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Priming of adult incision response by early life injury: neonatal microglial inhibition has persistent but sexually dimorphic effects in adult rats

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1   **Title**

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5   Neonatal microglia inhibitor and adult re-incision

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35   The authors declare no competing financial interests.

36   **Author contributions**

37   Study design/planning by O.M., S.B., M.W.S, and S.M.W. Study conduct and research

38   performed by O.M., Y-S T., A.S., S.B, S.M.W. Data analysis by O.M., Y-S T., A.S., S.B., S.M.W.

39   Writing and revising paper by O.M., S.B., M.W.S, S.M.W.

Abstract

40

41 Neonatal hindpaw incision primes developing spinal nociceptive circuitry, resulting in

42 enhanced hyperalgesia following re-injury in adulthood. Spinal microglia contribute to this

43 persistent effect and microglial inhibition at the time of adult re-incision blocks the

44 enhanced hyperalgesia. Here, we pharmacologically inhibited microglial function with

45 systemic minocycline or intrathecal SB203580 at the time of neonatal incision and evaluated

46 sex-dependent differences following adult re-incision. Incision in adult male and female rats

47 induced equivalent hyperalgesia and spinal dorsal horn expression of genes associated with

48 microglial proliferation (*Emr1*) and transformation to a reactive phenotype (*Irf8*). In control

49 adults with prior neonatal incision, the enhanced degree and duration of incision-induced

50 hyperalgesia and spinal microglial responses to re-incision were equivalent in males and

51 females. However, microglial inhibition at the time of the neonatal incision revealed sex-

52 dependent effects: the persistent mechanical and thermal hyperalgesia following re-incision

53 in adulthood was prevented in males but unaffected in females. Similarly, re-incision

54 induced *Emr1* and *Irf8* gene expression was downregulated in males, but not in females

55 following neonatal incision with minocycline. To evaluate the distribution of re-incision

56 hyperalgesia, prior neonatal incision was performed at different body sites. Hyperalgesia

57 was maximal when the same paw was re-incised, and was increased following prior incision

58 at ipsilateral, but not contralateral sites; supporting a segmentally restricted spinal

59 mechanism. These data highlight the contribution of spinal microglial mechanisms to

60 persistent effects of early-life injury in males, and sex-dependent differences in the ability of

61 microglial inhibition to prevent the transition to a persistent pain state spans developmental

62 stages.

63   *Impact statement:* Following the same surgery, some patients develop persistent pain.

64   Contributory mechanisms are not fully understood, but early-life experience and sex/gender

65   may influence the transition to chronic pain. Surgery and painful procedural interventions in

66   vulnerable preterm neonates are associated with long-term alterations in somatosensory

67   function and pain that differ in males and females. Surgical injury in neonatal rodents

68   primes the developing nociceptive system and enhances re-injury response in adulthood.

69   Neuroimmune interactions are critical mediators of persistent pain, but sex-dependent

70   differences in spinal neuroglial signaling influence the efficacy of microglial inhibitors

71   following adult injury. Neonatal microglial inhibition has beneficial long-term effects on re-

72   injury response in adult males only, emphasizing the importance of evaluating sex-

73   dependent differences at all ages in pre-clinical studies.

Introduction

74 **Introduction**

75 Early-life stress, adversity and pain can influence neurodevelopmental and health outcomes

76 throughout the lifespan (Nemeroff, 2016; Burke et al., 2017). The need to understand how

77 early-life experience influences chronic pain in later life has been highlighted (Price et al.,

78 2018). In preterm-born neonates, surgery and repeated painful procedures during intensive

79 care are associated with worse neurodevelopmental outcome (Ranger and Grunau, 2014;

80 Hunt et al., 2018), altered brain structure (Duerden et al., 2018), and differences in

81 somatosensory function and pain experience during childhood (Hermann et al., 2006;

82 Walker et al., 2009b) that persist, but with sex differences emerging in young adults (Walker

83 et al., 2018). While analgesia improves acute outcome, the long-term benefit of neonatal

84 analgesic interventions is debated (Walker, 2017; Schiller et al., 2018). Evaluating

85 mechanisms triggered by early-life tissue injury is essential to identify preventive strategies

86 that minimise long-term alterations in pain response.

87 Microglia are critical mediators of normal development, sculpting neuronal circuitry

88 in the developing central nervous system, and being implicated in diverse functions

89 including neurogenesis, synaptic pruning and synaptic plasticity (Kettenmann et al., 2013;

90 Salter and Stevens, 2017). Early-life stress and tissue injury can disrupt microglial sex-

91 dependent maturation or trigger long-term changes in microglial phenotype, altering

92 reactivity to future immune or environmental challenges and influencing responses to

93 physical and psychological stressors and susceptibility to neurological disorders (Perry and

94 Holmes, 2014; Burke et al., 2016; Hanamsagar and Bilbo, 2017). While brain injury

95 secondary to hypoxia/ischemia, hyperoxia, or trauma in neonatal rodents evokes a

96 neuroinflammatory response and increased microglial reactivity, the pathophysiological role

97 of microglia can vary with type of injury, time, and brain region (Hagberg et al., 2015; Salter

98   and Stevens, 2017). Microglial inhibition with minocycline has also been variably reported to

99   have no benefit (Cikla et al., 2016), paradoxically increase acute cell death (Strahan et al.,

100   2017) and worsen long-term function (Hanlon et al., 2017), or improve outcome (Wixey et

101   al., 2011; Schmitz et al., 2014), depending on the assessment method, type and age of brain

102   injury.

103   Plantar hindpaw incision during the first postnatal week in the rat produces activity-

104   dependent alterations in adult sensory threshold and increased hyperalgesia when the paw

105   is re-incised (Walker et al., 2009a; Moriarty et al., 2018). As this enhanced re-incision

106   hyperalgesia is reduced by microglial inhibitors (intrathecal minocycline or p38 inhibitor) in

107   adult males (Beggs et al., 2012; Schwaller et al., 2015), we hypothesized that spinal

108   microglia are involved in priming spinal nociceptive signaling and amplifying the subsequent

109   injury response. Neuroimmune signaling is sexually-dimorphic (Gutierrez et al., 2013; Nelson

110   et al., 2018), and microglial inhibitors in male, but not female, adult rodents reduce pain

111   behaviors following peripheral nerve injury, hindpaw inflammation (Sorge et al., 2011; Sorge

112   et al., 2015; Taves et al., 2016; Mapplebeck et al., 2018), and hyperalgesic priming (Paige et

113   al., 2018). As the efficacy of mechanism-based interventions may differ in males and

114   females, and sex-dependent differences in experimental pain sensitivity and chronic pain

115   prevalence are well-documented in clinical populations (Mogil, 2012; Fillingim, 2017),

116   considering sex as a biological variable (Shansky and Woolley, 2016) is particularly relevant

117   for pre-clinical pain studies (Rosen et al., 2017). In addition, evaluation following neonatal

118   tissue injury is required to identify developmentally-regulated and persistent effects of

119   early-life pain.

120   To further investigate the contribution of spinal microglia to persistent and

121   potentially sex-dependent differences in pain response following early-life injury, we now

6

122   adopted a preventive strategy. Microglial inhibitors (systemic minocycline or intrathecal p38

123   inhibitor SB203580) were administered concurrently with neonatal plantar hindpaw

124   incision, and our primary outcome was the impact on re-incision hyperalgesia in adult male

125   and female rats. To assess incision-induced spinal microglial response, expression of genes

126   related to microglial reactivity and proliferation were assessed in adult males and females.

127   Finally, to determine if re-incision hyperalgesia is restricted to the prior incision site or has a

128   segmental distribution, neonatal incision was performed at different body sites, and

129   hyperalgesia was compared following left hindpaw incision in adulthood.

130

131   **Materials and Methods**

132   *Animals.* All procedures were performed under personal and project licenses approved by

133   the UK Home Office in accordance with the Animal (Scientific Procedures) Act, 1986 or with

134   the approval of the Animal Care Committee of the Hospital for Sick Children, Toronto and in

135   accordance with the Canadian Council on Animal Care. Reporting of results follows the

136   ARRIVE Guidelines (Kilkenny et al., 2010). Behavioral and electrophysiology experiments

137   were performed in the UK with Sprague-Dawley (RRID: MGI:5651135) adult rats and litters

138   of rat pups obtained from the same colony, bred and maintained in-house by the Biological

139   Services Unit, University College London. Handling of rat pups and duration of maternal

140   separation was kept to a minimum with body temperature maintained on a heating blanket.

141   Spinal cord gene expression studies were performed in Toronto, with additional Sprague-

142   Dawley rats obtained from Charles River Laboratories (Boucherville, QC, Canada). All

143   animals were regularly monitored and maintained under standard environmental conditions

144   with food and water available *ad libitum.* All procedures were carried out during the light

145   phase (12 h light/dark cycle, lights on 08:00-20:00 h). Individual litters were reduced to a

146   maximum of 12 pups and weaned into same-sex cages at postnatal day (P) 21. Experimental

147   groups comprised male and female rats distributed across multiple litters and/or adult cage

148   groups (4-5/cage). Each rat was considered an experimental unit except in the case of PCR

149   tissue analysis where two animals were pooled per unit. Rats were randomly selected from

150   the litter or cage, numbered, and then allocated to treatment groups according to a

151   computer-generated randomization code. Animals and tissue samples were coded by an

152   independent colleague to ensure the experimenter was unaware of treatment allocation

153   during behavioral testing or tissue analysis.

154   *Surgical Procedures.* All procedures were performed under isoflurane (Isoflo®,

155   Abbott, UK) anesthesia (2-4% in 1L/min oxygen). Plantar hindpaw incision was chosen as a

156   clinically relevant and established model of surgical injury in infant and adult rodents,

157   (Brennan et al., 1996) with incision of skin and muscle producing acute hyperalgesia and

158   increased spinal excitability at all ages (Ririe et al., 2003; Ririe et al., 2008), and activity-

159   dependent long-term alterations in spinal reflex sensitivity, synaptic signaling and response

160   to re-injury (Walker et al., 2009a; Li et al., 2015; Li and Baccei, 2016). Skin incisions in

161   neonatal (P3) and adult (6-8 weeks age) rats were matched to the relative length of the

162   hindpaw from the midpoint of the heel to the first skin pad as previously described (Beggs

163   et al., 2012), with elevation and longitudinal incision of underlying plantaris muscle using a

164   number 11 blade scalpel. Neonatal non-incision minocycline or saline controls had injections

165   performed with the same depth and duration of anesthesia as incision groups, and the same

166   degree of handling and duration of maternal separation. We have previously shown that the

167   response to adult incision does not differ between littermates with prior neonatal handling

168   and anesthesia and naïve age-matched adults (Beggs et al., 2012).

169   Neonatal incisions were also performed at different sites: ipsilateral (left) hindpaw;

170   contralateral (right) hindpaw; and the left and right forepaw. Forepaw and hindpaw sizes

171   are more comparable in pups than adult rats, and we have previously shown incision at

172   either site produces the same degree of acute hyperalgesia (Walker et al., 2015). As

173   microglial reactivity in the lateral dorsal horn was increased following thigh incision for

174   exposure of the sciatic nerve in adult rats with prior neonatal incision (Beggs et al., 2012),

175   we also performed neonatal incisions on the left anterior thigh based on the skin-muscle

176   incision and retraction model (Flatters, 2008), but without retraction to minimize tissue

177   damage in neonatal animals.

