Oral Administration of Boldine Reduces Spare Nerve Injury- Induced Neuropathic Pain

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Abstract

Background: Chronic pain is present in about 20% of the population and is a major burden to the health care system. About 30-40% of these patients report neuropathic pain. Neuropathic pain is defined as pain caused by injury or disease of the somatosensory nervous system. Current available treatments for neuropathic pain have limited efficacy and substantial side effects. Methods: To address the need for more effective and safer treatments for neuropathic pain, this study aimed to test whether boldine, a naturally occurring alkaloid, could attenuate neuropathic pain in a murine model of spared nerve injury (SNI). Von Frey filament test, hot/cold plate test and dynamic weight bearing test were used to assess pain phenotypes following SNI.

Results: We found that boldine inhibited the lipopolysaccharide-induced overexpression of inflammatory markers in BV-2 microglial cells. Oral administration of boldine at 50 mg/kg body weight/day resulted in significant reduction of SNI-induced mechanical and thermal hypersensitivity. Boldine also corrected SNI-induced weight bearing deficits, which are an indication of spontaneous pain. Boldine significantly inhibited SNI-induced peripheral inflammation as indicated by reduced levels of inflammatory cytokines/chemokines in the serum. Immunofluorescence studies revealed that boldine reduced the number of reactive astrocytes and inhibited microglia activation in lumbar spinal cord.

Conclusion: Our findings suggest that boldine may be a promising therapeutic candidate for the treatment of neuropathic pain, possibly through inhibition of glia activation and neuroinflammation.

46 **Introduction**

47 As described by the International Association for the Study of Pain, neuropathic pain is
48 “pain caused by a lesion or a disease of the somatosensory nervous system". It affects 7-10% of
49 the general population globally [12]. Neuropathic pain can be central or peripheral, and tissue
50 damage is not always apparent. Studies have shown that neuropathic pain has multiple adverse
51 effects in both humans and rodents that include impaired sleep, depression, anxiety, and lower
52 quality of life [4; 14; 36; 42]. Common causes of neuropathic pain include trigeminal neuralgia,
53 post-amputation pain, persistent post-operative or post-traumatic pain, pain after peripheral nerve
54 injury, painful diabetic neuropathy, cancer pain, and chemotherapy-induced peripheral neuropathy.
55 Due to its subjective nature, diagnosing neuropathic pain is challenging. Typical characteristics of
56 neuropathic pain include allodynia, where normally non-painful stimuli cause pain; hyperalgesia,
57 an exaggerated response to painful stimuli; and spontaneous pain [2; 28; 34; 41].

58 Currently, the first line of choice for neuropathic pain treatment includes: anticonvulsants,
59 tricyclic antidepressants, serotonin and norepinephrine re-uptake inhibitors, and topical agents
60 [5]. Opioids, such as morphine and oxycodone, can be administered under careful medical
61 supervision for very severe cases, although their long-term efficacy in treating neuropathic pain
62 is uncertain, and they can be associated with severe side effects and risks, including sedation,
63 respiratory depression, potential dependence and addiction [17]. Thus, there is an urgent need to
64 develop novel, efficacious and safe interventions for treating neuropathic pain. Recently, the
65 beneficial effect of some nutraceuticals and herbal remedies have gained attention in combating
66 neuropathic pain [1]. Curcumin for instance, reduced neuropathic pain in streptozotocin-induced
67 diabetic neuropathy [51] and peripheral nerve injury rodent models [25].

68 Boldine is the most abundant alkaloid isolated from the leaves and bark from boldo
69 (*Peumus boldus*), a widely distributed evergreen tree native to Chile [48]. Boldo extracts
70 containing boldine has been used since the last century in Europe and North/South America, for
71 the treatment of digestive problems, headache, rheumatism and urinary tract inflammation [53].

72 Boldine has also been shown to have antioxidative activities that protect both biological and non-
73 biological systems against free radical-mediated oxidative damage [45]. In addition, boldine
74 exhibits hepatoprotective, anti-inflammatory and antipyretic activities in animal models [3; 8].
75 Recent studies conducted by us and others have shown the beneficial effect of boldine in
76 neurological conditions associated with neuroinflammation including spinal cord injury [16; 56],
77 delayed nerve repair [7], antiepileptic [43], and Alzheimer's Disease [55], suggesting that boldine
78 could pass the blood-brain barrier. Consistent with this interpretation, boldine accumulates in brain
79 tissues after oral administration [30]. Considering the anti-inflammatory activity of boldine and the
80 key role of neuroinflammation in the development and maintenance of neuropathic pain, we
81 evaluated the potential of boldine to attenuate neuropathic pain in a mouse model of spared nerve
82 injury.
83

84 **Material and Methods**

85 *Reagents*

86 Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), Dulbecco's Phosphate-
87 Buffered Saline (DPBS), penicillin and streptomycin, were purchased from GIBCO (Waltham, MA).
88 Lipopolysaccharides (LPS), boldine, dimethyl sulfoxide (DMSO), triton X-100, and
89 paraformaldehyde (PFA) were purchased from Sigma-Aldrich (St. Louis, MO). LPS was dissolved
90 in PBS and boldine was first dissolved in DMSO then diluted in culture medium.

91 *Cell culture, treatment, cytotoxicity, and ELISA.*

92 BV-2 cells were obtained from AcceGen (Fairfield, NJ) and maintained in DMEM supplemented
93 with 10% FBS and antibiotics (penicillin 100 U/mL, streptomycin 100 μg/mL) at 37°C with 5% CO_2.
94 Cells (in triplicates) were treated with 100 ng/mL LPS with or without boldine (25 or 50 μg/mL) for
95 6 hours, and 100 μL of the supernatant was sampled for cytotoxicity essay before cells were
96 harvested for RNA extraction or ELISA. Cell viability was assessed using the CytoTox 96 Non-
97 Radioactive Cytotoxicity Assay (Promega, Fitchburg, WI) following manufacturer's instructions.

98 For ELISA, BV-2 cells were harvested and lysed in RIPA buffer (Pierce Biotechnology, Waltham,
99 MA) and protein concentration was measured using the BCA Protein Assay Kits (Pierce
100 Biotechnology). IL-1β protein levels were assessed using the Mouse IL-1 beta/IL-1F2 Quantikine
101 ELISA Kit (R&D Systems, Minneapolis, MN) following the manufacturer's directions. Optical
102 densities were measured using a SpectraMax iD5 plate reader (Molecular Devices, San Jose,
103 CA).

104 *Animal, surgery, and boldine administration.*

105 Animals were maintained on a 12:12-h light/dark cycle in a temperature-controlled (20 ± 2°C)
106 vivarium, with food and water *ad libitum*. All procedures were approved by the Institutional Animal
107 Care and Use Committee at James J. Peters Veterans Affairs Medical Center (#1585874) and
108 conformed to all guidelines and regulations for the protection of the welfare of animals. C57BL6
109 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and pair-housed. Spared
110 nerve injury (SNI) surgery was conducted on the left hindlimb under anesthesia (3-5% isoflurane)
111 by cutting the peroneal and tibial nerves and leaving an intact sural nerve. Sham animals
112 underwent a similar procedure where the branches of the sciatic nerve were exposed but left
113 intact. Boldine was administered to the animal right after surgery via a customized chow
114 (Research Diets, New Brunswick, NJ) to reach a dose of 50 mg/Kg BW/day. A control diet
115 (OpenStandard Diet With 15 Kcal% Fat, Research Diets) was provided to sham-operated or SNI
116 group. Animals were euthanized at 15 days post-surgery. Lumbar spinal cords (L3-5) were
117 dissected and stored at -80 °C for further analysis. Blood samples were collected and left at room
118 temperature for 1 hour to allow it to clot. Blood was then centrifuged at 1,000 x g for 10 minutes
119 and supernatant (serum) was stored at -80 °C for further analysis.

