based on brightfield images. Integrated density of fluorescence within the ROI was used to quantify astrocyte/microglia density, and CGRP-immunoreactivity.

Serum Analysis by Multiplex Assay

At euthanasia, blood was obtained by cardiac puncture, coagulated for 30 min at room temperature, and centrifuged at 3 000 rpm for 10 min at 4°C to collect serum. Serum (n=5-6 mice per diet/per timepoint) was diluted 2-fold in DPBS and analyzed using the Luminex™ 200 system (Luminex, Austin, TX, USA) by Eve Technologies Corp. (Calgary, Alberta). Thirty-two markers were simultaneously measured in each serum sample using the MILLIPLEX Mouse Cytokine/Chemokine 32-plex kit (Millipore, St. Charles, MO, USA) according to the manufacturer's protocol. The multiplex assay quantified Eotaxin, G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNFα, and VEGF. The assay sensitivities range from 0.3 – 30.6 pg/mL for the 32-plex.

Statistical Analysis

For all assays except histopathological scoring of the joints, outcome measures for mice within each time point were compared between the different diet groups by one-way ANOVA with Tukey’s multiple comparisons test. For histopathological analysis, within each timepoint scores for mice were compared between the different diet groups by non-parametric Kruskal-Wallis test with Dunn’s multiple comparison test. $P < 0.05$ was considered significant. Statistical analysis was conducted using GraphPad Prism 8 (Graphpad Software: San Diego, CA, USA).

To assess the effect of diet, adiposity, knee OA and IVD degeneration on behavioral, molecular, and histological changes assessed, bivariate and multivariate linear regression models were used to identify

which variables remained independently associated with the other outcomes. Bivariate and multivariate modelling was conducted using STATA 16 (StataCorp LLC: College Station, TX, USA).

Results

Weight & Adiposity

As expected, following 12, 24- and 40-weeks mice fed the high-fat and western diets showed a significant increase in body mass and weight gain compared to age-matched chow fed controls (**Figure 1A**). Analysis of body composition by micro-CT **(Figure 1B**) demonstrated that the increase in adiposity in mice fed the experimental diets was accompanied by a significant decrease in the percentage of both lean and skeletal tissues at all time points (**Figure 1C**). This analysis also showed a significant increase in overall bone mineral density (BMD) in mice fed the high-fat diet at all timepoints, and at the 24- and 40-week timepoints for mice fed the western diet, compared to age-matched chow fed controls (**Figure 1C**).

Behavioral indicators of axial discomfort

We first investigated whether mice fed the high-fat or western diet showed behavioral indicators of stretch-induced axial discomfort using three complimentary assays established as indicators of discogenic back pain in a mouse model of degeneration (40): behavior during tail suspension, changes in spontaneous activity after tail suspension, and tolerance to axial stretching in the grip force assay. In the tail suspension test, no significant differences were observed between diet groups at any time point investigated (**Figure 2A**). Similarly, changes in spontaneously activity induced by the tail suspension assay (locomotion pre versus post tail suspension) were not altered between experimental diet groups and age-matched chow fed controls (**Figure 2B**). Grip force during axial stretch was reduced in mice fed the high-fat diet compared to age-matched chow fed controls at 12- and 40-weeks and in mice fed the western diet at all time points compared to control (**Figure 2C**), suggesting decreased tolerance to axial stretch.

Behavioral indicators of mechanical and cold sensitivity

Mechanical and cold sensitivity were assessed in the hind paw using the Von Frey assay and by measuring the response of mice to the evaporative cooling of acetone, respectively. Mice fed the western diet showed a significant increase in mechanical sensitivity at the 24- and 40-week time points compared to age-matched chow fed controls, while mice fed a high-fat diet showed a significant increase in sensitivity only at the 24-week timepoint compared to controls (**Figure 3A**). In contrast, no significant difference was observed in sensitivity to cold between mice in either experimental diet group compared to age-matched chow fed controls at any time point (**Figure 3B**).

Spontaneous locomotion

Behavior and locomotion in open field was assessed for all mice over a 2 h period. Mice fed the western diet showed a significant decrease in total distance travelled following 12 and 24 weeks compared to

age-matched chow fed controls. Mice fed the high-fat diet showed a significant reduction in locomotion following 24-weeks compared to age-matched controls (**Figure 3C**). The number of rearing events was also significantly decreased in mice fed the high-fat and western diets at the 40-week time point compared to age-matched chow fed controls (**Figure 3C**).

Assessment of IVD degeneration

The effects of the high-fat and western diets on IVD health were assessed using both histopathological evaluation and molecular analysis (**Figure 4**). On average, no overt differences were detected in the histological appearance of lumbar IVDs between mice fed the high-fat or western diet for 12- and 24-weeks compared to age-matched chow fed controls (**Figure 4A**). Accordingly, histopathological scoring using the modified Boos system showed no significant differences in degeneration between the groups. However, when data was analyzed by individual spinal level, mice fed the western diet showed significantly lower scores than their high-fat or chow fed counterparts at the L6S1 spinal level at the 12-week timepoint (**Figure 4B**). Following 40 weeks on the experimental diets, an accumulation of hypertrophic cells surrounded by a glycosaminoglycan-rich pericellular matrix was consistently detected in the inner annulus fibrosus of mice fed both the high-fat and western diets, but not in age-matched chow fed control mice (**Figure 4A** – black arrows). Despite this observation, histopathological scoring revealed no significant degeneration in the diet groups compared to chow fed controls at the 40-week time point (**Figure 4B**). Histopathological analysis was paired with qPCR to quantify expression of markers of inflammation, neural ingrowth, and matrix degrading enzymes in thoracic IVDs to further investigate molecular changes associated with diet-induced obesity. Mice fed the high-fat or western diets showed no significant differences in the expression of the genes investigated at the 12-week timepoint compared to age-matched chow fed controls (**Figure 4C**). Mice fed the high-fat diet showed no significant differences in gene expression compared to chow fed controls at the 24-week timepoint for any of the genes investigated. At the 40-week timepoint, mice fed the high-fat diet showed increased expression of inflammatory mediators (*Il-6*, *Ptsg2),* neurotrophins (*Bdnf*), as well as matrix degrading enzymes (*Adamts5)* compared to age-matched chow fed controls (**Figure 4C**). Mice fed the western diet showed increased expression of the inflammatory mediators *Il-1b* and *Ptgs2* compared to chow fed controls at 24 weeks; however, no significant differences in gene expression were detected at the 40-week timepoint (**Figure 4C**).