178   All adult incisions were performed on the plantar surface of the left hindpaw.

179   Incisions were closed in rat pups with a single loop of 5-0 silk suture (Mersilk #W595,

180   Ethicon, UK) to produce small stable knots in pups, and with two mattress 5-0 silk sutures in

181   adult animals to standardize the material at both ages. Animals were monitored daily to

182   ensure skin closure remained intact and residual sutures were removed at 5 days. Pups

183   were maintained on a warming blanket and returned to the dam following recovery from

184   anesthesia or between evaluations.

185   *Drug Administration.* All injections of drug or control solutions were performed

186   under brief isoflurane (Isoflo®, Abbott, UK) anesthesia (2-4% in 1L/min oxygen). Minocycline

187   hydrochloride (Sigma-Aldrich, UK Cat# M9511,) was diluted to 4mg/ml in sterile saline and

188   administered by intraperitoneal (i.p.) injection. P3 rats received 45mg/kg minocycline 30

189   min prior to incision, and 22.5mg/kg on day 1 (P4) and 2 (P5) post incision, as neonatal rats

190   have previously been shown to tolerate this dose regime (Buller et al., 2009; Wixey et al.,

191   2011). Control animals received an equivalent volume of saline.

192   The p38 mitogen-activated protein kinase (MAPK) inhibitor 4-(4-fluorophenyl)-2-(4-

193   methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580; EMD Millipore Corporation,

194   Temecula, CA Cat# 19-135) was solubilized in dimethyl sulfoxide (DMSO, Sigma-Aldrich

195   D2650 Hybri-Max® sterile-filtered, PubChem ID 24893703) and then diluted to a final

196   concentration of 0.8mg/ml in 8% DMSO. We have previously shown that intrathecal

197   SB203850 1mg/kg reduces mechanical hyperalgesia and spinal microglial expression of

198   phosphorylated-p38 following hindpaw incision in adult rats (Schwaller et al., 2015). Here,

199   percutaneous low lumbar injections were performed by the same investigator (SMW) as

200   previously described (Walker et al., 2010), with divided doses to match the timing of

201   minocycline experiments: 0.4mg/kg SB203850 (injectate volume 0.5mcl/g) 30 minutes prior

202   to P3 incision, and 0.3mg/kg SB203850 on day 1 and 2 post incision. As the developing

203   spinal cord is susceptible to high local concentrations of some drugs and diluents (Walker

204   and Yaksh, 2012), vehicle control animals received an equivalent volume of 8% DMSO.

205   *Behavioral testing.* In rat pups, hand held calibrated von Frey filaments (0.13g to

206   7.8g) were sequentially applied five times at one-second intervals and the number of

207   evoked flexion reflexes recorded. The maximum force applied was that which evoked five

208   withdrawal responses. A sigmoidal stimulus-response curve was generated for each animal

209   with the midpoint (50% effective force, EF_{50}) calculated as the threshold (Walker et al.,

210   2009a).

211   Adult rats were habituated on an elevated mesh platform for one hour prior to

212   testing. An electronic von Frey device (Dynamic Plantar Aesthesiometer, Cat# 37450, Ugo

213   Basile, Italy) applied increasing pressure to the plantar hindpaw (20g/s to a maximum of

214   50g), and mechanical withdrawal threshold was calculated as the mean of three measures

215   of the force producing brisk hindlimb withdrawal. For thermal latency, animals were

10

216 habituated to the heated glass surface of a modified Hargreaves apparatus (University

217 Anesthesia Research and Development Group, University of California San Diego, La Jolla,

218 CA), and the time for withdrawal from a heat stimulus directed at the mid-plantar paw was

219 recorded (maximum 20 s). The mean of three measures was designated as thermal

220 withdrawal latency.

221   For evaluation of spontaneous locomotor activity, animals were habituated to an

222 open field consisting of a 90cm square dark grey plastic arena (40 cm high) for 20 min on the

223 day prior to adult incision and testing was carried out 48h later (24h post incision). A video

224 camera placed above the open field tracked movement over a 3-min period for subsequent

225 analysis with Ethovision® behavioral tracking software (Version XT 11, Noldus, Wageningen,

226 Netherlands, RRID: SCR_004074).

227   *Electromyography (EMG) recording.* Flexor reflex EMG recordings were performed

228 24 hours after incision in neonatal and adult rats (Walker et al., 2009a). Briefly, animals

229 were anesthetized (2-4% isoflurane in 1L/min oxygen), and the trachea cannulated for

230 mechanical ventilation (Small Animal Ventilator, Harvard Apparatus Ltd., Cambridge, UK).

231 The inspired isoflurane concentration was reduced to 1.75% in P4 pups and 1.25% in adult

232 rats for 20 min to allow equilibration and was maintained at this level to provide stable

233 anesthesia during EMG recordings. The left hindpaw was secured with an adhesive pad on a

234 fixed platform and a bipolar EMG electrode comprising a stainless steel 30-gauge needle

235 with a central copper wire core was placed through a small skin incision into the biceps

236 femoris muscle. Von Frey hairs were applied to the plantar surface of the hindpaw for 1 s,

237 and the EMG response to the mechanical stimulus was processed (Neurolog, Digitimer,

238 Welwyn Garden City, UK) and recorded in 12-s epochs (PowerLab 4S, AD Instruments, Bella

239 Vista, Australia, RRID: SCR_001620). To evaluate responses to both threshold and

240   suprathreshold stimuli, Von Frey hairs were sequentially applied up to a maximum 60 g

241   bending force (von Frey hair number 17) at P4, and 180 g (von Frey hair number 20) in

242   adults, with a minimum of 60 s between stimuli. The duration of the EMG response was

243   outlined from the display of the raw data and the integral of the root mean square (RMS) of

244   the signal was calculated (EMG response)(Chart, Powerlab AD Instruments, Bella Vista,

245   Australia). The EMG response was plotted against the von Frey hair number (mechanical

246   stimulus) and the area under the stimulus-response curve (AUC) calculated to quantify the

247   overall "reflex response" (Walker et al., 2009a).

248   *Tissue Preparation and Analysis.* Rats were terminally anesthetized with

249   pentobarbital (i.p. 100mg/kg, Euthatal, Merial Animal Health Ltd., UK) and transcardially

250   perfused with heparinized saline followed by 4% paraformaldehyde (Fisher Scientific,

251   Loughborough, UK Cat# 10131580). Spinal cords were exposed, and the L4 to L5 spinal

252   segment dissected. Tissue was post-fixed in 4% paraformaldehyde, then cryoprotected in

253   sucrose (30% sucrose, 0.02% sodium azide in 0.1M phosphate buffer).

254   Neonatal L4/L5 spinal 20 micron free floating sections were mounted on SuperFrost®

255   slides (Fisher Scientific Loughborough, UK Cat# 10149870). P4 cords (24h post intervention)

256   were assessed for cell death with Fluoro-Jade C (FJ-C) staining, and P6 cords (3 d post

257   intervention) for microglial cell counts with ionized calcium binding adaptor molecule (Iba1)

258   immunohistochemistry.

259   For Iba1 immunohistochemistry, sections were washed initially and between

260   subsequent steps with phosphate-buffered saline (PBS) containing 0.1% Triton X-100,

261   blocked for 1h at room temperature (5% chicken serum in PBS), and then incubated for 24h

262   with primary goat anti-Iba1 antibody (1:400, AbCam, UK, Cat# ab5076, RRID: AB_2224402)

263   followed by AlexaFluor® 594-conjugated chicken anti-goat IgG (1:200, Invitrogen, USA, Cat#

264   A-21468, RRID: AB_2535871) for 24h at room temperature. Sections were coverslipped with

265   Prolong Gold fluorescent mounting media (Molecular Probes, USA, Cat# P36930, RRID:

266   SCR_015961). Negative controls omitting the primary antibody resulted in a complete

267   absence of positive staining.

268   For Fluoro-Jade C staining, slides were stained in the following sequence: washed in

269   0.1M PB, immersed in 1% sodium hydroxide in 80% ethanol, rinsed with 70% ethanol then

270   distilled water, incubated in 0.06% potassium permanganate for 10 min, stained with

271   0.0002% Fluoro-Jade® C (Millipore, USA, Cat# AG325-30MG) and 0.0001% 4, 6-diamidino-2-

272   phenylindole (DAPI, Molecular Probes, USA, Cat# D1306, RRID: 2629482) prepared in 0.1%

273   acetic acid, and then cleared and coverslipped.

274   Sections were visualized at 10X magnification for fluorescence (Leica DMR,

275   Germany) under FITC or TRITC filters, and images obtained using a Hamamatsu (ORCA-100

276   C4742-95) digital camera. Iba1-positive cells within a standard size region of interest over

277   the medial dorsal horn were counted (Image J software https://imagej.nih.gov/ij/, RRID:

278   SCR_003073, cell-counter plugin). Fluoro-Jade C-positive cells were counted in each

279   quadrant (dorsal: ipsilateral and contralateral; ventral: ipsilateral and contralateral). All

280   counts were averaged for a minimum of 6 sections (Iba1), or summed for 6 sections (FJ-C),

281   per rat and 'n' represents the number of rats per group.

282   Three days following adult incision, animals for qPCR experiments were

283   transcardially perfused with approximately 30ml of RNAlater® (Ambion®, Life Technologies,

284   UK Cat# AM7021), and a cylindrical biopsy tissue punch (2.0 mm internal diameter, Harvard

285   Apparatus, UK Cat# 72-5041) was used to isolate tissue from the ipsilateral L5 medial dorsal

286   horn and stored in RNAlater® until processing.

287   For Quantitative Real Time Polymerase Chain Reaction (RT-PCR), tissue from two

288   animals was pooled for each experimental unit, and total RNA was extracted using a

289   PureLink® RNA mini kit (Ambion®, Life Technologies, Canada, Cat# 12183018A). The

290   quantity, purity and quality of RNA were assessed with an ND-2000 Nanodrop

291   spectrophotometer. Samples were equalized to a concentration of 250ng/20µl by addition

292   of RNAase free water, and RNA extracts were reverse transcribed to cDNA using the

293   SuperScript VILO cDNA kit (Life Technologies, Canada Cat# 11754050). Gene expression of

294   target proteins was determined using commercially available Taqman® gene expression

295   assays (Applied Biosystems, Canada, Cat# 4331182) containing specific forward and reverse

296   target primers and FAM-labelled MGB probes. Assay IDs for the genes investigated were:

297   *Emr1*, Rn01527631_m1; *Irf8*, Rn01762214_m1. qPCR reactions were run with 12.5ng of

298   cDNA and Taqman® Master Mix (Applied Biosystems, Canada, Cat# 4324018) on a StepOne®

299   Plus real-time PCR machine (Life Technologies, Canada, Cat# 4376600, RRID: SCR_015805)

300   using the following parameters: one cycle of 95°C for 20 s, followed by 40 cycles at 95°C for

301   1 s and 60°C for 20 s. Reactions were performed in triplicate, and non-template controls

302   were included in each run. Amplification plots and copy threshold (Ct) values were

303   examined using StepOne® software (Version 2.3,Life Technologies, Canada, RRID:

304   SCR_014281). Expression was normalized to the average of three housekeeping genes

305   (*Abt1*, *Eef2* and *GAPDH*). Relative gene expression was calculated using the ΔΔCt method

306   and data are expressed relative to the naïve or the saline-treated double incision group.

