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MATERNAL DEPRIVATION IS ASSOCIATED WITH SEX-DEPENDENT
ALTERATIONS IN NOCICEPTIVE BEHAVIOUR AND NEUROINFLAMMATORY
MEDIATORS IN THE RAT FOLLOWING PERIPHERAL NERVE INJURY

Running title: Enhanced neuropathic pain in MD female rats

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Abstract

Early-life stress is associated with an increased risk of developing affective disorders and chronic pain conditions. This study examined the effect of maternal deprivation (MD) on nociceptive responding prior to and following peripheral nerve injury (L5-L6 spinal nerve ligation (SNL)). As neuroimmune signalling plays an important role in pain and affective disorders, associated alterations in glial and cytokine expression were assessed in key brain regions associated with emotional and nociceptive responding, the hippocampus and prefrontal cortex (PFC). MD female, but not male, rats exhibited thermal hypoalgesia and mechanical allodynia when compared to control (non-MD) counterparts. SNL resulted in mechanical and cold allodynia in MD and control rats of both sexes. However, MD females exhibited enhanced SNL-induced allodynic responding when compared to non-MD counterparts. IL-6 expression was reduced in the PFC of MD-SNL males when compared with non-SNL counterparts. GFAP and IL-1 β expression in the hippocampus of MD-SNL males was increased compared with non-MD controls. MD-SNL females exhibited reduced TNF α in the PFC with a concomitant increase in IL-6 and TNF α expression in the hippocampus, compared with either MD or SNL alone. In conclusion, MD female, but not male, rats exhibit enhanced nociceptive responding following peripheral nerve injury, effects which may relate to the distinct neuroinflammatory profile observed in female versus male rats.

Perspective: This study demonstrates that females rats exposed to early-life stress exhibit enhanced neuropathic pain responding, effects associated with alterations in neuroinflammatory mediators. Increased understanding of the interactions between early-life stress, gender and pain may lead to the identification of novel therapeutic targets for the treatment of chronic pain disorders.

Abbreviations: Co control; EPM Elevated Plus Maze; GFAP Glial fibrillary acidic protein; IB4 isolectin B4; IL-1 β Interleukin-1 beta; IL-6 interleukin 6; MD Maternal Deprivation; NSNL Non-Spinal Nerve Ligation; PND postnatal day; PFC prefrontal cortex; SNL Spinal Nerve Ligation; TNF α Tumour necrosis factor alpha.

Keywords: maternal deprivation, Spinal Nerve Ligation, mechanical allodynia, cold allodynia, cytokines.

Introduction

Adverse early-life events are associated with a predisposition to developing psychiatric disorders^{1,22,46} and chronic pain conditions^{10,16,38} in later life. Manipulation of mother-pup interactions has been used extensively as a model of early-life stress in rodents with which to study underlying neurobiological mechanisms⁷. One of the most widely used methods involves the separation of the mother from her pups during the first 2 weeks of life, a critical period in the development of nociceptive, sensory, emotional and social functions^{4,15,18,39}. In rodents, two experimental procedures are primarily used: a prolonged single period (24hrs) of maternal deprivation (MD)^{15,54} and episodic brief periods (3–6hrs) of maternal separation³⁰. A single episode of MD on postnatal day 9 results in behavioural changes in adolescence and adulthood which resemble those found in the affective disorders, including depressive-like responses³², enhanced impulsivity⁴⁰, disruption in pre-pulse inhibition^{14,15} and cognitive deficits^{35,41}. Despite the robust changes in affective behaviour, few studies have investigated the effect of early-life stress on nociceptive responding. Episodic maternal separation has been shown to result in enhanced affective pain behaviour⁵³, visceral hypersensitivity^{6,8,20,42} and inflammatory hyperalgesia⁵³. However, the effect of a single period of MD on nociceptive responding has not been examined, nor have there been studies investigating the effect of early-life stress on neuropathic pain responding. The present study sought to address these two gaps in knowledge.

Neuroinflammatory processes are now well recognised to play important roles in the pathophysiology of stress-related psychiatric disorders and chronic pain^{3,9}. We therefore hypothesised that MD-related alterations in sensory and affective responding would be accompanied by altered expression of neuroinflammatory molecules in discrete brain regions involved in pain and affect. Stressful rearing conditions are associated with a number of neuroimmune alterations such as downregulated expression of microglial markers, cytokines

(IL-10, IL-1 β), chemokines (CCL7) and receptors (IL-5 receptor-alpha, CCR4) in the brain¹³. In addition, early-life stress reportedly reduces the expression of astrocytic markers (S100 β , GFAP) in the anterior cingulate and precentral medial cortices⁴⁴ and increases astrocyte density in the hippocampus and cerebellum^{34,36,41}. However, it is unknown if alterations in pro-inflammatory cytokines occur in key brain regions involved in emotional and nociceptive processing in the MD model. It is well established that neuropathic pain induces alterations in inflammatory processes at the level of the spinal cord^{24,49}. However a number of recent studies have indicated that peripheral nerve injury is also associated with increased supraspinal neuroinflammatory processes. Specifically, chronic constriction injury is associated with increased hippocampal TNF α levels²³ while spared nerve injury is associated with increased IL-1 β expression in the frontal cortex and GFAP expression in the periaqueductal grey^{2,11,47}. Chronic stress prior to peripheral nerve injury has been shown to exacerbate mechanical allodynia, depressive behaviour and augment injury-induced IL-1 β expression in the frontal cortex⁴⁷, thus indicating a possible functional interaction between stress, neuroinflammation and pain. Thus, we hypothesised that early life stress-induced changes in affective and nociceptive behaviour may be accompanied by alterations in supraspinal neuroinflammatory processing.

The aims of the current study were to examine the effect of MD on nociceptive and neuropathic pain behaviour in adulthood. Glial activation and cytokine expression were assessed in the prefrontal cortex and hippocampus, key brain regions involved in emotional and nociceptive processing^{12,37}, in order to determine if MD-induced alterations in behavioural responding are associated with concomitant alterations at the neuroimmune level. As sexually dimorphic effects on behaviour and neuroendocrine function occur following MD^{41,54,55}, responses were evaluated in both male and female animals.

Materials and Methods

Animal husbandry

Experimental subjects were the offspring of Albino Wistar male and female rats purchased from Harlan Interfauna Ibérica S.A. (Barcelona, Spain) mated (one male × two females) in the animal facility approximately 2 weeks after their arrival. Animals were housed in standard facilities on a reverse light-dark cycle (lights on at 20:00 h). On the day of birth [postnatal day (PND) 0], litters were sex-balanced, weighed and culled to 8 pups per dam (4 males and 4 females). Testing began in adulthood, animals older than PND 69, and all testing was carried out during the dark phase. The experimental protocol was carried out in accordance with the guidelines and approval of the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 86/609.

Maternal deprivation

The maternal deprivation (MD) protocol took place on PND9 as previously described^{32,34}. In brief, on PND9, half of the litters were submitted to 24 hours of MD, i.e. dams removed from their home-cages at 09.00hrs and pups left undisturbed in their corresponding home-cage (in the same room), until PND10, when dams were returned to their corresponding home-cages. Based on results from our laboratory and others^{31,54,55,59}, we believe the MD model depends on sensorimotor, nutritional and temperature insults to the neonate, the combination of which synergise to establish the long term changes in the model. At weaning (PND22), animals were housed in pairs of sibling animals of the same sex. Body weight was recorded from

control (Co) and MD pups at PND9 and PND10, and thereafter every 6 days from PND22 to 64.

