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Everlasting Effects of Primary Life Pain on Osteoarthritis Pain in a Premature

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Abstract*: One in ten newborns are born premature, receiving an average of 14 painful procedures daily in the hospital. The developing nervous system readily alters in response to noxious stimuli due to a particular propensity for neuroplasticity and immature descending inhibitory mechanisms. Early-life, injury-induced alterations can lead to increased severity of subsequent painful events, but its effects on later osteoarthritis (OA) are unknown. Here, we investigate the impact of early repetitive needle prick (RNP) pain on subsequent adult OA pain. Neonatal Sprague-Dawley received a series of RNPs or tactile (TS) stimuli from postnatal day 1 to 7. At 17-weeks, OA (RNP-OA; TS-OA) and control (RNP-C; TS-C) groups were created. Stifle OA was induced via intra-articular monoiodoacetate injection. Limb use, reflexive, and behavioral assays were performed at intervals. During the 6-week period following OA-induction, RNP-OA had reduced ipsilateral limb use compared to others characterized by: decreased static weight-bearing, increased time to maximum paw- floor contact, and reduced print length during locomotion. RNP-OA showed less activity ($p=0.047$) and decreased time spent in the center of an open field ($p=0.023$). Histological scoring of stifles showed that OA caused more damage than controls ($p<0.005$), with the highest scores in female RNP-OA ($p<0.030$). Spinal microglia in RNP-OA were more activated later in life, as measured by intensity, quantity, and morphological features ($p<0.049$). We conclude that early RNP injury appears to heighten pain due to monoiodoacetate induced-OA, over OA alone, in adults, as defined by clinically relevant limb use, histology, and microglia activation, with similar trends in reflexive and complex behaviors.*

Keywords: premature, noxious stimuli, osteoarthritis, monoiodoacetate injection

Introduction

Worldwide the number of babies being born prematurely is at an all-time high (Blencowe et al., 2012). Preterm babies often require special medical care, spending the first weeks to months of their life in the neonatal intensive care unit (NICU). Newborns are exposed to an average of 14 ± 4 painful procedures daily if they are being cared for in the NICU (Carbajal et al., 2014). These procedures are related to stabilization, monitoring, and diagnostic evaluation. Needle pricks for blood procurement are the most commonly performed painful procedures (Barker and Rutter, 1995; Carbajal et al., 2008). The needle prick, also referred to as a heel-stick/lance, is used as a quick way to obtain blood for screening laboratory tests, monitoring glucose levels, and evaluating organ function and general health status. Squeezing the heel after being pricked is painful (Shah and Ohlsson, 2011) and frequently is performed without analgesia due to reported lack of analgesic efficacy with pharmacological and non-pharmacological interventions, such as continuous morphine (Carbajal et al., 2005) or sucrose (Gao et al., 2018).

Although clinical and laboratory studies have clearly shown that untreated pain early in life can have undesirable effects later in life (adolescence to young adult in humans and early adulthood in rodents), there appears to be a gap in understanding how early life painful injury impacts chronic pain much later in life. Chronic pain effects more than 1.5 billion people worldwide (Institute of Medicine Committee on Advancing Pain Research, 2011), and given the relative lack of effective therapeutic options for chronic pain (Hay et al., 2014), it is important to understand all the factors that contribute to this burden of chronic pain.
Collectively, the knowledge of the long-term effects of early life injury pain processing has led us to investigate the effects of early life painful injury on chronic osteoarthritis (OA) pain. The primary objective of this study was to assess the impact of RNP injury on subsequent OA pain during adulthood. We hypothesized that neonatal injury would lead to increased pain and disability associated with OA, exacerbated progression of OA, and long-term changes to microglial activation in the spinal cord. To test these hypotheses we 1) evaluated clinical, reflexive, and non-reflexive responses; 2) assessed histological changes; and 3) explored underlying mechanisms responsible for the changes in a combined RNP and OA model.
Materials and Methods

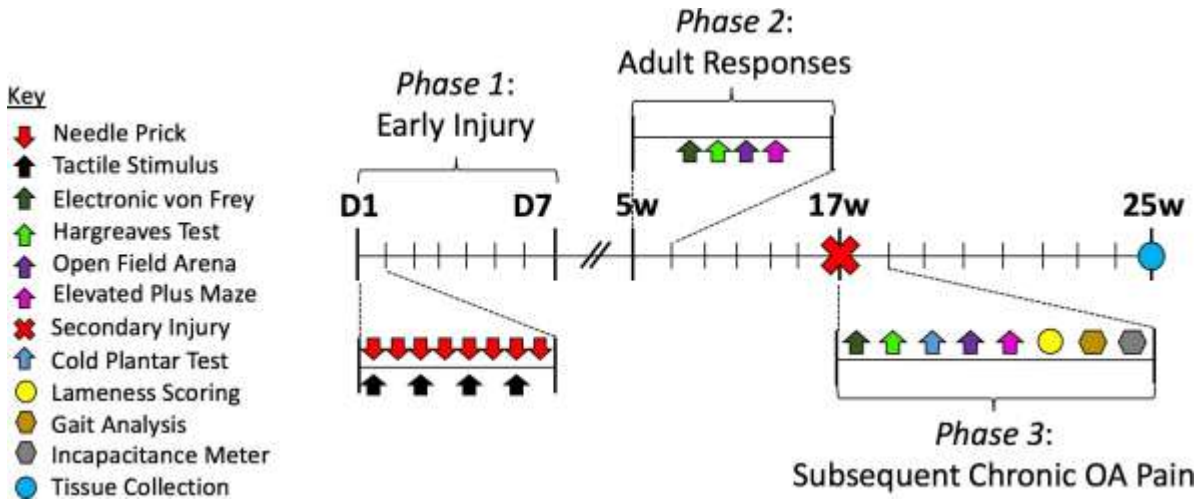
Rats used in this study were born from timed-pregnant SAS-Sprague Dawley dams (Charles River Kingston, Stone Ridge, NY). They were housed individually in standard rodent cages with food and water available *ab libitum* and were kept on a standard 12:12 light/dark cycle. The cages were housed in temperature (19–24°C) and humidity (55 ± 15%) conditioned rooms. At birth, pups were tattooed and placed in injury or control groups using stratified randomization based on sex. Pups remained with their litter until weaning. After weaning at postnatal day 21 (P21), rodents were sex-housed in pairs when possible or in groups of three until the end of experiments.

Experimental Study Design

The schematic of the experimental study design is shown in Figure 1. The experiment was divided into three phases: 1) early life injury, 2) adulthood, and 3) secondary adult injury.

During phase 1, rodents were exposed to either a noxious or non-noxious insult at pre- determined intervals for the first 7 days of life. Phase 2 began at 5 weeks of life, when rodents underwent biweekly behavioral and reflexive testing in a pre-determined testing order to evaluate the impacts of early life injury. Finally, phase 3 started when the rodents were 17 weeks old and OA or control (C) groups were created using monoiodoacetate (MIA) or saline injected intra-articularly (stifle), respectively. Post-secondary injury, lameness scoring, limb use, and reflexive testing were conducted weekly until the end of the study (25 weeks of life). rodents were then euthanized and tissues were collected, processed, and analyzed as described later.

Figure 1: Schematic overview of experimental study design.



Ipsilateral hindpaws of neonatal rodents were stimulated with a needle prick (RNP) or tactile stimulus (TS) eight or four times per day, respectively, for the first 7 days of life (D1-D7) (phase 1). Beginning at 5 weeks of age, pain sensitivity and behavioral responses were determined biweekly until secondary injury (phase 2). At 17 weeks of age, osteoarthritis (OA) was induced in the ipsilateral knee via intraarticular injection of monoiodoacetate (OA) or saline (C) (secondary injury). Following OA induction, pain sensitivity, behavioral responses, and limb use were determined weekly (phase 3). At 25 weeks, rodents were euthanized, and tissues were collected.