307   *Experimental design.* To assess the long-term impact of microglial inhibition

308   restricted to the time of neonatal incision, and minimize potential disruption of normal

309   development, pharmacological microglial inhibitors were administered 30 minutes prior to

310   incision and at 24 and 48 hours. Male and female P3 rat pups were randomly assigned to

311 four experimental groups: neonatal saline (ns); neonatal minocycline (nm); neonatal saline

312 plus incision (nsIN); and neonatal minocycline plus incision (nmIN) (Figure 1). The number of

313 animals per group was based on our previous studies using similar methodology (Beggs et

314 al., 2012; Schwaller et al., 2015). Several outcomes were compared across treatment

315 groups. 1) Changes in hindlimb reflex withdrawal assessed behavioral hyperalgesia. In

316 neonatal animals, mechanical withdrawal thresholds were measured at baseline on P3, and

317 then 4, 24, 48 and 72h post intervention. At 7-8 weeks of age, baseline mechanical

318 threshold and thermal withdrawal latency were measured prior to and at regular intervals

319 to 21 days after left hindpaw incision (ns-IN; nm-IN; nsIN-IN; nmIN-IN). 2) EMG recordings in

320 anesthetized animals quantified reflex sensitivity 24 h post neonatal or adult incision. 3)

321 Spontaneous locomotor activity was assessed in adults by movement in an open field 24 h

322 following incision. 4) Tissue analysis was performed on lumbar spinal cord. In neonates,

323 sections were collected on P4 for FJ-C staining or on P6 for Iba1 immunohistochemistry. In

324 adults, punch biopsies for qRT-PCR were taken from the medial superficial dorsal horn 3

325 days following adult incision.

326   In additional experiments, male and female P3 rat pups were randomly assigned to

327 three experimental groups: neonatal DMSO vehicle (nv, n=8); neonatal vehicle plus incision

328 (nvIN, n=8); and neonatal intrathecal SB203580 plus incision (nSBIN, n=8 males + 8 females).

329 Mechanical withdrawal thresholds were measured at baseline on P3, and then 4, 24, 48 and

330 72h post neonatal intervention. At 7-8 weeks of age, mechanical thresholds were measured

331 at baseline and at regular intervals to 21 days after left hindpaw incision (nv-IN; nvIN-IN;

332 nSBIN-IN). To evaluate potential tissue effects of repeat intrathecal SB203580 in 8% DMSO,

333 spinal cords were collected for FJ-C staining following P3 injection of 0.4mg/kg 24 hours

334 previously plus 0.3mg/kg 6 hours prior to sacrifice on P4.

335   To assess the anatomical distribution of re-incision hyperalgesia, P3 incision was

336   performed at 5 sites: left or right hindpaw (nIN ipsilateral or contralateral), left anterior

337   thigh (nThi), left or right forepaw (nFor ipsilateral or contralateral) in P3 male rat pups.

338   Littermate controls received the same duration of neonatal anesthesia, handling and

339   maternal separation. Mechanical and thermal hindlimb thresholds were measured in

340   adulthood (7-8 weeks of age) and at regular intervals to 21 days after incision of the left

341   hindpaw in all groups (nIN-IN ipsilateral or contralateral, nThi-IN, nForIN-IN ipsilateral or

342   contralateral). As behavioral responses to hindpaw incision did not differ between males

343   and females, and no additional intervention was performed, these experiments were

344   performed only in males.

345   *Statistical analysis.* Our primary outcome was the impact of neonatal minocycline on

346   re-incision hyperalgesia in adult male and female rats. Based on comparisons of sensory

347   threshold in male and female adult rats from the same in-house colony using the same test

348   protocol (Walker et al., 2015), a sample size of 8 was chosen (80% power at $P<0.01$ for

349   detecting a 20 and 25% difference in mechanical withdrawal threshold in males and females

350   respectively; 80% power at $P<0.05$ for detecting a 35% difference in thermal withdrawal

351   latency). Sensory threshold data is also presented as percentage of baseline [(post-incision

352   threshold)/pre-incision baseline threshold) x 100] plotted against time. To incorporate

353   differences in both the degree and duration of hyperalgesia, the hyperalgesic index for each

354   animal was calculated as the area over the percentage change sensory threshold versus

355   time curve from baseline (0) to 21 days, such that a larger area over the curve represents a

356   greater change from baseline and greater degree and/or duration of hyperalgesia. Based on

357   our previous data (Beggs et al., 2012; Schwaller et al., 2015), a sample size of 8 has 90%

358   power for detecting a 30% difference (P< 0.05) in mechanical hyperalgesic index following

359   adult incision.

360   Behavioral data were normally distributed (D'Agostino and Pearson omnibus

361   normality test), and analyzed by unpaired Student's t-test (baseline thresholds) or three-

362   way analysis of variance (ANOVA) with sex, incision, and drug (minocycline or saline) as

363   factors. Data are graphed separately for males and females and analyzed with 2-way ANOVA

364   with group and sex as variables, and timeline data with repeated measures; time as the

365   within-subjects factor and treatment group as between-subject factors. Dunnett's post *hoc*

366   *tests* were used to assess changes relative to baseline and Bonferroni post *hoc tests* to

367   assess between-group differences, with *p* values adjusted for multiple comparisons.

368   Normalized RT-PCR data was analyzed by two-way ANOVA with sex and drug as factors,

369   followed by Bonferroni *post hoc* tests as appropriate. Cell counts (Iba1, FJ-C) were analyzed

370   by three-way ANOVA (sex, surgery and drug) with Bonferroni *post hoc* tests.

371   For clarity of behavioral timelines, data points are represented as mean +/- SEM. For

372   other outcomes, individual data points are shown with bars representing mean +/- SD. Data

373   were analyzed with GraphPad Prism (Version 7, San Diego, USA, RRID: SCR_002798) or IBM

374   SPSS Statistics (Version 22, Portsmouth, UK, RRID: SCR_002865). *p*<0.05 was considered

375   statistically significant; *p* values are reported in the text apart from very small values below

376   0.001, which is designated as *p* <0.001.

377

378   **Results**

379   **Re-incision hyperalgesia following neonatal incision is equivalent in adult males and**

380   **females**

381   To support our previous finding of enhanced re-incision hyperalgesia following neonatal

382   incision (Beggs et al., 2012; Schwaller et al., 2015), larger groups of males and females were

383   compared to evaluate potential sex-differences in behavioral response. Mechanical

384   withdrawal thresholds following adult incision (nsIN-IN *vs* ns-IN) were influenced by prior

385   neonatal incision ($F_{(1,28)}$ =11.2, p=0.002) but not sex ($F_{(1,28)}$ =1.7, p=0.20); and similarly,

386   thermal withdrawal latency was influenced by prior neonatal incision ($F_{(1,28)}$ = 6.1, p=0.02)

387   but not sex ($F_{(1,28)}$ =0.7, p=0.41). Differences in the degree of hyperalgesia following adult

388   incision were not solely due to alterations in adult baseline values, as nsIN-IN groups had

389   both higher pre-incision and lower post-incision thresholds for mechanical withdrawal

390   threshold (nsIN-IN *vs* nsIN: baseline mean±SD 30.2±5.4 *vs* 24.9±3.1g; [t(29)=3.3, p=0.002];

391   4hrs post-incision (7.4±1.4 *vs* 11.1±1.3g; t(29)=7.6, p<0.001). Differences in thermal latency

392   were less marked (nsIN-IN vs nsIN: baseline 12.1±2.1 *vs* 10.8±1.8 s t(29)=1.9, p=0.056; 4hrs

393   post incision 3.3±1.1 *vs* 4.1±1.1 s; t(29)=2.0, p=0.052). Expression as percentage change

394   from baseline facilitated comparison of the relative change across groups and demonstrated

395   increased hyperalgesia following neonatal incision at time points to 21 days in males and

396   females (nsIN-IN *vs* nsIN; Figures 2*A-D*). Prior neonatal incision increases mechanical and

397   thermal behavioral hyperalgesia to an equivalent degree in males and females.

398

399   **Neonatal perioperative minocycline prevents enhanced re-incision hyperalgesia in adult**

400   **males but not females.**

401   We next evaluated the potential for a neonatal intervention to prevent long-term

402   alterations in injury response. While there was a significant main effect of prior incision in

403   both males ($F_{(1,31)}$ =33.3, p<0.001) and females ($F_{(1,24)}$ =48, p<0.001), minocycline had a

404   significant effect in males ($F_{(1,31)}$ =6.6, p=0.02) but not females ($F_{(1,24)}$ =2.8, p=0.10). In males,

405   neonatal mincocycline prevented re-incision hyperalgesia (nmIN-IN did not differ from ns-

406   IN) and significant differences between nsIN-IN and nmIN-IN groups emerged after 7 days

407   (Figure *2A*). In females, mechanical hyperalgesia did not differ from the re-incision saline

408   group (nmIN-IN vs nsIN-IN) and enhanced hyperalgesia persisted (nmIN-IN vs ns-IN from 7

409   days post-incision; Figure *2B*). Thermal withdrawal latencies similarly showed a main effect

410   of prior incision in both males ($F_{(1, 31)}$ = 38.5, p<0.01) and females ($F_{(1, 24)}$ = 27.0, p<0.001). At

411   time points after 10 days, thermal latency in the male nmIN-IN group differed from nsIN-IN

412   (ie. reduced re-incision hyperalgesia; Figure 2*C*), whereas in females nmIN-IN differed from

413   the ns-IN group (ie. re-incision hyperalgesia persisted; Figure 2*D*). Summary figures for

414   mechanical threshold (Figure 2*E*) and thermal latency (Figure 2*F*) highlight the differences in

415   male and female nmIN-IN groups, with sex-dependent differences particularly apparent 7-

416   10 days after adult re-incision.

417   Neonatal minocycline alone, in the absence of neonatal incision (nm) did not alter

418   the response to adult incision in males or females. The degree and duration of incision-

419   induced hyperalgesia in adulthood did not differ between neonatal minocycline and

420   neonatal saline control groups (nm-IN *vs* ns-IN; Figures 2*A-D*).

421   To provide a composite measure encompassing both the degree and duration of

422   behavioral response, hyperalgesic indices (area over threshold *vs* time 0-21 days) were

423   calculated. For mechanical hyperalgesic index there were significant main effects of incision

424   ($F_{(1,55)}$ =88.1; p<0.001), sex ($F_{(1,55)}$ =9.3, p=0.003) and drug ($F_{(1,55)}$ =9.4, p=0.003). Similarly,

425   thermal hyperalgesic index showed a main effect of incision ($F_{(1,55)}$= 68.1, p<0.001), sex

426   ($F_{(1,55)}$ =7.7, p=0.007) and drug ($F_{(1,55)}$ =4.6, p=0.036)(3-way factorial ANOVA). Re-incision

427   mechanical hyperalgesia was modulated by neonatal minocycline in males (nmIN-IN < nsIN-

428   IN, p=0.002) but in females an enhanced response persisted (nmIN-IN > ns-IN, p=0.008 and

429   nmIN-IN > nm-IN, *p*=0.002)(Figure 2*G*). Similar results were seen with thermal hyperalgesic

430   index in males (nmIN-IN < nsIN-IN, *p*=0.009) and females (nmIN-IN > ns-IN, *p*=0.004 and

431   nmIN-IN > nm-IN, *p*=0.002)(Figure 2*H*).