Assessment of degenerative changes in the knee

Since diet-induced obesity leads to other arthropathies, such as knee OA (23), we investigated degenerative changes to the knee joint as a potential contributor to the pain-related behavioral outcomes assessed. We focused this analysis on the 24- and 40-week timepoints where changes in behavioral measures were most consistently identified. No overt histological differences were detected in the knee joints of mice fed either the high-fat or western diet for 24 weeks compared to age-matched chow fed controls (**Figure 5A**). Histopathological scoring using the OARSI system supported these observations (**Figure 5B**). At the 40-week timepoint, mice fed the western diet showed decreased proteoglycan staining

in the medial femoral condyle (MFC) compared to age-matched controls (**Figure 5A**), resulting in significantly higher OARSI scores (**Figure 5B**) indicating early signs of osteoarthritis. However, no significant differences were seen in the other compartments of the knee joint or in the cumulative OARSI scores for the whole joint. In addition to histopathological analysis, cartilage thickness was measured by histomorphometry. Although no significant differences in cartilage thickness were detected in mice following 24 weeks on the experimental diets, mice fed the high-fat diet for 40 weeks showed a significant decrease in cartilage thickness specific to the lateral femoral condyle compared to age-matched chow fed controls (**Figure 5C**). Histomorphometry was similarly used to measure the relative surface area of trabecular bone between the articular cartilage and growth plate. Mice fed the western diet for 40 weeks showed a significant increase in subchondral trabecular bone compared to age-matched chow fed controls specifically in the medial tibial plateau (**Figure 5D**), suggesting subchondral bone sclerosis.

Analysis of sensory neuroplasticity within the lumbar spinal cord

To assess neuroplastic changes associated with neuroinflammation and chronic pain, lumbar spinal cords from mice at the 40-week time point were assessed for markers of astrocytes (glial fibrillary acidic protein, GFAP), microglia (ionized calcium-binding adapter molecule 1, IBA-1), and nociceptive innervation (calcitonin gene-related peptide, CGRP; **Figure 6A**). Although multiple mice in both the high-fat and western diet groups showed increased GFAP and IBA-1 staining in the upper and lower lumbar spinal cord compared to the average values for chow fed controls, quantification of fluorescent intensity within the dorsal horn showed no significant difference between diet conditions for any of the proteins investigated (**Figure 6B**).

Circulating inflammatory factors

Luminex xMAP multiplex assays were used to quantify a panel of 32 cytokines, chemokines, and growth factors in the serum of experimental mice. Mice fed a western diet showed increased levels of interleukin (IL)-1β, IL-6, IL-10, and tumor necrosis factor alpha (TNFα) at the 12- and 40-week timepoints in their serum, however, no significant differences were seen when compared to chow fed control due to variability between animals (**Table 1**). Despite this variability, at the 40-week timepoint a significant increase in circulating monocyte chemoattractant protein 1 (MCP-1) was observed in mice fed a western diet compared to chow-fed control (**Table 1**). Animals fed a western diet showed for 24-weeks also showed significantly lower levels of circulating eotaxin and IL-1α compared to chow fed controls, but no other differences in any of the other cytokines investigated (**Supplementary Table 3**).

Linear regression analysis

To directly examine associations in the context of the observed variability within each experimental group in our study, linear regression modeling was conducted to determine whether diet, adiposity or age were predictors of behavioral, histological, or systemic outcomes **(Table 2**). Bivariate modelling demonstrated that both adiposity and age are independent predictors of multiple indicators of pain including stretch-

induced axial discomfort (grip force), mechanical sensitivity (Von Frey assay) and spontaneous locomotion (open field). Despite their association with behavioral alterations, neither adiposity nor age were associated with measures of joint degeneration or levels of most circulating factors assessed (**Table 2**). Exceptions to this were TNFα and MCP-1, which were associated with age (bivariate), and the western diet (multivariate), respectively (**Table 2**). When diet, adiposity and age were accounted for, age was found to be the most robust predictor of all outcomes measured (**Table 2**). Aside from age, the multivariate model also showed that diet and adiposity are covariates for grip force and rearing in open field, respectively (**Table 2**). To determine if histopathological scores for joint damage were independently associated with behavioral indicators of pain, bivariate and multivariate regression modeling was completed. In this study, no significant association was detected between histopathological scores for IVD degeneration and pain-related behaviors. A weak but significant association was detected between histopathological scores for knee OA and grip force, but not for any of the other behavioral metrics assessed **(Supplementary Table 4**).

Discussion

Obesity is one of the largest modifiable risk factors for the development of both IVD degeneration and LBP (35, 36), yet the biological mechanisms underlying this association are currently unknown. With the prevalence of obesity on the rise (1), it is necessary to address this problem and identify underlying pathogenic mechanisms. The current study was designed to investigate the longitudinal effects of diet-induced obesity on inflammation, IVD degeneration, knee OA, sensory neuroplasticity, and pain using the mouse as a preclinical model. We show that obesity induced by both a high-fat and high-fat/high-sugar western diet led to behavioral indicators of pain in as little as 12-weeks, preceding gross structural changes to the IVD and knee joints. Following 40 weeks, changes in cellular morphology within the inner AF of the IVD were detected in mice fed both obesogenic diets compared to chow-fed controls; however, these changes were not associated with increased histopathological degeneration. Chronic consumption of the high-fat diet was associated with increased expression of *Il-6*, *Ptgs2*, *Bdnf*, *Adamts-5*, and *Mmp-3* within the IVD and a decrease in articular cartilage thickness within the lateral tibial plateau. In contrast, chronic consumption of the western diet was associated with increased expression of *Il-1b* and *Ptgs2* within the IVD, histopathological features of early OA, subchondral bone sclerosis, and increased serum MCP-1 levels. These findings highlight the complex interplay between diet, adiposity, pain, inflammation, and joint health.