120 *RNA Isolation, cDNA synthesis and quantitative PCR.*

121 BV-2 cells were harvested and RNAs were extracted using the RNeasy Kit (Qiagen, Hilden,
122 Germany) following the manufacturer's directions. Frozen tissues were homogenized in TRI-
123 Reagent (Sigma-Aldrich, St. Louis, MO) using a FastPrep-24™ 5G Bead Beating Grinder and

124 Lysis System (MP Biomedicals, Solon, OH). Total RNA was isolated following the manufacturer's
125 instructions. RNA concentrations were measured using a NanoDrop Spectrophotometer (Thermo
126 Fisher Scientific, Waltham, MA). One microgram of total RNA was reverse transcribed with High-
127 Capacity cDNA Reverse Transcription Kit using random hexamers in a 20 μL reaction according
128 to the manufacturer's protocol. Quantitative PCR (qPCR) was performed using the Taqman
129 Universal PCR Master Mix on a ViiA 7 Real-Time PCR System. Relative expression levels were
130 calculated using the $2^{-\Delta\Delta Ct}$ method with hypoxanthine phosphoribosyltransferase 1 (HPRT) as an
131 internal control. A list of the Taqman probes used in this study is provided in Supplemental Table
132 1 (all from Applied Biosystems, Waltham, MA).

133 *Behavioral Tests.*

134 Mechanical pain responses were assessed by Von Frey filament test performed on the ipsilateral
135 and contralateral hind paws [15]. Briefly, The Von Frey hair (Bioseb, Vitrolles, France) is inserted
136 perpendicularly from below an elevated wire platform into the plantar surface of the hind paw,
137 starting with the smallest gauge filament. The pressure was increased slowly, until the hair just
138 began to bend. The test was then repeated with the next largest gauge filament until withdrawal
139 responses including paw flicking, paw lifting, paw licking or possibly vocalizing was observed. The
140 withdrawal threshold, defined as the smallest gauge wire that produced a withdrawal response,
141 was recorded.

142 Thermal pain responses were measured using a hot/cold pate apparatus (Maze Engineers,
143 Skokie, IL). Mice were allowed to move freely on a hot/cold plate enclosure for a maximum of 30
144 seconds. For assessing pain responses to heat, plate temperature was set at 50 ˚C. For testing
145 pain responses to cold, plate temperature was set at 15 ˚C. Once paw retraction, paw licking, or
146 escaping behaviors were observed, mice were immediately taken out of the testing apparatus
147 and the time latency was recorded [63].

148 Spontaneous pain responses were measured using the Dynamic Weight Bearing 2.0
149 system (DWB2.0) (Bioseb, Vitrolles, France) [50]. Briefly, the animal was placed into the DWB

150 enclosure for a test duration of 5 minutes. The animal was recorded from above using a high-
151 definition camera and the captured video feed was analyzed in real-time using tracking software.
152 An observer manually validated each automatically presumed paw position to avoid subsequent
153 error identification afterward.

154 *Multiplex ELISA.*

155 MILLIPLEX® Mouse Cytokine/Chemokine Magnetic Bead Panel (Millipore, Billerica, MA) was
156 used to measure the levels of 32 cytokines/chemokines in mouse serum as previously described
157 [59; 60; 66]. Briefly, 25 μL of serum sample was diluted with assay buffer and incubated with
158 magnetic beads overnight at 4°C. The samples were then washed, incubated with detection
159 antibodies for 1 hour at room temperature followed by streptavidin-phycoerythrin incubation for
160 30 minutes. The plate was run on a Luminex 200 instrument (Millipore, Billerica, MA) and the
161 median fluorescent intensity was used to calculate the concentration of each cytokine/chemokine.

162 *Immunostaining and microscopy.*

163 At 15 dpi, mice were transcardially perfused with saline followed by 4% paraformaldehyde (PFA).
164 Lumbar spinal cords (L3-5) were harvested and transversely sectioned using a LEICA CM3050
165 Cryostat (LEICA, Wetzlar, Germany). For immunostaining, slides were first incubated with
166 blocking buffer (1 x PBS + 5% goat serum + 0.3% Triton X-100) for 1h at room temperature,
167 followed by incubation with primary antibodies of astrocyte marker glial fibrillary acidic protein
168 (GFAP, Abcam, Cambridge, UK) or microglia marker IBA1 (Abcam) overnight at 4°C. After
169 incubation, the slides were washed 3 times in PBS and incubated with goat anti-mouse Alexa
170 Fluor 488 secondary antibody (Abcam) for 1 hour at room temperature. Slides were then washed
171 with PBS three times and mounted with VECTASHIELD Antifade Mounting Medium (Vector
172 Laboratories, Newark, CA). Images were acquired using the ZEISS LSM 700 confocal imaging
173 system (Carl Zeiss, Oberkochen, Germany) with 20× objective. Mean fluorescent intensity was
174 quantified using ImageJ.

175 *Statistical analysis.*

176 Data were analyzed using GraphPad Prism 10 (San Diego, CA) and were presented as the mean
177 ± standard error of the mean (SEM). Two-way repeated-measures analysis of variance (ANOVA)
178 followed by multiple comparison test were performed to compare the mean differences in
179 mechanical thresholds, thermal thresholds and weight bearing among the experimental groups.
180 One-way ANOVA followed by multiple comparison test was performed for the comparison of mean
181 difference in cytotoxicity and RNA or protein expression levels among experimental groups. p-
182 value of < 0.05 was considered statistically significant.
183
184 **Results**
185 *Boldine inhibits LPS-induced inflammation in microglial cells.*
186 Neuroinflammation acts as both a driver and amplifier of pain states, particularly in chronic
187 conditions. Boldine has been shown to exhibit anti-inflammatory activities [3; 8]. To test whether
188 boldine could reduce neuroinflammation, BV-2 microglia cells were treated with LPS with or
189 without boldine. After treatment, cell medium was collected and subjected to a cell viability assay.
190 Neither the 25 μg/mL or the 50 μg/mL dose of boldine showed any cytotoxicity (Supplemental
191 Figure 1A). Cells were then harvested and subjected to gene expression analysis by quantitative
192 PCR. LPS treatment led to significantly elevated mRNA levels of chemokines (CCL2, CCL3 and
193 CCL5), cytokines (IL-1β, IL-6, and IL-18), macrophage and microglia marker CD11b, toll-like
194 receptors (TLR2 and TLR3), and NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3)
195 (Figure 1). Co-incubation with boldine inhibited the LPS-induced upregulation of the above-
196 mentioned molecules, suggesting a beneficial role of boldine in LPS-induced inflammation. We
197 also found that IL-1β protein levels were drastically increased after LPS treatment, which was
198 prevented by boldine co-treatment (Supplemental Figure 1B).
199 *Boldine alleviates SNI-induced mechanical allodynia.*
200 To assess the role of boldine in peripheral nerve injury-induced neuropathic pain, we
201 conducted studies in a mouse model of SNI, which produces persistent peripheral hypersensitivity

202 to mechanical stimuli [13]. Briefly, 10 weeks old female C57BL6 mice were subjected to SNI or
203 sham surgery. Customized diet containing boldine or control diet was administered immediately
204 after surgery. Pain phenotyping tests including Von Frey filament test, hot/cold plate test and
205 dynamic weight bearing test were performed before injury for baseline and starting from 3 days
206 after injury (Figure 2A, experiment design). The dosage of boldine was monitored by weekly
207 measurement of food consumption (Figure 2B). The average food intake is 2.5 ~ 2.7 gram per
208 animal per day, which is equivalent to 50 ~ 54 mg/kg BW/day of boldine. No significant difference
209 in food intake was observed among experimental groups (Figure 2B).

210 As expected, SNI significantly induced mechanical pain hypersensitivity on the ipsilateral
211 hindlimb at 3-, 7-, and 14- days post injury (dpi) when compared to sham-operated animals. Oral
212 administration of boldine completely prevented this injury-induced mechanical allodynia (Figure
213 2C). There was no change of responses to mechanical stimuli after nerve injury or boldine
214 treatment on the contralateral hind limb (Figure 2D), suggesting that the boldine-mediated
215 prevention of mechanical-induced allodynia is specific to the region innervated by the injured
216 nerve. We also observed a similar effect of boldine in preventing SNI-induced mechanical
217 hypersensitivity in male mice (Supplemental Figure 2). We choose to focus on female mice for
218 subsequent studies because women are more likely to have chronic pain and high-impact chronic
219 pain compared with men [65].