432   To confirm differences were not restricted to behavioral withdrawal thresholds,

433   reflex sensitivity to threshold and suprathreshold stimuli was quantified by

434   electromyographic recordings in anesthetized animals 24 hours following adult incision.

435   There were significant main effects of incision ($F_{(1, 68)}$ = 87.6, *p*<0.001) and interactions

436   between incision and sex ($F_{(1, 68)}$ =5.8, *p*=0.004) and incision and drug ($F_{(1, 68)}$ =8.8, *p*=0.019).

437   Minocycline at the time of neonatal incision (nmIN-IN) reduced re-incision hyperalgesia in

438   males (nmIN-IN < nsIN-IN, *p*=0.024), but in females reflex sensitivity was enhanced (nmIN-IN

439   > ns-IN, *p*<0.001)(Figure 2*I*).

440   These data demonstrate that while reflex sensitivity is enhanced by prior neonatal

441   incision in both males and females, administering minocycline at the time of neonatal injury

442   prevents the long-term re-incision hyperalgesia in males only, and the same dose is

443   ineffective in females.

444

445   **Adult incision increases spinal cord microglial-specific gene expression, but modulation by**

446   **neonatal minocycline is sex-dependent**

447   As spinal expression of genes associated with microglial proliferation (*Emr1*) and

448   transformation to a reactive phenotype (*Irf8*) increase following peripheral nerve injury in

449   adult rodents (Masuda et al., 2012; Sorge et al., 2015), we first determined if expression of

450   these genes was also increased in the medial superficial dorsal horn following hindpaw

451   incision in adult rodents. Expression was assessed 3 days following incision, as spinal

452   microglial reactivity (Iba1 immunohistochemistry) increased at this time point in adults

453   without prior neonatal injury (Beggs et al., 2012). *Emr1* expression showed a main effect of

454   incision ($F_{(1,26)}$ =30.4, *p*<0.001) but not of sex ($F_{(1,26)}$ =0.4, *p*=0.55) (Figure 3*A*). Similarly, there

455   was a main effect of incision ($F_{(1,26)}$ =21.5, *p*<0.001) but not sex ($F_{(1,26)}$ =4.1, *p*=0.053) on *Irf8*

456   expression (two-way ANOVA; Figure 3*B*).

457   As prior neonatal incision alters the time course, degree and distribution of spinal

458   microglial response (Beggs et al., 2012; Schwaller et al., 2015), effects of neonatal

459   minocycline following adult incision (nmIN-IN) were normalized against the re-incision saline

460   group (nsIN-IN). There were significant effects of sex and sex by drug interactions for both

461   *Emr1* ($F_{(1,27)}$ =5.5, *p*=0.027) and for *Irf8* ($F_{(1,27)}$ =5.7, *p*=0.024). In neonatal minocycline groups,

462   expression following adult re-incision (nmIN-IN) was significantly lower in males than in

463   females for *Emr1* (*p*=0.023; Figure 3*C*) and *Irf8* (*p*=0.019; Figure 3*D*). Therefore, in addition

464   to sex-dependent long-term effects on behavioral hyperalgesia, neonatal minocycline

465   specifically effects the spinal microglial response following re-incision, but in males only.

466

467   **Neonatal intrathecal p38 inhibitor prevents enhanced re-incision mechanical hyperalgesia**

468   **in adult males but not females**

469   As microglial P2X$_4$ receptors are a key point of divergence for sex-dependent responses in

470   neuroglial signaling in adult rodents (Mapplebeck et al., 2018), we also evaluated the effect

471   of inhibition of the downstream p38 MAPK signaling pathway. Prior neonatal incision

472   increased adult-incision induced expression of microglial phospho-p38 and anti-allodynic

473   efficacy of the p38 MAPK inhibitor SB203580 (Schwaller et al., 2015). Here, SB203580 was

474   administered intrathecally at the time of neonatal incision (nSBIN; 1mg/kg in divided doses

475   30 mins pre- and 24 and 48 hours post-incision). In vehicle control animals, prior neonatal

476   incision was again associated with higher baseline mechanical withdrawal threshold in

477   adulthood in both the ipsilateral (nvIN *vs* nv; 29.3 ± 3.6 *vs* 24.1 ± 2.2g; *t* (14)=3.5, *p*=0.010)

478   and contralateral paw, and an increased degree and duration of re-incision hyperalgesia.

479   Changes in mechanical withdrawal threshold following adult incision (nvIN-IN *vs* nv-IN) were

480   influenced by time ($F_{(7,84)}$ = 47, *p*<0.001) and prior neonatal incision ($F_{(1,12)}$ =36, *p*<0.001). As

481   there was no main effect of sex ($F_{(1,12)}$ =0.48, *p*=0.5) or sex by group interaction ($F_{(1,12)}$ = 0.3,

482   *p*=0.6), male and female data were combined in subsequent analyses of nv-IN and nvIN-IN

483   groups (Figure 4).

484   Following neonatal incision with intrathecal SB203580 (nSBIN), baseline mechanical

485   withdrawal thresholds in adulthood were higher and more variable in females than males

486   (35.1 ± 8.9g *vs* 25.3 ± 1.2g; *t* (14)=3.1, *p*=0.01). Despite this higher baseline, raw mechanical

487   withdrawal thresholds following re-incision (nSBIN-IN) were significantly lower in females

488   than males at time points from 7 to 21 days post-incision (*p*<0.05; two-way repeated

489   measures ANOVA with Bonferroni *post-hoc* comparisons). Expression as percentage change

490   from baseline facilitated comparison across all groups (nv-IN *vs* nvIN-IN *vs* nSBIN-IN males *vs*

491   nSBIN-IN females; *n*=8 per group; Figure 4). There was a significant main effect of group (*F*

492    $_{(3,28)}$=29, *p*<0.001) with differences between male and female nSBIN-IN groups initially at 3

493   days (*p*=0.02) that were more marked from 7 to 21 days post incision (*p*<0.001; two-way

494   repeated measures ANOVA with Bonferroni *post-hoc* comparisons; Figure 4*A*). Neonatal

495   microglial inhibition with intrathecal SB203580 prevented re-incision hyperalgesia in males

496   (nSBIN-IN males *vs* nvIN-IN, *p*<0.001) and this group did not differ from adults without prior

497   incision (nsSBN-IN males *vs* nv-IN, *p*=0.9). By contrast, in females re-incision hyperalgesia

498   was evident (nSBIN-IN females *vs* nv-IN, p<0.001) and values did not differ from the vehicle

499   re-incision group (nSBIN-IN females *vs* nvIN-IN, *p*=0.4).

500   The composite measure of mechanical hyperalgesic index (0-21 days) similarly

501   highlighted a main effect of prior neonatal incision ($F_{(1,26)}$ = 27, $p<0.001$), sex ($F_{(1,26)}$ =14,

502   p=0.002), and sex by drug interaction ($F_{(1,26)}$ =13, p=0.001). Enhanced re-incision

503   hyperalgesia (nv-IN *vs* nvIN-IN, $p<0.001$) was prevented by neonatal SB203580 in males

504   (nSBIN-IN males *vs* nvIN-IN, p <0.001), but was still evident in females (nSBIN-IN females *vs*

505   nv-IN, p <0.001)(Figure 4*B*).

506

507   **Enhanced hyperalgesia is not restricted to re-incision of the same paw**

508   Neonatal incision produces baseline *hypo*algesia and re-incision unmasks *hyper*algesia in

509   adulthood. We have previously shown that elevated baseline thresholds have a generalized

510   distribution, with enhanced descending inhibition from the rostroventral medulla

511   influencing reflex sensitivity irrespective of prior incision on the ipsi- or contralateral

512   hindpaw or forepaw (Walker et al., 2015). Hindpaw carrageenan inflammation in the first

513   postnatal week, but not at older ages, is similarly associated with generalized hypoalgesia in

514   adulthood, whereas an enhanced hyperalgesic response is restricted to re-inflammation of

515   the same, but not contralateral hindpaw (Ren et al., 2004). As we have previously assessed

516   re-incision in the same paw only, we now evaluated the degree and distribution of

517   *hyper*algesia following neonatal incision at different body sites. The same length of initial

518   incision was performed either on the left or right hindpaw (nIN ipsilateral or contralateral),

519   left anterior thigh (nThi), left or right forepaw (nFor ipsilateral or contralateral) at P3, and

520   we have previously shown that forepaw and hindpaw incisions produce similar acute

521   hyperalgesia at this age (Walker et al., 2015). Hindlimb reflex thresholds were then assessed

522   at baseline and following incision of the left hindpaw in adulthood (Figure 5). As our

523   previous experiments had shown no difference in behavioral response in males and

524   females, these experiments were performed in males only.

525   At 6-7 weeks of age, baseline mechanical withdrawal threshold in the left hindpaw

526   was significantly altered following prior neonatal incision (main effect of group $F_{(5,50)}$ =5.7,

527    $p<0.001$) with thresholds higher following prior incision in all sites, apart from the

528   contralateral forepaw; Figure 5*A*). Thermal latency was increased following all prior

529   neonatal incisions with a main effect of group ($F_{(5,50)}$=5.9, $p<0.001$); Figure 5*B*).

530   In all adults, the left hindpaw was incised to facilitate comparison across groups

531   (Figure 4). Mechanical withdrawal thresholds and thermal latency were plotted against time

532   and expressed as percentage change from baseline (data not shown) for calculation of

533   hyperalgesic indices. The mechanical hyperalgesic index (0-21 days) was increased following

534   prior ipsilateral (na-IN *vs* nIN-IN, nThi or nFor-IN; all $p<0.001$) but not contralateral incision

535   (contralateral hindpaw nIN-IN, $p=0.07$; contralateral forepaw nFor-IN, $p=0.9$)(one way

536   ANOVA with Dunnett's comparison to na-IN). Thermal data demonstrated enhanced

537   hyperalgesia following prior incision of the same paw and ipsilateral thigh (na-IN *vs* nIN-IN

538   or nThi, $p<0.001$), but not ipsilateral forepaw ($p=0.14$) or contralateral hindpaw ($p=0.35$) or

539   forepaw ($p=0.16$). The relative changes in hyperalgesic index highlight that increased

540   mechanical hyperalgesia was maximal with re-incision in the same paw (nIN-IN *vs* IN

541   mean±SD: 97±25% increase), but also increased following prior ipsilateral anterior thigh

542   (37±15%) or ipsilateral forepaw (35±11%) incision (Figure 5*C*). Similarly, thermal

543   hyperalgesia was enhanced following prior ipsilateral incision, with maximal effect when the

544   same hindpaw was incised (82±28%), but prior contralateral hindpaw incision had no effect

545   (Figure 5*D*). In adults with prior neonatal incision, we have previously shown that enhanced

546   hyperalgesia and spinal microglial reactivity is independent of peripheral re-injury and can

547   be induced by lateral thigh incision and tibial nerve stimulation (Beggs et al., 2012), and

548   these current data further support a role for segmentally restricted spinal mechanisms in

549   the primed response to injury following neonatal surgical incision.

550

551   **Neonatal incision produces acute hyperalgesia and a spinal microglial response in male**

552   **and female rat pups**

553   To evaluate acute effects of microglial inhibition and incision, we also present data from the

554   neonatal period. Plantar incision at P3 acutely reduced mechanical withdrawal threshold

555   with lower mechanical withdrawal threshold 4 hours after incision in males (nsIN < ns,

556   p=0.011; nmIN < ns, p=0.045; Figure 6*A*) and females (nsIN < ns, p=0.046; nmIN < ns,

557   p=0.003; two-way repeated measures with Bonferroni post-hoc comparisons; Figure 6*B*).