Experimental design

The experimental design is presented in Figure 1, with testing beginning in adulthood. Essentially, animals were tested in the holeboard test, elevated plus maze and open field test to assess exploration, anxiety-like behaviour and locomotor activity, respectively, while nociceptive responding was assessed using the hot plate test (noxious thermal stimulus), von Frey test (mechanical stimulus) and the acetone-drop test (cold stimulus). Animals were then allocated to one of four groups: Co-non-spinal nerve ligation (NSNL) (male n=8, female n=10), Co-spinal nerve ligation (SNL) (male n=10, female n=10), MD-NSNL (male n=10, female n=10), MD-SNL (male n=9, female n=9). Mechanical allodynia was examined on days 1, 3, 7, 10 and 14 post-surgery. Cold allodynia was assessed on days 7 and 14 post-surgery. Animals were reassessed in the holeboard test and elevated plus maze on day 16, and in the open field test on day 18, post-surgery to examine if persistent pain had an effect on affective and exploratory behaviours. Animals were sacrificed by decapitation 48hrs following the final behavioural test (day 21 post-surgery, around PND103). The prefrontal cortex and hippocampus were dissected out on an ice cold plate and stored at -80°C until quantitative RT-PCR analysis was performed for the expression of inflammatory mediators.

Behavioural testing

Holeboard test

The holeboard test is used to measure exploratory behaviour, with increased duration of head dips indicative of increased exploration. On the testing day, animals were habituated to a quiet room for a 30 min period prior to experimental testing. The holeboard was a box (60 cm × 60 cm × 45 cm) with matte-painted metallic walls and a plastic-covered wooden floor bearing four equally spaced holes (3.8 cm in diameter) and divided into 36 squares (10 cm × 10 cm). Animals were placed in the centre of the holeboard area and the duration of head-dipping, an independent measure of directed exploration, and distance moved (Ethovision XT, Noldus Netherlands) was assessed over a 5 min testing period. Testing occurred under red-light conditions (lux 0-1).

Elevated plus maze

Immediately following exposure to the holeboard test, animals were placed in the elevated plus maze which consisted of two open (50 cm × 10 cm) and two enclosed arms with a central square area (10 cm × 10 cm), elevated to a height of 62 cm from the floor of the room. Frequency of entries and duration of time spent in the different arms was assessed over a 5 min test period. A greater percentage of time and entries in the open arms of the elevated plus maze has been reported to be indicative of reduced anxiety-like behaviour, while the number of closed arms entries provides a measure of locomotor activity. Percentage open arm entries was calculated as number of open arm entries / total arm entries × 100.

Open field test

The open field test was used to assess locomotor activity and anxiety-related behaviour in a novel aversive environment. On the experimental day, animals were removed from the home cage and placed singly into a brightly lit (lux 180) novel circular open field arena (diameter 75cm) where locomotor activity (distance moved, cm) and latency to enter and time spent in

the inner zone (diameter 40cm) was assessed using a computerised video tracking system (EthoVision XT, Noldus Netherlands) for a 5 minute period.

Hot plate test

The hot plate test was used to assess nociceptive responding to a noxious thermal stimulus. On the test day, the animals were moved to the testing room to habituate for 30 minutes after which they were placed individually onto a hot plate (Harvard Apparatus) heated to $55\pm 1^{\circ}\text{C}$. Thermal nociception was measured as the time elapsed, i.e. latency to respond (seconds), between placement of the animal on the surface of the hot plate and when the animal first licked either of its hind-paws, or jumped. A cut-off time of 40s was employed in order to avoid any tissue damage.

von Frey test

The arena used for von Frey testing consisted of a six-compartment Perspex arena (11cm x 20cm x 15cm) with wire mesh flooring. Rats were habituated to the arena for 20 minutes prior to testing, following which von Frey filaments (Touch-Test ®Sensory Evaluators, North Coast Medical, Inc.) of different forces (0.16g – 180g) were used to determine the 50% withdrawal threshold as previously described⁴³. Briefly, filaments were applied perpendicular to the plantar surface of the hind-paw, with sufficient force to cause slight buckling of the filament, for up to a maximum of 5 seconds or until flinching, licking or withdrawal of the paw occurred. Filaments of increasing forces were applied to both left and right hind-paws five times (alternating between paws) until a 100% positive response (5 positive responses to 5 applications) for two consecutive filaments was observed. The filament force eliciting 50% response was calculated by plotting the percentage response versus filament force for each

rat. Testing was carried out under red light with a low white light illumination (lux = 5) in order for the tester to visualise the paw responses to filament application.

Acetone-drop Test

The acetone-drop test was used to measure responding to an innocuous cold stimulus as previously described⁴³. Animals were placed in the same apparatus used for the von Frey test and allowed to habituate for 20 minutes. A drop of acetone was applied to the plantar surface of the hind-paw and the response (flinch, lick or withdrawal of the hind-paw) frequency was recorded. Each animal received 4 trials per paw alternating between left and right, with at least a 3 minute interval. The response frequency to acetone was expressed as a percentage withdrawal frequency [number of paw withdrawals/number of trials (four) × 100] as previously described²⁷.

L5-L6 spinal nerve ligation (SNL) surgery

L5-L6 spinal nerve ligation (SNL) is a well-characterized model of chronic neuropathic pain and was carried out as previously described^{28,43}. Briefly, rats were anaesthetized with isoflurane (2.5% in 0.6 L/min O₂), the fur lateral to the midline on the left-hand side at the lower lumbar and sacral regions was clipped and an incision was made through the skin between the spinal column and the left iliac crest. Paraspinal muscles were removed using a toothed forceps to visualise the L6 transverse process, which was removed and the L5 and L6 nerves were tightly ligated using 6-0 (0.07mm diameter) silk suture. Sham (NSNL) rats were treated in the same manner however the L5 and L6 nerves were exposed but not ligated. Rats were allowed to recover from anaesthesia in heated recovery cages and subsequently returned to their home cage.

Quantitative real-time PCR for the expression of inflammatory mediators

RNA was extracted from prefrontal cortex and hippocampal tissue using NucleoSpin RNAII total RNA isolation kit (Macherey-Nagel, Germany). Genomic DNA contamination was removed by adding DNase to the samples according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using a High Capacity cDNA Archive kit (Applied Biosystems, UK). Taqman gene expression assays (Applied Biosystems, UK) containing forward and reverse primers and a FAM-labelled MGB Taqman probe were used to quantify the gene of interest and real-time PCR was performed using an ABI Prism 7500 instrument (Applied Biosystems, UK), as described previously ²⁶. Assay IDs for the genes examined were: IL-1 β (Rn00580432_m1), TNF α (Rn99999017_m1), IL-6 (Rn00561420_m1), GFAP (Rn00566603_m1) and CD11b/Itgam (Rn00709342_m1). PCR was performed using Taqman Universal PCR Master Mix. Cycling conditions were 90°C for 10 minutes and 40 cycles of 90°C for 15 minutes, then 60°C for 1 minute. The endogenous control used to normalise gene expression data was β -actin. Relative gene expression was calculated using the $\Delta\Delta$ CT method and data expressed as % control NSNL males.

Data analysis

SPSS statistical package was used to analyse all data. Kolmogorov and Levene tests were used to determine normality and homogeneity of variance, respectively, and data were analysed using two-way (factors of MD and sex or MD and SNL) or three-way (factors of MD, SNL and sex) analysis of variance (ANOVA) for parametric data, and Kruskal-Wallis for non-parametric data. Repeated measures ANOVAs were used to assess changes over

time. *Post-hoc* analysis was performed using Duncan's test for parametric data, and Mann-Whitney U-test for non-parametric data, where appropriate. Data were considered significant when $P < 0.05$. Results are expressed as group means \pm standard error of the mean (SEM). The results of ANOVA or Kruskal-Wallis tests are given in the main body text of the Results section while the results of *post-hoc* testing are presented in the figures and explained in the figure legends.

Results

Maternally deprived animals demonstrate sex-dependent alterations in nociceptive responding depending on stimulus modality

As previously reported, MD induced long-lasting reductions in body weight in both male and female rats (Supplementary data table 1), and sex-specific changes in affective responding (Supplementary data Figure 1). Examining the effect of MD on nociceptive responding revealed a significant MD \times sex interaction ($F_{(1,76)} = 6.16$ $P = 0.015$) on latency to jump in the hot plate test with *post-hoc* analysis revealing that MD females, but not MD males, exhibit an increased latency to respond in comparison to control counterparts, indicating that MD females exhibit reduced sensitivity to a noxious thermal stimulus (Fig. 2A). In the von Frey test, MD female, but not male, rats exhibited a significant reduction in the threshold to respond to a mechanical stimulus (MD $F_{(1,76)} = 13.31$ $P < 0.001$; MD \times sex interaction $F_{(1,76)} = 4.2$ $P = 0.044$), indicative of mechanical allodynia when compared to control counterparts (Fig. 2B). There was no effect of MD or sex on percentage withdrawal frequency to an innocuous cold stimulus in the acetone-drop test (Fig. 2C).