220 *Boldine reduces SNI-induced thermal pain.*

221 Rodents with SNI often exhibit increased sensitivity to thermal stimuli, manifesting as decreased
222 withdrawal latencies in response to heat or cold compared to control animals [13]. To test the
223 effect of boldine in altering thermal pain phenotypes, we performed the hot plate test at and the
224 cold plate test following SNI. At 7- and 14-dpi, SNI animals developed increased sensitivities to
225 both warm (Figure 3A) and cold stimuli (Figure 3B). Administration of boldine significantly
226 attenuated the SNI-induced hypersensitivity to warm and cold temperatures.

227 *Boldine prevents SNI-induced weight bearing deficits.*

228 Alterations in weight-bearing behavior can be indicative of pain, including those resulting from
229 peripheral nerve injury. In the context of SNI, changes in weight-bearing distribution are often
230 observed as part of the pain-related behavioral phenotype [44]. To evaluate the effect of boldine
231 on potential SNI-induced weight bearing deficits, we performed weight bearing tests using the
232 dynamic weight bearing 2.0 system. As compared to sham-operated mice, injured mice tended to
233 use the ipsilateral hindlimb less for weight support at 3 and 7 dpi, and boldine administration
234 significantly alleviated the SNI-induced weight bearing deficit at 7dpi (Figure 4A). We also
235 measured some additional parameters including the hindlimb weight support area ratio and
236 rearing duration. Rearing behavior, where a mouse stands on its hind legs with its forelimbs raising
237 and head extending, has been used as a spontaneous pain indicator in mice [62]. SNI led to
238 significantly decreased ipsilateral/contralateral weight support area ratio (Figure 4B) and
239 markedly lower rearing duration (Figure 4C). However, the administration of boldine did not
240 prevent SNI-induced deficits in weight support area or rearing duration (Figure 4B and C).

241 *Boldine reduces SNI-induced peripheral inflammation.*

242 To evaluate the effect of boldine on peripheral inflammation, serum samples were subjected to a
243 multiplex ELISA using the MILLIPLEX® Mouse Cytokine/Chemokine Magnetic Bead Panel
244 (Millipore). SNI induced the elevation of interleukin-12 (IL-12p40), interleukin-15 (IL-15),
245 macrophage colony-stimulating factor (M-CSF) and chemokine ligand 5 (CCL5) in the serum,
246 which was prevented by boldine treatment (Figure 5).

247 *Boldine inhibits glia activation in the lumbar spinal cord.*

248 In pain conditions, nociceptive signals are transmitted from peripheral nerves to the spinal cord.
249 Glial cells in the spinal cord dorsal horn play complex and dynamic roles in pain processing. Both
250 activated microglia and reactive astrocytes have been implicated in pathogenesis of pain following
251 nerve injuries [9; 10; 24; 31; 39]. To test the effect of boldine on glia activation, transverse lumbar
252 spinal cord sections were immuno-stained with the astrocyte marker GFAP or the microglia
253 marker IBA1. As compared to sham-operated mice, significantly more reactive astrocytes were

254 detected in the lumbar spinal cord after SNI (Figure 6A), and boldine treatment prevented the
255 increase in number of GFAP-positive cells in the ipsilateral dorsal horn (Figure 6B) and ventral
256 horn (Figure 6C). Consistent with previous reports [20; 46], microglia in the spinal cord dorsal
257 horn and ventral horns were activated after SNI (Figure 7A), and boldine treatment significantly
258 reduced the SNI-induced microglia proliferation/activation in the ipsilateral dorsal horn (Figure 7B)
259 and ventral horn (Figure 7C).

260 *Boldine inhibits the elevation of neuroinflammation markers in the spinal cord.*

261 Glia mediators, such as IL-1β and tumor necrosis factor-α (TNF-α), are key factors in the glial
262 control of pain. It has been reported that overexpression of IL-1β in the injured sciatic nerve
263 following nerve injury may be a common mechanism underlying the generation of neuropathic
264 pain [21]. C-C motif chemokine ligand 2 (CCL2), also known as monocyte chemotactic protein 1
265 (MCP-1), and its receptor CCR2 may play a role in neuroinflammation by participating in neuron-
266 glial cell interactions [6; 40; 57]. To evaluate the effect of boldine on these inflammatory markers
267 in the spinal cord following SNI, we extracted RNA from lumbar spinal cord collected at 15 dpi
268 and performed quantitative PCR using the standard Taqman method. Significantly elevated levels
269 of TNF-α, IL-1β and CCL2 (Figure 8A-C) mRNA were detected in the lumbar spinal cord following
270 SNI. Oral administration of boldine decreased the levels of TNF-α (Figure 8A) and IL-1β (Figure
271 8B). We also examined the mRNA levels of the receptors of TNF-α, IL-1β and CCL2, and found
272 no difference (Figure 8D-F).

273

274 **Discussion**

275 The SNI model is a commonly used model in rodents to study neuropathic pain. SNI leads
276 to mechanical allodynia, which develops 3 days after injury and lasts between 4 weeks to 12
277 months in mice [22]. SNI also leads to thermal hypersensitivity and weight bearing deficits. In this
278 study, we administered boldine via a customized diet to avoid potential stress associated with
279 gavage or injections. Boldine is a bitter alkaloid and there is no added flavor in the diet, one

280 potential risk is that the bitter taste might negatively affect food intake in the boldine treatment
281 group. We monitored food consumption and did not observe any difference between experimental
282 groups, suggesting that the bitter taste does not have any influence on food intake. We found that
283 oral administration of boldine could prevent SNI-induced mechanical allodynia at 3, 7, and 14 dpi.
284 A potential confound is whether boldine could impair locomotor function which could slow or
285 weaken paw withdrawal responses to mechanical stimulus. Several lines of evidence argue
286 against this possibility. First, boldine did not alter the withdraw threshold on the contralateral hind
287 paw; Second, a recent study from our lab showed no effect of boldine on paralyzed muscle
288 weights [47]; Third, the pharmacological effects of boldine have been studied in naive and multiple
289 rodent models of disease without any reported defects in motor function [48]. Boldine prevented
290 SNI-induced thermal hypersensitivity and weight bearing deficit at 7 dpi. We did not observe
291 boldine-mediated prevention of weight bearing deficits at 3 dpi, which might be partially attributed
292 to possible neophobia to the diet (i.e. receiving a lower dose of boldine for the first several days).
293 We also noticed that animals started to recover from thermal or weight bearing pain responses at
294 14 dpi, which is similar to previous report that thermal hypersensitivity and weight bearing share
295 similar pain recovery profiles, whereas mechanical allodynia persists in a sciatic nerve entrapment
296 injury model [52]. At 14 dpi, the ipsilateral/contralateral hindlimb weight support ratios were similar
297 among experimental groups, while the support area ratios remained significantly lower in injured
298 animals, suggesting a more persistent and slower recovery of weight support area deficits.

299 Inflammatory responses are of great significance in the development of neuropathic pain.
300 A large body of evidence indicates that inflammatory mediators in local inflamed tissues,
301 peripheral nerves, and spinal cord make important contributions to the initiation and maintenance
302 of chronic pain [27; 29]. In this study, we have observed significantly elevated expression of
303 cytokine/chemokines including IL-12p40, IL-15, CCL5, and M-CSF in the serum after SNI.
304 Subcutaneous injection of IL-12 leads to mechanical hyperalgesia in rats [58]. IL-15 has been
305 linked with nerve injury-induced neuropathic pain in a rodent CCI models [18] as well as

306 rheumatoid arthritis- and osteoarthritis- induced joint pain in humans [61]. Intradermal injection of
307 CCL5 has been shown to induce pain and administration of Met-CCL5, an antagonist of CCL5
308 receptor CCR5, attenuates inflammatory and nociceptive responses following peripheral nerve
309 injury [38]. In humans, upregulated CCL5 has been detected in patients with various pain
310 conditions including small fiber neuropathy [32], atypical facial pain and trigeminal neuralgia [35],
311 and discogenic back pain [19; 33]. M-CSF is also an important contributing factor to the
312 development of neuropathic pain [64] and arthritic pain [11]. Our data suggested that boldine
313 administration prevented the SNI-mediated induction of these pain-related inflammatory
314 mediators in the periphery.