Rodent models have proven useful to study IVD and joint biology since they allow insight into biological processes that regulate tissue homeostasis and degeneration. Numerous models have been described in which IVD degeneration is induced through genetic manipulation, surgical disruption, chemical injection or aberrant mechanical loading (46). Although these studies contribute to understanding the cellular and molecular basis of IVD degeneration, few investigate the association with pain (47). Given the discordance between structural IVD degeneration and pain development in humans (10, 48, 49) and pre-clinical models (50), it is important to investigate both pain and structural IVD degeneration and their relation to one another. Although pain cannot be directly measured in animals, several indirect,

quantitative behavioral assays have been developed to evaluate pain-like behaviors for a variety of different pain states (51, 52). Many of these behavioral metrics are not specific to back pain and are used to assess joint, inflammatory, and neuropathic pain (53, 54). Stretch-induced axial discomfort has however been established as a reliable measure of axial low back pain (40). In the current study, mice fed obesogenic diets showed significant impairments in grip force at all timepoints compared to control mice, suggesting axial discomfort. In contrast, no significant difference was detected in the tail suspension assay between mice fed obesogenic diets and controls. This potentially contradictory data may be influenced by the nature of the tail suspension assay, which was originally used to test depressive behavior in mice (55). In addition to musculoskeletal pathologies, obesity is also associated with depression (33). In fact, recent studies demonstrated that mice fed a high-fat diet for 8 weeks showed increased time immobile in tail suspension, interpreted as a characteristic of depression-like behavior (56). This behavior is in contrast to both genetic and age-related mouse models of IVD degeneration and axial pain which show increased time rearing in tail suspension (57, 58). This confounding factor may impact the outcome of tail suspension in the diet-induced obesity model, and consequently the interpretation of our findings.

In addition to indicators of stretch-induced axial discomfort, obese mice also displayed mechanical but not thermal (cold) hypersensitivity of the hind-paw. These alterations are consistent with results seen in a model of surgically induced IVD degeneration, where intradiscal injection of PBS induced mechanical hypersensitivity in rats (59). However, these results contrast those reported in a genetic model of IVD degeneration, where SPARC-null mice show thermal but not mechanical hypersensitivity compared to WT mice (39). While the mechanisms underlying these differences are unclear, mechanical sensitivity is common in both inflammatory and neuropathic pain models (60) and different models of pain are likely to impact nociception through different pathogenic mechanisms (61). Obesity also appeared to impact non-reflexive (spontaneous) behaviors including distanced travelled and rearing in open field. These behaviors are decreased in both inflammatory and neuropathic pain models (62). Taken together, our findings indicate that mice fed a high-fat or western diet display pain-related behaviors starting at the 12-week timepoint that persist until the 40-week timepoint.

Previous studies established that high-fat diet-induced obesity accelerates OA in mice (23, 26); however, pain-related behaviors were found to be independent of OA severity (23). As IVD degeneration has been associated with many of the pain-related behaviors reported for OA (38), we characterized both IVD degeneration and OA-associated knee degeneration in our experimental mice to account for both as factors contributing to pain. Histopathological scoring of lumbar IVDs did not reveal significant degeneration caused by the high-fat or western diet at any timepoint. However, mice fed both obesogenic diets showed a consistent accumulation of hypertrophic cells in the inner annulus fibrosus at 40 weeks, suggesting early degenerative change. Moreover, increased expression of inflammatory mediators (*Il-1b*, *Il-6, Ptgs2*), matrix degrading enzymes (*Adamts5*) and neurotrophins (*Bdnf*) were detected in mice fed the high-fat and western diets. These inflammatory cytokines are known drivers of IVD pathogenesis associated with ECM degeneration and expression of neurogenic factors such as NGF and BDNF that can contribute to pain (63). Similarly, significant histopathological degeneration in the knee was only detected

at the 40-week timepoint in mice fed the western diet. Linear regression modeling indicates that the behavioral indicators of pain assessed are independent of joint degeneration, except for the grip force assay for which knee OA was a significant predictor of impairment. Together these findings indicate that diet-induced obesity may potentially accelerate the progression of structural IVD degeneration and knee OA at the later timepoints; however, these changes are mild and are likely independent of most pain-related behaviors.

Findings from the current study suggesting that pain-related behaviors precede molecular and structural alterations in joint tissues raise questions related to the source of the pain observed. Our findings are however consistent with the hypothesis that pain is multifactorial. Obesity is considered a state of chronic inflammation associated with increases in circulating inflammatory cytokines such as TNFα and IL-6 in humans (64). Inflammation has been shown to contribute to peripheral and central sensitization and may lead to hyperexcitability of the nervous system and chronic pain (65). Consistent with these findings, we demonstrate that consumption of the western diet led to increased levels of circulating MCP-1 in mice, a pro-algesic mediator that can increase primary afferent neuron activity (66, 67). Obesity may also impact central pain processing; in mice consumption of a high-fat diet increases the activation of microglia (68) while exposure of cultured astrocytes to saturated fatty acids induces cytokine release and astrocyte inflammation (69). These neuroplastic changes can contribute to central sensitization through multiple mechanisms, including increased release of inflammatory factors contributing to modulation of synaptic activity (65). Although the averaged values of GFAP and IBA-1 expression in the spinal cord were not significantly different between diet groups in our study, multiple mice in both the high-fat and western diet groups showed increased activation of both microglia and astrocytes at the 40-week timepoint. These alterations to the nociceptive pathways at either the peripheral or central level may contribute to the pain response seen. While not assessed, obesogenic diets also lead to painful diabetic neuropathy, or nerve damage, which may also contribute to the pain phenotype observed (70). Given the diverse impacts of obesity, it could be postulated that obesity-related pain may arise either from hyperexcitability or damage to nociceptive pathways in conjunction with tissue damage, and these contributions may differ between animals and over time. A limitation of this study is the utility of behavioral tests used to assess pain in our model. While they serve as indicators of pain in other models, many assays used in the current study also have been used to test muscle strength (71) and psychiatric disorders (55) which may be impacted by obesity (33, 72). To explore whether these behavioral responses are due to pain, future studies may wish to pharmacologically inhibit nociceptive pathways to confirm that behavioral differences are due to pain.

Although the average weight gain was similar between the two obesogenic diets evaluated in this study, important differences in outcomes were detected. Mice fed the western diet showed a more consistent pain response compared to control at all timepoints than mice fed the high-fat diet. Furthermore, at the 40-week timepoint knee osteoarthritis and increases in systemic inflammation were only seen in mice fed the western diet. These findings highlight the importance of dietary composition in the study of obesity. In the context of OA, dietary fatty acid and carbohydrate composition can significantly impact joint health (24, 73). Diets high in saturated fatty acids or ω-6 polyunsaturated fatty acids (PUFAs) induce more

severe metabolic dysregulation and OA progression than diets enriched with ω-3 PUFAs (24). These findings may explain our results, as the composition of the western diet is higher in saturated fats than the high-fat diet. Dietary composition has also been shown to impact IVD health. Diets rich in advanced glycation end-products (AGE) precursors accelerate IVD degeneration in mice in parallel with insulin resistance(74).