SNL-induced mechanical and cold allodynia is enhanced in MD female rats

Analysis of mechanical withdrawal thresholds revealed that SNL induces mechanical allodynia of the ipsilateral (left) hind-paw in both control and MD rats of both sexes when compared to NSNL counterparts (SNL: $F_{(1,65)} = 95.92$ $P < 0.001$, Fig. 3A-C). Further temporal analysis indicated a significant time \times MD \times sex \times SNL interaction ($F_{(4,260)} = 2.45$ $P = 0.047$) and *post-hoc* analysis revealed that SNL induced mechanical allodynia in both MD

and control male and control female rats from day 1 post surgery, while mechanical allodynia was evident in MD females from day 3 post-surgery, when compared to pre-surgery values. MD did not alter mechanical withdrawal thresholds in SNL male rats at any of the time-points examined. However, on day 14 post-SNL, MD-SNL females exhibited lower withdrawal thresholds of the ipsilateral hind-paw compared to their control counterparts (MD-SNL vs. Co-SNL females). Analysis of the number of responses to the 1g von Frey filament at this time-point MD-SNL females exhibit an increased number of responses indicative of enhanced mechanical allodynia, when compared to both NSNL and control counterparts ($\chi^2 = 14.76$ P = 0.002, Fig.3B inset). It should be noted that similar to the responses observed prior to surgery, both male and female MD animals which underwent sham surgery (NSNL) exhibited mechanical allodynia when compared to corresponding controls ($F_{(1,69)} = 10.42$ P = 0.002; MD-NSNL vs. Co-NSNL Fig. 4C). In addition, control and MD-NSNL females demonstrated reduced withdrawal thresholds compared to their male counterparts ($F_{(1,69)} = 19.53$ P < 0.001, Fig. 3C).

Assessment of mechanical withdrawal thresholds of the contralateral (right) hind-paw revealed that MD females exhibited reduced thresholds, prior to and following NSNL or SNL surgery, compared to their control counterparts ($F_{(1,36)} = 31.18$ P < 0.001, Fig. 4A-C). SNL did not alter mechanical withdrawal thresholds of the contralateral hind-paw in either male or female animals.

Cold allodynia, expressed as an increase in the percentage withdrawal frequency following the application of acetone to the hind-paw, was observed in the ipsilateral hind-paw of both control and MD rats, 7 and 14 days following SNL, when compared to NSNL controls (SNL: $F_{(1,67)} = 186.42$ P < 0.001, Fig 5A&C). Subsequent analysis within female comparisons revealed that MD-SNL female rats exhibited increased withdrawal frequencies on day 14 when compared to control counterparts, indicating an MD-induced exacerbation of cold

allodynia in female rats ($MD \times SNL F_{(1,34)} = 9.49 P = 0.004$, MD-SNL vs. Co-SNL; Fig. 5C). SNL did not alter the withdrawal frequency of the contralateral hind-paw in control animals (Fig 5B&D). However, on day 14 post-SNL, MD-SNL females, but not males, exhibited slight cold allodynia of the contralateral hind-paw when compared to control-SNL counterparts, ($F_{(1,34)} = 5.73 P = 0.022$, MD-SNL vs. Co-SNL; Fig 5D), an effect much less pronounced than that observed in the ipsilateral hind-paw.

Animals were re-exposed to the holeboard test, elevated plus maze and open field test following assessment of nociceptive behaviours in order to determine the effect of MD-SNL interactions on affective responding and locomotor activity. SNL *per se* did not alter the behavioural responding of male or female control animals but reduced locomotor activity of female MD rats in the open field test when compared to NSNL counterparts (Supplementary data Fig 2C). In addition, MD-SNL males exhibited decreased percentage open arm entries in the elevated plus maze when compared to MD-SNL female counterparts (Supplementary data Fig 2B).

MD results in reduced expression of proinflammatory cytokines in the prefrontal cortex following SNL in a sex-dependent manner

Analysis of the expression of glial activation markers and pro-inflammatory cytokines revealed that control females exhibit less GFAP (Three-way ANOVA effect of Sex: $F_{(1,66)} = 5.55 P = 0.022$) and IL-1 β (Sex: $F_{(1,67)} = 19.20 P < 0.001$) mRNA expression when compared to male counterparts (Co-NSNL female vs. Co-NSNL male; Figure 6 B-C). This lower level of IL-1 β mRNA expression in female vs. male rats was also evident following SNL (Co-SNL female vs. Co-SNL male; Figure 6 C). MD *per se* did not alter the expression of any of the inflammatory markers in either male or female rats (MD-NSNL vs. Co-NSNL). However, IL-

6 mRNA was reduced in MD-SNL males when compared to control-SNL counterparts (MD: $F_{(1,64)} = 8.26$, $P = 0.005$; MD-SNL male vs. Co-SNL male; Fig 6E). In addition, the expression of TNF α mRNA was reduced in MD-SNL females when compared to male counterparts (MD \times sex $F_{(1,65)} = 4.13$, $P = 0.046$; MD-SNL females vs. MD-SNL males; Fig 6D), an effect not observed in NSNL animals. Sex, MD or SNL did not alter CD11b expression in the PFC.

MD-SNL exhibit increased expression of neuroimmune markers in the hippocampus in a sex-dependent manner

Although neither MD nor SNL alone altered the expression of any of the inflammatory mediators under investigation, MD-SNL male rats exhibited increased expression of GFAP ($F_{(1,64)} = 4.9$, $P = 0.023$) and IL-1 β (SNL: $F_{(1,62)} = 5.55$, $P = 0.041$; SNL \times sex: $F_{(1,62)} = 7.77$, $P = 0.007$) in the hippocampus, compared with NSNL counterparts (MD-SNL male vs. MD-NSNL male; Fig 7 B-C). In comparison, MD-SNL female rats displayed increased expression of IL-6 when compared to NSNL counterparts (MD \times SNL \times sex: $F_{(1,61)} = 4.56$, $P = 0.037$, MD-SNL vs. MD-NSNL; Fig 7E) and increased TNF α expression when compared to control counterparts (MD: $F_{(1,62)} = 4.10$, $P = 0.048$; MD \times SNL: $F_{(1,61)} = 5.4$, $P = 0.023$; MD-SNL vs. Co-SNL; Fig 7D).

Discussion

The present study demonstrates that early-life stress, in the form of maternal deprivation (MD), results in sex-dependent alterations in nociceptive responding to mechanical and thermal stimuli prior to and following peripheral nerve injury in adulthood. In particular, MD female, but not male, rats exhibit thermal heat hypoalgesia and reduced nociceptive threshold to mechanical stimuli, and display enhanced mechanical and cold allodynia following SNL. Furthermore, the expression of pro-inflammatory cytokines and glial markers in key brain areas involved in affective and nociceptive processing, the prefrontal cortex and hippocampus, is differentially altered in male and female rats exposed to a combination of MD and SNL. These data provide evidence for sexually dimorphic effects of early-life stress on nociceptive processing and suggest that alterations in neuroinflammatory processes may, in part, underlie these effects.

Although several studies have demonstrated the effect of early-life stress on visceral pain^{6,8,42}, relatively few have examined its effect on thermal or mechanical nociceptive responding. Maternal separation has been demonstrated to increase the thermal response latency of female⁵⁶, but not male⁵³, rats in the hot plate test, correlating with clinical reports where childhood abuse is associated with reduced sensitivity to noxious thermal stimuli in women only¹⁶. In accordance with these findings, the present study demonstrated that in tests of acute nociception, MD female, but not male, rats exhibit increased nociceptive thresholds to a noxious heat, but not cold, stimulus. In addition, MD females displayed reduced paw withdrawal thresholds to mechanical stimulation, indicative of mechanical hypersensitivity/allodynia in MD female but not male animals. The effect of early life stress on mechanical thresholds has only been reported in male animals. Exposure to the neonatal limited bedding paradigm results in lower muscle mechanical nociceptive thresholds in male rats during adulthood²¹. In comparison, mechanical withdrawal thresholds were unaltered in

maternally separated adult males⁵³, results correlating with the current study. To our knowledge, the present study is the first to demonstrate divergent sexually-dimorphic effects of MD on nociceptive responding to thermal and mechanical stimuli.

