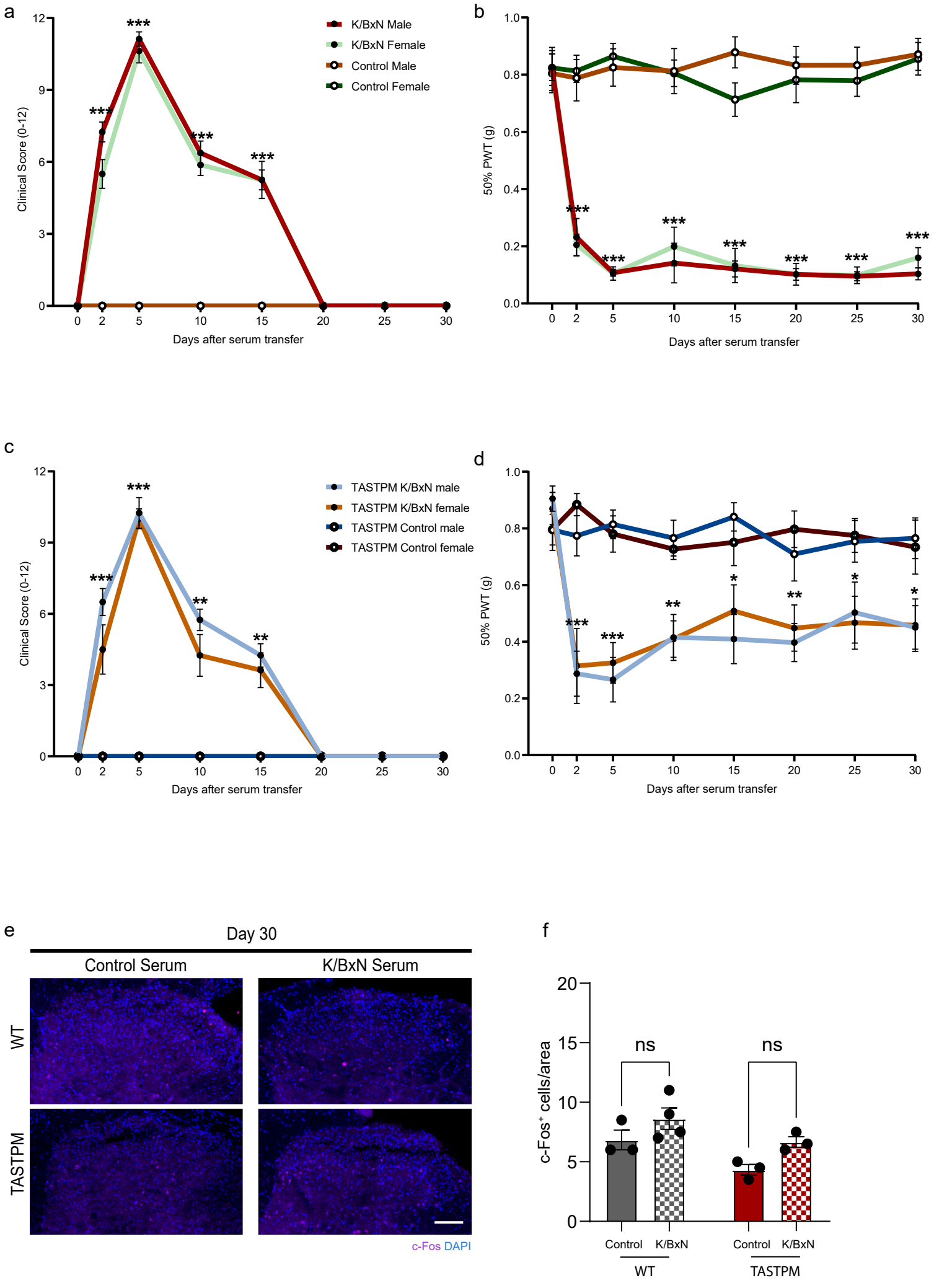
Supplementary Information

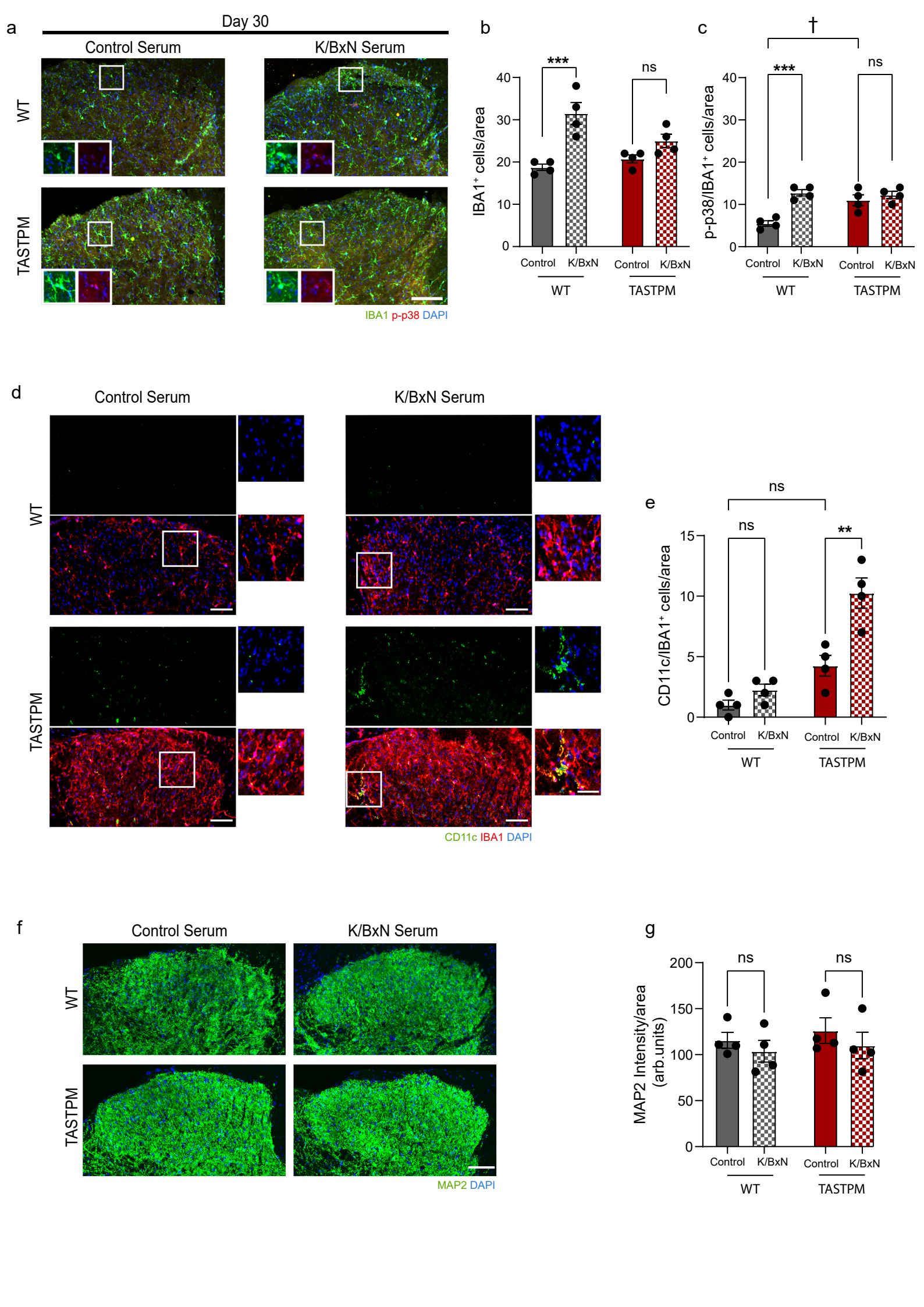
Galectin-3 activates spinal microglia to induce inflammatory nociception in wild type but not in mice modelling Alzheimer's Disease