315 Neuroglial cells, including microglia, astrocytes, oligodendrocytes, and ependymal cells,
316 play complex and dynamic roles in pain processing. Their activation and interactions with neurons
317 contribute to the amplification and maintenance of pain signals, as well as the development of
318 chronic pain conditions [26]. Our immunofluorescence data confirmed an increased number of
319 reactive astrocytes and activated microglia in the ipsilateral spinal cord. Boldine treatment
320 significantly prevented the injury-induced glia activation. Notably, we also observed some levels
321 of reactive astrocytes in the contralateral spinal cord sections at 15 dpi (Figure 7A), which could
322 possibly be attributed to the central sensitization process. Recent studies have shown that after
323 nerve injury, toll-like receptors (TLRs) are involved in the generation of neuropathic pain. For
324 example, TLR2 and TLR4 modulate glial activation in the spinal cord and contribute to neuropathic
325 pain development [37; 54]. TLRs are responsible for the stimulation of astrocytes and microglia,
326 which will induce proinflammatory mediators and cytokines, therefore leading to the generation
327 and maintenance of neuropathic pain. The majority of TLRs can signal primarily through the
328 myeloid differentiation 88 (MyD88) pathway, and then trigger the activation of ERK/JNK/NFκB
329 signaling. Nod-like receptors (NLRs) are cytoplasmic sensors that oligomerize to form the
330 inflammasome, a multi-subunit complex that processes the maturation of pro-IL-1β and pro-IL-18
331 via caspase-1 [23]. A well-characterized outcome of TLR and inflammasome interaction is the

332 secretion of IL-1β and IL-18 [49]. In this study, we report that boldine prevented the LPS-induced
333 overexpression of multiple inflammatory cytokines and chemokines including CCL2, IL-1β, IL-6,
334 and IL-18 in BV-2 microglial cells. We have also found decreased expression of toll-like receptors
335 (e.g. TLR2, TLR3), and NLRP3 after boldine treatment, suggesting that boldine might prevent
336 neuroinflammation through inhibiting the TLR/NLRP3 inflammasome signaling. Significant
337 decrease of TNF-α and IL-1β mRNA in the lumbar spinal cord was detected in boldine-treated
338 animals at 15 dpi. The boldine-mediated changes at the protein level and the direct target of
339 boldine still remain to be characterized.

340 In summary, we report for the first time that oral administration of boldine, a naturally-
341 occurring alkaloid, attenuated neuropathic pain after spared nerve injury, suggesting that boldine
342 might be considered as a novel therapeutic agent for the treatment of neuropathic pain. Future
343 studies will explore how the boldine and its derivatives differentially influence pain perception in
344 the central nervous system and peripheral nervous system across sexes and extend our research
345 to other pain conditions, including arthritic pain, back pain, and diabetic pain.

346

347 **Conflict of Interest**

348 Authors CAT, JCS, CPC, and WZ are co-inventors for US patent no. 11,707,459 for the use of
349 boldine to treat neurological injuries. This study falls within the claims of the patent. The remaining
350 authors declare no conflict of interest.

351

352 **Author Contributions**

353 Experiment design: WZ, CPC; experiment execution and data collection: JP, WZ, CAT; data
354 analysis: WZ, JP, CC, CAT; manuscript writing and editing: WZ, CPC, JCS, YD.

355

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363 **FIGURE LEGEND**

364 **Figure 1. Boldine prevents LPS-induced inflammation *in vitro*.** BV-2 microglia cells were
365 treated with 100 ng/mL lipopolysaccharides (LPS) with or without boldine (25 μg/mL or 50 μg/mL)
366 for 6 hours. Cells were harvested and subjected to RNA extraction followed by cDNA synthesis
367 and quantitative PCR using standard Taqman methods. Taqman probes include CCL2, CCL3,
368 CCL5, CD11b, IL-1β, IL-6, IL-18, TLR2, TLR3, and NLRP3. Relative levels were calculated using
369 the $2^{-\Delta\Delta Ct}$ method with HPRT as an internal control. Data represent Mean ± SEM, N = 3 per
370 condition, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by one-way ANOVA followed by
371 Tukey post-tests.

372

373 **Figure 2. Boldine prevents spared nerve injury-induced mechanical allodynia.** Female
374 C57BL6 mice were subjected to spared nerve injury (SNI). Boldine was administered right after
375 injury via a customized diet (Research Diet) to reach a dose of 50 mg/kg/day. A control diet was
376 provided to sham or SNI group. Von Frey filament tests were performed to evaluate mechanical
377 pain responses. (A) Diagram of experiment design; (B) Food intake was monitored weekly and
378 average daily intake was calculated and plotted in GraphPad Prism 10. (C) Withdraw threshold
379 to mechanical stimuli on the ipsilateral hind paw before and post injury; (D) Paw withdraw
380 threshold to mechanical stimuli on the contralateral hind paw. Data represent mean ± SEM, N =
381 8-10 per group, *** $p < 0.001$ (SNI vs. sham), ### $p < 0.001$ (SNI+boldine vs. SNI) by two-way
382 ANOVA with Tukey post-tests

383

384 **Figure 3. Boldine reduces SNI-induced thermal pain hypersensitivity.** Hot/cold plate tests
385 were performed to evaluate thermal pain responses before (Pre) and after (7dpi and 14dpi) injury
386 in sham animals (Sham) and animals with (SNI + Boldine) or without (SNI) boldine treatment. (A)
387 Latency to nociceptive responses by hot plate test (50°C). (B) Latency to nociceptive responses

388 by cold plate test (15°C). Data represent Mean ± SEM, N = 8-10 per group, * $p < 0.05$, ** $p < 0.01$,
389 *** $p < 0.001$ by Two-way ANOVA with Tukey post-test.
390
391 **Figure 4. Effect of boldine on SNI-induced weight bearing deficits.** Assessment of
392 spontaneous pain measuring the changes in postural equilibrium was achieved using the
393 Dynamic Weight Bearing System 2.0 (Bioseb) in sham animals (Sham) and animals with (SNI +
394 Boldine) or without (SNI) boldine treatment. (A) Weight support ratio of the ipsilateral hind limb vs.
395 the contralateral hind limb; (B) Support area ratio of the ipsilateral hind limb vs. the contralateral
396 hind limb; (C) Rearing duration. Data represent Mean ± SEM, N = 8-10 per group, *$p < 0.05$, ** p
397 < 0.01, *** $p < 0.001$, **** $p < 0.0001$ by two-way ANOVA with Tukey post-tests.
398
399 **Figure 5. Peripheral inflammation changes after SNI and boldine treatment.** Serum samples
400 from sham (Sham) animals and mice subjected to spared nerve injury (SNI) or SNI plus 50 mg/kg
401 boldine (SNI+Boldine) collected at 15 dpi were subjected to a multiplex ELISA using the
402 MILLIPLEX® Mouse Cytokine/Chemokine Magnetic Bead Panel (Millipore) and the median
403 fluorescent intensity was used to calculate the concentration of each cytokine/chemokine. (A) IL-
404 12p40; (B) IL-15; (C) CCL5; (D) M-CSF. Data represent Mean ± SEM, N = 8-10 per group, * $p <$
405 0.05, ** $p < 0.01$ by one-way ANOVA with Tukey post-test.
406
407 **Figure 6. Boldine decreases levels of reactive astrocytes in the lumbar spinal cord after**
408 **SNI.** Sixteen micrometer thick transverse lumbar spinal cord sections were immuno-stained with
409 astrocyte marker GFAP to evaluate the effect of SNI and boldine treatment on glia activation.
410 Images were acquired using the ZEISS LSM 700 confocal imaging system with 20 × objective.
411 (A) Representative images of the lumbar spinal cord. Red box: ipsilateral dorsal horn; Blue box:
412 ipsilateral ventral horn. Scale bar: 10 μm. Quantification of GFAP expression at the (B) ipsilateral