Due to the link between early-life stress and chronic pain, a further aim of this study was to determine the effect of MD on a chronic, persistent, clinically relevant neuropathic pain state, using the L5-L6 spinal nerve ligation (SNL) model. In agreement with previous reports^{5,43}, SNL induced robust mechanical and cold allodynia, an effect observed in both control and MD animals. However, MD females were slower to develop mechanical allodynia following SNL when compared to controls (day 3 vs. day 1). Moreover, MD females also exhibited enhanced mechanical (ipsilateral) and cold (bilateral) allodynia 14 days following SNL compared with control females. Mechanical allodynia following peripheral-nerve injury has been shown to be enhanced following chronic restraint stress and in a genetic model of depression (Wistar-Kyoto rat)^{47,58}, however, to our knowledge, this is the first study to examine the effect of early-life stress on neuropathic pain behaviour in rodents. Although the neurobiological mechanisms underlying the effects of MD on nociceptive responding in adulthood remain unknown, it has been shown that MD induces a range of sex-dependent developmental and psychoneuroimmunendocrine alterations⁵⁴. It has been proposed that disruptions in stress regulation caused by early-life stress may explain the reduced pain thresholds seen in fibromyalgia³⁸. Noxious painful stimuli in early life have been demonstrated to increase the plasticity of nociceptive systems, altering the development of somatosensory pathways and subsequently leading to long-lasting alterations in nociceptive responding to thermal and inflammatory stimuli in adulthood, with females displaying greater responses compared to males^{18,29}. Thus, stressful events at critical developmental periods may cross-sensitise nociceptive circuits leading to enhanced susceptibility to chronic pain in later life. Therefore, it is possible that MD at a critical developmental stage (PND9) induces

alterations in sensory and affective processing resulting in enhanced nociceptive responding in later life. The exacerbation of SNL-induced mechanical and cold allodynia in MD females suggests that adverse early-life events may elicit a more profound impact in females, accounting for the increased incidence and severity of chronic pain conditions reported in this gender ¹⁷.

Alterations in affective responding such as anxiety- and depressive-like behaviours have been shown to be induced in a number of models of peripheral nerve injury ^{45,47,51,57}, although in the present study SNL failed to modulate affective behaviour. The short period following surgery over which these behaviours were examined (Day16), may account for the lack of effects as previous studies have indicated that alterations in affective behaviour only manifest after at least a month post-surgery ^{45,51,57}.

The PFC and hippocampus are key regions in both emotion and pain processing ^{12,37}. Sex-dependent changes in neuronal and non-neuronal functioning in these regions may underlie the higher incidence of certain psychiatric and pain disorders in women ^{17,48}. Over the past decade, there has been increased interest in the role of non-neuronal cells such as glia, in emotional and nociceptive processing. The present study demonstrated that female rats (control-NSNL) exhibit less GFAP and IL-1 β expression in the PFC, but not hippocampus, when compared to male counterparts. Similarly, we have recently demonstrated lower GFAP density in adolescent female rats in the frontal cortex, but not hippocampus ⁴¹. Furthermore, astrocyte number and density are lower in the amygdala ²⁵ and cerebellum ⁵⁰, of female rats when compared to males. Although further studies are required to determine the functional significance of such sex-dependent alterations, it is possible that reduced astrocyte activity may underlie the lower withdrawal thresholds to mechanical stimuli in female vs. males rats observed in the current study.

In the present study, MD *per se* did not alter GFAP or CD11b mRNA expression, markers of astrocyte and microglial activation respectively, in the hippocampus or PFC of adult rats. Previous reports have described increased astrocyte density in the hippocampus and cerebellum, with a slight increase in the PFC, of neonatal MD male rats^{34,36,41}. Different methods of evaluation of GFAP (qRT-PCR vs. immunohistochemistry) and time of evaluation (neonate vs. adult) may account for the discrepancies between the current study and previous reports.

Nerve injury has been shown to increase GFAP and cytokine expression (IL-1 β) in the PFC, hippocampus and brain stem^{2,12,47}. Recent studies from our laboratory have demonstrated that SNL decreases IL-6 expression in the amygdala but does not alter expression of pro-inflammatory cytokines in other brain regions, including the PFC and hippocampus (unpublished observations). Similarly, SNL *per se* did not alter the expression of any of the inflammatory mediators under investigation in the PFC or hippocampus in the present study. It is possible that the post-surgery timepoint at which brain tissue was collected was not optimal to detect SNL-induced alterations in these inflammatory mediators. However, the combination of peripheral nerve injury and MD resulted in sexually dimorphic alterations: IL-6 expression was reduced in the PFC while GFAP and IL-1 β expression was increased in the hippocampus of MD males following SNL. In comparison, MD-SNL female rats exhibited lower TNF α in the PFC but enhanced IL-6 and TNF α expression in the hippocampus. To our knowledge, this is the first study to examine and demonstrate the effect of early-life stress on behavioural and supra-spinal neuroimmune changes following peripheral-nerve injury. Thus, the differential effect of MD-SNL on cytokine expression in male versus female brain regions involved in regulating emotion and pain may underlie the sexually dimorphic effects observed on nociceptive processing.

In conclusion, MD induces sexually dimorphic effects on nociceptive behaviour. Specifically, MD results in heightened mechanical and cold allodynia following peripheral-nerve injury in female rats, effects accompanied by increased cytokine (IL-6 and TNF α) expression in the hippocampus. These findings expand our understanding of the interaction between early-life stress and pain.

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Disclosures

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Figure Legends

Fig 1: Experimental Protocol

Abbreviations: AT Acetone-drop test; EPM Elevated Plus Maze, HB Holeboard, MD Maternal Deprivation, NSNL non-Spinal Nerve Ligation, PND Postnatal day, SNL Spinal Nerve Ligation, VF von Frey.

Fig 2: Maternally deprived female, but not male, rats exhibit thermal hypoalgesia and mechanical allodynia

(A) MD female, but not male, rats exhibit increased thermal thresholds (latency to jump) and (B) reduced mechanical withdrawal thresholds when compared to control (Co) counterparts. (C) Neither MD nor sex altered the percentage withdrawal frequency to an innocuous cold stimulus. ⁺P < 0.05 vs. Co, [#]P < 0.05 vs. males. Data expressed as Mean ± SEM, n=20. Co Control, MD Maternal Deprivation.

Fig. 3: MD females exhibit exacerbated mechanical allodynia of the ipsilateral hind-paw post-SNL

(A) SNL results in lower mechanical thresholds of the ipsilateral hind-paw of control and MD males from day 1 to day 14 post-SNL, when compared to pre-surgery levels. Inset: SNL results in increased percentage withdrawal response to 1g filament of the ipsilateral hind-paw of males on Day 14 post surgery (B) SNL results in lower mechanical thresholds in the ipsilateral hind-paw of control females from day 1 – 10 and in MD females from day 3 - 14. Inset: SNL results in increased percentage withdrawal response to 1g filament of the

ipsilateral hind-paw of MD females on Day 14 post surgery, when compared to control and NSNL counterparts. (C) Area under the curve of mechanical thresholds in the ipsilateral hind-paw. *P < 0.05 **P < 0.01 vs. NSNL, ⁺P < 0.05 ⁺⁺ P < 0.01 vs. Co, [#]P < 0.05 vs. males. Data expressed as Mean ± SEM, n=8-10. Co Control, MD Maternal Deprivation, SNL Spinal Nerve Ligation, NSNL non-Spinal Nerve Ligation.