Sideris-Lampretsas et al.

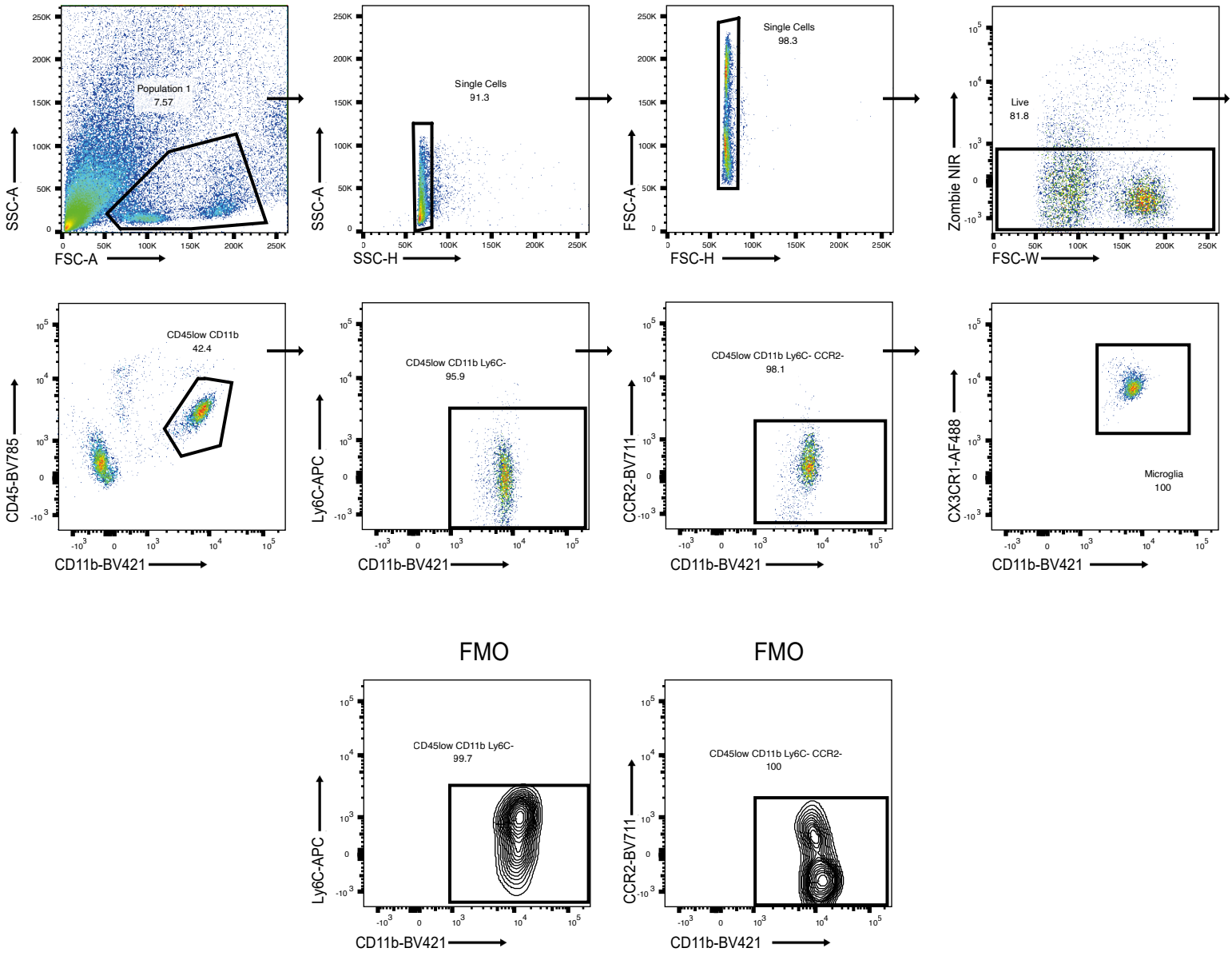

a
Clinical Score (0-12)
Days after serum transfer
K/BxN Male
K/BxN Female
Control Male
Control Female
b
50% PWT (g)
Days after serum transfer
c
Clinical Score (0-12)
Days after serum transfer
TASTPM K/BxN male
TASTPM K/BxN female
TASTPM Control male
TASTPM Control female
d
50% PWT (g)
Days after serum transfer
e
Day 30
Control Serum
K/BxN Serum
WT
TASTPM
c-Fos DAPI
f
c-Fos⁺ cells/area
ns
ns
Control
K/BxN
Control
K/BxN
WT
TASTPM

Supplementary figures

Supplementary Figure 1: No sex-dependent changes in clinical scores and mechanical hypersensitivity in WT and TASTPM K/BxN ST. (a) Clinical scores of fore and hind paws and (b) mechanical hypersensitivity in male and female WT after K/BxN ST. (c) Clinical scores of fore and hind paws and (d) mechanical hypersensitivity in male and female TASTPM after K/BxN ST. n = 8 male and female mice per group. Data represent mean ± SEM. ns – not significant, *p < 0.05, **p < 0.01, ***p < 0.001, Two-Way RM ANOVA, Tukey's multiple comparisons test. (e) Representative images of c-Fos$^+$ neurons in DH. Scalebar, 100µm (f) Quantification of c-Fos$^+$ neurons in WT and TASTPM K/BxN ST at Day 30. n = 3 (WT and TASTPM Control ST and TASTPM K/BxN ST), n = 4 (WT K/BxN ST). Data represent mean ± SEM. Two-way ANOVA, Tukey's multiple comparisons test.