413 dorsal horn (DH) and (C) ipsilateral ventral horn (VH). Data represent Mean ± SEM, N = 3 mice
414 per group. ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA with Tukey post-test.
415
416 **Figure 7. Boldine decreases levels of activated microglia in the lumbar spinal cord after**
417 **SNI.** Sixteen micrometer thick transverse lumbar spinal cord sections were immuno-stained with
418 microglia marker IBA1 to evaluate the effect of SNI and boldine treatment on glia activation.
419 Images were acquired using the ZEISS LSM 700 confocal imaging system with 20 × objective.
420 (A) Representative images of the lumbar spinal cord. Red box: ipsilateral dorsal horn; Blue box:
421 ipsilateral ventral horn. Scale bar: 10 μm. Quantification of IBA1 expression at the (B) ipsilateral
422 DH and (C) VH. Data represent Mean ± SEM, N = 3 mice per group. ** $p < 0.01$, *** $p < 0.001$ by
423 one-way ANOVA with Tukey post-test.
424
425 **Figure 8. Boldine decreases mRNA levels of inflammation markers in the spinal cord.** Total
426 RNA was extracted from lumbar spinal cords collected at 15 dpi and subjected to gene expression
427 analysis by quantitative PCR using standard Taqman methods. Relative levels were calculated
428 using the $2^{-\Delta\Delta Ct}$ method with HPRT as an internal control. (A) TNF-α; (B) IL-1β; (C) CCL2; (D)
429 TNFR; (E) IL1R; (F) CCR2 mRNA levels. Data represent Mean ± SEM, N = 6 per group, * $p <$
430 0.05, ** $p < 0.01$, *** $p < 0.001$ by unpaired t-test.
431

432 **Abbreviations**

433 ANOVA: Analysis of variance

434 BW: body weight

435 CCI: Chronic constriction injury

436 CCL: C-C Motif Chemokine Ligand

437 CCR: C-C Motif Chemokine receptor

438 CXCL: C-X-C motif chemokine ligand

439 DH: dorsal horn

440 DMEM: Dulbecco's Modified Eagle Medium

441 Dpi: Days post injury

442 DWB: Dynamic Weight Bearing

443 ELISA: enzyme-linked immunosorbent assay

444 FBS: fetal bovine serum

445 GFAP: Glial fibrillary acidic protein

446 HPRT: Hypoxanthine phosphoribosyltransferase 1

447 IL: Interleukin

448 IL1R: IL-1 receptor

449 LPS: Lipopolysaccharides

450 M-CSF: macrophage-colony stimulating factor

451 NFκB: nuclear factor-kappa B

452 NLRP3: Nod-like receptor pyrin containing 3

453 PBS: Phosphate-buffered saline

454 PFA: paraformaldehyde

455 SEM: standard error of the mean

456 SNI: Spared nerve injury

457 TNF-α: Tumor Necrosis Factor Alpha

TNFR: Tumor Necrosis Factor Receptor

TLR: Toll-like receptor

VH: ventral horn

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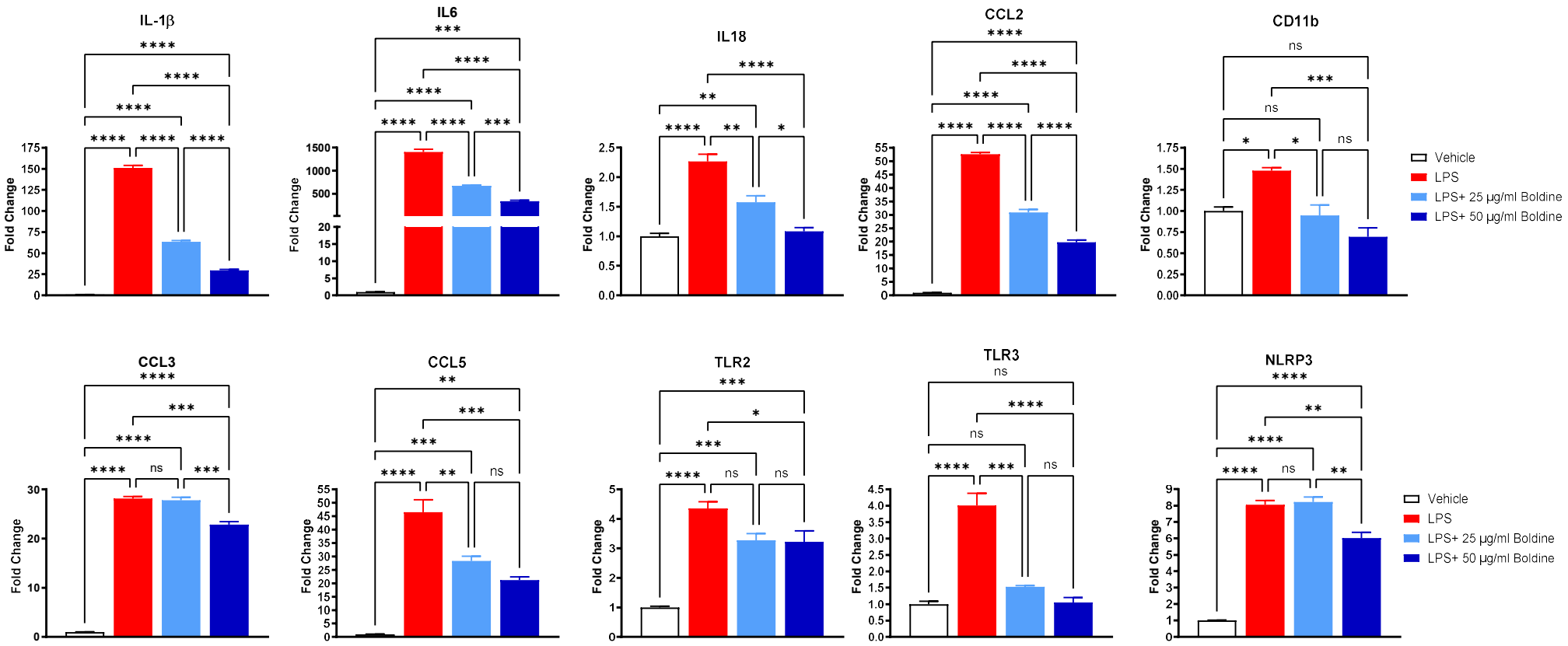
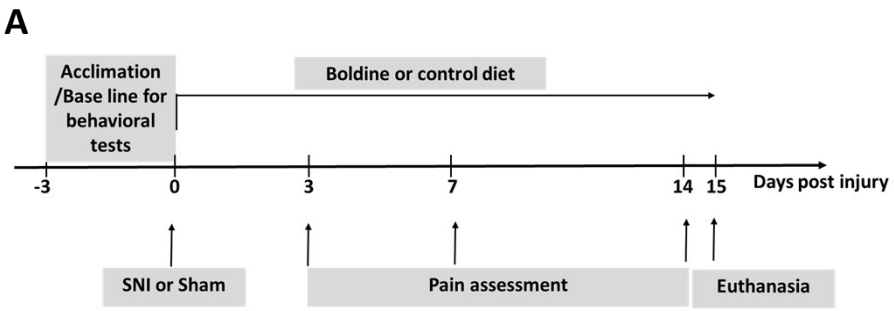
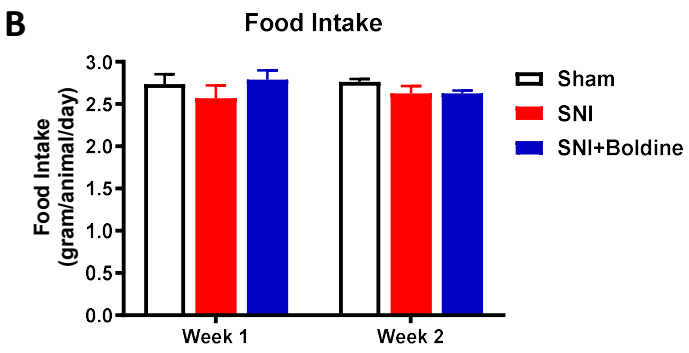
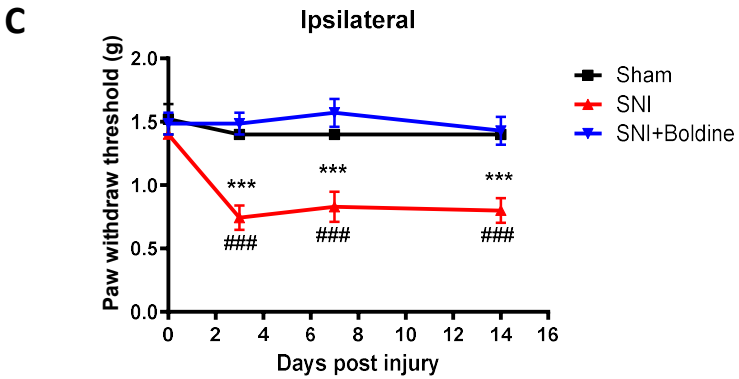
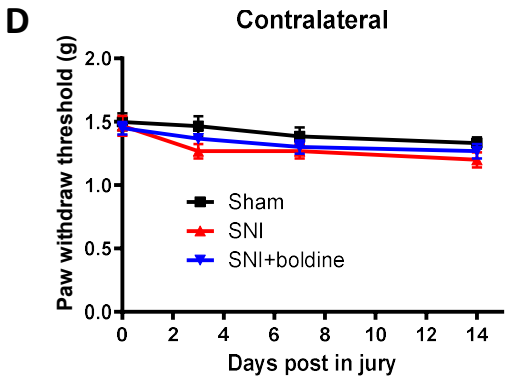
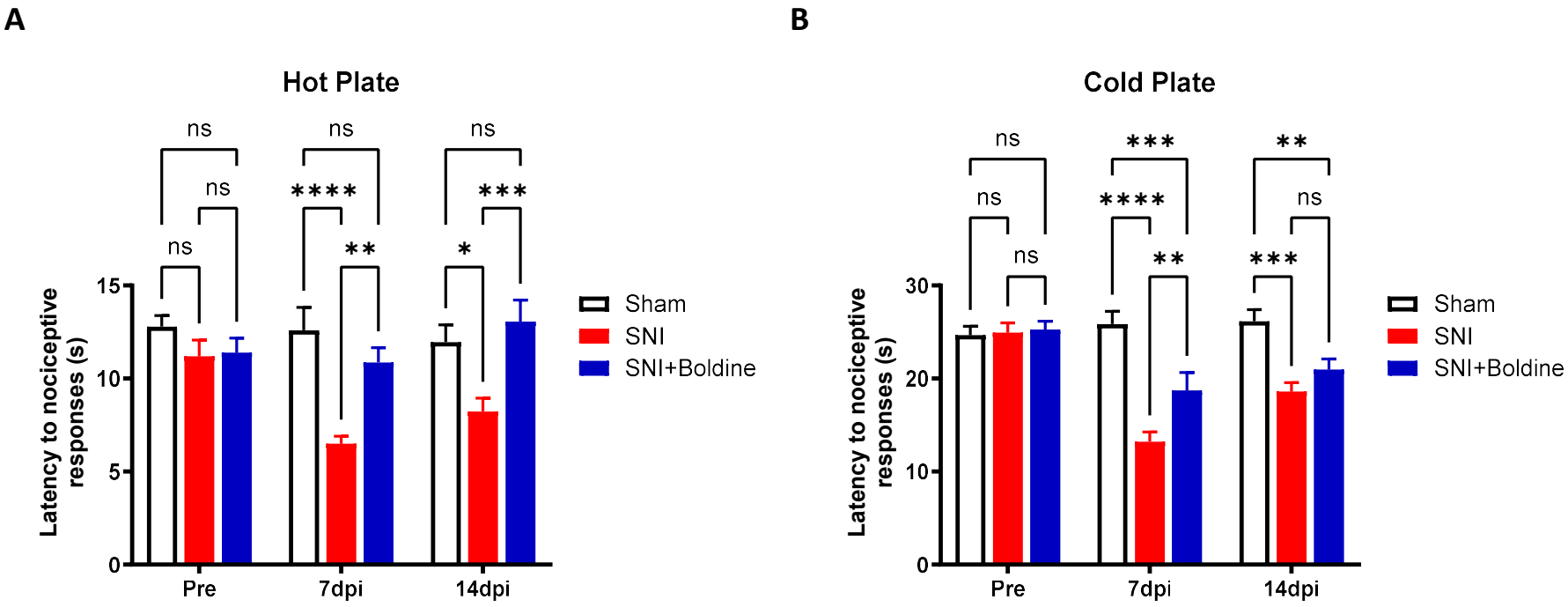