Fig. 4: MD female, but not male, rats exhibit reduced mechanical withdrawal thresholds of the contralateral hind-paw

(A) SNL did not alter the mechanical withdrawal thresholds of the contralateral hind-paw of control or MD males. (B) MD females displayed lower mechanical withdrawal thresholds of the contralateral hindpaw, an effect not altered by SNL. (C) Area under the curve of mechanical thresholds of the contralateral hind-paw. ⁺P < 0.05 vs. Co. Data expressed as Mean ± SEM, n=8-10. Co Control, MD Maternal Deprivation, SNL Spinal Nerve Ligation, NSNL non-Spinal Nerve Ligation.

Fig. 5: MD females exhibit exacerbated cold allodynia following SNL

(A) SNL results in increased percentage withdrawal frequency to an innocuous cold stimulus of the ipsilateral, but not (B) contralateral, hind-paw in both control and MD males. (C) MD females exhibit increased percentage withdrawal frequency of the ipsilateral hindpaw on day 14 post-SNL, indicating exacerbated cold allodynia. (D) MD-SNL females exhibit a slight increase in percentage withdrawal frequency to an innocuous cold stimulus of the contralateral hind-paw on day 14 post-SNL. *P < 0.05 vs. NSNL, ⁺P < 0.05 vs. Co. Data

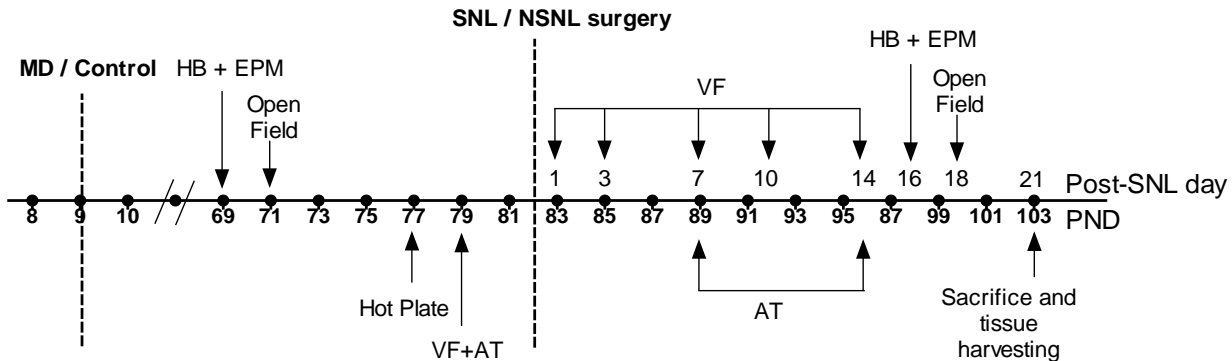
expressed as Mean \pm SEM, n=8-10. Co Control, MD Maternal Deprivation, SNL Spinal Nerve Ligation, NSNL non-Spinal Nerve Ligation.

Fig. 6: The expression of neuroinflammatory mediators in the prefrontal cortex

The expression of (A) CD11b, (B) GFAP, (C) IL-1 β (D) IL-6 and (E) TNF α mRNA in the prefrontal cortex. [#]P < 0.05 vs. males, ⁺P < 0.05 vs. control. Co Control; GFAP Glial fibrillary acidic protein; IL-1 β Interleukin-1 beta; MD Maternal Deprivation; NSNL non-Spinal Nerve Ligation; PFC prefrontal cortex; SNL Spinal Nerve Ligation; TNF α Tumour necrosis factor alpha. Data expressed as Mean \pm SEM, n=8-10.

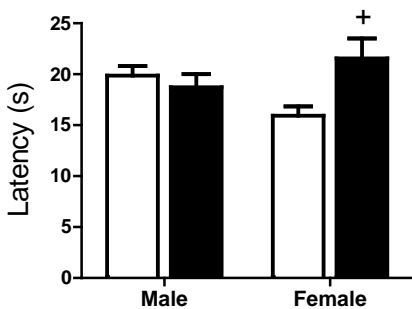
Fig. 7: The expression of neuroinflammatory mediators in the hippocampus

The expression of (A) CD11b, (B) GFAP, (C) IL-1 β (D) IL-6 and (E) TNF α mRNA in the hippocampus. *P < 0.05 vs. NSNL, ⁺P < 0.05 vs. control. Co Control; GFAP Glial fibrillary acidic protein; IL-1 β Interleukin-1 beta; MD Maternal Deprivation; NSNL non-Spinal Nerve Ligation; PFC prefrontal cortex; SNL Spinal Nerve Ligation; TNF α Tumour necrosis factor alpha. Data expressed as Mean \pm SEM, n=8-10.

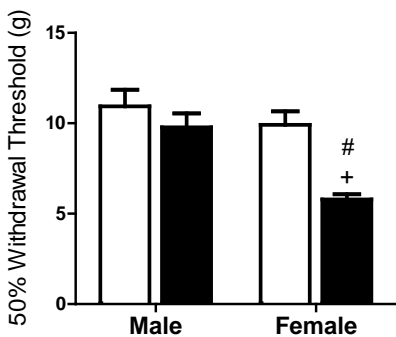


□ Co
■ MD

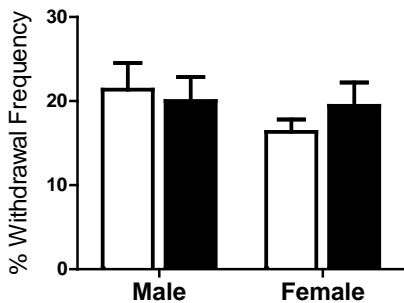
A.



B.

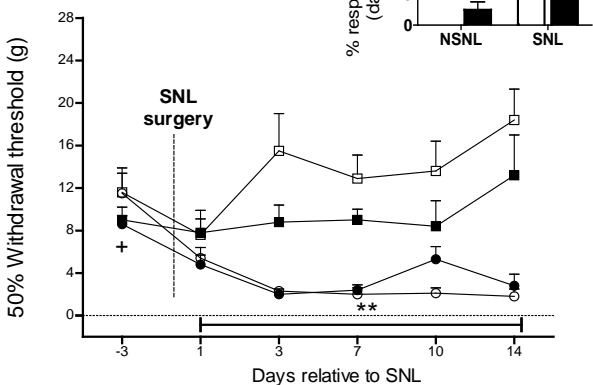


C.