Table. 1: Experimental groups and distribution of male and female

Groups	N	Male	Female
RNP-C	17	9	8
RNP-OA	17	9	8
TS-C	14	7	7
TS-OA	16	9	7
Total	64	34	30

Statistical Analysis

For data besides RNA-seq, statistical analysis was performed by using computer software (JMP®, Version 14, SAS Institute Inc., Cary, NC). The Tukey Kramer HSD test (parametric data) or Steel-Dwass method (non-parametric data) was used to analyze the difference between means of two or more independent groups and to analyze multiple comparisons, controlling for overall experiment-wise error rodent. All assessments were evaluated as independent measures, and a P value of less than 0.05 was considered significant. Data are expressed as mean ± SEM.

Results

Clinical Assessment

Neonatal maintained normal attitude, suckling behavior, and body weight during and after RNP injury. Maternal rejection did not occur during the early life injury phase. Three pups, one from TS and 2 from RNP group, were euthanized or found dead within the first 4 days of life due to failure to thrive (n=2) or bite wounds (n=1) and were not included in Table 1. Early life injury did not have a significant effect on body weight ($p=0.965$). Body weight rapidly increased over the first week of life, indicative of general growth. Over the duration of the study, sex differences in body weight were apparent with males being heavier than females ($p<0.001$), indicative of expected sexual dimorphism. During experimental phases 2 and 3, when accounting for sex, no differences were detected in body weight between groups ($p>0.79$), but there was a time effect with weight steadily increasing over time ($p<0.001$).These data suggest that there are no negative effects on body weight with early RNP and/or adult OA injury in rodents.

RNA Sequencing of Lumbar Dorsal Root Ganglion

We used RNA-Seq to explore gene expression profiles of ipsilateral L4–6 DRGs 23 weeks after RNP injury and 6 weeks after OA-induction. We used the hierarchical clustering analyses to obtain an overview of gene expression profiles of the RNP or OA models compared with their respective controls (TS and C). All samples were examined in relationship to each other using a correlation analysis (LaPaglia et al., 2018) showing no outliers based on auto-association. Autosegregation and hierarchical clustering analysis of all genes showed that each sample was largely separated by sex with rare exceptions.

Furthermore, the replicates in each group appeared to be well correlated (Figure10b); however, the distance between each sample was very large indicating a great deal of variance unexplained by the experimental groupings. The clear segregation between sex but unreliable clustering of the sequencing data by injury group indicated that within this small sample size a distinct RNA expression profile differentiating the early RNP injury or adult OA induction was not found.

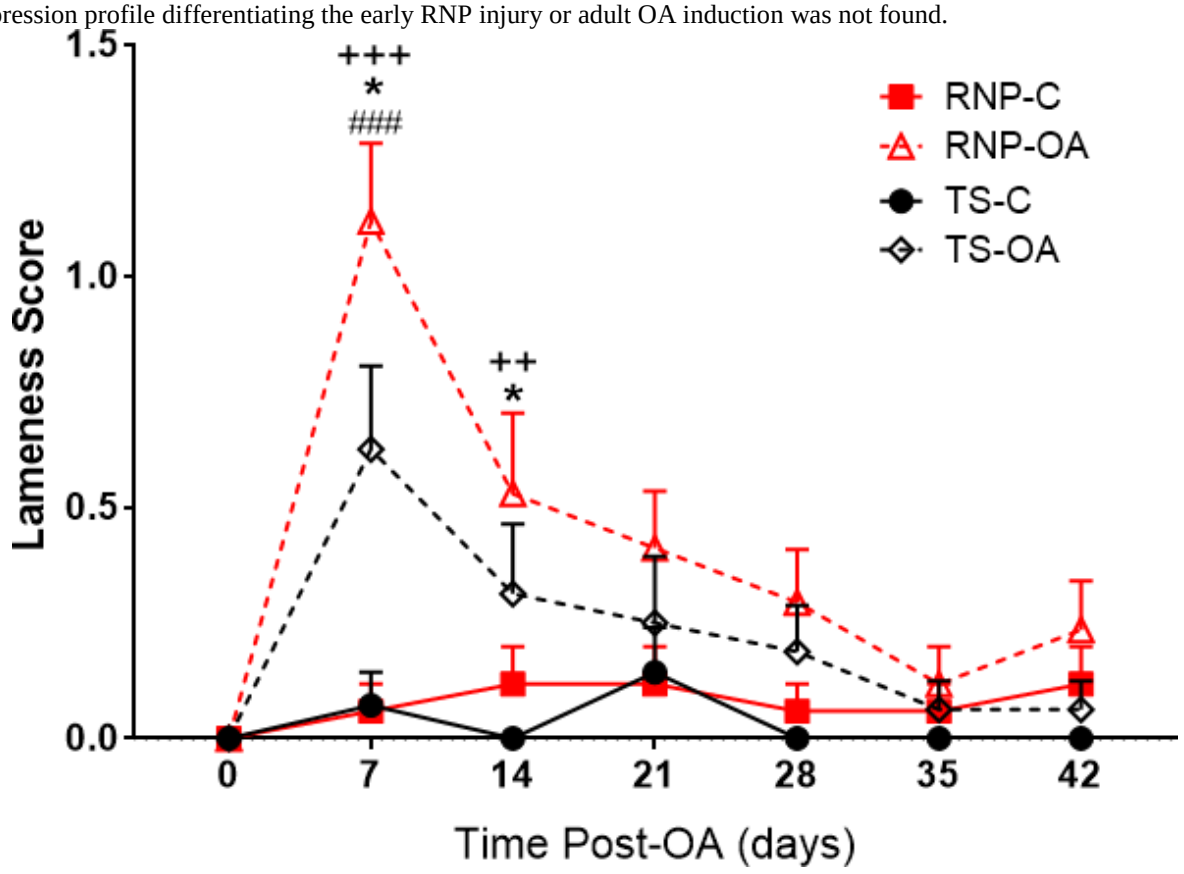


Figure 2: Lameness score over time post-OA injury (phase 3).

Plot of subjectively assessed lameness scores showing group differences over time (phase 3). Data are expressed as the mean ± SEM. Statistical significance indicates differences between respective groups at each time point: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ for RNP-OA vs TS- OA; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ for RNP-OA vs RNP-C or TS-OA vs TS-C; $^{\S}p<0.05$, $^{\S\S}p<0.01$, $^{\S\S\S}p<0.001$ for RNP-OA vs TS-C or TS-OA vs RNP-C; $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ compare to baseline (T0).