IL-1β
IL6
IL18
CCL2
CD11b
CCL3
CCL5
TLR2
TLR3
NLRP3
Fold Change
Vehicle
LPS
LPS+ 25 µg/ml Boldine
LPS+ 50 µg/ml Boldine

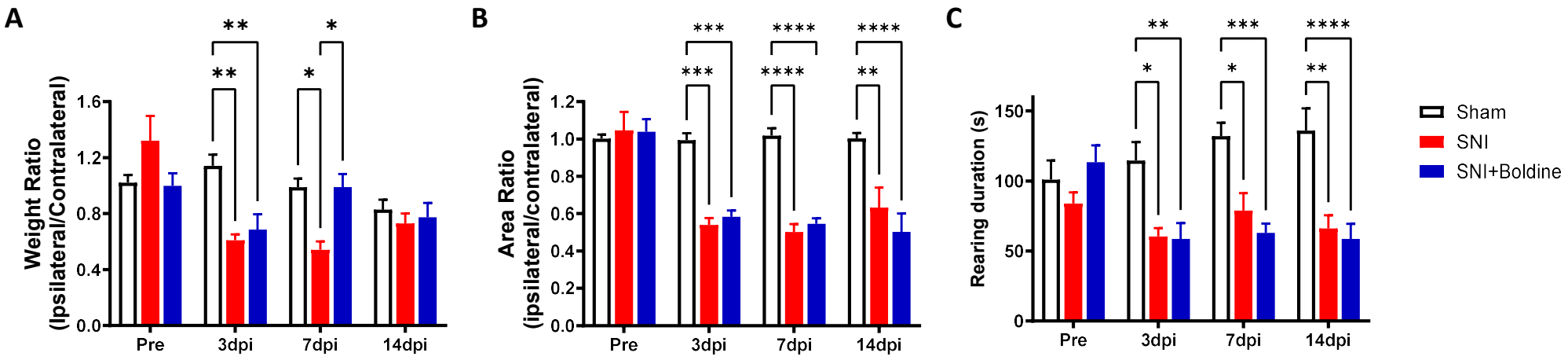

A
Acclimation /Base line for behavioral tests
Boldine or control diet
-3
0
3
7
14
15
Days post injury
SNI or Sham
Pain assessment
Euthanasia