A confounding factor in the interpretation of our findings was the substantial variability for many of the outcomes investigated, including substantial differences in weight gain between mice on both obesogenic diets. Despite controlling for genetics using an inbred strain, susceptibility to diet-induced obesity can be affected by social stress, microbiome composition and epigenetic mechanisms (75–77). Accounting for this variability, previous studies in mice demonstrated that cartilage damage induced by a high-fat diet is proportional to adiposity (23). In the current study, adiposity did not predict histopathological measures of joint degeneration; however, adiposity and age were significant predictors of behavioral indicators of pain. Highlighting the complexity of these models, increased adiposity was also not directly associated with increased circulating cytokine levels in our study. In humans, obese individuals can be classified as metabolically healthy (metabolically healthy obese (MHO)). MHO individuals are at less risk for developing obesity-related complications including OA than metabolically abnormal obese individuals (78). The mechanisms underlying MHO are not well understood; however, genetic, epigenetic and environmental factors are thought to play a role (79, 80). As adiposity does not directly correlate with systemic or neuroinflammation in the current model, it is important to investigate all aspects of metabolic syndrome (i.e. circulating lipids, glucose, cytokines/adipokines, blood pressure) on both the musculoskeletal and nervous system in future models to determine their contribution to disease progression.

Taken together, this study highlights the complexity of the relationship between obesity, IVD degeneration and pain. Mice fed a high-fat or western diet showed pain-related behaviors that preceded structural joint degeneration in both the IVD and knee. The chronology of these findings may be of clinical importance, as pain may affect the progression of radiographic joint degeneration. While not directly investigated in IVD degeneration, knee pain has shown to be a predictor of accelerated radiographic OA through inflammation and reduced mobility (81), which is also seen in IVD degeneration (82). This raises the intriguing possibility that back pain may be both a consequence of structural IVD degeneration in the current model, and a contributor to it.

Declarations

Ethics approval and consent to participate

All aspects of this study were conducted in accordance with the policies and guidelines set forth by the Canadian Council on Animal Care and were approved by the Animal Use Subcommittee of the University of Western Ontario (protocol 2017-154).

Consent for publication

Not applicable

Availability of data and material

The data associated with the current study is available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors contributions

G.J.K. contributed to study design, directed data collection/analysis of all aspects of the study and drafted the manuscript. B.T. participated in the collection and analysis of data from the knee joints. I.W. participated in the collection and analysis of data from the spinal cords. M.M and L.S.S. contributed to the experimental design and analysis of data from the pain-related behavior assays and participated in the interpretation of all study results. F.B. contributed to the experimental design and analysis of the data from the knee joint and participated in the interpretation of all study results. C.A.S. contributed to the study design, data analysis and interpretation and manuscript preparation. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, table 1,2 is only available as a download in the Supplemental Files section.

Figures

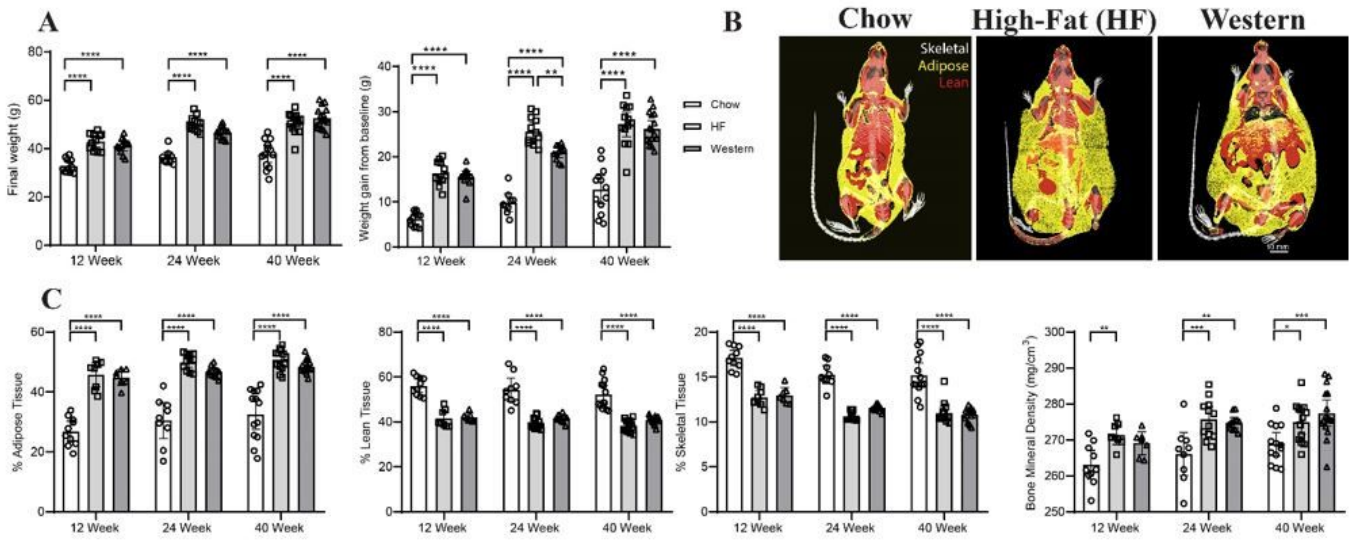

Figure 1

Chronic consumption of the high-fat and western diets increases adiposity in C57BL/6N mice. (A) At all timepoints, mice fed the high-fat and western diets showed significant increases in overall weight and in weight-gain from baseline compared to age-matched chow fed controls. (B) Representative reconstructed micro-CT images of mice following 40-weeks of experimental diets. Isotropic surface-rendering of skeletal tissue (indicated in white) is overlaid with a mid-coronal slice where lean tissue is indicated in red and adipose tissue is indicated in yellow. (C) Quantitative micro-CT analysis of whole-body composition showed a significant increase in adiposity and significant decreases in both percentage of lean and skeletal and in mice fed the high-fat and western diet mice compared to age-matched chow fed controls at all time points. A significant increase was also seen in bone mineral density in mice following consumption of the high-fat and western diets at the 24- and 40-week timepoints. n=9-16 mice per timepoint, per diet. Data are displayed as mean ± 95% CI; data points for each mouse are graphed within each group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 by 2-way ANOVA.