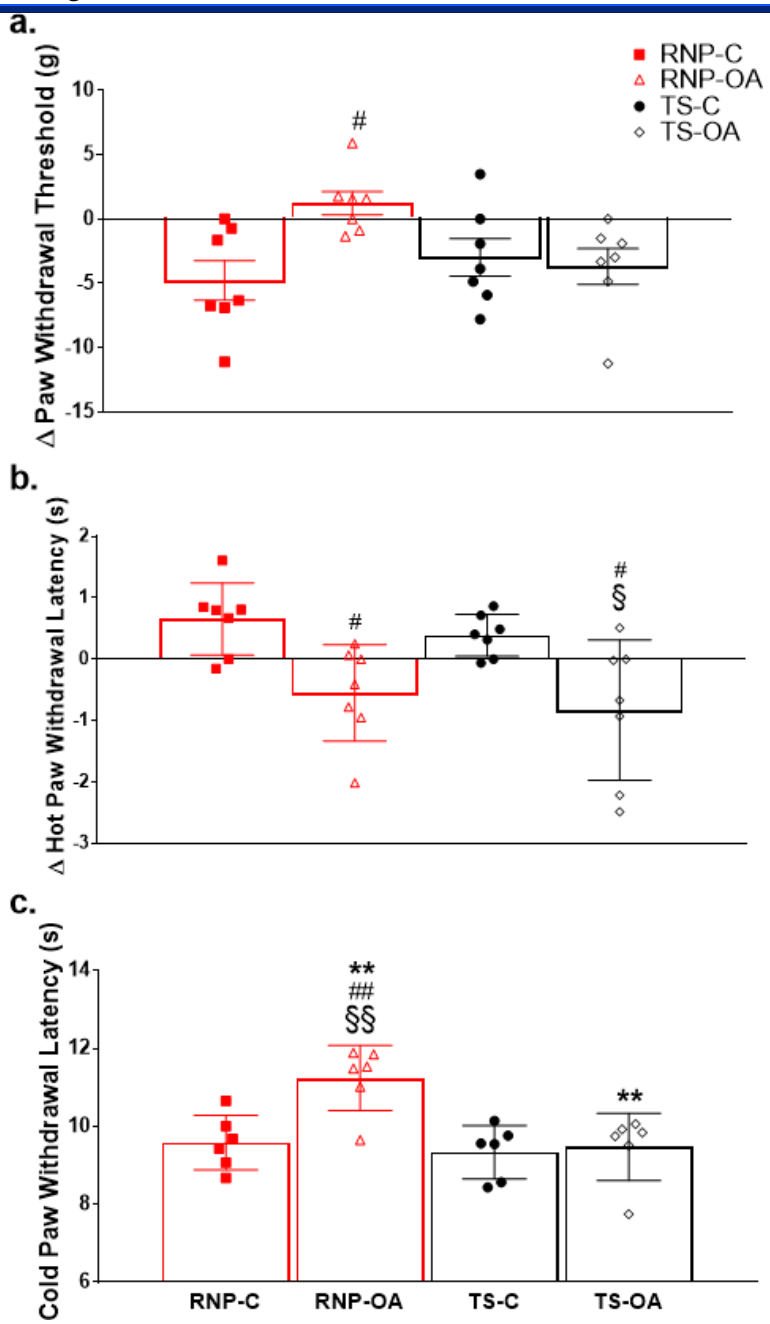


Figure 3: Reflexive nociception responses on ipsilateral hindlimb following secondary OA pain.
Bar-plots of overall change from baseline following OA injury (phase 3) for (a) mechanical paw withdrawal threshold and (b) hot thermal withdrawal latency. Overall raw values for (c) cold thermal latency during phase 3 are shown. Data are expressed as the mean ± SEM. Each individual symbol represents the mean at different time points. *p<0.05, **p<0.01,

***p<0.001 for RNP-OA vs TS-OA; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 for RNP-OA vs RNP-C

or TS-OA vs TS-C; §p<0.05, §§p<0.01, §§§p<0.001 for RNP-OA vs TS-C or TS-OA vs. RNP- C.

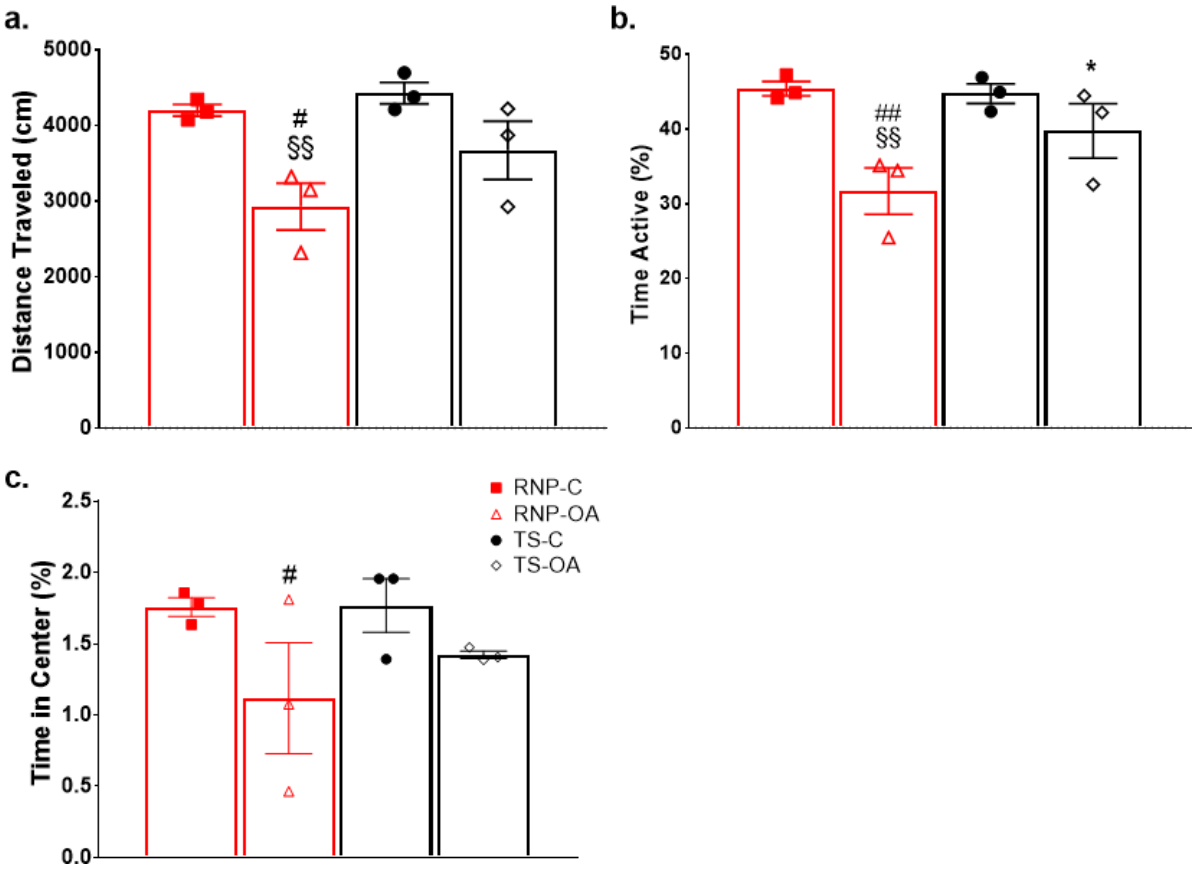


Figure 4: Behavioral responses in the open field arena following secondary OA pain (phase 3).
Bar-chart plots of (a) total distance traveled, (b) time spent active, and (c) time spent in the center for the open filed arena. Data are expressed as the mean ± SEM. Each individual symbol represents the mean at different time points. *p<0.05, **p<0.01, ***p<0.001 for RNP-OA vs TS-OA; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 for RNP-OA vs RNP-C or TS-OA vs TS- C; §p<0.05, §§p<0.01, §§§p<0.001 for RNP-OA vs TS-C or TS-OA vs. RNP-C.