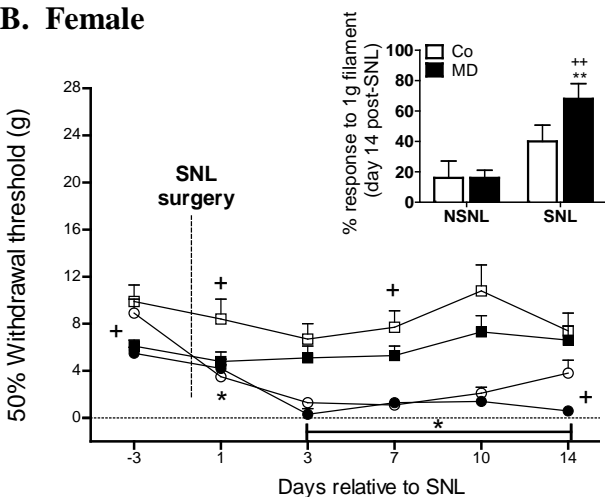


□ Co-NSNL ■ MD-NSNL
 ○ Co-SNL ● MD-SNL

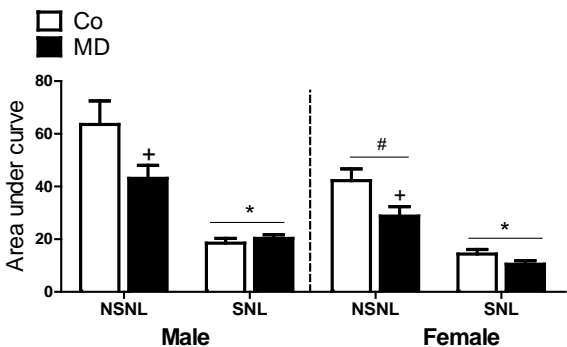
A. Male



B. Female

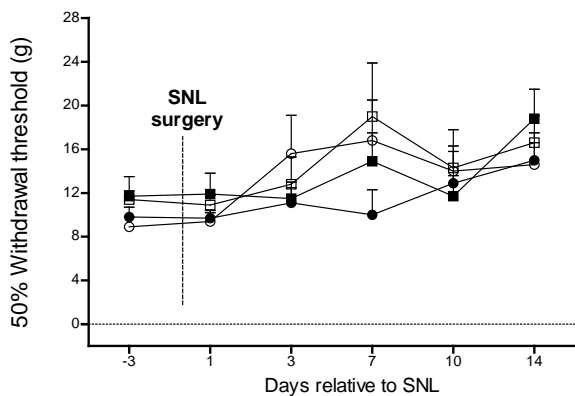


C. Area under the curve

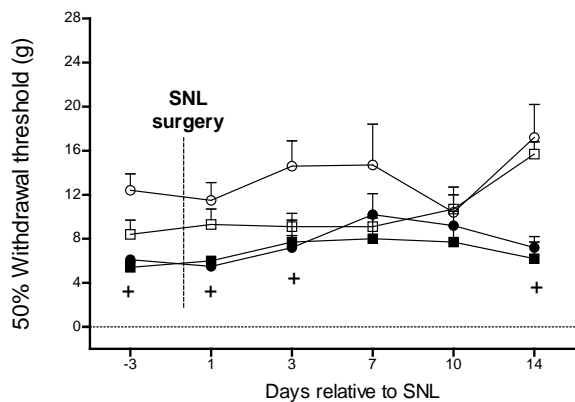


□ Co-NSNL ■ MD-NSNL
 ○ Co-SNL ● MD-SNL

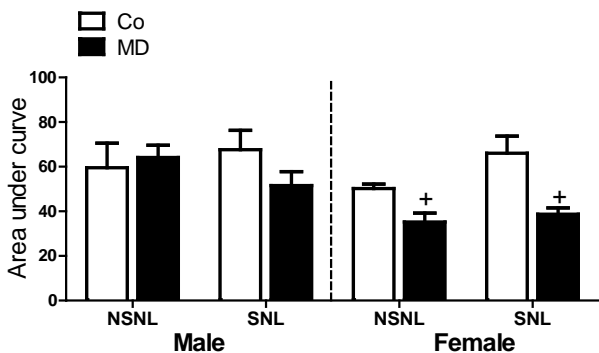
A. Male



B. Female



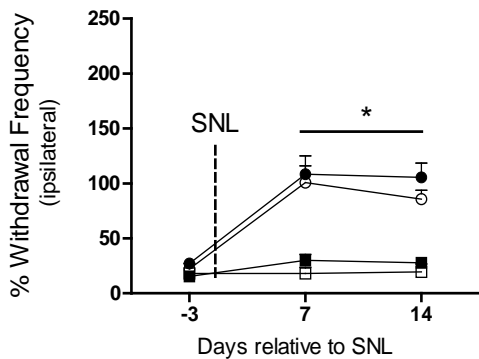
C. Area under the curve



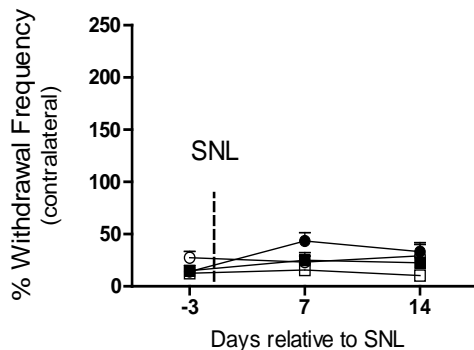
- ⊕ Co-NSNL ■ MD-NSNL
- ⊖ Co-SNL ● MD-SNL

Male

A.

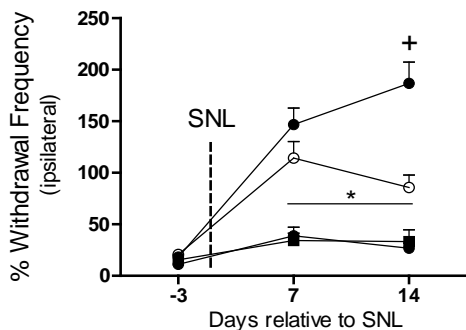


B.

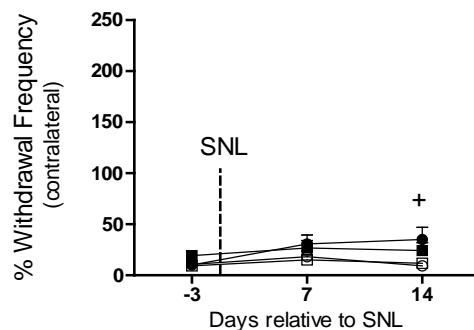


Female

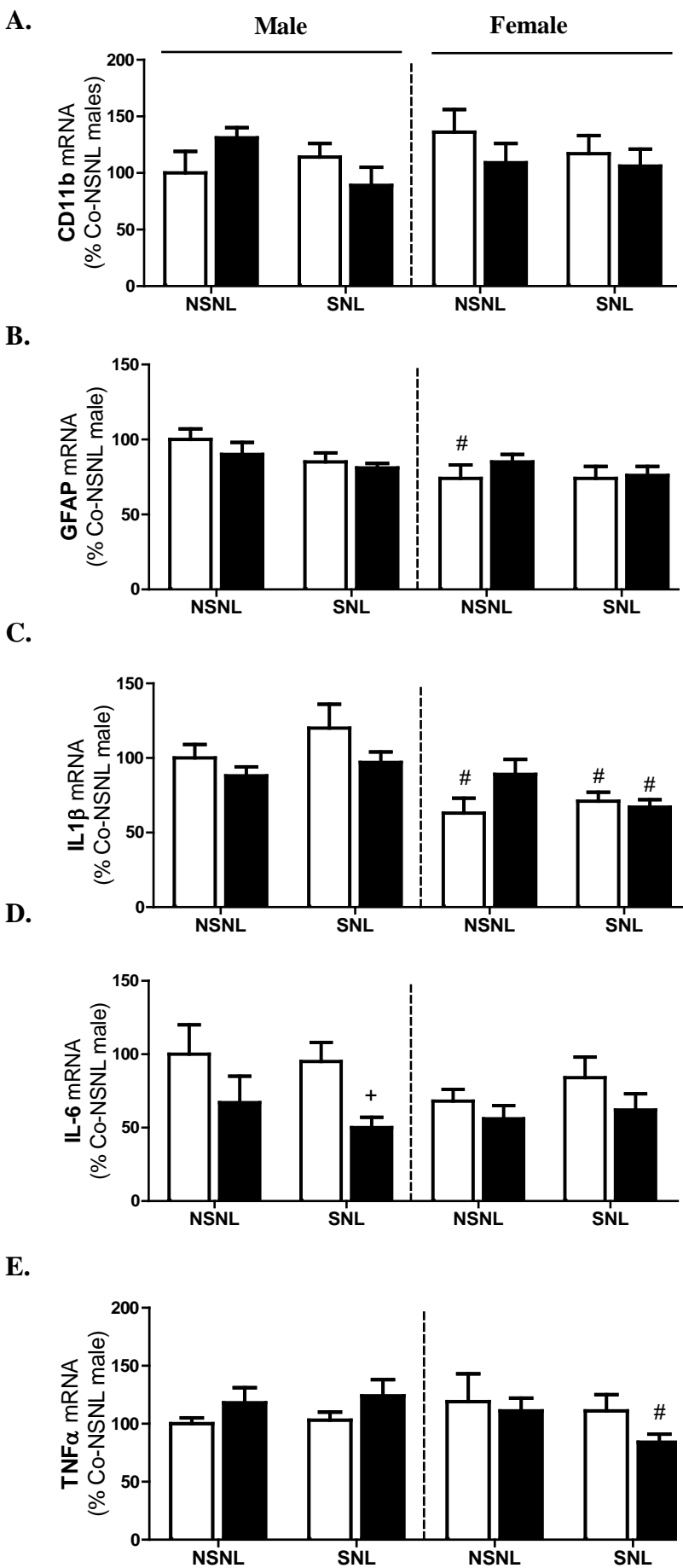
C.