558   Withdrawal thresholds in non-incised saline and minocycline groups did not differ at any

559   time point. Reflex sensitivity to both threshold and more intense suprathreshold mechanical

560   stimuli (quantified by EMG response 24 hours post P3 incision) showed a main effect of

561   treatment group (F $_{(3,55)}$ =10.4, p <0.001), but not sex (F $_{(1,55)}$ =1.6, p=0.21)(two-way ANOVA

562   with sex and group as variables; Figure 6*C*). Minocycline did not prevent acute hyperalgesia

563   in incised rats, and values did not differ between minocycline alone and saline controls

564   (Figures 6*A-C*). This suggests that effects of systemic minocycline are not due to the non-

565   specific acute peripheral anti-inflammatory effects shown with higher doses of systemic

566   minocycline in adult animals (Beggs et al., 2012).

567   Four hours following neonatal incision, mechanical withdrawal thresholds were

568   reduced from baseline in intrathecal vehicle (nvIN, p=0.04) and female SB203580 groups

569   (nSBIN, p=0.04) but to a reduced degree in males (nSBIN males, p=0.38; two-way repeated

570   measures with Bonferroni *post-hoc* comparisons). Overall, values did not differ significantly

571   across groups at each time point, and the normal developmental increase in mechanical

572   withdrawal threshold with postnatal age was evident (P6 > P3 baseline, all groups, $p<0.001$).

573   Three days following neonatal incision, analysis of the number of Iba1-positive cells

574   in the medial superficial ipsilateral dorsal horn (Figure 6*D*) demonstrated a main effect of

575   incision ($F_{(1,48)}$ =25.3, $p<0.001$) but not sex ($F_{(1,48)}$ =3.4, $p=0.07$) or drug ($F_{(1,48)}$ =0.01, $p=0.72$).

576   Minocycline did not prevent incision-induced increases in Iba1-positive cell counts in males

577   or females, although analysis was limited by variability in this outcome (Figure 5*E*).

578   In   neonatal   rodents,   normal   developmental   neuronal   apoptosis   occurs

579   predominantly in the dorsal horn of the spinal cord (Lowrie and Lawson, 2000), but can be

580   increased by injury and anesthesia/analgesia (Walker and Yaksh, 2012; Chiarotto et al.,

581   2014), and in the developing brain, systemic minocycline has been reported to paradoxically

582   increase brain cell death in an age- (Arnoux et al., 2014), and dose-dependent manner (5

583   fold increase in somatosensory cortex following 5 x 45mg/kg between P3 to P5)(Strahan et

584   al., 2017). Therefore, we used FJ-C staining to assess cell death 24 hours following P3

585   interventions. At P4, FJ-C counts were higher in the dorsal versus ventral horn (ns 28±7 *vs*

586   9±4; mean±SD, summed from 6 sections per animal). In the ipsilateral dorsal horn, FJ-C cell

587   counts showed a main effect of incision ($F_{(1,56)}$ =26.7, $p<0.001$) but not sex ($F_{(1,56)}$ =0.13,

588    $p=0.72$) or minocycline administration ($F_{(1,56)}$ =0.18, $p=0.68$)(Figure 6*G*). FJ-C cell counts

589   increased 24 h following incision in males (nsIN *vs* ns: 41±14 *vs* 25±7) and females (nsIN *vs*

590   ns: 43±9 *vs* 31±10). This relative increase (40-60%) was lower than following intrathecal

591   ketamine doses at P3 (>300% increase) that were also associated with long-term alterations

592   in adult hindlimb sensory thresholds and gait (Walker et al., 2010). Dorsal horn FJ-C counts

593   following 2 intrathecal doses of SB203580 in 8% DMSO did not significantly differ from

594   saline or minocycline non-incision groups (nSB *vs* ns *vs* nm: 33±5 *vs* 28±7 *vs* 29±7, $p=0.36$).

595   Therefore, the pharmacological interventions used here did not cause paradoxical cell death

596   in the neonatal spinal cord.

597   To exclude effects of injury or minocycline on growth and sensorimotor function,

598   body weight and spontaneous locomotor activity were measured in adulthood. Males were

599   heavier than females (mean ± SD: 312 ± 17 g *vs* 218±21 g; $t(59)=19.2$, $p<0.001$), but within

600   sexes, weight did not differ markedly across treatment groups (data not shown).

601   Spontaneous locomotor activity was assessed by distance travelled during 3 minutes in a 90

602   x 90cm open field 24 hours following adult incision. Males were less active than females

603   (distance travelled mean±SD: 11.9±3.8 m *vs* 14.1±4.8 m) resulting in a main effect of sex ($F_{(1,}$

604    $_{64)}$ = 6.14, 0.016), but there was no effect of incision ($F_{(1, 64)}$ = 0.06, $p=0.81$) or minocycline

605   ($F_{(1, 64)}$ = 2.71, $p=0.81$) on distance travelled.

606

607   **Discussion**

608   Prior neonatal incision has a long-term impact on somatosensory processing, and enhanced

609   post-surgical hyperalgesia following adult re-incision is abolished by microglial inhibitors in

610   adult males (Beggs et al., 2012; Schwaller et al., 2015). We now demonstrate persistent

611   sexually-dimorphic effects following microglial inhibition in early development: neonatal

612   peri-incision minocycline prevents re-incision hyperalgesia only in adult males, and dorsal

613   horn genes related to microglial function are down-regulated in males but up-regulated in

614   females. MAPK signaling is involved as neonatal intrathecal SB203850 also prevented re-

615   incision hyperalgesia in males only. Following neonatal incision at different sites, adult re-

616   incision hyperalgesia is restricted to prior ipsilateral injury and is maximal when the same

617   paw is re-injured, supporting a segmentally-restricted spinal mechanism.

618   Hindpaw incision produces equivalent acute hyperalgesia in male and female rat

619 pups, and enhanced hyperalgesia following subsequent adult re-incision is also independent

620 of sex. In adults, spinal microglial inhibition selectively minimized re-incision hyperalgesia at

621 doses that were ineffective following adult-only incision (Beggs et al., 2012; Schwaller et al.,

622 2015), but experiments were predominantly in males. Sexually-dimorphic responses to

623 microglial inhibition in adult rodents follow peripheral nerve injury and inflammation (Sorge

624 et al., 2015; Mapplebeck et al., 2018), hindpaw formalin (Taves et al., 2016), and

625 hyperalgesic priming to prostaglandin E2 (Paige et al., 2018). Here, the key finding is that

626 microglial inhibition with systemic minocycline or intrathecal SB203580 at the time of

627 neonatal injury has a long-term preventive effect: modulating re-incision hyperalgesia in

628 males only, with significant sex-dependent group differences following adult incision. These

629 data suggest the transition from acute to persistent post-incision pain state is mediated by

630 different mechanisms (Echeverry et al., 2017; Price et al., 2018), and more effectively

631 modulated by neonatal microglial inhibition in males.

632 Sex-dependent responses to microglial inhibition following tissue injury in adult rodents are

633 spinally-mediated (Taves et al., 2016; Mapplebeck et al., 2018). While intrathecal LPS

634 induced mechanical allodynia only in adult males, intracerebroventicular or intraplantar LPS

635 produced equivalent allodynia in both sexes (Sorge et al., 2011). However, there has been

636 limited evaluation of age-dependent changes in microglial function in the spinal cord.

637 Compared to brain microglia, spinal microglia have a reduced *in vitro* inflammatory

638 response to LPS (Baskar Jesudasan et al., 2014). Behavioral and microglial responses in

639 juvenile rodents also vary with type of injury. In male P10 rats, intrathecal LPS but not

640 spared nerve injury produced acute hyperalgesia and increased spinal microgliosis, and age-

641 dependent shifts between anti-inflammatory and pro-inflammatory spinal microglial

642 responses influenced the delayed emergence of behavioral allodynia following nerve injury

643 (Moss et al., 2007; McKelvey et al., 2015). Plantar incision induces microgliosis in the

644 ipsilateral dorsal horn in both neonatal and adult rats, and prior incision increases the

645 degree, duration and distribution of the adult response (Beggs et al., 2012). To investigate

646 potential sex differences in the underlying molecular pathway, we first confirmed that

647 hindpaw incision upregulated *Emr1* a marker of microglial proliferation, and *Irf8* a

648 transcription factor critical for adoption of a reactive phenotype (Masuda et al 2012) in the

649 ipsilateral dorsal horn of adult males and females. The response following neonatal

650 microglial inhibition in the re-incision groups was sex-dependent, with *Emr1* and *Irf8*

651 upregulated in minocycline-treated females, but downregulated in males. Spinal $P2X_4R$-

652 signaling pathways underlie sexually-dimorphic effects to microglial inhibitors in adults

653 (Sorge et al., 2015; Taves et al., 2016; Mapplebeck et al., 2018), and we now demonstrate a

654 role for downstream MAPK signaling in the long-term preventive effects following neonatal

655 incision in males, but not females.

656 Directly activating spinal microglia by intrathecal LPS produces testosterone-

657 dependent allodynia in male but not female mice (Sorge et al., 2011) and manipulating sex

658 hormone levels also alters efficacy of microglial inhibitors which become ineffective in

659 castrated males (Sorge et al., 2015). Microglia in the developing and adult mouse brain

660 show sex-specific transcriptomic and proteomic differences that can be influenced by, or

661 independent of, circulating sex hormones (Hanamsagar et al., 2017; Guneykaya et al., 2018;

662 Nelson et al., 2018; Villa et al., 2018). The molecular mechanisms underlying this sex

663 dichotomy are not well-established (Villa et al., 2018), and following brain injury the

664 response to microglial inhibitors varies across studies. Minocycline efficacy following

665 traumatic injury at P11 varied across brain regions, but sex had minimal impact (Hanlon et

666 al., 2017). While minocycline improved outcome following brain hypoxia/ischemia in adult

667 males only (Spychala et al., 2017), benefit in P3 rats has not been separately assessed in

668 males and females (Wixey et al., 2009; Wixey et al., 2011). Using a similar repeat dose

669 regimen, that was well-tolerated and improved outcome in rat pups, minocycline alone did

670 not alter spinal reflex sensitivity or cell death in male or female rat pups. Peri-incision

671 minocycline did not block acute injury-induced microgliosis or hyperalgesia following

672 neonatal incision, suggesting doses were insufficient to produce anti-hyperalgesic effects

673 seen with high systemic doses in adults (Beggs et al., 2012). Nevertheless, systemic

674 minocycline modulated priming by neonatal incision, producing long-term preventive

675 effects on re-incision hyperalgesia in adult males, but not females; and more selective

676 inhibition with intrathecal SB203580 produced the same sexually-dimorphic effects.