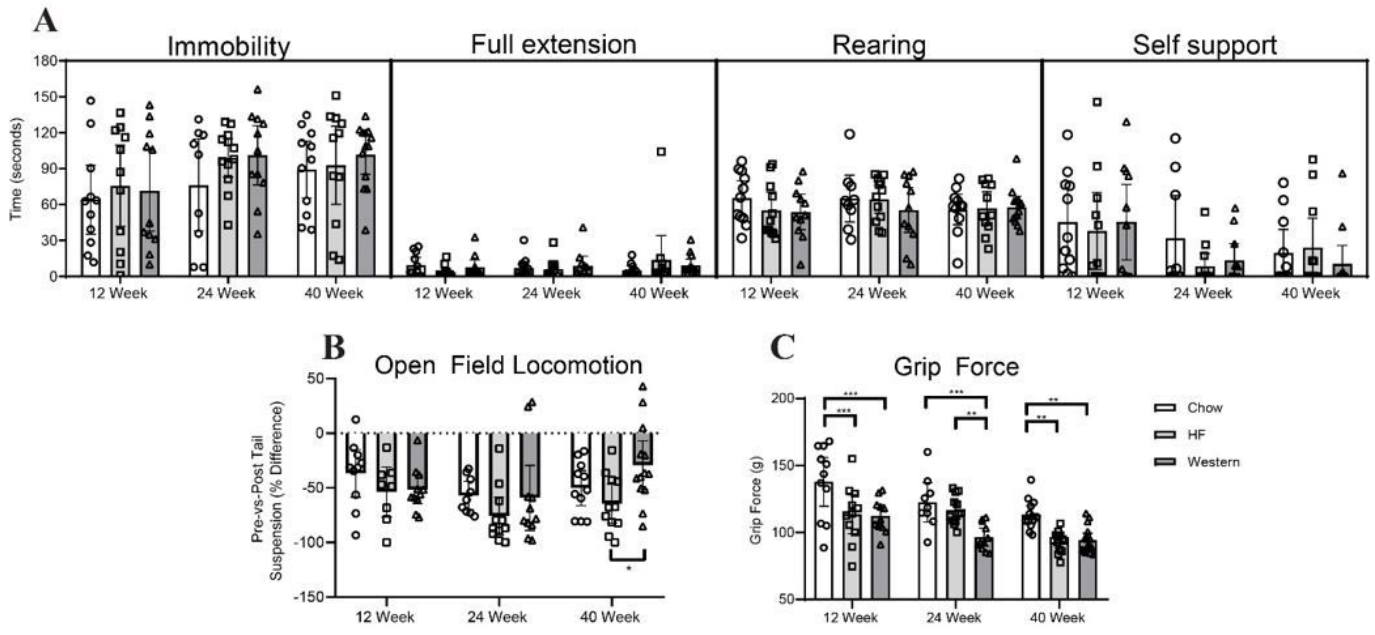

Figure 2

Diet-induced obesity reduces grip strength but does not alter behavior in tail suspension. (A) During tail suspension, the duration of time spent by mice immobile, in full extension, rearing or self-supported was quantified. No significant differences were detected between diet groups at any of the time points assessed. (B) Stretch-evoked discomfort was assessed using the open field assay, in which the total distance covered in 5 min immediately before (pre) and after (post) the 3 min tail suspension assay was quantified. Obesity induced by the high-fat and western diets did not alter behavior of mice in open field compared to age-matched chow fed controls at any of the time points assessed. However, a significant difference was seen between mice fed a high-fat and western diet at the 40-week timepoint. (C) Grip force during axial stretch was reduced in obese mice. Mice fed the high-fat diet showed a significant decrease in grip force at the 12- and 40-week time points compared to age-matched chow fed controls. Mice fed the western diet showed a significant decrease in grip force compared to age-matched chow-fed controls at all time points. n=9-16 animals per timepoint, per diet. Data are plotted mean ± 95% CI; data points for each mouse are graphed within each group. *P<0.05, **P<0.01, ***P<0.001 by 2-way ANOVA.