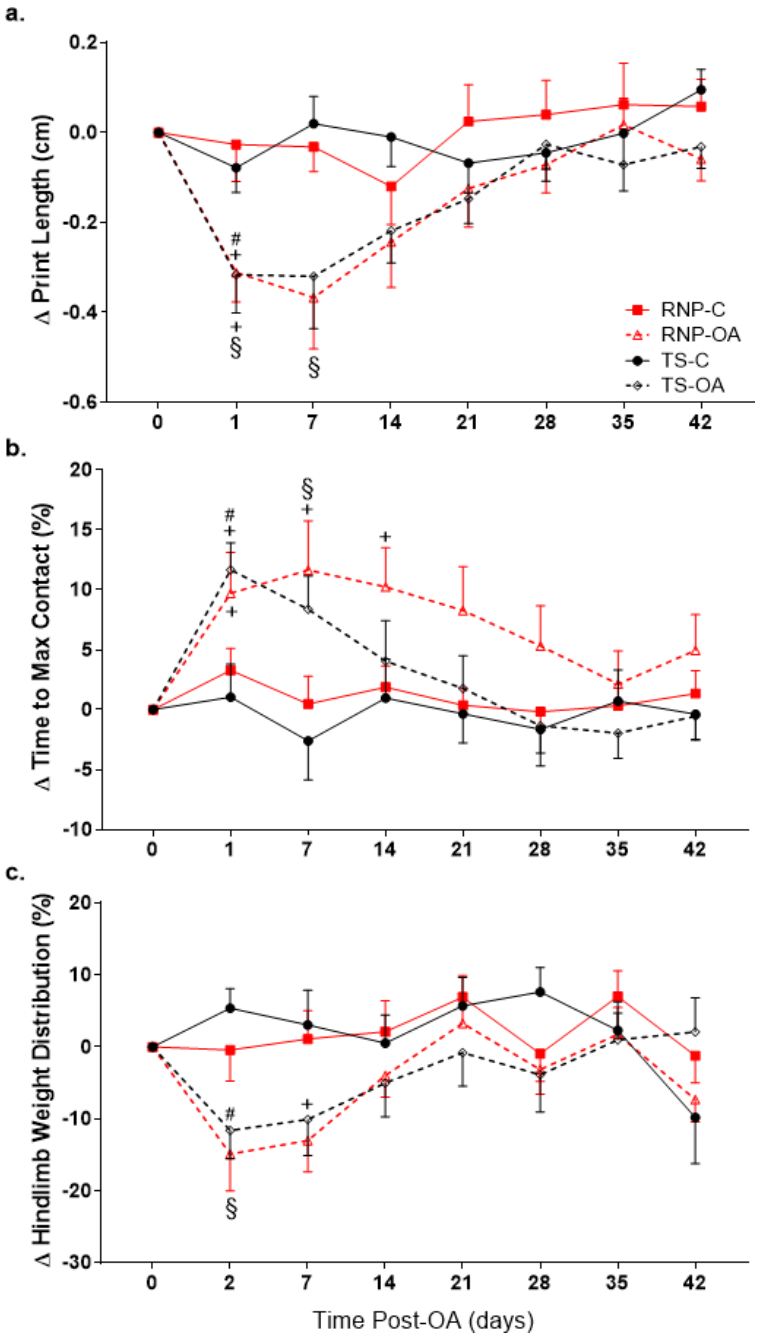


Figure 5: Changes from baseline over time in ipsilateral hindlimb limb use following OA induction (phase 3).
Plot of dynamic gait analysis outcome measures (a) print length and (b) % time to max paw- floor contact on the ipsilateral hindlimb over phase 3. Plot of static weight-bearing on ipsilateral limb during phase 3 is shown in (c). Data are expressed as the mean ± SEM. Statistical significance indicates differences between respective groups at each time point:

*p<0.05, **p<0.01, ***p<0.001 for RNP-OA vs TS-OA; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 for RNP-OA vs RNP-C or TS-OA vs TS-C; §p<0.05, §§p<0.01, §§§p<0.001 for RNP-OA vs TS-C

or TS-OA vs RNP-C; $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001 compare to baseline (T0).

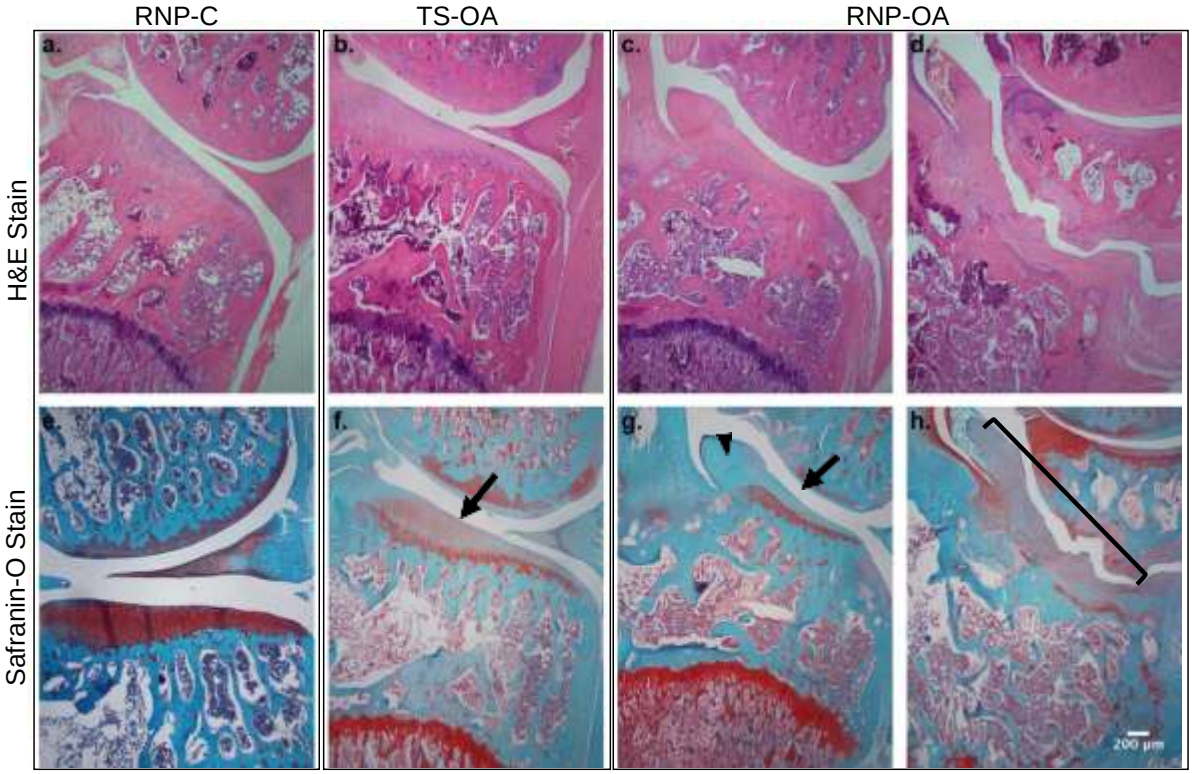


Figure 6: Representative frontal plane images of the medial aspect of the femorotibial joint with or without early RNP injury and/or secondary OA injury 6 weeks post-induction.
Representative histologic images of the femorotibial joint in the RNP-C, TS-OA and RNP-OA groups. Stained sections show progressively worsened articular cartilage of medial tibial plateau and subchondral bone degeneration, representative of increased severity of OA from left to right. Loss of proteoglycan stain and cellularity (arrows), osteophyte formation (arrowhead) and complete loss of articular cartilage and extensive bone remodeling (bracket) are observed. 10x magnification.