677   Priming of microglial responses may reflect intrinsic phenotypic changes with

678 exaggerated responses to subsequent challenges (Perry and Holmes, 2014; Burke et al.,

679 2016), and perinatal insults can alter the normal sex-dependent trajectory of microglial

680 development (Hanamsagar et al., 2017). Microglia have multiple roles in normal activity-

681 dependent refinement of sensory system circuitry (Salter and Stevens, 2017). Induction of

682 long-term changes in re-incision response is both developmentally-regulated and activity-

683 dependent. Blocking primary afferent input at the time of neonatal incision prevents early

684 alterations in glutamatergic signaling (Li et al., 2009) and subsequent re-incision

685 hyperalgesia (Walker et al., 2009a; Moriarty et al., 2018). Neonatal plantar incision produces

686 long-term increased gain in spinal nociceptive circuitry (Li et al., 2015; Li and Baccei, 2018),

687 including increased monosynaptic input from low-threshold mechanoreceptors onto spinal

688 projection neurons (Li and Baccei, 2016). As postnatal refinement of A-fiber distribution

689 (Beggs et al., 2002) and maturation of local inhibitory circuitry in the spinal dorsal horn

690   (Baccei and Fitzgerald, 2004; Koch et al., 2012) are sensitive to altered afferent input,

691   microglial involvement in developing normal sensory circuits may also be influenced by

692   injury-induced alterations in microglial reactivity.

693   A key consideration is whether priming by neonatal incision is dependent upon both

694   neonatal and adult surgeries being performed at the same site. Adult incision rapidly

695   induced extracellular signal-related kinase phosphorylation in spinal dorsal horn neurons,

696   but the distribution and degree was not influenced by prior neonatal incision (Schwaller et

697   al., 2015). Effects are also not dependent on peripheral re-injury, as tibial nerve electrical

698   stimulation increased the degree of hyperalgesia and microglial reactivity in adults with

699   prior neonatal incision. Microglial reactivity extended beyond the somatotopic afferent field

700   of the initial hindpaw injury, with enhanced microgliosis related to the ipsilateral mid-thigh

701   incision required to expose the nerve (Hathway et al., 2009; Beggs et al., 2012), and

702   microglial phospho-p38 expression in adults with prior neonatal incision was also more

703   extensive (Schwaller et al., 2015). While enhanced hyperalgesia was greatest when neonatal

704   and adult incisions were at the same location, behavioral responses to adult hindpaw

705   incision were also primed following neonatal incision at other ipsilateral but not

706   contralateral sites; supporting a segmental mechanism that differs from effects on baseline

707   threshold. Following neonatal hindpaw incision or inflammation, baseline hypoalgesia

708   emerges after the fourth postnatal week and is generalized to ipsilateral and contralateral

709   paws in adulthood (Ren et al., 2004; Walker et al., 2015; Moriarty et al., 2018). We

710   hypothesize these phenomena are mediated by two distinct mechanisms, with centrally-

711   mediated increased descending inhibition from the rostral ventromedial medulla

712   contributing to generalized hypoalgesia (Zhang et al., 2010; Walker et al., 2015), while the

713   restricted ipsilateral distribution of re-incision hyperalgesia is spinally-mediated.

714   The present study provides further evidence for the role of microglia in persistent

715 effects of early-life injury and the transition from acute to chronic pain following

716 subsequent injury. In addition, we identify a novel preventive mechanism: pharmacological

717 microglial inhibition at the time of neonatal injury prevented subsequent re-incision

718 hyperalgesia in a sex-dependent manner. Following preterm birth, male sex is an

719 independent risk factor for adverse neurodevelopmental outcome (Linsell et al., 2018), but

720 repeated procedural pain exposure has a greater impact on brain volume and connectivity

721 in females (Schneider et al., 2018), and both somatosensory function and pain experience

722 differ in young adult males and females born extremely-preterm (Walker et al., 2018). In

723 later life, chronic pain conditions are more prevalent in females (Mogil, 2012; Fillingim,

724 2017). From a clinical perspective, our data highlight the need to consider early-life

725 experience when assessing risk for persistent pain in later life, and to compare efficacy in

726 males and females enrolled in clinical trials of microglial inhibitors (Tong et al., 2012). In line

727 with NIH Federal Pain Research Strategy priorities (Price et al., 2018), our data identify an

728 important contribution of early-life experience to pain in later life, and further highlight the

729 importance of sex as a biologic variable when evaluating mechanism and efficacy of

730 therapeutic interventions in preclinical pain research.

731

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Figure Legends

Figure 1: Schematic of experimental design**.** Treatment groups included: neonatal saline, ns; neonatal minocycline, nm; neonatal saline and incision, nsIN; neonatal minocycline and incision, nmIN. Injections were performed on postnatal day (P) 3, 4 and 5. All animals the underwent incision in adulthood (ns-IN, nm-IN, nsIN-IN, nmIN-IN). Evaluations included: measures of reflex sensitivity with mechanical withdrawal threshold, thermal withdrawal latency and electromyography (EMG) recordings; spontaneous activity in open field; neonatal spinal tissue analysis with Fluoro-Jade C (FJ-C) staining and Iba1 immunohistochemistry; and spinal gene expression with Quantitative Real Time Polymerase Chain Reaction (RT-PCR) following adult incision. In additional experiments, treatment groups included intrathecal injection at the same neonatal time points of 8% DMSO vehicle (nv), and neonatal incision with vehicle (nvIN) or SB203580 (nSBIN). Mechanical withdrawal thresholds were compared following incision 6-7 weeks later (nv-IN *vs* nSBIN-IN *vs* nvIN-IN).

Figure 2: Mechanical and thermal hyperalgesia following adult re-incision is sex-dependently influenced by minocycline at the time of neonatal incision. *A-D,* Changes in behavioral thresholds following incision are normalized as percentage change from baseline (adult pre-incision). Data points are mean ± SEM; *n* =8-9 animals per group analyzed by two-way repeated measures ANOVA with Bonferroni *post-hoc* comparisons. Mechanical withdrawal threshold (MWT) in male *(A)* and female (*B*) rats and thermal withdrawal latency (TWL) in male (*C*) and female (*D*) rats are plotted against time points to 21 days post-incision. Hyperalgesia is enhanced by prior incision (nsIN-IN vs nsIN; ***$p<0.001$; **$p<0.01$, *$p<0.05$) in both males and females. In male rats (*A*), neonatal minocycline treatment significantly attenuated the enhanced mechanical hyperalgesia from 7 to 21 days

950   (nmIN-IN *vs* nsIN-IN: §§ *p*<0.01, § *p*>0.05). In females (**B**) mechanical hyperalgesia following

951   re-incision was enhanced 7-21 days following incision despite neonatal minocycline (nm-IN

952   *vs* nmIN-IN: [###]*p*<0.001, [##]*p*<0.01, [#]*p*<0.05). Differences in thermal latency in males (**C**) and

953   females (**D**) were less marked but followed the same overall pattern with neonatal

954   minocycline attenuating re-incision hyperalgesia in males but not females. **E,F,** Summary

955   figures of mechanical (**E**) and thermal (**F**) hyperalgesia highlight sex-dependent differences

956   following neonatal incision with minocycline (nmIN-IN). Within the ns-IN and nsIN-IN

957   groups, data did not differ between males and females and are combined to minimize

958   overlap in the figure. The impact of prior neonatal incision is highlighted by the clear

959   separation in both the degree and duration of hyperalgesia (ns-IN *vs* nsIN-IN). In males,

960   minocycline at the time of neonatal incision prevents adult re-incision hyperalgesia (♂

961   nmIN-IN differs from nsIN-IN) and this group more closely approximates animals with no

962   prior neonatal injury (ns-IN). In females, minocycline at the time of neonatal incision (♀

963   nmIN-IN) has no effect; enhanced adult re-incision hyperalgesia persists and this group

964   approximates the nsIN-IN group. **G,H,** Behavioral data are expressed as the hyperalgesic

965   index (area over the curve to 21 days post-incision) of mechanical threshold (**G**) or thermal

966   latency (**H**). Re-incision hyperalgesia is apparent in males and females (nsIN *vs* nsIN-IN:

967   *********p*<0.001, *******p*<0.01) and is reduced by neonatal minocycline in males only (nsIN-IN *vs*

968   nmIN-IN: §§ *p*<0.01). In females, enhanced re-incision hyperalgesia persists despite

969   minocycline (nmIN *vs* nmIN-IN: ## *p*<0.01). Data points are individual animals (*n*=8-9 per

970   group) with bars = mean ± SD analyzed by 2-way ANOVA with Bonferroni *post-hoc*

971   comparisons. **I,** Reflex sensitivity 24 hours following adult incision quantified as the area

972   under the stimulus (hindpaw mechanical von Frey hair) versus response curve

973   (electromyography recording biceps femoris; EMG area under curve reflex response)

974   demonstrates re-incision hyperalgesia in males and females (nsIN-IN>ns-IN **p<0.01).

975   Neonatal minocycline (nmIN-IN) reduced the re-incision response in males only (nmIN-IN <

976   nsIN-IN §P<0.05), but enhanced hyperalgesia persisted in females (nmIN-IN > nsIN

977   **P<0.001). Data points are individual animals (n=9-10 per group) and bars = mean ± SD

978   analyzed by 2-way ANOVA with sex and group as variables and Bonferroni *post-hoc*

979   comparisons. Groups = ns-IN, neonatal saline plus adult incision; nm-IN, neonatal

980   minocycline plus adult incision; nsIN-IN, neonatal saline and incision plus adult re-incision;

981   nmIN-IN, neonatal minocycline and incision plus adult re-incision.

982

983   **Figure 3:** Incision, neonatal minocycline and sex influence expression of microglial related

984   genes in the medial ipsilateral dorsal horn. *A,* Expression of *Emr1* increased 3 days following

985   single adult incision (IN) in males and females. *B,* Expression of *Irf8* increased following IN.

986   *A,B,* Data normalized to age- and sex-matched naïve rats. *C*, Expression of *Emr1* was lower

987   in males than females following neonatal minocycline and re-incision (nmIN-IN). Male nmIN-

988   IN *vs* female nmIN-IN, p=0.023. *D*, Expression of *Irf8* was lower in males than females

989   following neonatal minocycline and re-incision (male nmIN-IN *vs* female nmIN-IN p=0.019).

990   *C,D,* nmIN-IN data normalized to saline repeat incision (nsIN-IN). *A,B,C,D,* Data points are

991   individual units with each including 2 animals (n = 6-9 units per group); Bars = mean ± SD.

992   *p<0.05,**p<0.01,***p<0.001 analyzed by two-way ANOVA with sex and group as variables

993   and Bonferroni *post-hoc* comparisons.

994

995   **Figure 4.**   Mechanical hyperalgesia following adult re-incision is sex-dependently

996   influenced by intrathecal SB203580 at the time of neonatal incision. *A,* Changes in

997   mechanical withdrawal threshold following incision are normalized as percentage change

998   from baseline (adult pre-incision). Within the nv-IN and nvIN-IN groups, data did not differ

999   between males and females, and are combined. The impact of prior neonatal incision is

1000   highlighted by the clear separation in both the degree and duration of hyperalgesia (nv-IN *vs*

1001   nvIN-IN; **$p<0.001$). In males, SB203580 at the time of neonatal incision attenuated the

1002   enhanced re-incision mechanical hyperalgesia from 7 to 21 days (♂ nSBIN-IN *vs* nvIN-IN: §§

1003 $p<0.001$, § $p>0.05$). In females, neonatal SB203850 has no effect; enhanced adult re-incision

1004   hyperalgesia persists 3 to 21 days following incision (♀ nSBIN-IN *vs* nv-IN: ##$p<0.001$). Data

1005   points are mean ± SEM; $n=8$ animals per group; analyzed by two-way repeated measures

1006   ANOVA with Bonferroni *post-hoc* comparisons. **B,** The mechanical hyperalgesic index (area

1007   over the curve 0 to 21 days post-incision) identifies enhanced re-incision hyperalgesia (nv-IN

1008   *vs* nvIN-IN, **$p<0.001$) that is reduced by neonatal SB203580 in males only (nSBIN-IN males

1009   *vs* nvIN-IN, §§ $p <0.01$). In females, enhanced hyperalgesia persists (nSBIN-IN female *vs* nv-

1010   IN, ## $p <0.01$). Data points are individual animals ($n=8$ per group) with bars = mean ± SD

1011   analyzed by 2-way ANOVA with Bonferroni *post-hoc* comparisons.