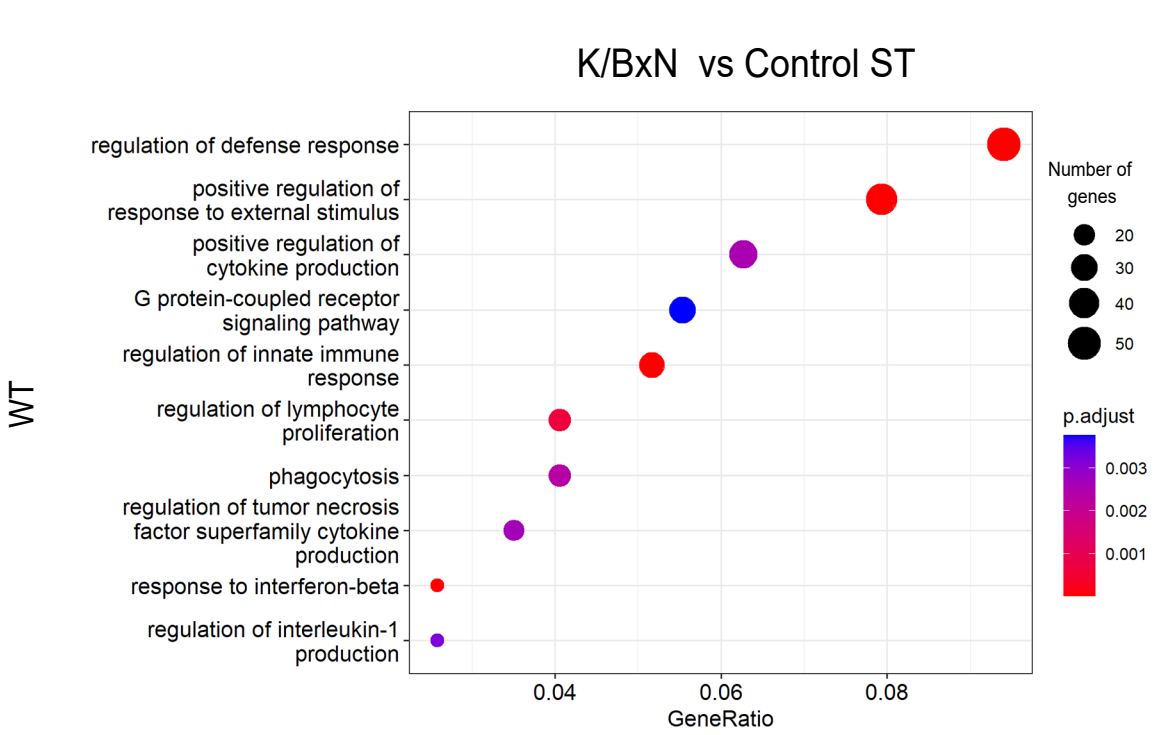

a
Day 30
Control Serum
K/BxN Serum
WT
TASTPM
IBA1 p-p38 DAPI
b
IBA1+ cells/area
***
ns
Control
K/BxN
WT
TASTPM
c
†
p-p38/IBA1+ cells/area
d
CD11c IBA1 DAPI
e
CD11c/IBA1+ cells/area
**
f
MAP2 DAPI
g
MAP2 Intensity/area (arb.units)

Supplementary Figure 2: Changes in microglial markers in WT and TASTPM after K/BxN ST. (a) Increased number of pp38^{+} microglia in the dorsal horn of WT and TASTPM K/BxN ST at day 30. Scalebar, 100μm (b, c) Quantification of IBA1^{+} and p-p38/IBA1^{+} microglia in DH at Day 30. Data represent mean ± SEM, n = 4 mice per group. ns – not significant, ***p < 0.001, ****p < 0.0001, †p < 0.05. Two-way ANOVA, Tukey's multiple comparisons test. (d) Increased number of CD11c^{+} microglia in DH of TASTPM at day 5 K/BxN ST. Scalebar, 100 μm (e) Quantification of CD11c/IBA1^{+} microglia. Data represent mean ± SEM, n = 4 mice per group. **p = 0.0011. Two-way ANOVA, Tukey's multiple comparisons test. (f) No change in MAP2 intensity in DH of WT and TASTPM at day 5 K/BxN ST. Scalebar, 100 μm (e) Quantification of MAP2 intensity. Data represent mean ± SEM, n = 4 mice per group. ns – not significant, Two-way ANOVA, Tukey's multiple comparisons test.

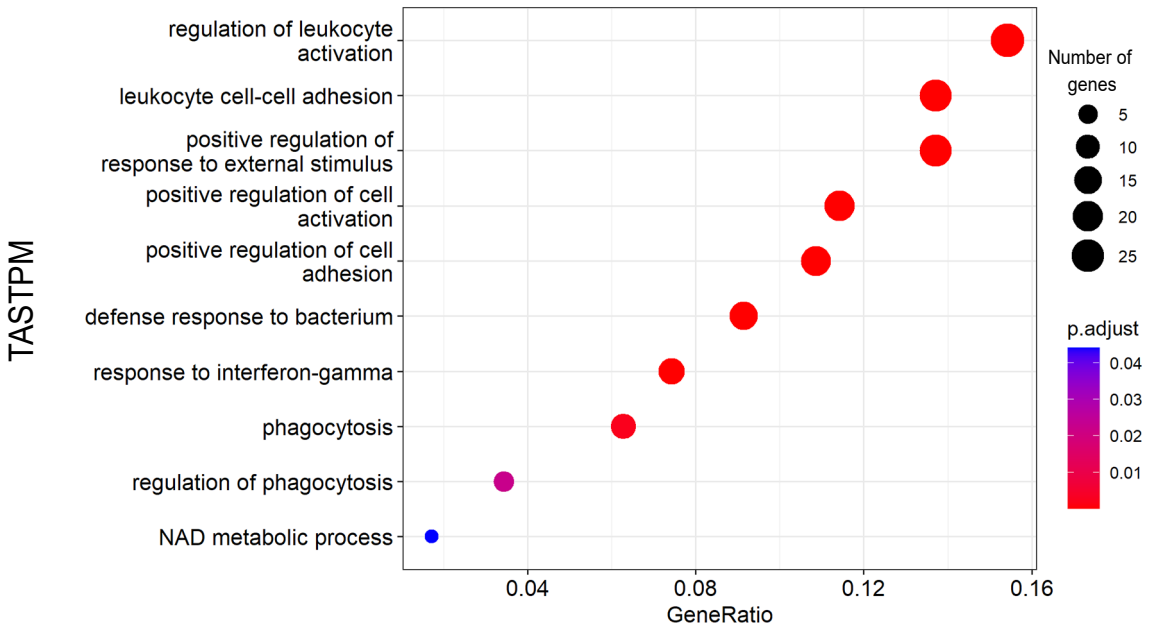

Population 1
7.57
SSC-A
FSC-A
Single Cells
91.3
SSC-H
Single Cells
98.3
FSC-H
Live
81.8
Zombie NIR
FSC-W
CD45low CD11b
42.4
CD45-BV785
CD11b-BV421
CD45low CD11b Ly6C-
95.9
Ly6C-APC
CD45low CD11b Ly6C- CCR2-
98.1
CCR2-BV711
Microglia
100
CX3CR1-AF488
FMO
CD45low CD11b Ly6C-
99.7
FMO
CD45low CD11b Ly6C- CCR2-
100

Supplementary Figure 3: Gating Strategy for microglia isolation using FACS. $CD45^{low}$ $CD11b^{+}$ $Ly6C^{-}$ $CCR2^{-}$ $CX3CR1^{+}$ cells were isolated per dorsal spinal cord.