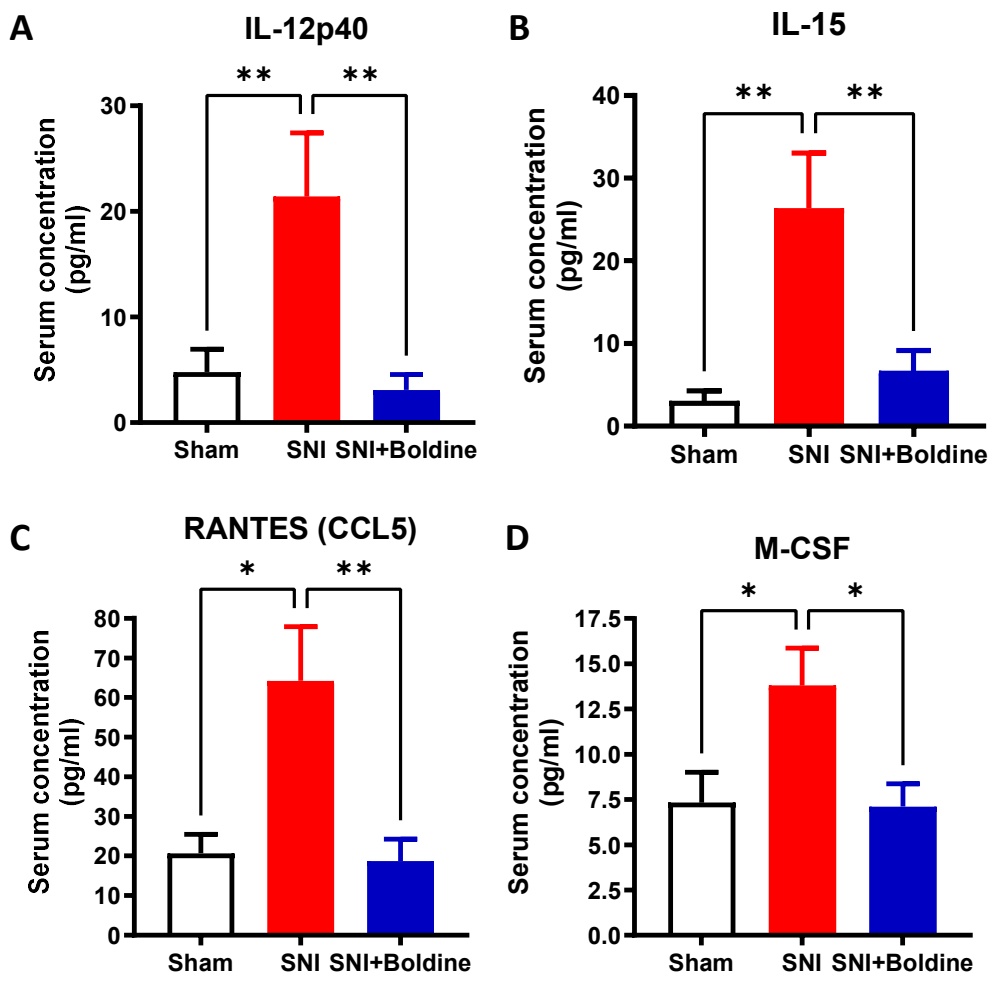

B
Food Intake
Food Intake (gram/animal/day)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Week 1
Week 2
Sham
SNI
SNI+Boldine

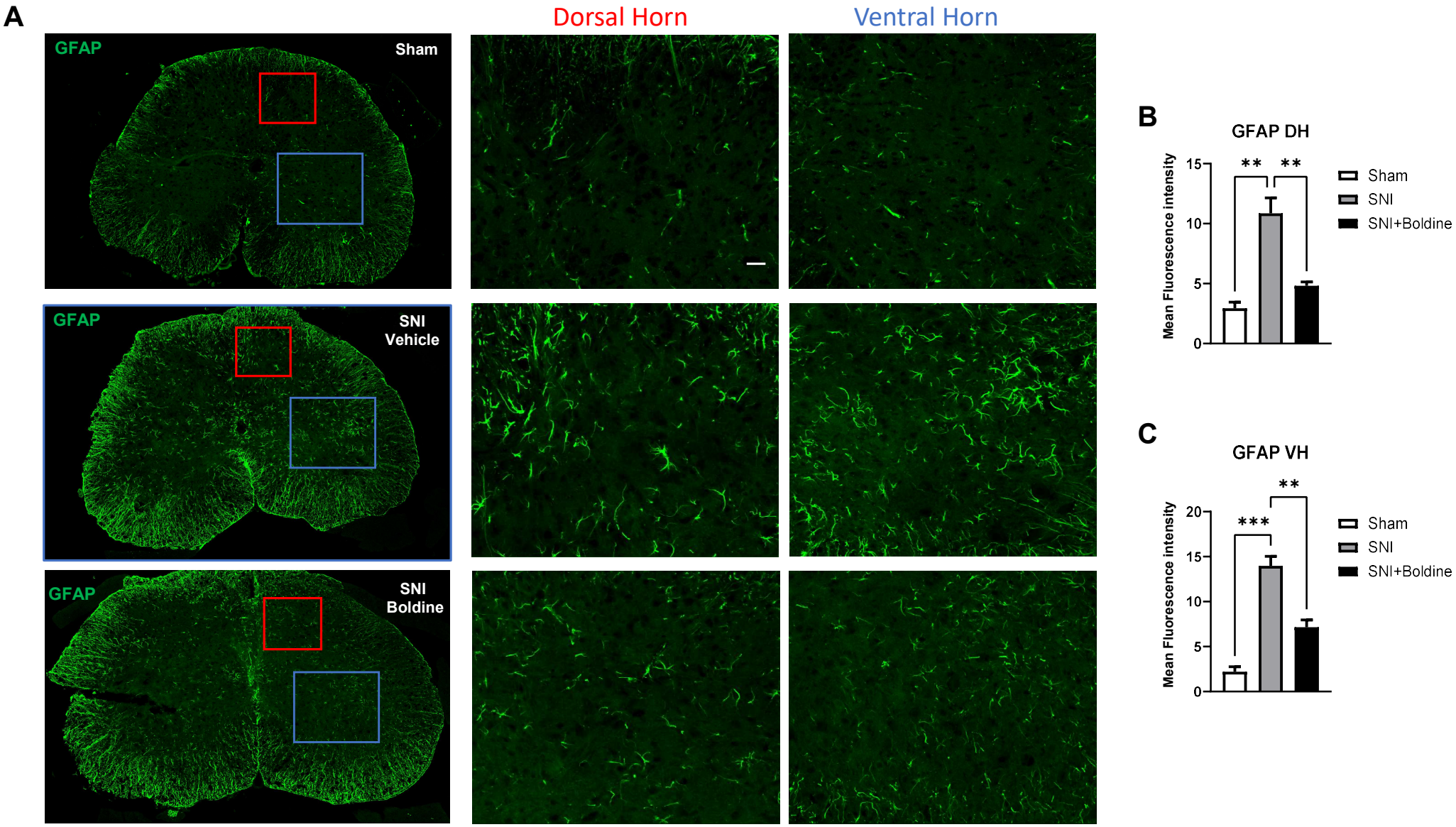

C
Ipsilateral
Paw withdraw threshold (g)
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
14
16
Days post injury
***
###
Sham
SNI
SNI+Boldine