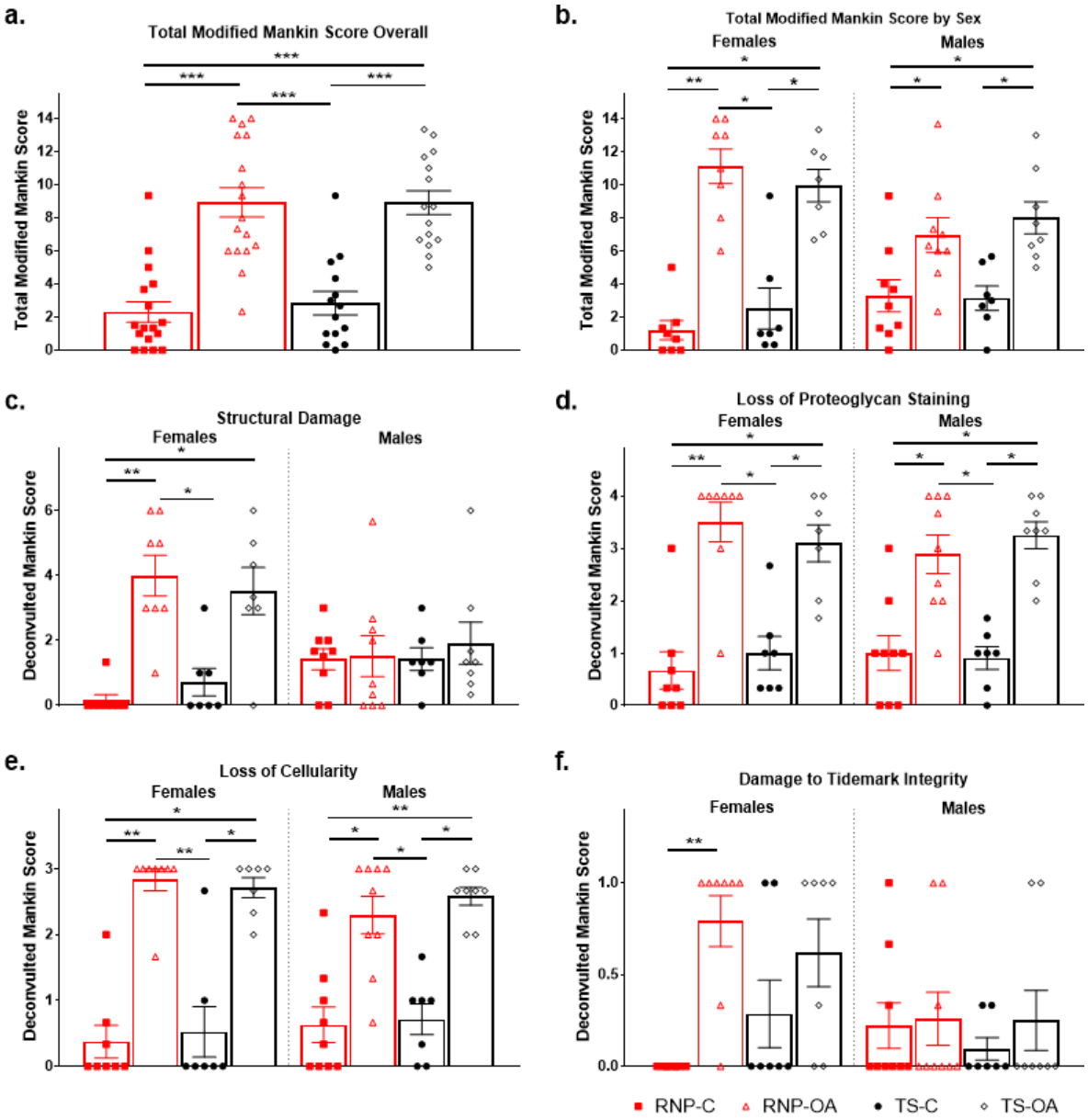


Figure 7: Graphical representation of modified Mankin scores, reflecting the degree of cartilage damage, in stifles with or without early RNP injury and/or secondary OA injury.

Modified Mankin scores of the medial tibial plateau of ipsilateral hindlimbs from RNP-C, RNP-OA, TS-C, and TS-OA groups are shown. (a) Overall modified Mankin scores, (b) overall scores separated by sex or as individual parameters, reflecting (c) increased structural damage, (d) loss of cellularity, (e) loss of proteoglycan staining, and (f) decreased tidemark integrity at 25 weeks post-OA induction. Data are expressed as the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 for group comparisons.

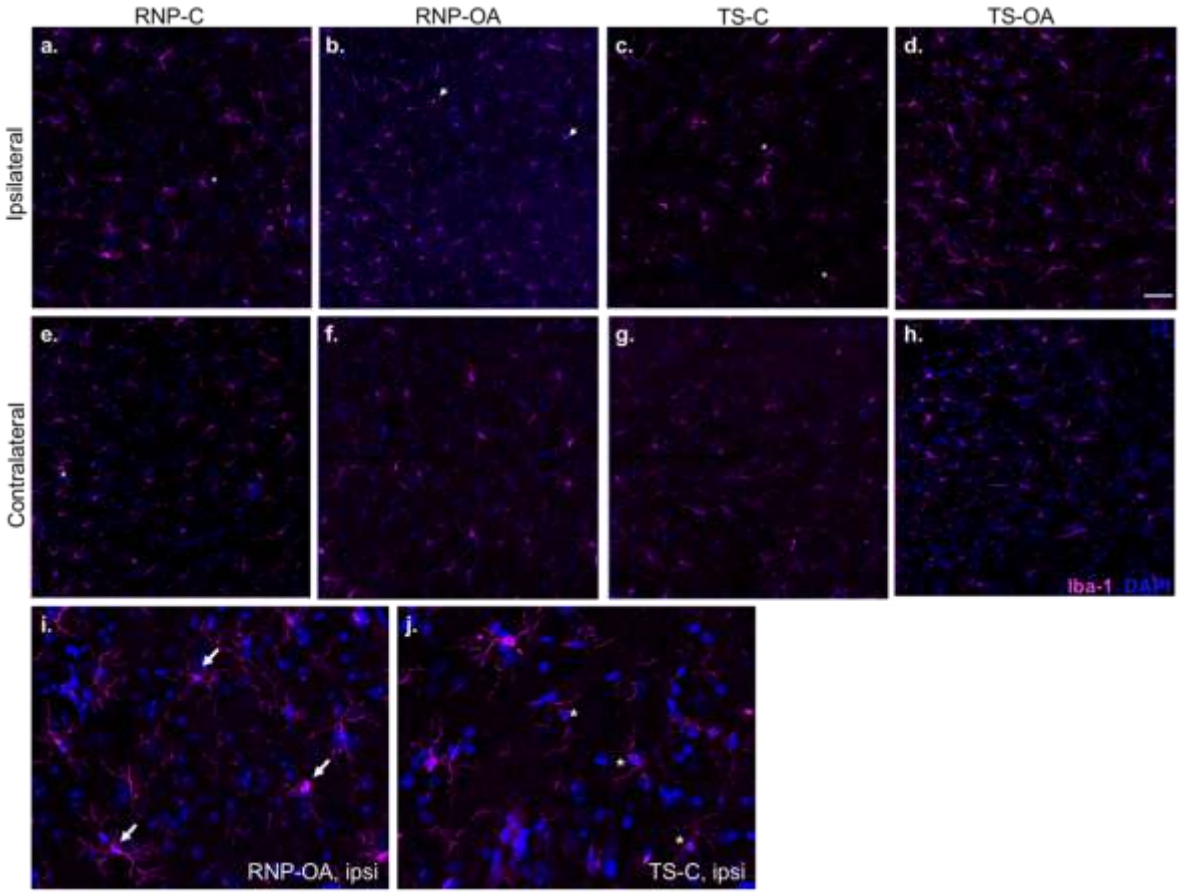


Figure 8: Photomicrographs of immunostaining against ionized calcium binding adaptor molecule 1 (Iba-1) in dorsal horns of the spinal cord

Representative confocal images show microglial activation in (a-d) ipsilateral and (e-h) contralateral dorsal horn of spinal cord from RNP-C, RNP-OA, TS-C, and TS-OA groups. Images show the increase in microglial marker Iba-1 (magenta); activated microglia cells exhibiting hypertrophy bodies with increased and thickened process (arrows); and resting microglia exhibiting small bodies with thin, compact processes (asterisk). (a-h) 10x magnification. (i-h) 20x magnification, Scale bar 50µm. Iba-1, magenta; 40,6-diamidino-2- phenylindole (DAPI), blue.