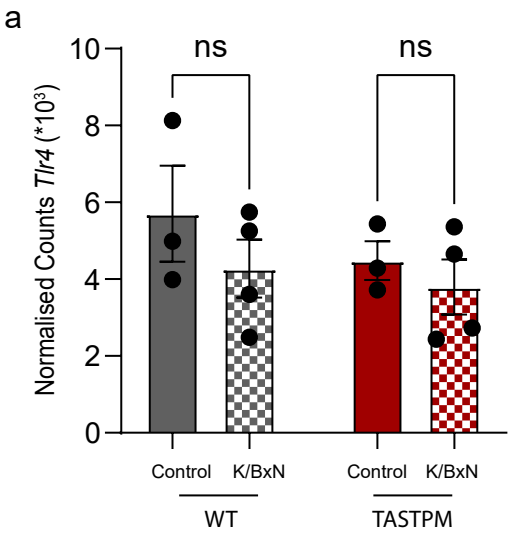
a

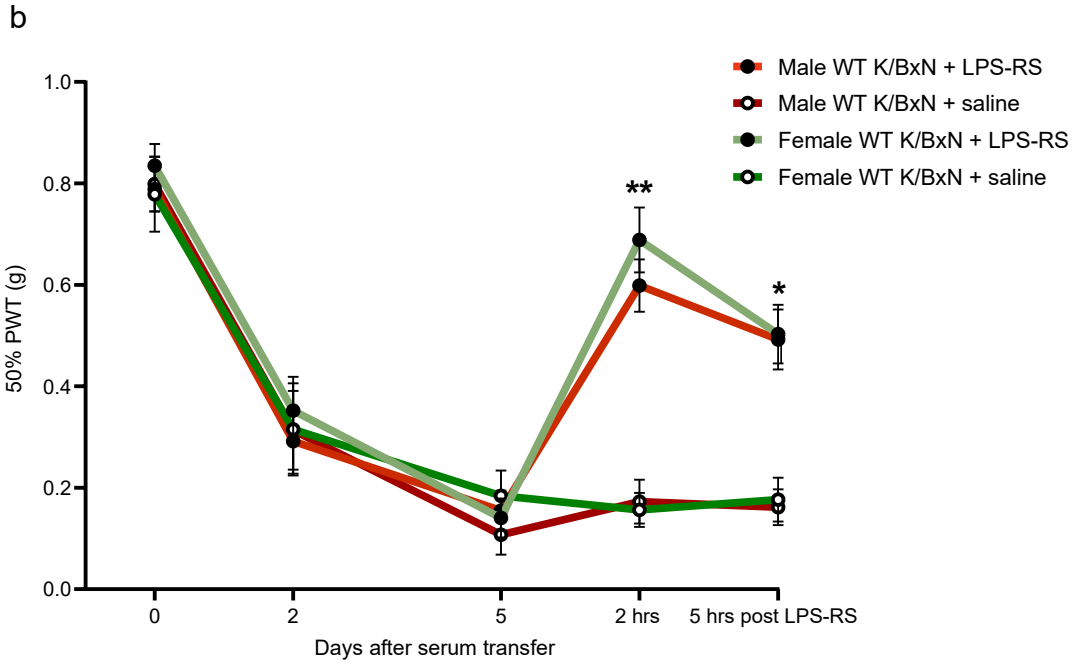

K/BxN vs Control ST
WT
regulation of defense response
positive regulation of response to external stimulus
positive regulation of cytokine production
G protein-coupled receptor signaling pathway
regulation of innate immune response
regulation of lymphocyte proliferation
phagocytosis
regulation of tumor necrosis factor superfamily cytokine production
response to interferon-beta
regulation of interleukin-1 production
0.04
0.06
0.08
GeneRatio
Number of genes
20
30
40
50
p.adjust
0.003
0.002
0.001