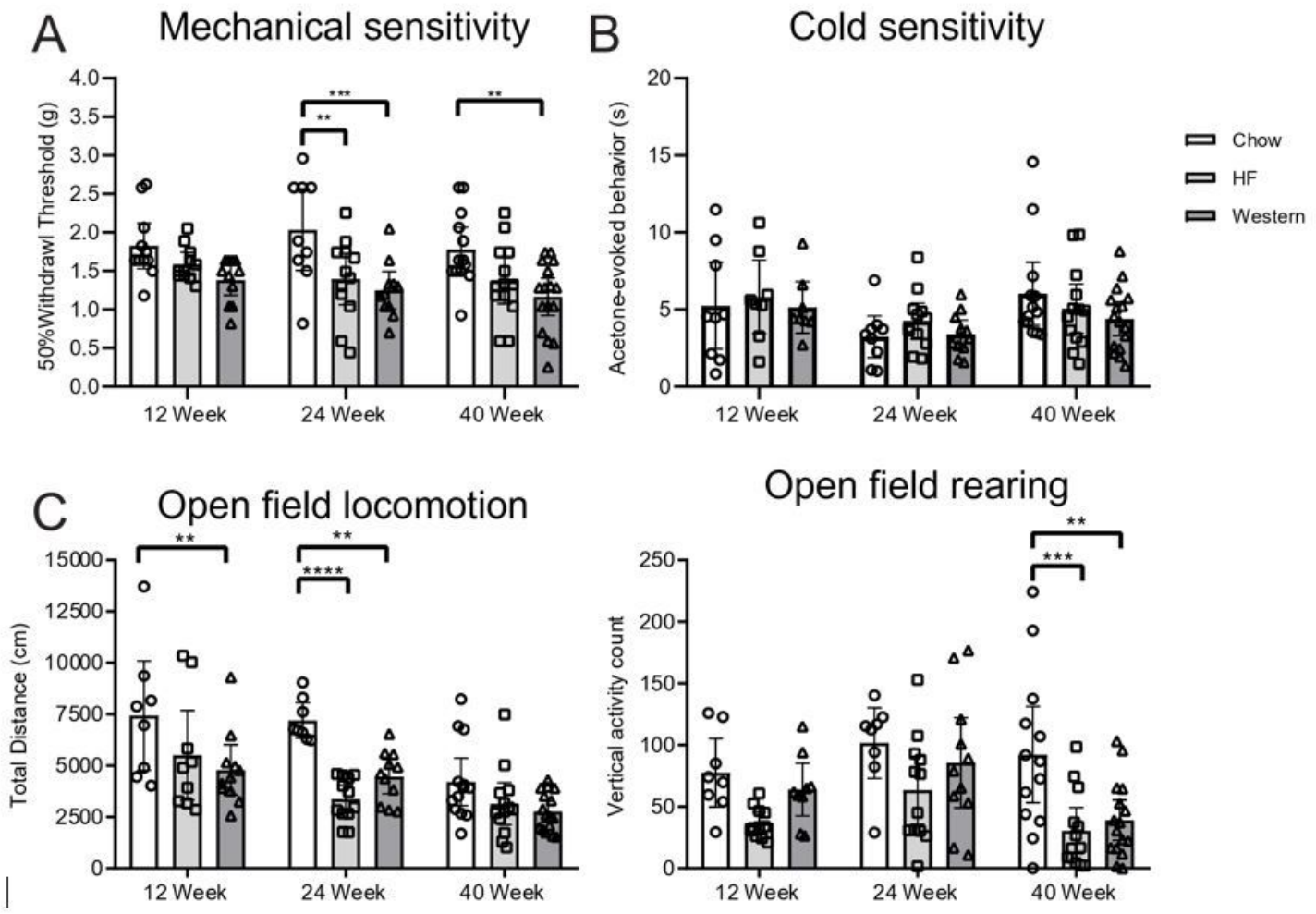

Figure 3

Diet-induced obesity increases sensitivity to mechanical stimulation and alters spontaneous locomotion. (A) Mechanical Sensitivity of the hind paw was assessed by manual application of Von Frey filaments using the Chaplin up-down method. Mice fed the western diet showed a significant decrease in withdrawal threshold at the 24- and 40-week time points compared to age-matched chow fed controls, indicative of increased mechanical sensitivity. Mice fed a high-fat diet showed a significant decrease in withdrawal threshold at the 24-week timepoint compared to control. (B) Sensitivity to cold was assessed by measuring the time spent in behavior evoked by evaporative cooling of acetone (flicking, stamping or licking of ventral surface of the paw) during the first 40s following application of acetone to the ventral surface of the hind paw. No significant differences were seen between the diet groups at any timepoint. (C) Spontaneous locomotor activity was recorded over three 2 hr sessions and averaged. Mice fed the western diet showed a significant decrease in the total distance travelled at the 12- and 24-week timepoint compared to age-matched chow fed controls, while mice fed the high-fat diet showed a decrease at the 24-week timepoint. The number of rearing events was significantly decreased in mice fed the high-fat and western diets compared to controls at the 40-week timepoint. n=9-16 animals per

timepoint, per diet. Data are plotted mean ± 95% CI; data points for each mouse are graphed within each group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 by 2-way ANOVA.