1012

1013   **Figure 5.**   Distribution of baseline hypoalgesia and re-incision hyperalgesia in adults

1014   differs following neonatal incision. **A,** The schematic demonstrates different ipsilateral or

1015   contralateral incision sites (nIN, hindpaw; nThi, thigh; nFor, forepaw) performed in neonatal

1016   (postnatal day 3) animals, that are followed by incision of the left hindpaw in adulthood. **B,**

1017   Mechanical withdrawal thresholds of the left hindpaw in young adult rats were higher than

1018   neonatal anesthesia (na) controls following neonatal incision of the ipsilateral hindpaw

1019   ($p<0.001$), thigh ($p=0.003$) and forepaw (nFor $p=0.002$) and contralateral hindpaw ($p=0.015$)**.**

1020   **C,** Thermal withdrawal latency of the left hindpaw was prolonged following prior incision at

1021   all sites (na *vs* nIN $p=0.006$, *vs* nThi $p<0.001$, *vs* nFor $p=0.033$, *vs* contralateral nIN $p=0.003$,

1022   *vs* contralateral nFor *p*=0.001). ***D,*** Mechanical hyperalgesic index (HI) following adult incision

1023   (area over behavioral withdrawal curve versus time to 21 days post incision) was increased

1024   by prior ipsilateral incision. ***E,*** Thermal HI was similarly increased following prior ipsilateral

1025   hindpaw or thigh incision, but not contralateral hindpaw incision. Forepaw incisions did not

1026   significantly alter thermal HI. ***B-E,*** Data are presented for individual animals (*n*=8-10 per

1027   group), with bars = mean ± SD; ***p<0.001, **p<0.01, *p<0.05 analyzed by one-way ANOVA

1028   with Dunnett's comparison to neonatal anesthesia (na: ***A,B***) or neonatal anesthesia plus

1029   adult incision (na-IN: ***C,D,***).

1030

1031   **Figure 6.**   Acute neonatal effects of incision and/or minocycline. ***A,B,*** Mechanical

1032   withdrawal threshold (MWT) is reduced 4 hours following neonatal saline and incision (nsIN)

1033   in male (***A***) and female (***B***) rat pups compared to non-incised saline (ns) and minocycline

1034   (nm) controls. Minocycline at the time of neonatal incision (nmIN) has no effect. Data are

1035   means ± SEM (*n*=6 ns animals, *n*=10 all other groups); ns *vs* nsIN: *p<0.05; ns *vs* nmIN:

1036   [##]p<0.01, [#]p<0.05 analyzed by 2-way repeated measures ANOVA with Bonferroni *post-hoc*

1037   comparisons. ***C,*** Twenty-four hours following P3 interventions, reflex sensitivity was

1038   quantified as the area under the curve (AUC) of the stimulus (von Frey hair to hindpaw)

1039   versus biceps femoris electromyography (EMG) response. Data points are individual animals

1040   (*n*=7-9 per group) with bars = mean ± SD. Male nmIN > ns *p*=0.043, nmIN> nm *p*=0.035;

1041   Female nsIN > ns *p*=0.007, nmIN> ns *p*=0.008 analyzed by 2-way ANOVA with Bonferroni

1042   *post-hoc* comparisons. ***D,*** Representative low- and high-power images of the dorsal horn of

1043   male and female rats 3 days following incision with perioperative saline (nsIN) or

1044   minocycline (nmIN). Bar= 210 micron. ***E,*** Iba1+ve cells within a fixed region of interest (ROI)

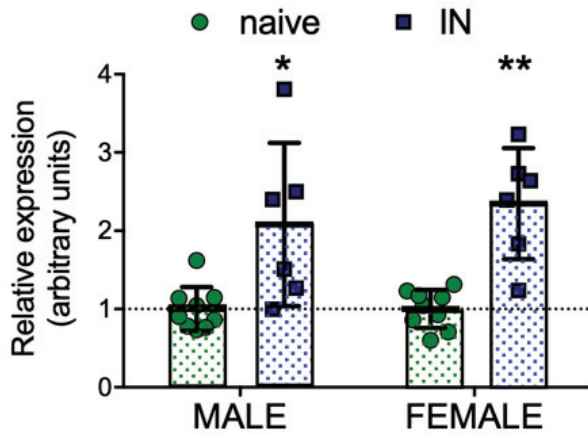1045   in the medial superficial dorsal horn were significantly increased following incision in males

1046   (ns *vs* nsIN, *p*=0.007), and females given saline (ns *vs* nsIN*, p*=0.011) or minocycline (ns *vs*

1047   nmIN, *p*=0.005). Data points = average of at least 6 spinal L4/5 sections for each individual

1048   animal (*n*=4 ns or nm; *n*=10 nsIN or nmIN). *F,* Fluoro-Jade C (FJ-C) positive cell counts in the

1049   ipsilateral (left) lumbar cord (L4,5 segments) were increased following incision in males (ns

1050   *vs* nsIN, *p*=0.008, ns *vs* nmIN *p*=0.002, nm *vs* nmIN *p*=0.024) and females (nm *vs* nsIN

1051   *p*=0.019). Data points = sum of FJ-C +ve counts from 6 L4/5 spinal cord sections per animal

1052   (*n* = 8 animals per group). *E,F,* Bars = mean ± SD; **p*<0.05 analyzed by 2-way ANOVA with

1053   Bonferroni *post-hoc* comparisons. Groups = ns, neonatal saline; nm, neonatal minocycline;

1054   nsIN, neonatal saline plus incision; nmIN, neonatal minocycline plus incision.

saline
OR
minocycline

P3　P4　P5

6 WEEKS

3 WEEKS

mechanical &
thermal thresholds

ns
nm

nsIN
nmIN

P6: Iba1

P4: FJ-C & EMG

P3: neonatal
incision

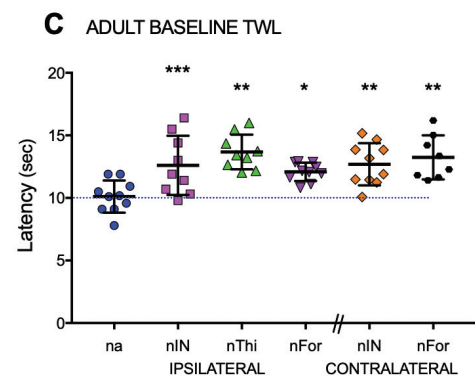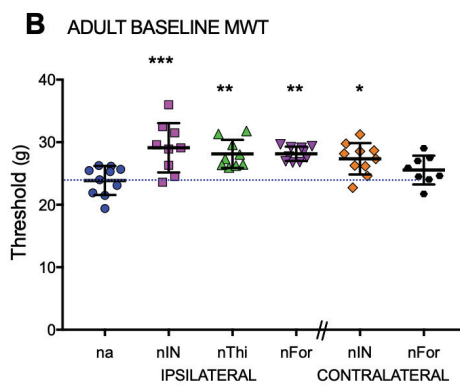24h: EMG
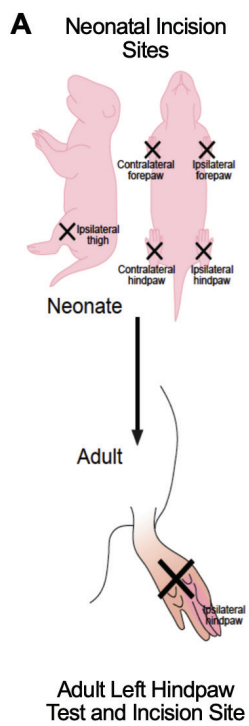& open field