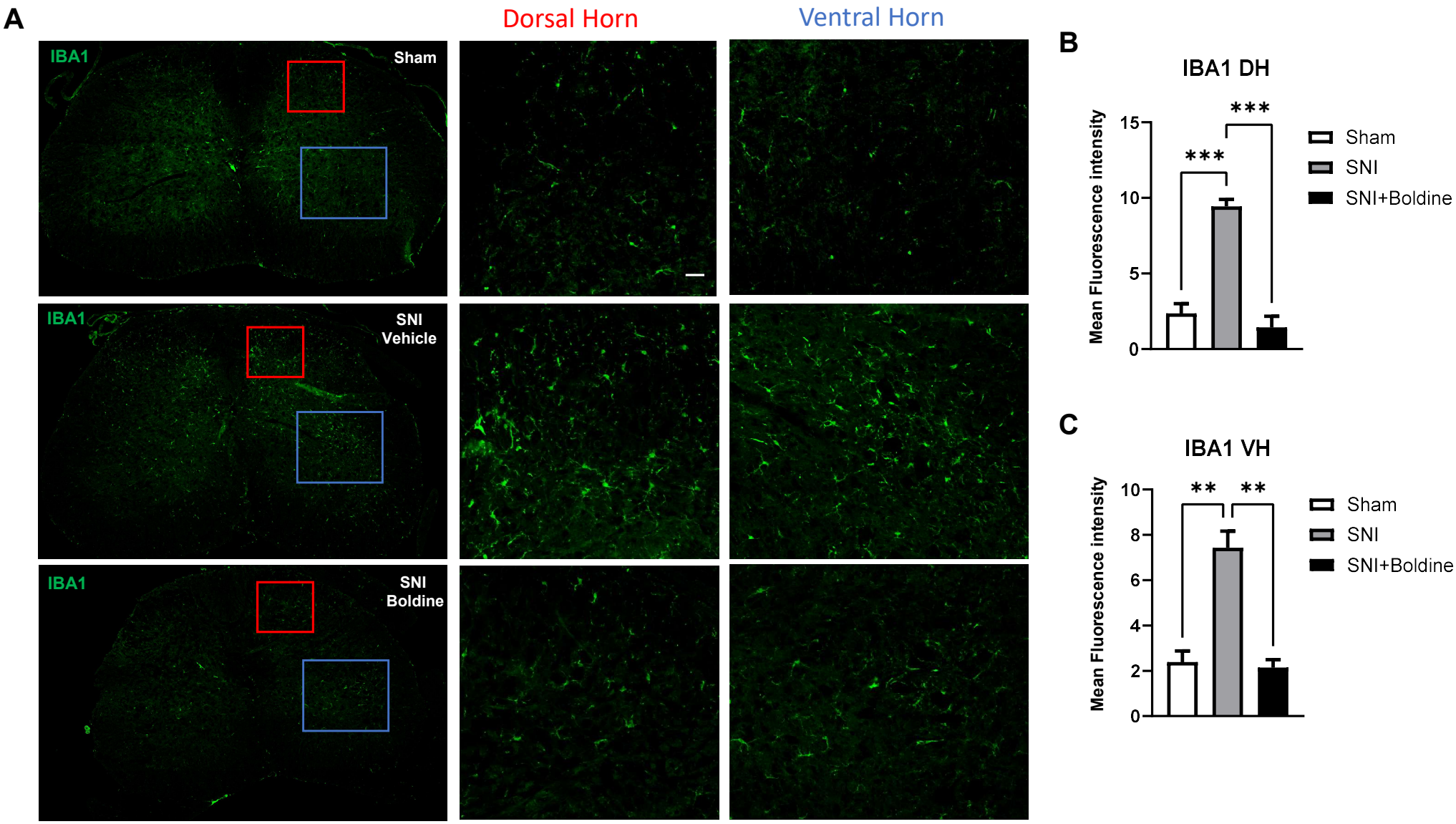

D
Contralateral
Paw withdraw threshold (g)
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
14
Days post in jury
Sham
SNI
SNI+boldine

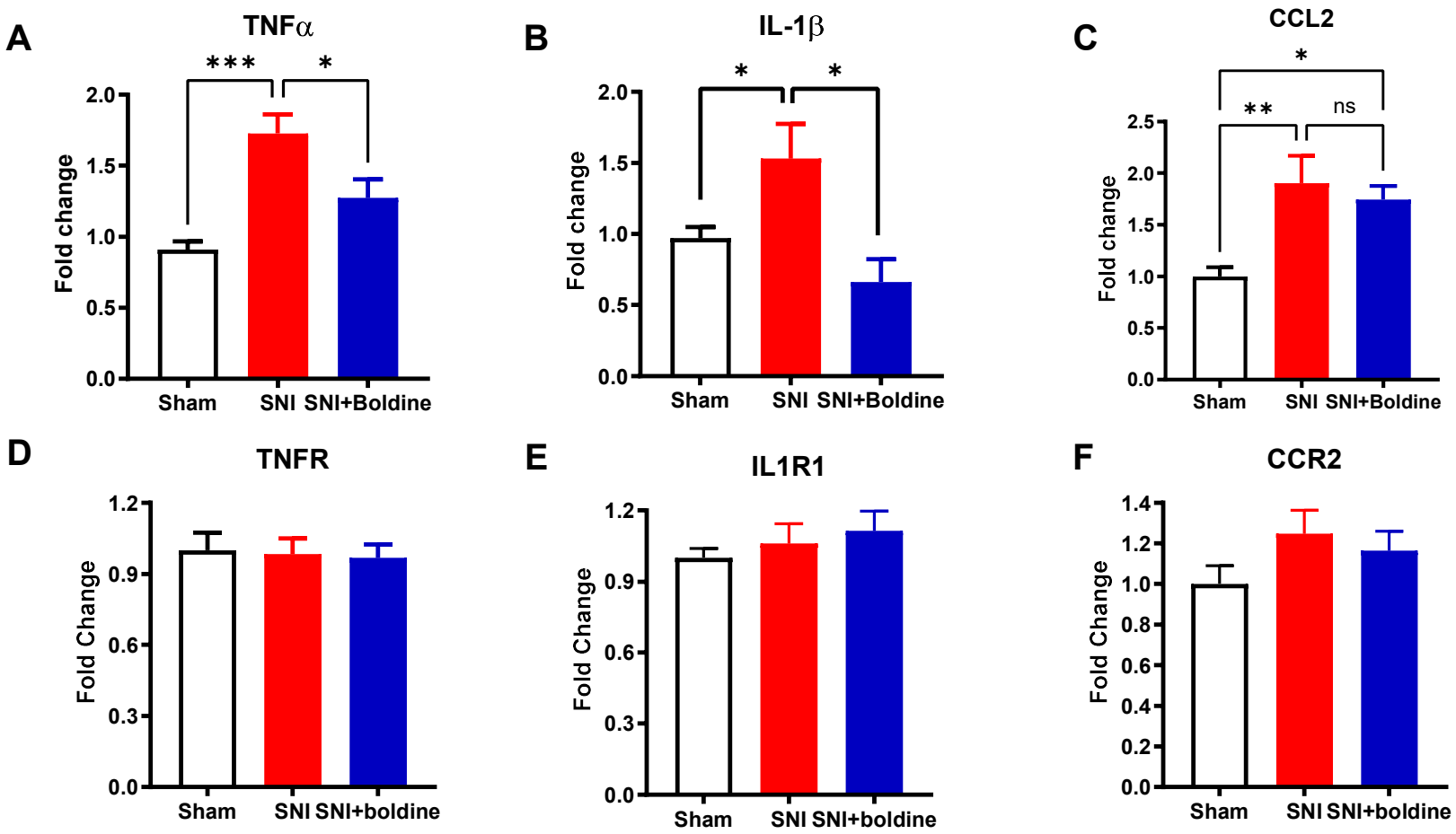

A
Hot Plate
ns
ns
ns
ns
****
ns
***
**
*
Latency to nociceptive responses (s)
15
10
5
0
Pre
7dpi
14dpi
Sham
SNI
SNI+Boldine
B
Cold Plate
ns
ns
ns
***
****
**
**
ns
***
Latency to nociceptive responses (s)
30
20
10
0
Pre
7dpi
14dpi
Sham
SNI
SNI+Boldine


A
Weight Ratio
(Ipsilateral/Contralateral)
1.6
1.2
0.8
0.4
0.0
**
**
*
*
Pre
3dpi
7dpi
14dpi
B
Area Ratio
(ipsilateral/contralateral)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
****
****
****
**
Pre
3dpi
7dpi
14dpi
C
Rearing duration (s)
150
100
50
0
**
*
***
*
****
**
Pre
3dpi
7dpi
14dpi
Sham
SNI
SNI+Boldine


A
IL-12p40
Serum concentration (pg/ml)
30
20
10
0
**
**
Sham
SNI
SNI+Boldine
B
IL-15
Serum concentration (pg/ml)
40
30
20
10
0
**
**
Sham
SNI
SNI+Boldine
C
RANTES (CCL5)
Serum concentration (pg/ml)
80
70
60
50
40
30
20
10
0
*
**
Sham
SNI
SNI+Boldine
D
M-CSF
Serum concentration (pg/ml)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
*
*
Sham
SNI
SNI+Boldine


A
Dorsal Horn
Ventral Horn
GFAP
Sham
GFAP
SNI
Vehicle
GFAP
SNI
Boldine
B
GFAP DH
Mean Fluorescence intensity
15
10
5
0
**
**
Sham
SNI
SNI+Boldine
C
GFAP VH
Mean Fluorescence intensity
20
15
10
5
0
***
**
Sham
SNI
SNI+Boldine


A
Dorsal Horn
Ventral Horn
IBA1
Sham
IBA1
SNI
Vehicle
IBA1
SNI
Boldine
B
IBA1 DH
Mean Fluorescence intensity
15
10
5
0
***
***
Sham
SNI
SNI+Boldine
C
IBA1 VH
Mean Fluorescence intensity
10
8
6
4
2
0
**
**
Sham
SNI
SNI+Boldine


A
TNFα
***
*
Fold change
2.0
1.5
1.0
0.5
0.0
Sham
SNI
SNI+Boldine
B
IL-1β
*
*
Fold change
2.0
1.5
1.0
0.5
0.0
Sham
SNI
SNI+Boldine
C
CCL2
*
**
ns
Fold change
2.5
2.0
1.5
1.0
0.5
0.0
Sham
SNI
SNI+Boldine
D
TNFR
Fold Change
1.2
0.9
0.6
0.3
0.0
Sham
SNI
SNI+boldine
E
IL1R1
Fold Change
1.2
0.9
0.6
0.3
0.0
Sham
SNI
SNI+boldine
F
CCR2
Fold Change
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sham
SNI
SNI+boldine