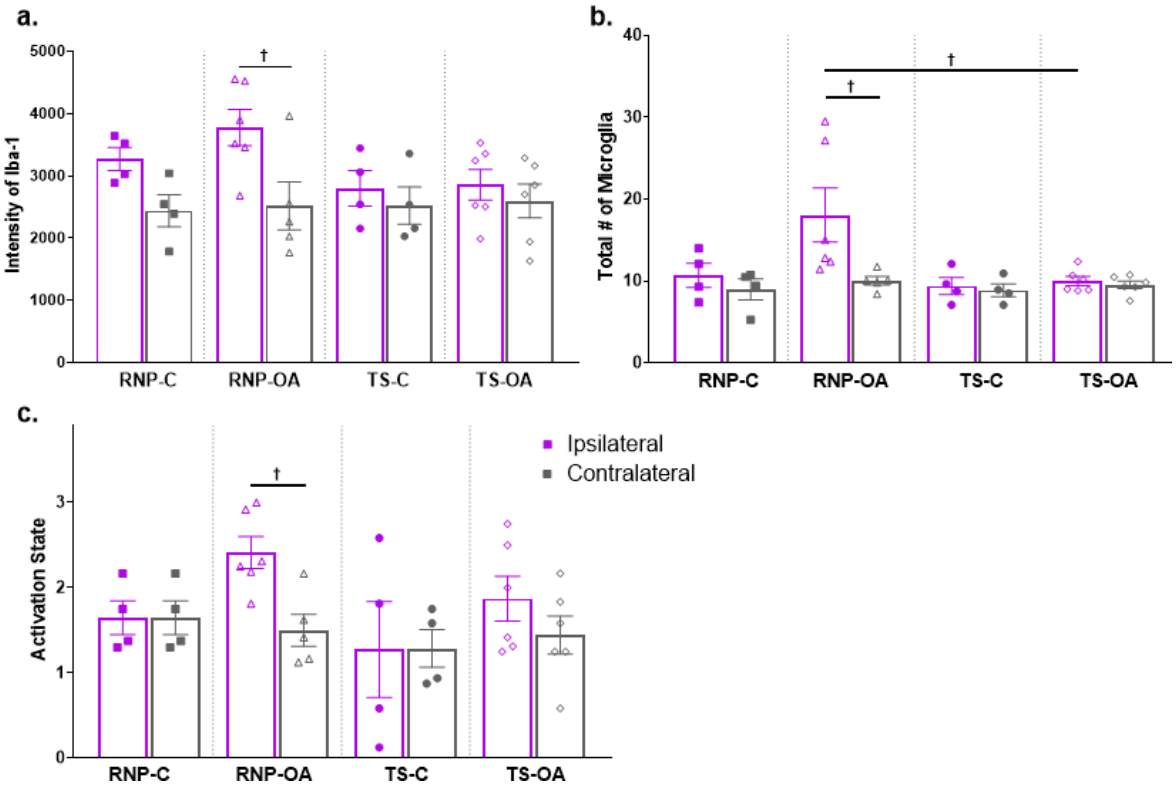


Figure 9: Immunohistochemical analysis of microglia in the dorsal horn of the lumbar spinal cord post-secondary injury.

Immunohistochemical analysis of ipsilateral and contralateral microgliosis in the dorsal horn of L4-L5 spinal cord in RNP-C, RNP-OA, TS-C, and TS-OA groups. Qualitative rating of microglial activation evaluated by (a) mean intensity of Iba-1 staining and (b) total number of microglia cells in the defined region of interest. Qualitative rating of microglial activation assessed by (c) activation state based on microglia morphology. Data are expressed as the mean ± SEM of 3-5 spinal cord sections. Differences between ipsilateral (magenta bar) and contralateral (grey bar) spinal cord tissue within each experimental group: †$p<0.05$, ††$p<0.01$. Differences between ipsilateral spinal cord between RNP-OA and TS-OA (*$p<0.05$, **$p<0.01$), RNP-C (#$p<0.05$), and TS-C (§$p<0.05$) are identified.

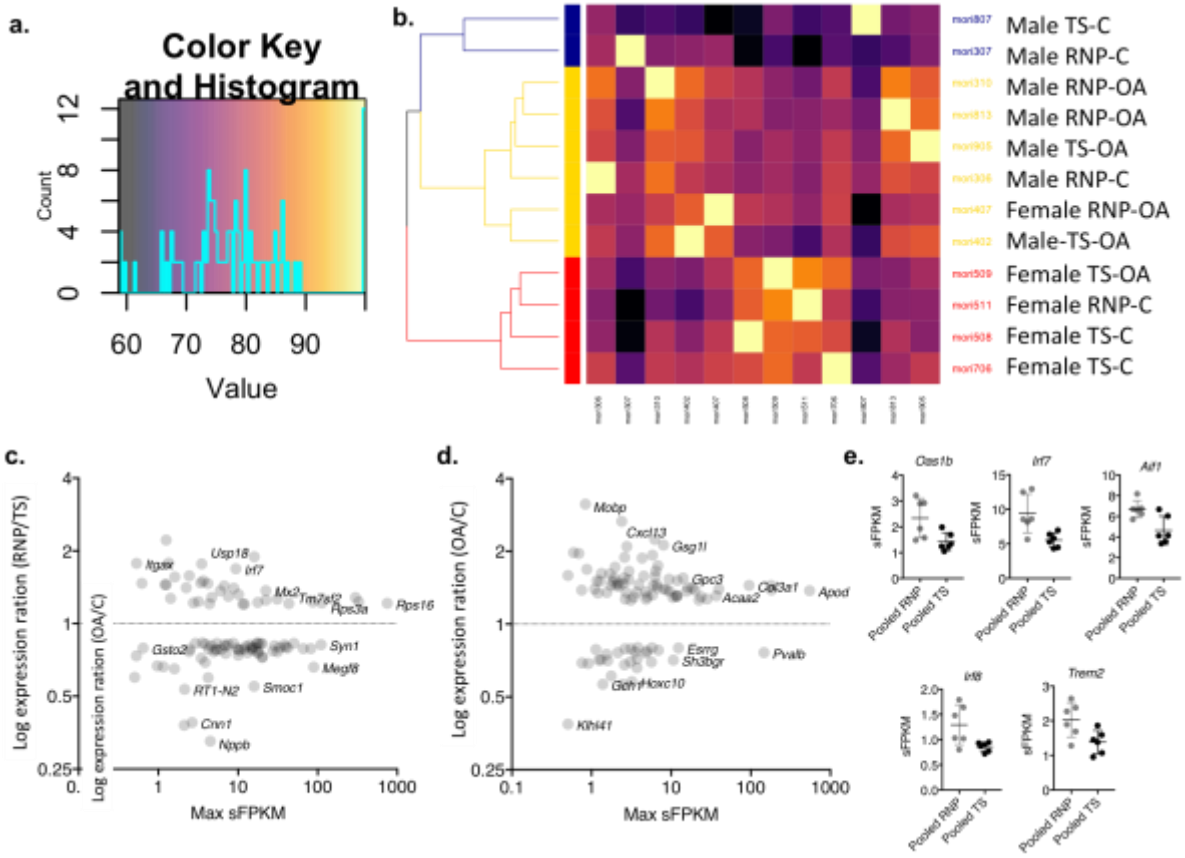


Figure 10: RNA-Seq reveals transcriptome profile of gene expression changes in DRGs following early RNP injury or OA induction.

RNA-Seq analysis of lumbar DRGs from the 4 groups (n = 9-12 DRGs per group). (a) A color key and histogram of all the values from the transcriptome profile show how they correspond to the heatmap color range. (b) Quality control correlation heatmap of hierarchical clustering of overall gene expression profile of DRGs from male and female of each group compared to same sex control. A summary of the top 100 genes with Max sFPKM >0.5 are shown with gene changes plotted for (c) early RNP vs TS injury and (d) adult OA induction vs adult C. Significant genes (MAGIC pipeline) are ranked by degree of change (sFPKM ratio) and are indicated accordingly. In these comparisons, the sample sizes were too small to reach significance. However, nominally significant ($p < 0.01$, uncorrected) genes were examined as part of a pilot study. (e) Several genes responsible for innate immune system show a trend towards enrichment in the RNP dataset relative to the TS dataset.