ns-IN
nm-IN

nsIN-IN
nmIN-IN

3 days:
RT-PCR

adult
incision
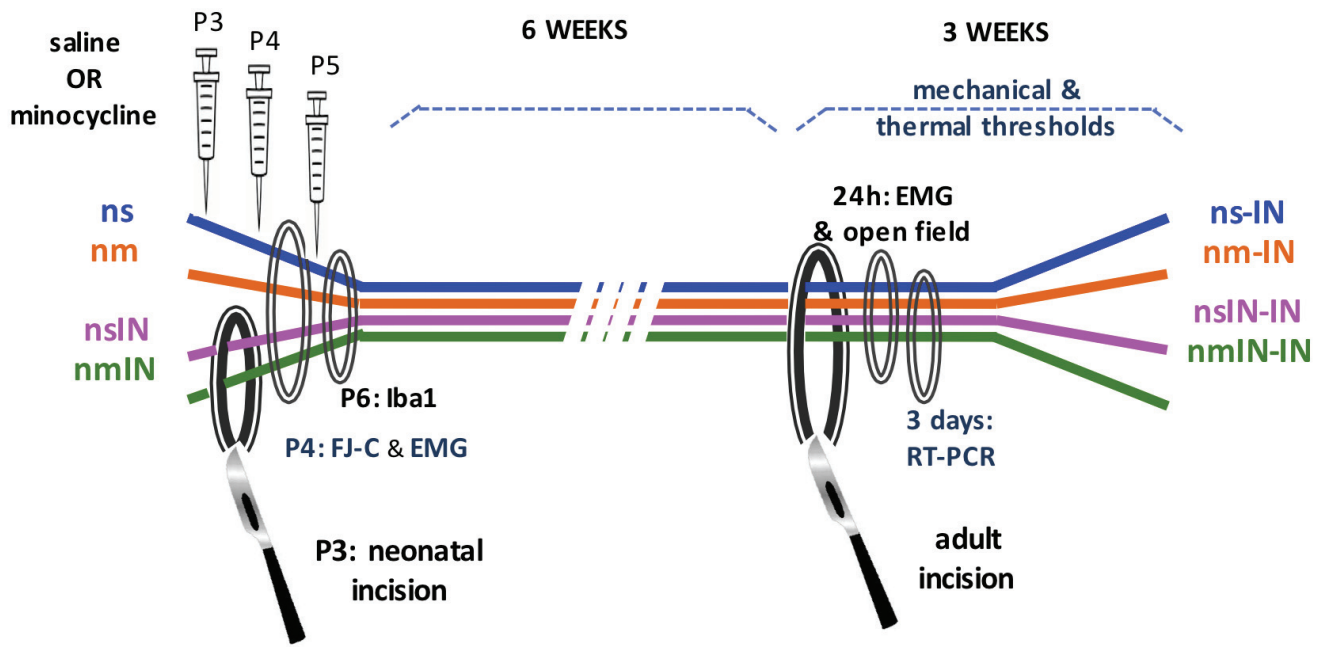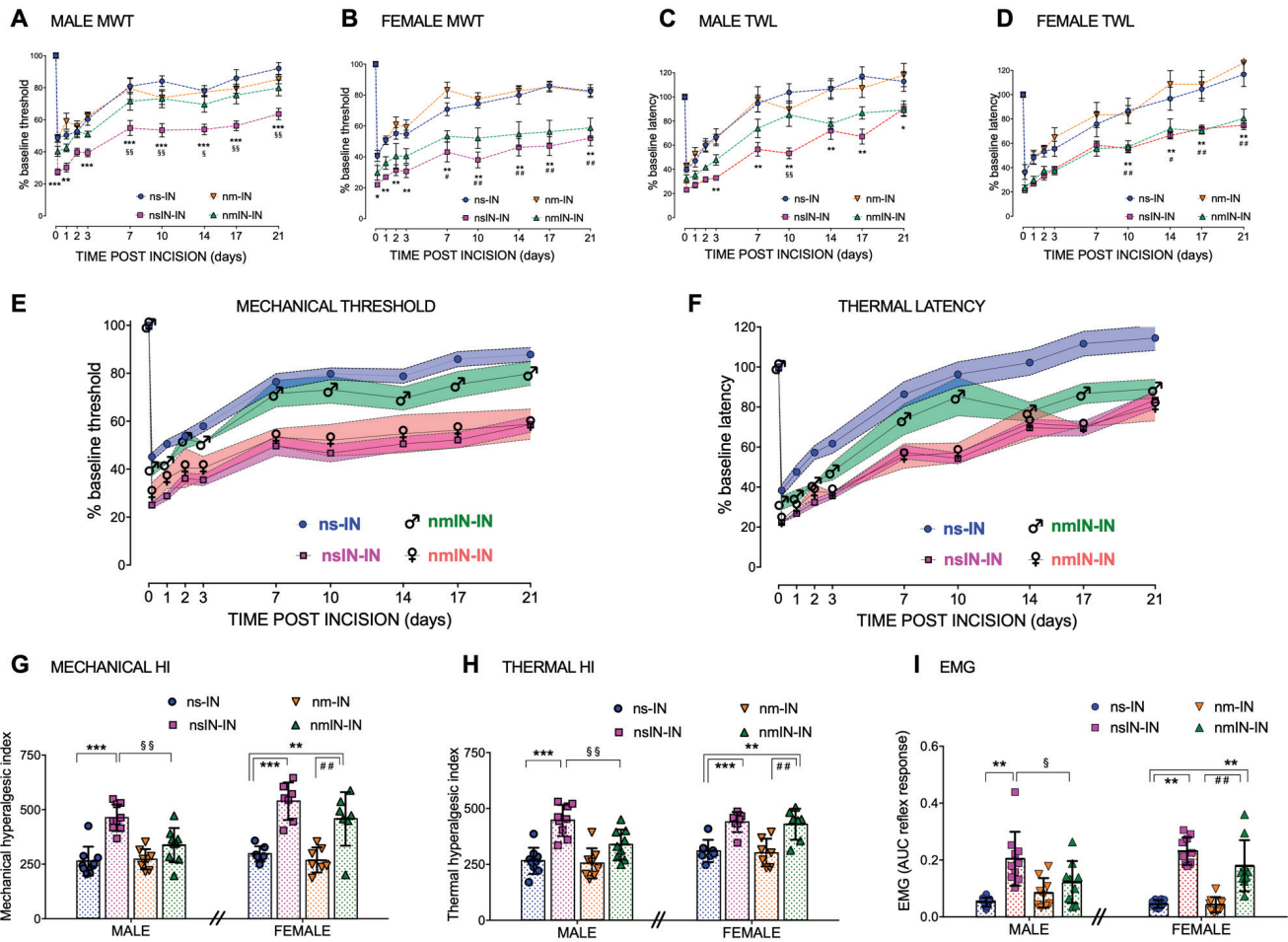
A *Emr1*

B *Irf8*

C *Emr1*

D *Irf8*

A MWT

B MECHANICAL HI

A Neonatal Incision Sites

Neonate

Adult

Adult Left Hindpaw Test and Incision Site

B ADULT BASELINE MWT

C ADULT BASELINE TWL

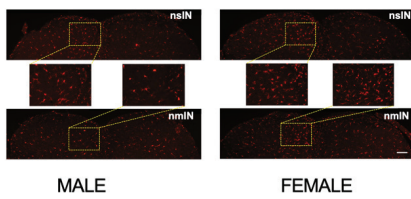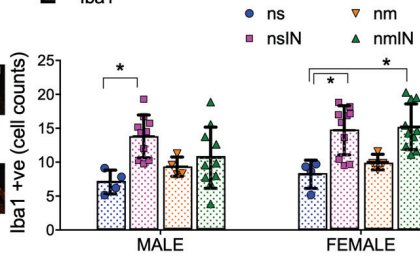**D** MECHANICAL HI

E THERMAL HI
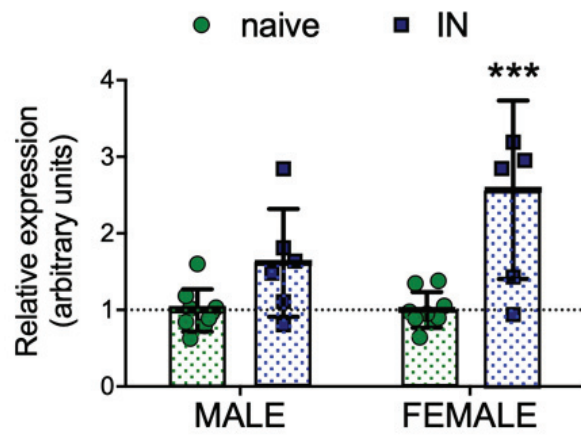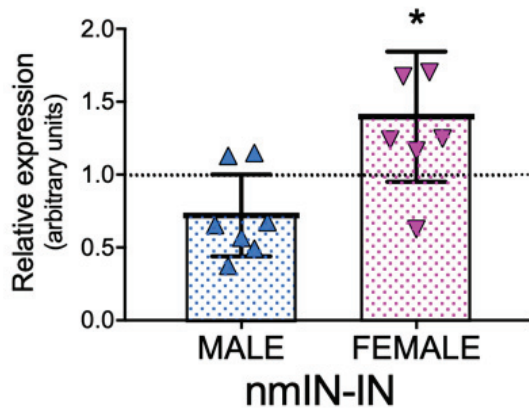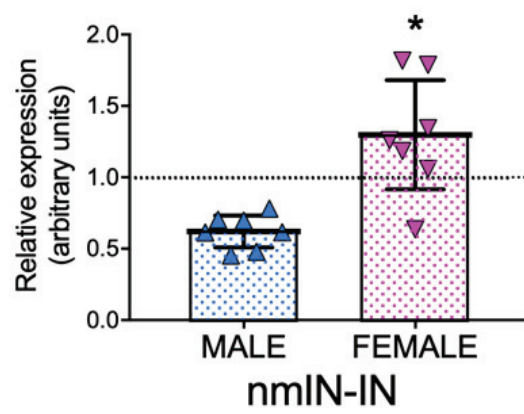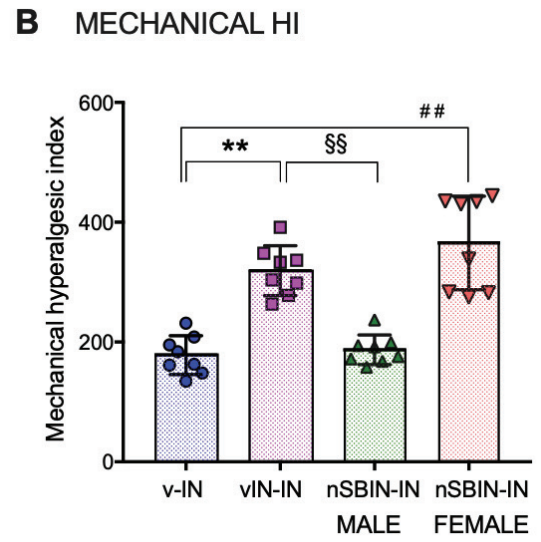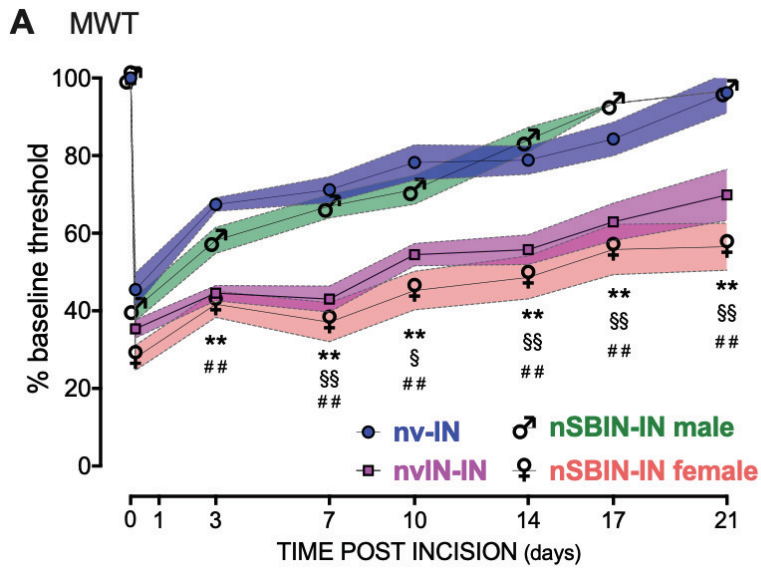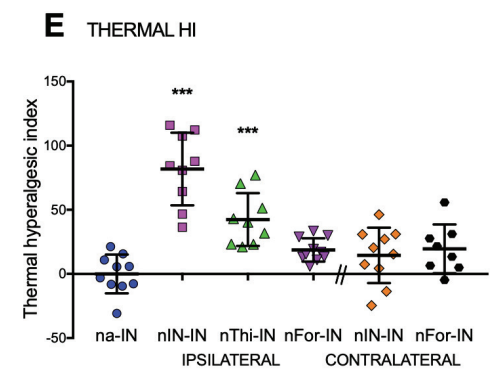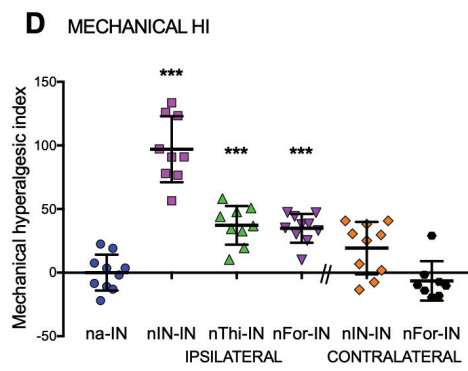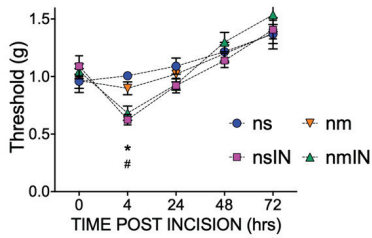
A MALE MWT

B FEMALE MWT
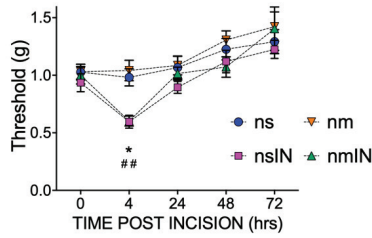
C EMG
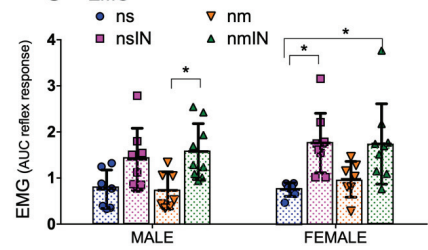
D Iba1 IHC

E Iba1

F Fluoro-Jade C