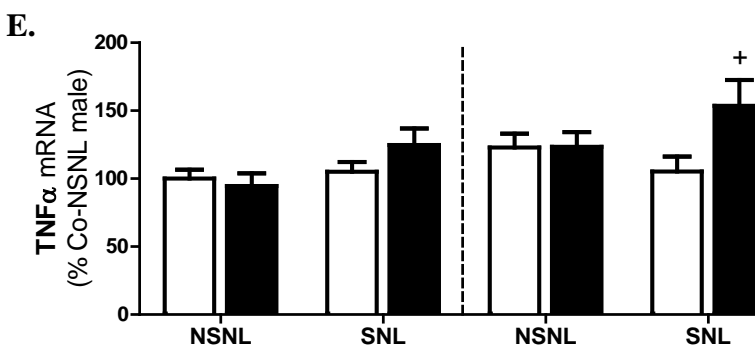
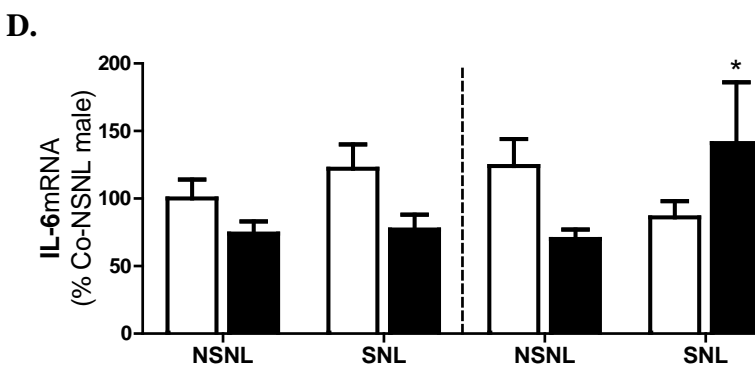
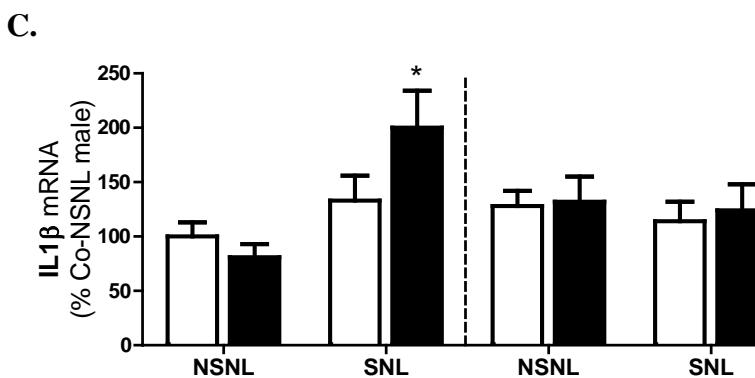
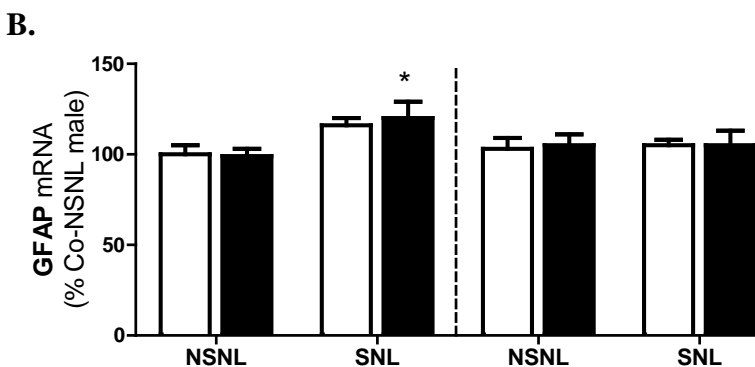
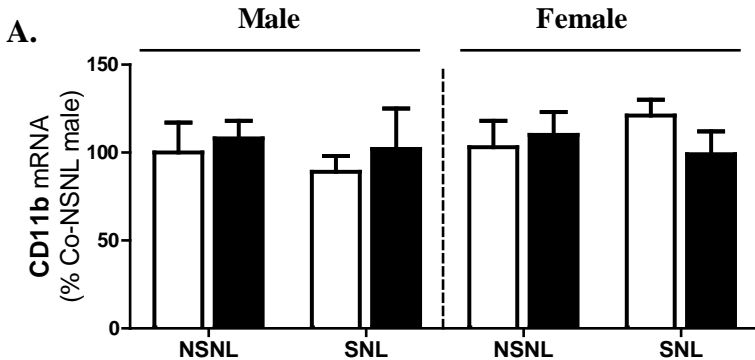
D.



Co
 MD



□ Co
■ MD

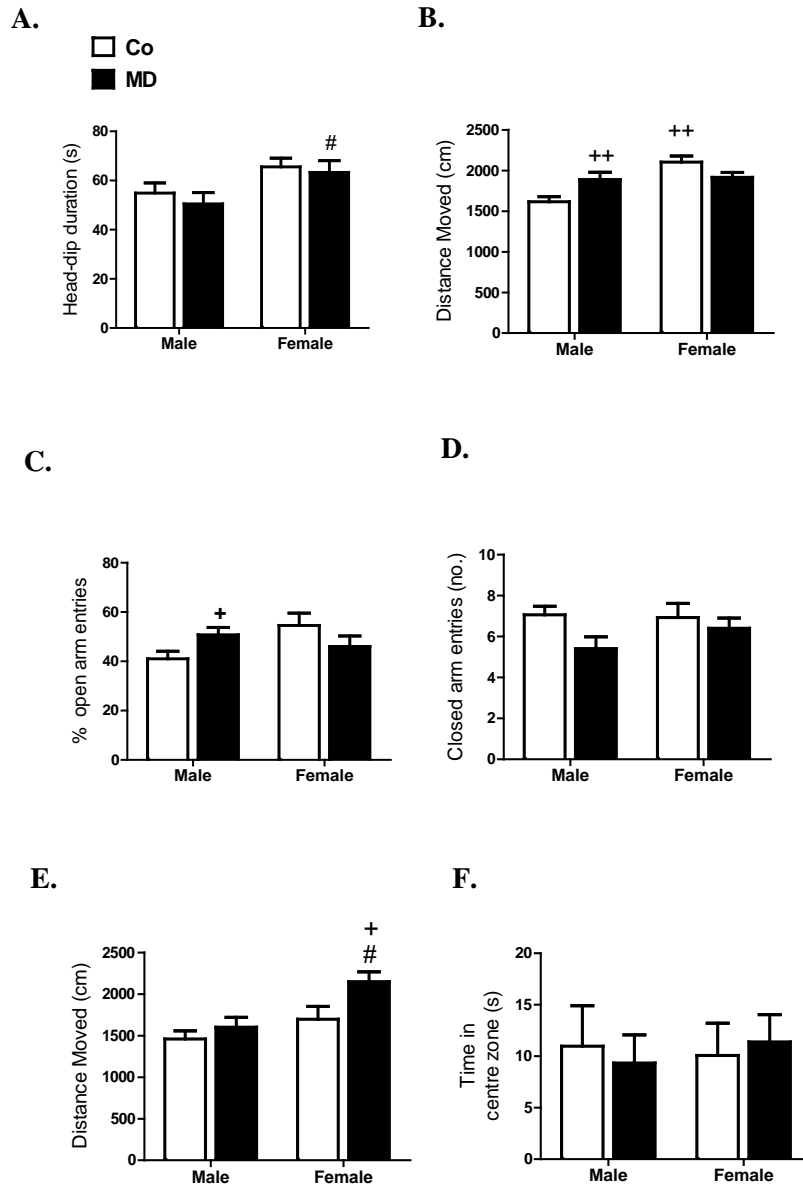


Supplementary data Table 1: MD results in long-lasting reductions in body weight (g)