Discussion

The RNP model is a clinically relevant, well-studied model in newborns replicating the painful heel-stick process of obtaining blood samples in the human NICU setting (Knaepen et al., 2012). However, there are limited experimental data on the long-term effects and no experimental or human clinical data on the effects on chronic pain conditions. In the present study, using this rodent model in conjunction with an established model of OA induction later in life ('two-hit' model), we examined the effects of early RNP injury on behavior and sensitivity responses in the adult and behavioral pain responses to secondary injury, specifically chronic OA pain, later in life. We demonstrate that early repetitive noxious stimuli modifies nociception, pain responses, and exploration in adults. There were long-lasting and significant effects on mechanical sensitivity and behavioral activity indicating that early repetitive tissue-breaking injury alters baseline ipsilateral sensory nociception, indicating residual peripheral and/or central neuroplasticity resulting in pain, which outlasts the initial insult and healing period. Further, for the first time, we investigated and demonstrated that early life pain (RNP) enhanced pain behaviors and nociceptive responses, and negatively impacted exploration and functional limb use associated with subsequent chronic OA in the adult. Spinal microgliosis in the ipsilateral dorsal horn significantly increased in the 'two-hit' injury model compared to all other groups, suggesting widespread, cumulative changes in spinal sensory processing as a result of the early life injury (RNP).

Persistent Effects of Early RNP on Reflexive Nociceptive Responses and Exploratory Activity on Adulthood

The presence of altered nociception, i.e., peripheral mechanical hypersensitivity, that we saw post-weaning (P24) through adulthood (P105) is in agreement with other studies using the RNP model (Chen et al., 2016; Page et al., 2013; van den Hoogen et al., 2019). Other early life insults, such as complete Freud's adjuvant (CFA) or carrageen (inflammatory insults), produce shorter lasting changes in mechanical sensitivity. A single injection of CFA at P1 produced acute mechanical hypersensitivity at 2 hours post-injection, but no alterations in mechanical or thermal nociception through P56 (Walker et al., 2003). Likewise, multiple injections of carrageenan at P1 and P4 did not influence mechanical or thermal thresholds later in life (P24, 45, or 66) (Davis et al., 2018). Interestingly, lower thresholds at first exposure to mechanical stimuli, as early as D35, were detected in control and may reflect the novelty of the testing. In contrast to mechanical hypersensitivity, we found thermal (hot) nociceptive responses were not altered following repetitive tissue-breaking injury (Davis et al., 2018; Page et al., 2013). Different effects on mechanical and thermal nociceptive sensitivity are likely associated with differential reorganization of the Aδ mechanosensitive and Aδ mechanothermal nociceptors following early life injury.

Lower baseline mechanical and thermal (hot) thresholds were detected in females compared to males, which may be due to overall smaller body weight and/or surface area of the paw (stimulated site), since females are generally smaller than males due to sexual dimorphism. These results are supported by several pre-clinical studies revealing female rodents have a lower pain thresholds in various experimental pain models (Hurley and Adams, 2008). Similarly, in human studies, females appear more sensitive (lower thresholds or tolerances) to heat and cold pain than males, with some discrepancies among pain outcomes (Fillingim et al., 2009). Although we found mechanical hypersensitivity at the site of prior injury in both males and females, mechanical thresholds in males were significantly impacted by early RNP injury but the reduction in thresholds was not significant in females. Similar findings were seen in another study, where only males developed mechanical hypersensitivity after neonatal RNP (Page et al., 2013). Interestingly, with the exception of reflexive responses, other functional and behavioral analyses in our study revealed females experiencing RNP had a more pronounced nociceptive/pain phenotype suggesting sexual dimorphism in the response to early life injury. Potential mechanisms responsible for sex differences may be related to differences in the ascending pain transmission pathways, descending endogenous pain modulatory system, or countless other mechanism that affect pain (Fillingim et al., 2009; Mogil, 2012).

We found females had higher levels of horizontal exploration and activity compared to males in both the OFA and EPM, similar to other work (Belviranli et al., 2012; Simpson and Kelly, 2012). Exploration and overall activity were significantly reduced in experiencing early tissue-breaking injury, and these effects were more pronounced in females. Decreased exploration is indicative of higher levels of anxiety-like behaviors, therefore, our results suggest that females may be at a greater risk of developing anxiety-like conditions. These data are in line with human literature describing females being generally two times more likely to develop anxiety disorders compared to males (Catuzzi and Beck, 2014). Further, females with associated chronic pain (e.g., joint, neck, pelvic) have higher anxiety levels than those without pain (Sale et al., 2008; Siqueira-Campos et al., 2019; Yalcinkaya et al., 2017). Mechanisms for these sex differences are unknown, therefore, the sex differences demonstrated in our study may be attributed to various factors, including, but not limited to: influence of gonadal hormones, presence/absence of estrus cycle, reactivity of the hypothalamic-pituitary-adrenal (HPA) axis, and differences in the neural circuits involved with emotional reactivity (Maeng and Milad, 2015).

Presumed Uninterrupted Developmental Maturation following Early RNP Injury

Identifying delayed development in newborns can be challenging, especially within the first week of life, therefore body weight is calculated as an adjunct evaluation of health in pediatric practice. In our study, though noxious stimulation and handling reshaped the adult pain phenotype, pup growth, measured by body weight, was unaffected during maturation, corroborated by other studies (Anand et al., 1999). Body weight alone is not a direct predictor of poor development, therefore conclusive interpretations of weight loss/gain should be avoided or done with caution as an auxiliary assessment tool. Achievement of development milestones, such as maintaining a fist or starting to lift head, in newborns can be extremely hindered in premature infants who experience repetitive pain. As a clinically relevant, translational adjunct to weight gain, our initial study documented the achievement of normal milestones (e.g., righting reflex, placing response, tactile startle, grasping reflex) at least 3 times a week for the first month, as in other studies (Paluch et al., 2014). However, measuring these behaviors was not performed due to the potential effects of maternal and pup stress on other outcome measures. Future studies incorporating feasible developmental assessments may provide additional translational evidence into developmental changes and nociception alterations following early life injury.

Long-Term Effects of Early RNP on Secondary Chronic OA in Adulthood

To investigate the global effects of early life injury on chronic OA, we evaluated the pain behaviors, nociceptive reflexes, and microglial responses. Effects on Subjective and Objective Measures of Sensory Processing and Limb Use Chronic ongoing joint pain can lead to significant sensory and functional impairments, triggering a patient to adjust activities of daily living to minimize or avoid pain such as shifting static weight distribution, adjusting gait, or decreasing activity. We found subjective lameness scores following OA injury were consistently higher among rodents that previously experienced RNP injury, especially so in femal. These findings are consistent with our hypothesis that pain-associated lameness would be more severe following OA injury, and further exacerbated by early RNP injury. Subjective lameness scores, reflecting a mixture of spontaneous and limb-use-induced pain, were significantly lower in males than females, reflective of human OA pain studies (Bartley et al., 2016; Glass et al., 2014),

where, regardless of pain experience history, females generally have higher pain scores associated with symptomatic OA. A number of factors including role of dominance in the colony (Jones and Monfils, 2016), and/or lower overall activity (Belviranli et al., 2012) may explain this sex difference. Additionally, all rodents received equal volumes of intraarticular MIA to induce OA pain. Therefore, the disruption of chondrocyte glycolysis with associated chondrocyte death, inflammation, and pain may have been lower in males due to a disproportionate ratio of chemical to joint space. However, other studies using this experimental model of OA describe using equal dosages/volumes of chemical agent across body weights and sex (Wang et al., 2016).