b

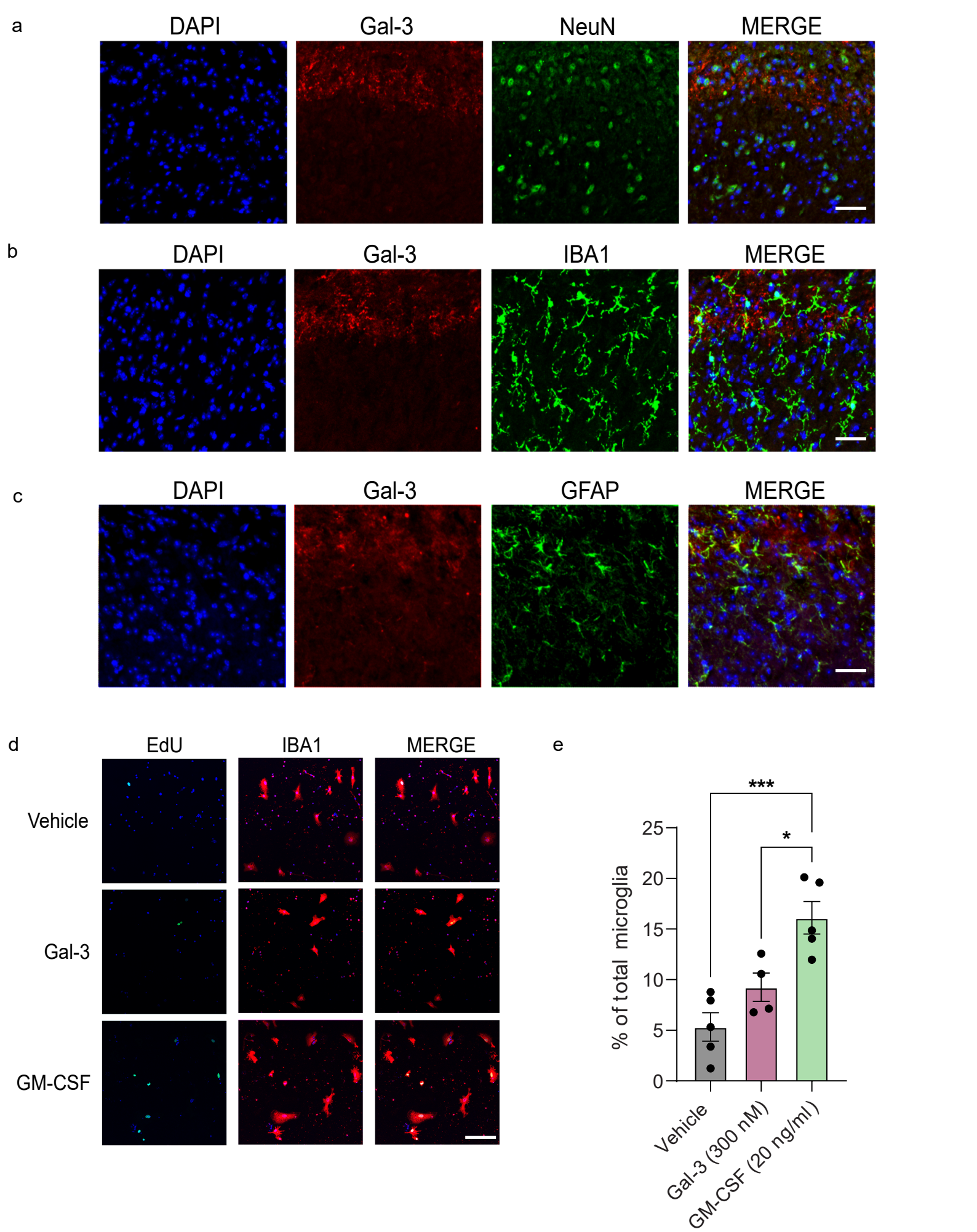

TASTPM
regulation of leukocyte activation
leukocyte cell-cell adhesion
positive regulation of response to external stimulus
positive regulation of cell activation
positive regulation of cell adhesion
defense response to bacterium
response to interferon-gamma
phagocytosis
regulation of phagocytosis
NAD metabolic process
0.04
0.08
0.12
0.16
GeneRatio
Number of genes
5
10
15
20
25
p.adjust
0.04
0.03
0.02
0.01

Supplementary Figure 4: Transcripts expressed by dorsal horn microglia in the WT K/BxN ST are enriched in pathways associated with IL-1β, TNF-α and IFN-β signalling, while microglia in the TASTPM K/BxN ST show upregulation of transcripts involved in glycolysis and metabolism. (a) Top 10 Gene Ontology (GO) pathways overrepresented in DEGs in DH microglia in the WT K/BxN ST. (b) Top 10 Gene Ontology (GO) pathways overrepresented in DEGs in DH microglia in the TASTPM K/BxN ST. GeneRatio is the number of observed genes divided by the number of expected genes from each GO. n = 3 (WT and TASTPM Control), n = 4 (WT and TASTPM K/BxN ST).

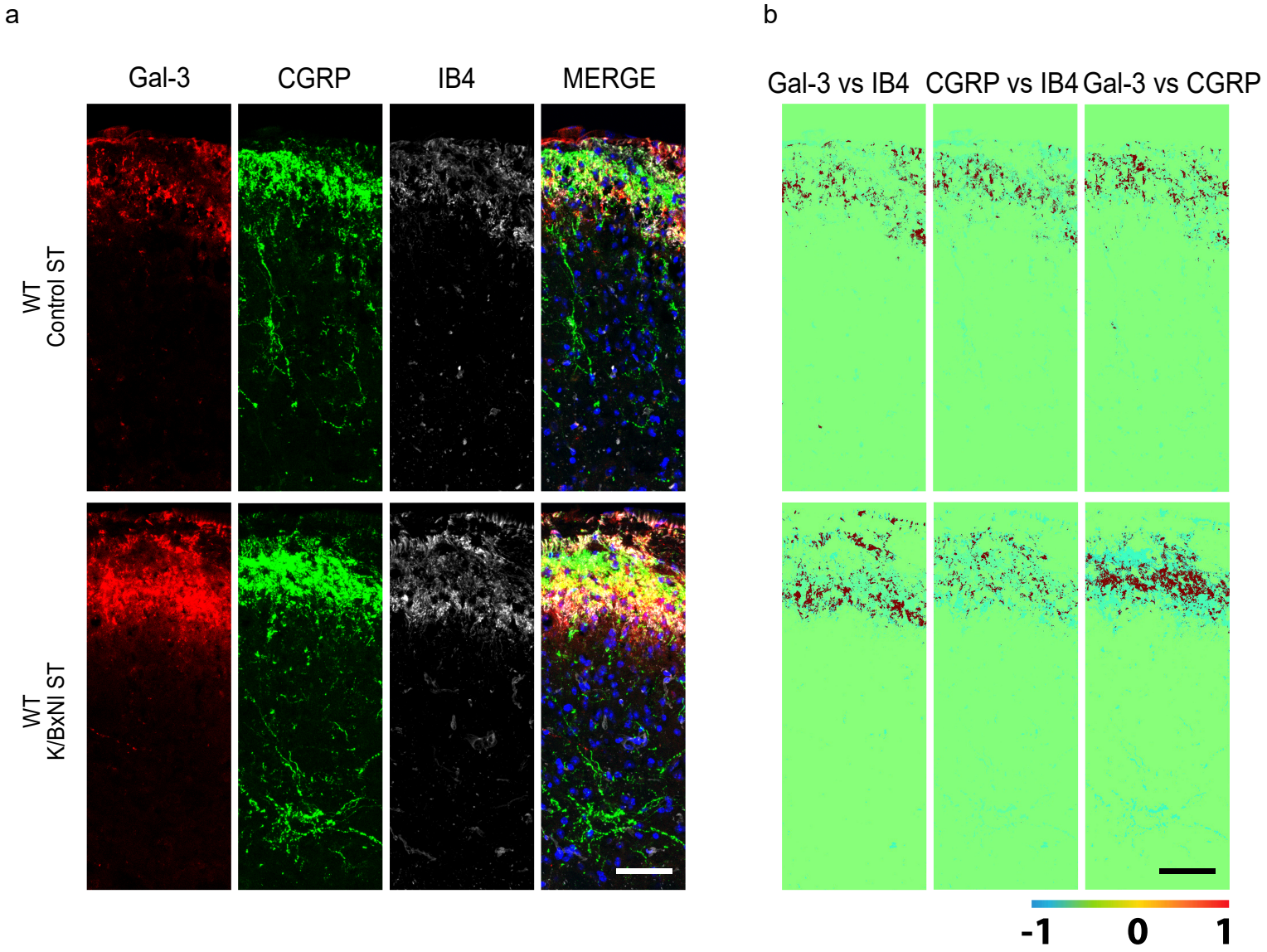

a
ns
ns
10
8
6
4
2
0
Normalised Counts Tlr4 (*10³)
Control
K/BxN
Control
K/BxN
WT
TASTPM