	Co-Male	MD-Male	Co-Female	MD-Female
PND9	21.0±0.5	21.4±0.3	20.4±0.5	21.0±0.3
PND10	23.4±0.5	20.6±0.3 ⁺	22.9±0.6	20.0±0.3 ⁺
PND22	58.1±0.9	52.7±0.5 ⁺	56.3±1	50.7±0.4 ⁺
PND28	87.9±1.4	79.3±0.8 ⁺	82.4±1.4 [#]	74.8±0.8 ^{+, #}
PND34	129.1±1.9	117.9±1.4 ⁺	115.5±1.8 [#]	106.3±1.11 ^{+, #}
PND40	173.1±2.3	157.6±2 ⁺	142.9±2.1 ^{###}	132.2±1.4 ^{+, ###}
PND48	210.9±2.9	193.1±3.0 ⁺	162.2±2.4 ^{###}	151.0±2.2 ^{+, ###}
PND52	253.9±3.4	235.3±3.9 ⁺	181.6±2.6 ^{###}	170.5±2.5 ^{+, ###}
PND58	291.3±4.7	268.6±5.4 ⁺	196.1±3.0 ^{###}	183.5±3.1 ^{+, ###}
PND64	317.8±4.7	293.4±5.6 ⁺	206.9±2.9 ^{###}	194.1±3.2 ^{+, ###}

Body weight (g) did not differ between the groups prior to MD on PND9. However, body weight of both male and female MD animals was significantly reduced following MD (PND10), until the final assessment on PND64 (Two-way ANOVA effect of MD: $F_{(1,75)} = 29.30$ $P < 0.001$, Table 1). In addition, female rats (both control and MD) weighed significantly less than their male counterparts from PND28 onwards (Two-way ANOVA effect of Sex: $F_{(1,75)} = 343.70$ $P < 0.001$). ⁺ $P < 0.05$ vs. Co. [#] $P < 0.05$, ^{###} $P < 0.01$ vs. corresponding male. Co Control, MD Maternal Deprivation, PND Postnatal day.

Supplementary data Fig 1

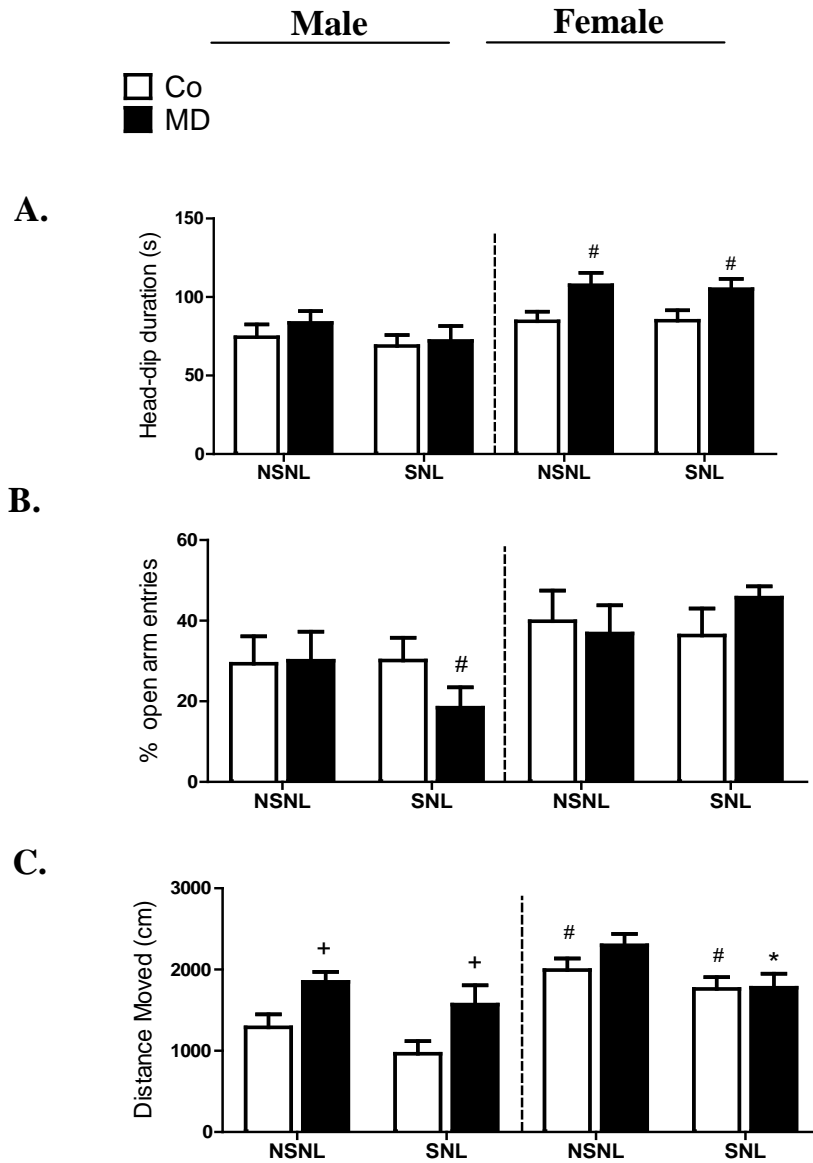


Maternally deprived animals demonstrate altered affective behaviour in a sex-dependent manner

(A) Head-dip duration in the holeboard is increased in MD females when compared to MD males (Two-way ANOVA effect of Sex: $F_{(1,71)} = 7.27$, $P=0.009$). (B) There was no significant difference in distance moved in the hole board test between MD males and females, however MD males (MD \times sex interaction: $F_{(1,71)} = 10.37$ $P = 0.002$) and Co females (Sex: $F_{(1,71)} = 13.00$

$P < 0.001$) exhibit increased distance moved in the holeboard test when compared to Co males. (C) Percentage open arm entries in the elevated plus maze is increased in MD males when compared to control males (MD \times sex interaction: $F_{(1,62)} = 4.61$ $P = 0.035$, Fig. 2C). (D) Number of closed arm entries in the elevated plus maze is unaltered by MD or sex. (E) MD females show increased distance moved over 5 minutes in the open field when compared to Co females and MD males ($F_{(1,75)} = 5.87$ $P = 0.018$). (F) Time spent in the centre zone of the open field did not differ between the groups. ⁺⁺ $P < 0.01$ ⁺ $P < 0.05$ vs. Co, [#] $P < 0.05$ vs. MD. Data expressed as Mean \pm SEM, $n=20$. Co Control, MD Maternal Deprivation.

Supplementary data Fig 2



Effect of MD-SNL interactions on affective behaviour and locomotor activity

(A) Re-exposure to the holeboard test revealed increased duration of head dipping in MD female, but not male, rats (MD: $F_{(1,67)} = 6.75$ $P = 0.012$; Sex: $F_{(1,67)} = 15.49$ $P < 0.001$). NSNL female rats, both control and MD, exhibited increased distance moved in the holeboard test when compared to male counterparts (Sex: $F_{(1,67)} = 43.79$ $P < 0.001$, data not shown), an effect attenuated in MD-SNL females (MD \times SNL interaction: $F_{(1,67)} = 5.58$ $P = 0.021$; MD-NSNL:

2525 ± 133cm vs. MD-SNL: 1976 ± 108cm) (B) Females exhibited higher percentage open arm entries compared to males (Sex: $F_{(1,62)} = 7.13$ $P = 0.01$), with further *post-hoc* analysis revealing that MD-SNL males exhibited decreased percentage open arm entries compared to MD-SNL female counterparts. There was no effect of MD, SNL or sex on the number of closed arm entries, demonstrating no effect on locomotor activity (data not shown) (C) Control females exhibited increased distance moved in the open field when compared to male counterparts ($F_{(1,67)} = 22.45$ $P < 0.001$, Co-NSNL male vs. Co-NSNL female), an effect not altered by SNL. MD enhanced distance moved in males, but not females ($F_{(1,67)} = 10.56$ $P = 0.002$, Co-NSNL male vs. MD-NSNL male), an effect not altered by SNL. Furthermore, MD-SNL females exhibited decreased distance moved when compared to their NSNL counterparts ($F_{(1,67)} = 8.89$ $P < 0.01$). Time spent in the centre zone of the open field was not altered by MD, sex or SNL (data not shown) * $P < 0.05$ vs. NSNL, + $P < 0.05$ vs. Co, # $P < 0.05$ vs. males. Data expressed as Mean ± SEM, n=8-10. Co Control, MD Maternal Deprivation, SNL Spinal Nerve Ligation, NSNL non-Spinal Nerve Ligation.