As seen in humans with OA pain (Hart et al., 2015) and experimental (Moreau et al., 2013; Smith et al., 2005) and spontaneous (Moreau et al., 2013) models of OA pain in dogs, OA showed decreased limb use during ambulation and standing. Repetitive needle prick resulted in greater impairment of limb use during ambulation, especially in female. Static weight-bearing on the ipsilateral hindlimb was reduced in OA groups, but RNP did not further decrease weight distribution. A possible explanation for these differences is enhanced limb disability due to movement-evoked nociceptive behavior versus resting spontaneous pain, which may be driven by different mechanisms (He et al., 2017). Human pain studies have shown that movement evoked pain is often greater than pain at rest, making it easier to measure the impact of movement-evoked pain compared to measuring pain at rest (Gilron et al., 2017). During ambulation stifle (knee) pain is intensified due to the demands of weight- bearing through a gait cycle (initial foot contact, mid-stance, and propulsion) and flexion/extension factors of the knee. Conversely, spontaneous pain at rest, measured using the SHIM apparatus in this study, is not influenced by these taxing demands reducing the probability of detecting pain-associated limb impairments at rest, as exhibited in this study.

Collectively, our results align with human studies showing, when compared to men with equal radiographic OA, women report increased levels of knee pain severity and lower function measured using visual analogue scale (VAS) and pain questionnaire (WOMAC), respectively (Glass et al., 2014). Female chronic knee OA patients also exhibit greater sensitization to thermal, mechanical, and temporal summation than males (Bartley et al., 2016). Women were almost 5 times more likely to report moderate to severe pain intensity post knee surgery than men (Solheim et al., 2017). These studies suggest body mass index, psychosocial characteristics (e.g. depression), presence of widespread pain, and enhanced central sensitization may be contributors to pain severity and intensity in females (Bartley et al., 2016; Glass et al., 2014). Our data indicate females experiencing repetitive pain early in life are predisposed to develop greater chronic pain with the onset of OA later in life. Additional studies, using this relevant model, should be performed to understand the mechanisms driving this.

Our analysis of gene expression in the DRG was very much preliminary work. However, even these preliminary results are in general agreement with the microglia immunostaining findings from the dorsal horn of the spinal cord. Our preliminary transcriptomic results suggest long-term changes to gene expression may be caused by early RNP injury.

Irf7 and Trem2 genes, both closely associated with microglia activation, were also upregulated in the DRG of experiencing RNP injury. Similar gene expression profiles have been seen with chronic exposure to transforming growth factor-ß1 (TGFß1) (Cohen et al., 2014; Hagemeyer and Prinz, 2014), a major differentiation factor for adult microglia (Butovsky et al., 2014). Both Irf7 and Trem2 have been identified as principle drivers that regulate phenotypic switching from M1 (pro-inflammatory) and M2 (anti-inflammatory) to a "disease-associated microglia" phenotypic expression pattern (Cohen et al., 2014; Konishi and Kiyama, 2018; Krasemann et al., 2017; Tanaka et al., 2015). Interestingly, the IRF7 pathway, based on upregulation of gene signatures in the spinal cord, was found to be altered in brain-derived neurotrophic factor (Bdnf; a crucial trophic factor for nociceptive afferents) knockout who had heat and cold nociception impairments (Sapio et al., 2019). Sapio et al. also found a larger number of highly differentially expressed genes in the dorsal horn of the spinal cord than the DRG in the Bdnf +/- pain insensitive (Sapio et al., 2019).

Together, these genes may be interconnected with mechanisms responsible for microglial activation and the associated pain that occurs downstream. Further work is needed to confirm these findings, and determine the potential of these as new targets for future clinical interventions for the reduction of CNS inflammation and pain (Cohen et al., 2014; Hagemeyer and Prinz, 2014; Krasemann et al., 2017).

Although the preliminary transcriptomic analysis revealed some interesting changes in gene expression and signaling receptors, there was a lack of clustering indicating that the experimental groupings do not fully explain the variance of the samples. This is in part due to the multifactorial design where multiple group comparisons and strong sex effects are considered simultaneously. It is also difficult to achieve clustering as few samples are biological replicates of the same combinatorial factors. Notably, the samples that are biological replicates appear to have minimal distance assigned by the hierchical clustering algorithm, suggesting that all of the experimental factors in the model impact variance, although the sample size is too small to address this definitively. Since RNA levels were assessed from a small sample size, which included both males and females, interpretation of these data is done cautiously, but provides a framework for future experimental investigation. We cannot make strong claims about how these genes or receptors are being effected; however, early RNP injury does appear to cause long-term molecular changes that may negatively impact chronic pain conditions later in life. Further, this preliminary analysis suggests that a future experiment could be designed to investigate this by powering each of these conditions to detect an effect in each dimension.

A single experiment without replication and without strongly significant genes is likely to be unreliable. Therefore, a repeated experiment of ample power should allow sufficient clustering and potentially demonstrate significant differentially expressed

genes. If replication does not support these preliminary findings or provide additional information then it is probable that a change in cellular expression in this two-hit model does not exist.

However, if no evidence points towards a gene induction event occurring, these changes in pain and immune-like responses are most likely driven by altered microglia or satellite cell numbers or size. But in the absence of cellular analyses it is unknown whether the observed changes represent an increase in transcription of the gene or an increase in the number of cells that express it. Therefore, we cannot prove whether the cellular activation (e.g., microglia, satellite cells, infiltrating immune cells) is a result of early nociceptor activity, degeneration of primary afferents and/or central neurons, or inflammation during RNP and/or OA injury. But these data support further investigation into the underlying mechanisms responsible for microglial activation, as it may shed light into targets for improving the quality of life for individuals exposed to early life injury. Preliminary evidence support further investigation into upstream neuronal effects on gene expression in the dorsal root ganglion.

Limitation of Experimental Design

Limitations of this study include repeated exposure to behavioral and reflexive testing, potentially impacting anxiety-like and pain sensitivity outcome measures with each repeated exposure. Because the rodents were handled frequently, rodents were expected to become more relaxed and amenable to testing (Malfait et al., 2013), so the outcome measures were likely not significantly affected. Additionally, in a long-term longitudinal study, such as the one we performed, rodents continue to grow in size and gain weight, thus as the paw grows so does print length and the amount of downward force on the paw, and so the amount and intensity of paw contact with the glass surface of the CatWalk. This complicates data analysis. However, using symmetry indices might overcome this issue. We did not use these indices because the compensation by the other 3 limbs is poorly understood, and weight may not be shifted over to the contralateral side, but rather may be shifted forwards, minimizing changes in symmetry indices. This needs further study in rodents. In human studies, limb symmetry indexes are commonly used however, they have been shown to overestimate knee function following knee injury (Wellsandt et al., 2017). Limitations for RNA Seq were previously described in detail but the primary limitation was the small sample size couple with multiple group comparisons. However, this was a preliminary analysis that provided substantial results that indicate the need for further investigation.

Implications of Findings and Future Directions

In conclusion, our study using a clinically relevant two-hit injury model demonstrates that the clinical and neuroplastic effects of neonatal injury are long-term and contribute to enhanced chronic pain in adulthood. Specifically, early RNP injury appears to heighten pain due to OA-induced by MIA, over MIA alone in mature, as defined by clinically relevant limb use, with similar trends reflected in reflexive behaviors and complex behaviors measured. These effects are most pronounced in females revealing a predisposition to suffer from the negative effects of early life injury, such as developing chronic pain and/or psychological disorders. Altering an individual's pain phenotype early in life can have lifelong deleterious outcomes, consequentially impacting their quality of life. Therefore, future studies should assess the underlying mechanisms responsible for these chronic effects of RNP on later chronic pain, which can aid in developing targeted individualized interventions to minimized pain experiences in early and later life.

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