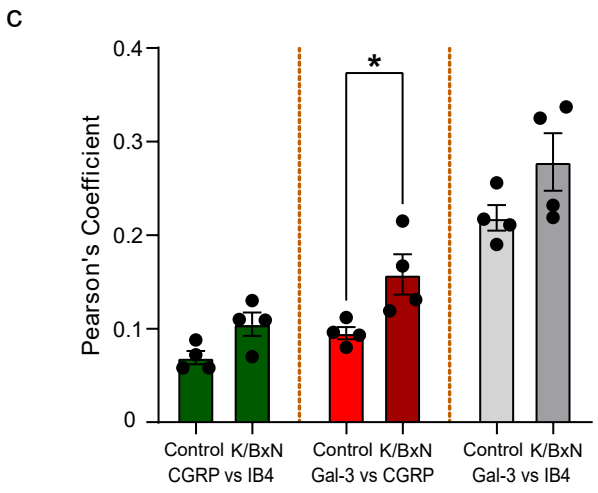

b
Male WT K/BxN + LPS-RS
Male WT K/BxN + saline
Female WT K/BxN + LPS-RS
Female WT K/BxN + saline
1.0
0.8
0.6
0.4
0.2
0.0
50% PWT (g)
**
*
0
2
5
2 hrs
5 hrs post LPS-RS
Days after serum transfer

Supplementary Figure 5: Spinal TLR4 in K/BxN ST-induced inflammatory allodynia. (a) Quantification of normalised counts from RNA-seq for *Tlr4*. Normalised counts were calculated using variance stabilizing transformation from R package DESeq2. ns – not significant. n = 3 (WT and TASTPM Control), n = 4 (WT and TASTPM K/BxN ST). (b) Intrathecal injection of a TLR4 antagonist (LPS-RS) (10 µg/ 5 µl/mouse) reversed mechanical hypersensitivity both in male and female WT K/BxN ST. Data represent mean ± SEM, n = 8 mice per group. *p < 0.05, **p < 0.01, compared to saline-treated same sex WT K/BxN ST, Two-Way RM ANOVA, Tukey's multiple comparisons test.

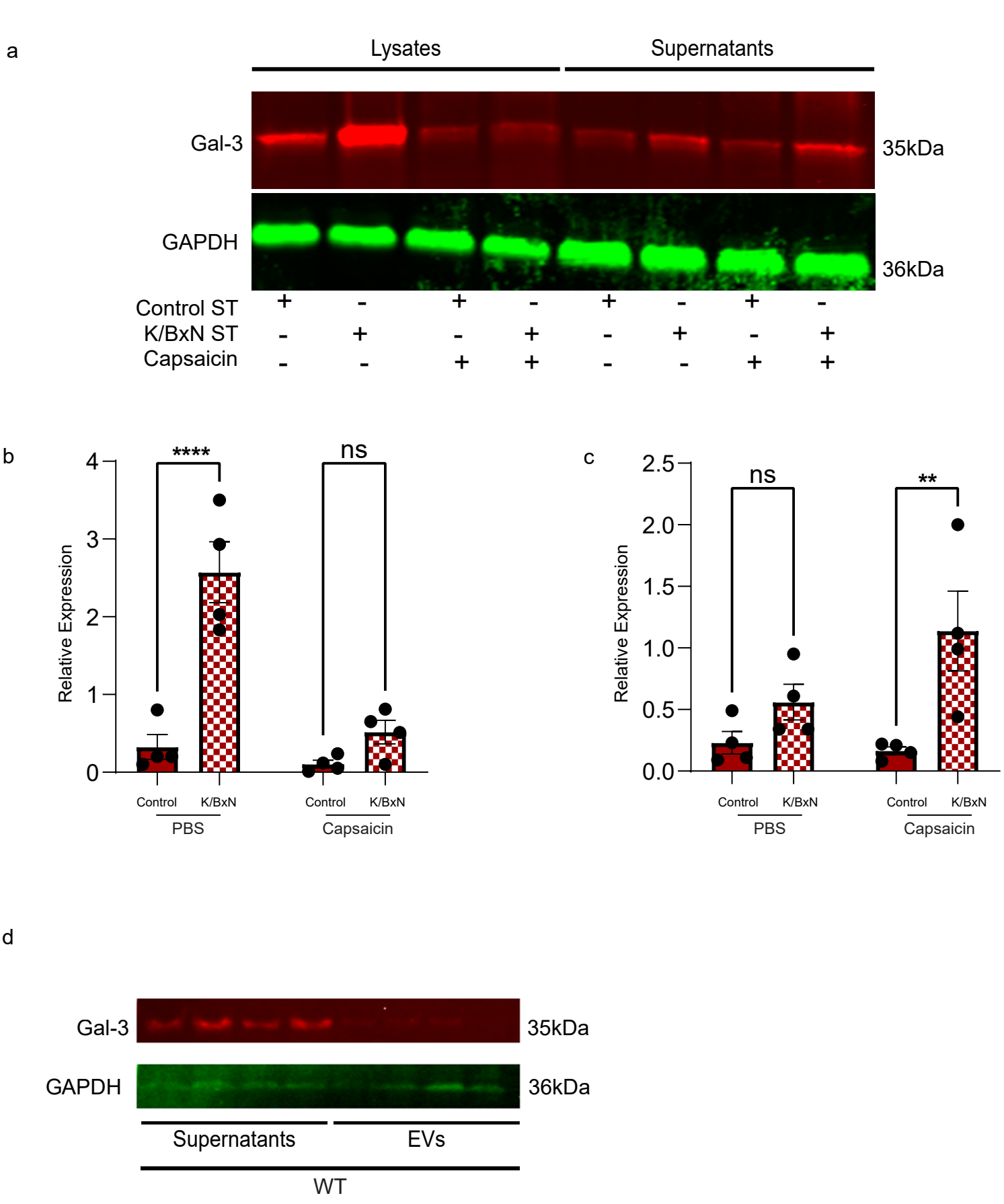

a
DAPI
Gal-3
NeuN
MERGE
b
DAPI
Gal-3
IBA1
MERGE
c
DAPI
Gal-3
GFAP
MERGE
d
EdU
IBA1
MERGE
Vehicle
Gal-3
GM-CSF
e
***
*
% of total microglia
25
20
15
10
5
0
Vehicle
Gal-3 (300 nM)
GM-CSF (20 ng/ml)

Supplementary Figure 6: Gal-3 is minimally expressed by microglia and astrocytes in WT mice, and not expressed by dorsal horn projection neurons. (a) Representative images of Gal-3 and NeuN in the dorsal lumbar spinal cord. (b) Representative images of Gal-3 and IBA1 in DH. (c) Representative images of Gal-3 and GFAP in DH. Scalebar, 20 µm. (d) Gal-3 (300 nM) did not induce proliferation in in vitro cultured primary microglia. (e) Quantification of percentage of EdU/IBA1^{+} microglia. Data represent mean ± SEM, n = 4 (Gal-3), n = 5 (GM-CSF was used as a positive control). *p = 0.0264, ***p = 0.0008. One-way ANOVA, Bonferroni's multiple comparisons test.