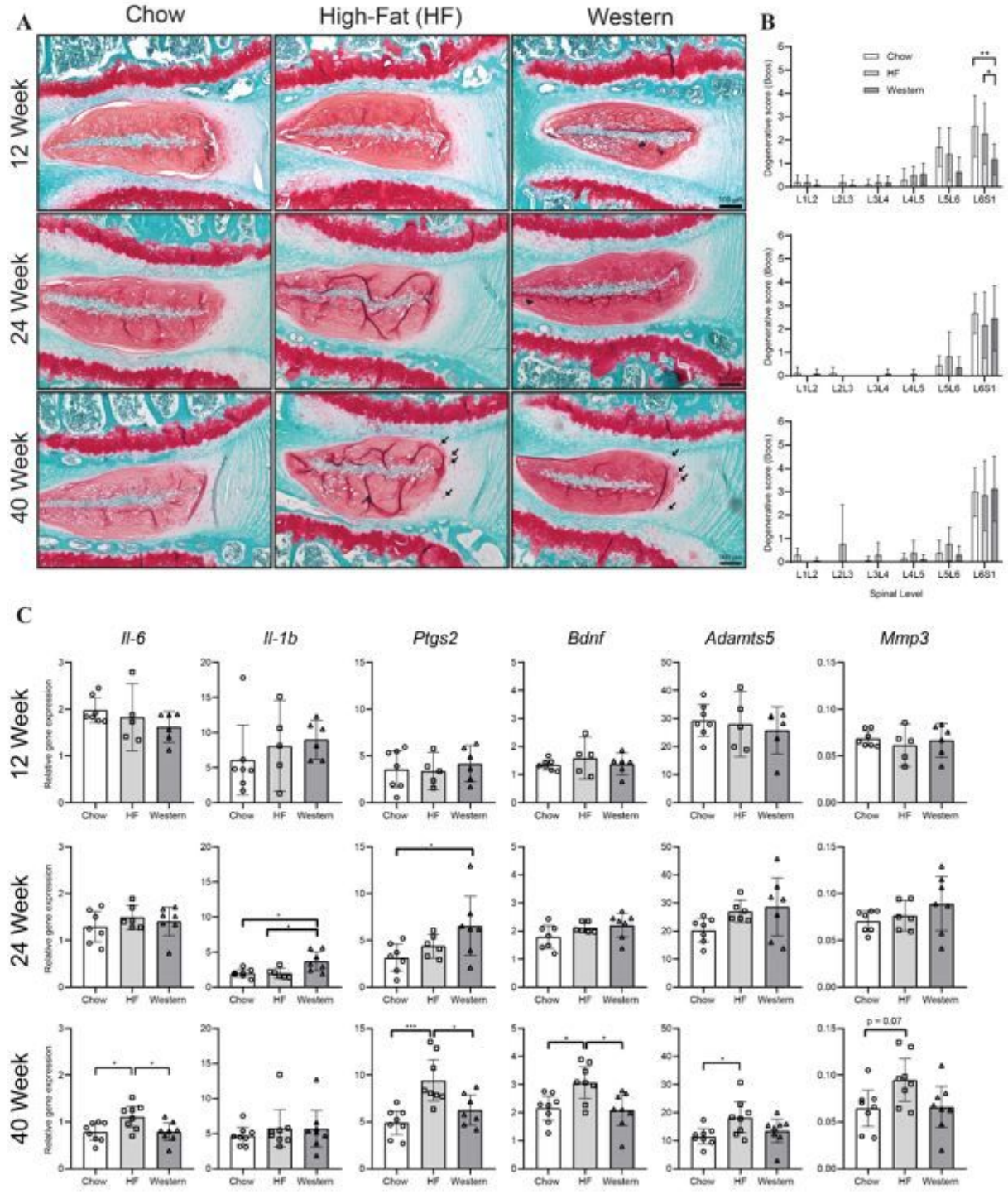

Figure 4

Effect of diet-induced obesity on the intervertebral disc. (A) Representative histological sections of the L6/S1 spinal level of the lumbar spine stained with safranin-o/fast green from mice fed control chow, high-fat, or western diet for 12, 24, or 40 weeks. The accumulation of hypertrophic cells was detected

within the inner annulus fibrosus of mice fed the high-fat and western diets for 40 weeks (highlighted by black arrows). (B) Evaluation of the grade of histopathological IVD degeneration using the modified Boos scoring system showed no significant differences between mice fed the control chow, high-fat or western diets at the 24- and 40-week timepoints. At the 12-week timepoint, mice fed the western diet showed a significant decrease in the degenerative score compared to mice fed chow and high-fat diets. n=9-16 animals per timepoint, per diet. Data are analyzed by Kruskal-Wallis test. (C) SYBR-based qPCR of intact thoracic intervertebral discs showed no significant difference between mice fed a chow, high-fat or western diet at the 12-week timepoint for any genes investigated. At the 24-week timepoint, significant increases in Il-1b and Ptgs2 expression were seen in mice fed the western diet compared to control. By 40-weeks, significant increases in Il-6, Ptgs2, Bdnf and Adamts5 expression were seen in mice fed the high-fat diet compared to control. n=5-8 animals per diet/per timepoint. Analyzed by one-way ANOVA. All data are plotted mean ± 95% CI; data points for each mouse are graphed within each group. *P<0.05, ***P<0.001.

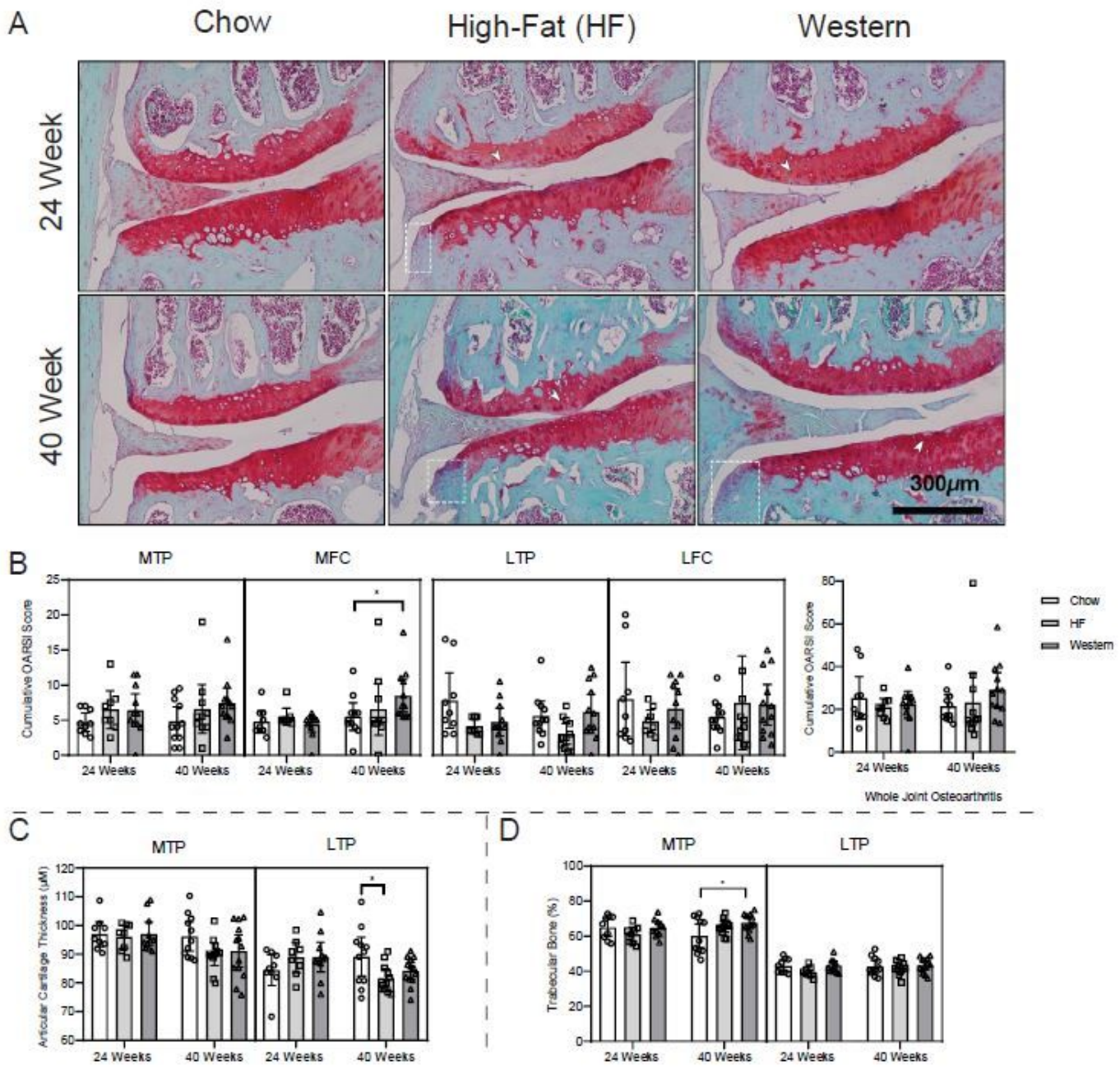

Figure 5

Effect of diet-induced obesity on the knee joint. (A) Representative histological coronal sections of the medial knee compartment stained with safranin-o/fast green from mice fed either control chow, high-fat, or western diet for 24- or 40- weeks. Images are oriented with the medial femoral condyle (MFC) located superiorly, and the medial tibial plateau (MTP) inferiorly. White arrows indicate cartilage damage, presenting as loss of proteoglycan staining and focal fibrillation of the cartilage, and white boxes indicate osteophyte formation. (B) Histopathological grading of the knee joints using the murine Osteoarthritis Research Society International (OARSI) scale corresponding to MTP, MFC, lateral tibial plateau (LTP), and lateral femoral condyle (LFC), combined to generate the summed score for the whole joint. Mice fed the