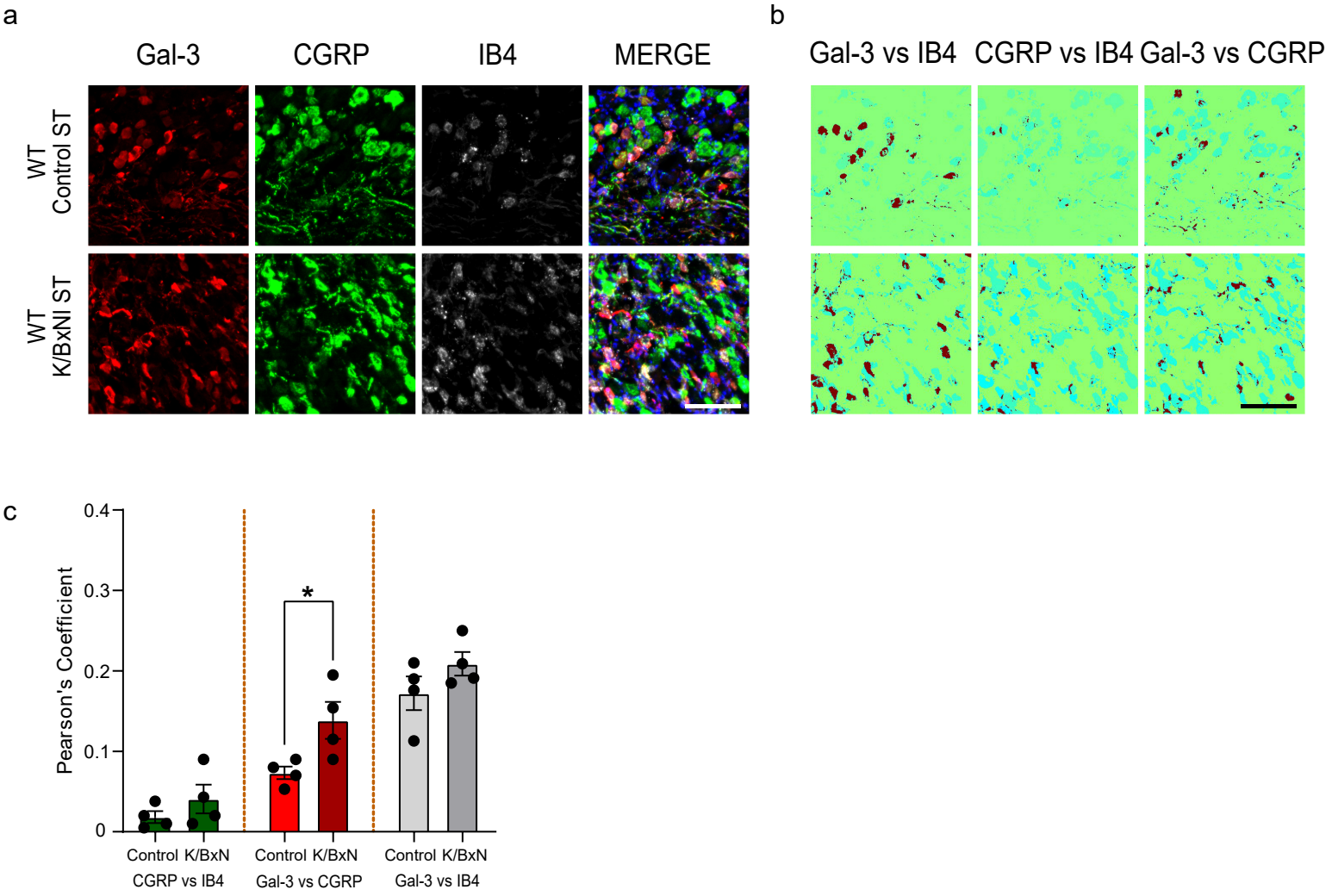

a
Gal-3
CGRP
IB4
MERGE
WT
Control ST
WT
K/BxNI ST
b
Gal-3 vs IB4
CGRP vs IB4
Gal-3 vs CGRP
-1
0
1


c
Pearson's Coefficient
0.4
0.3
0.2
0.1
0
*
Control
K/BxN
CGRP vs IB4
Control
K/BxN
Gal-3 vs CGRP
Control
K/BxN
Gal-3 vs IB4

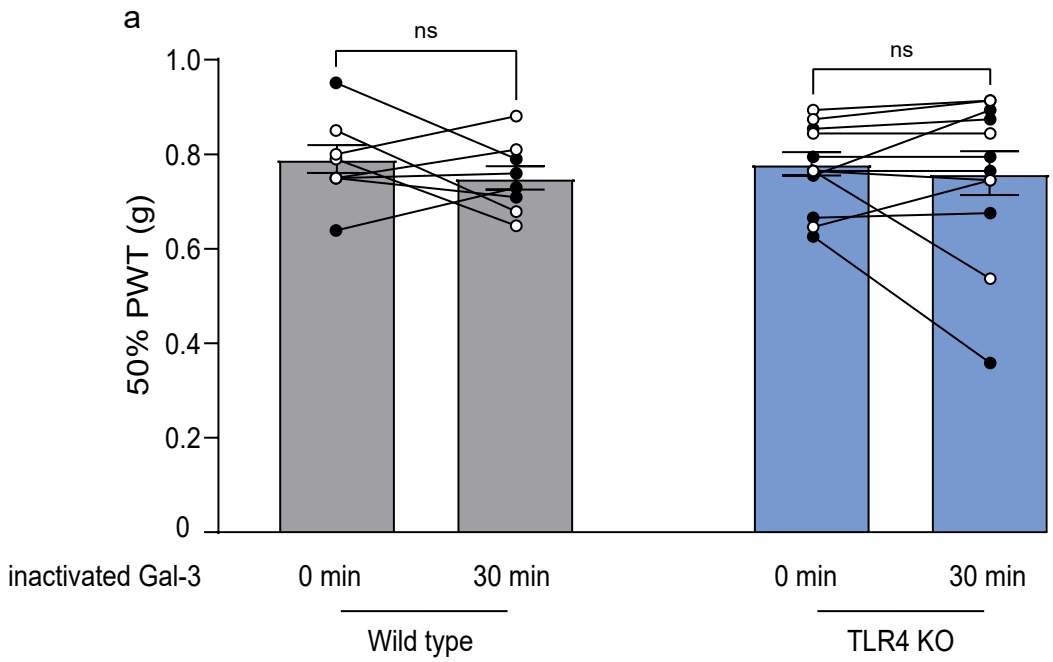
Supplementary Figure 7: Gal-3 is normally expressed in non-peptidergic IB4^{+} terminals in DH superficial laminae, but also in peptidergic CGRP terminals in K/BxN ST at day 5. (a) Representative images of immunostaining in primary afferent nerve terminals of control and K/BxN ST DH. Scalebar, 20μm, (b) Representative colocalization heatmaps generated by Colocalization Colormap. (c) Quantification of colocalising pixel proportion shows more Gal-3^{+} terminals colocalised with CGRP^{+} terminals in the superficial laminae of the dorsal lumbar spinal cord in WT K/BxN ST. Data represent mean ± SEM. n = 4 mice per group. *p = 0.0319, Unpaired Student's *t*-test, two-tailed.