western diet for 40-weeks showed a significant increase in the degenerative score in the MFC compared to those fed the control chow. However, no difference was seen in the whole joint score between any of the groups at either timepoint. (C) Average articular cartilage thickness. After 40 weeks on the high fat diet, mice presented with decreased articular cartilage thickness on the LTP. No other differences were seen in any other joint compartment. Data analyzed by Kruskal-Wallis test. (D) Percent trabecular bone in the medial and lateral subchondral compartments of the tibia. Mice enrolled on the western diet for 40 weeks exhibited significantly more trabecular bone in the medial compartment for the tibia. n=9-16 animals per diet/per timepoint. Analyzed by one-way ANOVA. All data are plotted mean ± 95% CI, *P<0.05.

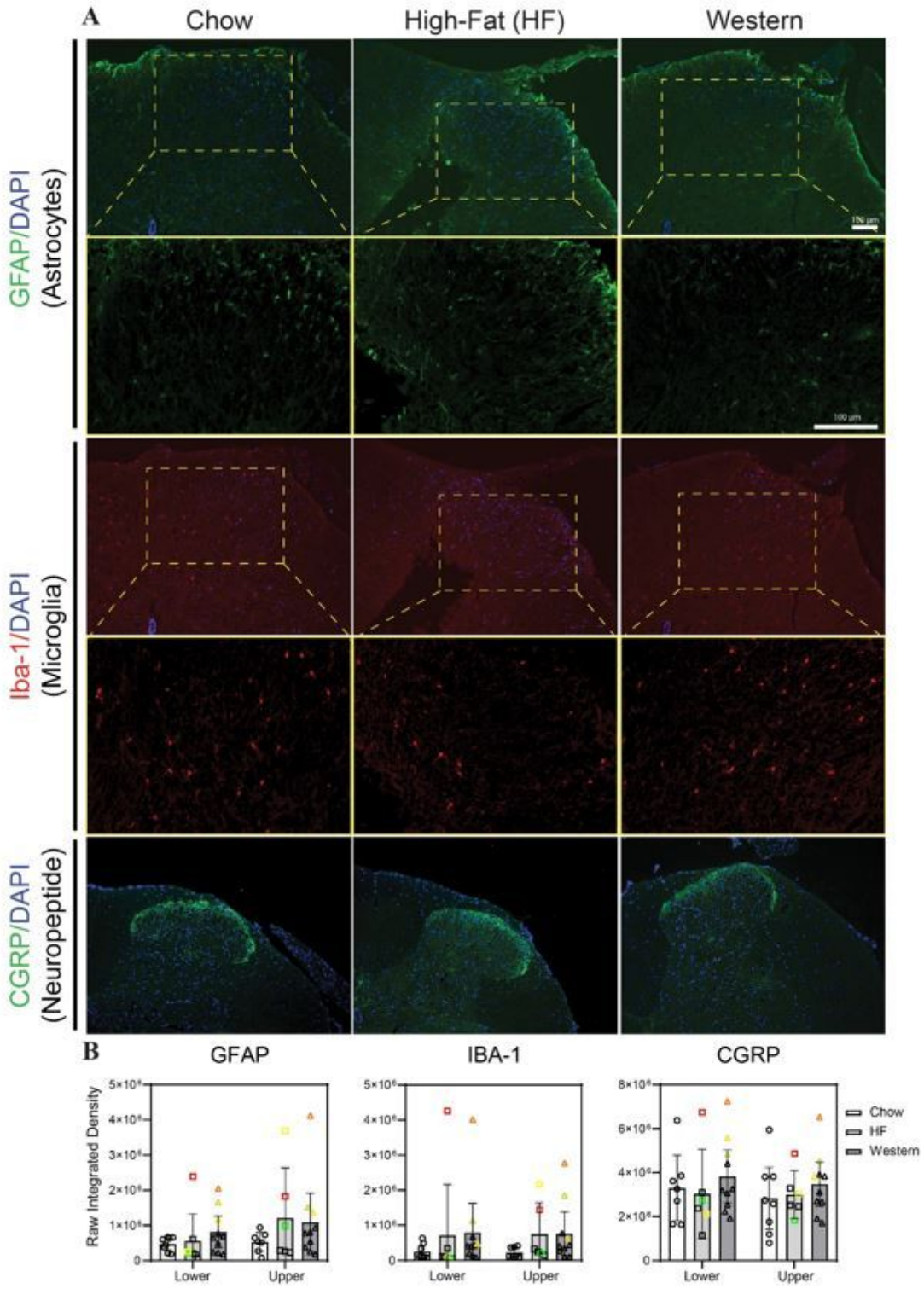

Figure 6

Effect if diet-induced obesity on neuroplastic changes within the lumbar spinal cord. (A) Representative images showing transverse sections of the dorsal horn of the lumbar spinal cord used for immunohistochemical analysis of neuroplastic changes associated with chronic pain. Tissues were isolated from mice following 40 weeks of experimental diet. Slides were stained for glial fibrillary acidic protein (GFAP), ionized calcium-binding adapter molecule 1 (IBA1) and calcitonin gene-related peptide

(CGRP). Yellow boxes indicate the region of interest for high magnification images (second row) for GFAP and IBA-1 (B) The fluorescence intensity averaged across the region of interest (lamellae 1-4 of the dorsal horn) of the upper (L1-L2) and lower (L3-L6) lumbar spinal cords. Mice fed a high-fat or western diet for 40-weeks showed no significant differences in immunoreactivity for any of the proteins investigated. n = 6-8 animals/group. Individual data points of the same colour indicates the same animal. Data are plotted mean ± 95% CI.

Supplementary Files

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- SUPPLEMENTARYFIGURELEGENDS.docx
- SupplementaryTable4.docx
- SupplementaryTable3.docx
- SupplementaryTable2.docx
- SupplementaryTable1.docx
- SupplementaryFigure2.docx
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- Table1.JPG
- Table2.JPG