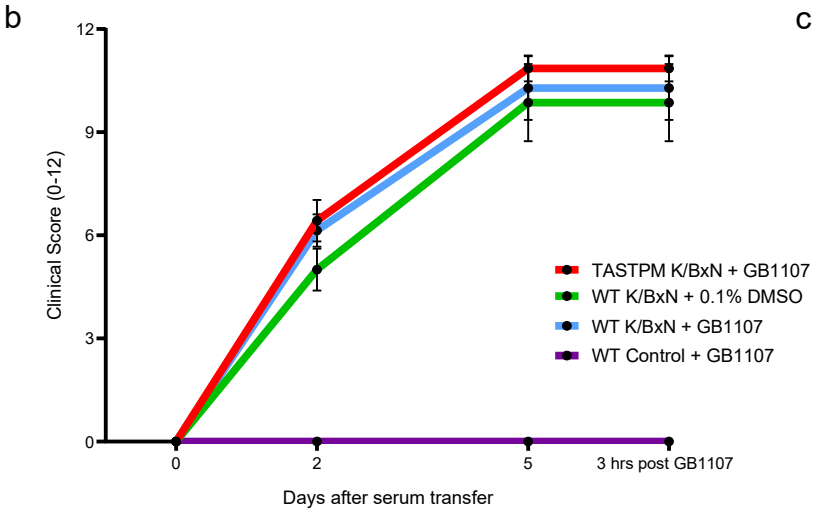

a
Lysates
Supernatants
Gal-3
35kDa
GAPDH
36kDa
Control ST
K/BxN ST
Capsaicin
b
Relative Expression
****
ns
Control
K/BxN
PBS
Capsaicin
c
Relative Expression
ns
**
Control
K/BxN
PBS
Capsaicin
d
Gal-3
35kDa
GAPDH
36kDa
Supernatants
EVs
WT

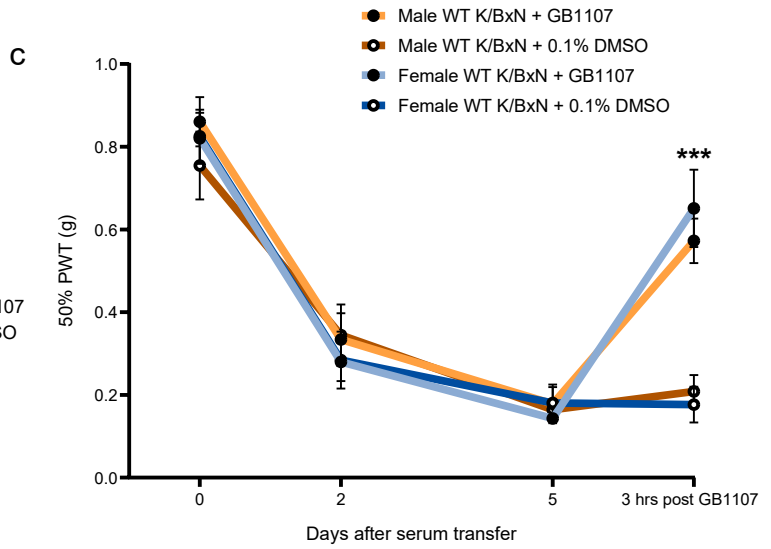
Supplementary Figure 8: Gal-3 is released by capsaicin in TASTPM control and K/BxN ST DH synaptosomes. (a) Western blots and quantification of Gal-3 in synaptosome lysates (b) and supernatants (c) after incubation of capsaicin (1 μM for 3 h). Data represent mean ± SEM, n = 4 mice per group. ns – not significant, **p = 0.0056, ****p < 0.0001, Two-way ANOVA, Tukey's multiple comparisons test. (d) Representative Western blots showing no Gal-3 detection in the EV fraction of synaptosome preparations from WT K/BxN ST.


a
Gal-3
CGRP
IB4
MERGE
WT Control ST
WT K/BxNI ST
b
Gal-3 vs IB4
CGRP vs IB4
Gal-3 vs CGRP
c
Pearson's Coefficient
0.4
0.3
0.2
0.1
0
*
Control K/BxN
CGRP vs IB4
Control K/BxN
Gal-3 vs CGRP
Control K/BxN
Gal-3 vs IB4

Supplementary Figure 9: In the dorsal root ganglia, Gal-3 is normally expressed in non-peptidergic IB4$^+$ neurons, and expression is also evident in peptidergic CGRP neurons in K/BxN ST at day 5. (a) Representative images of control and K/BxN ST lumbar DRGs. Scalebar, 20 μm, (b) Representative colocalization heatmaps generated by Colocalization Colormap. (c) Quantification of colocalising pixel proportions shows that more Gal-3$^+$ neurons colocalised with CGRP$^+$ neurons in day 5 K/BxN ST than control ST DRGs. Data represent mean ± SEM, n = 4 mice per group, *p = 0.0363, Unpaired Student's *t*-test, two-tailed.


a
ns
ns
1.0
0.8
0.6
0.4
0.2
0
50% PWT (g)
inactivated Gal-3
0 min
30 min
0 min
30 min
Wild type
TLR4 KO


b
12
9
6
3
0
Clinical Score (0-12)
TASTPM K/BxN + GB1107
WT K/BxN + 0.1% DMSO
WT K/BxN + GB1107
WT Control + GB1107
0
2
5
3 hrs post GB1107
Days after serum transfer


c
Male WT K/BxN + GB1107
Male WT K/BxN + 0.1% DMSO
Female WT K/BxN + GB1107
Female WT K/BxN + 0.1% DMSO
1.0
0.8
0.6
0.4
0.2
0.0
50% PWT (g)
***
0
2
5
3 hrs post GB1107
Days after serum transfer

Supplementary Figure 10: Intrathecal injection of heat-inactivated Gal-3 shows no effect on WT and TLR4 KO mechanical thresholds. (a) Mechanical thresholds were assessed before and 30 min after intrathecal injections of heat-inactivated Gal-3 (1 μM). Data represent mean ± SEM, n = 7 (WT Control ST), n = 8 (WT K/BxN ST), and n = 4 (TASTPM Control and K/BxN ST). Two-Way RM ANOVA, Tukey's multiple comparisons test. (b) clinical scoring in both WT and TASTPM K/BxN ST was not affected by GB1107 (10 nM intrathecal), Two-Way RM ANOVA, Tukey's multiple comparisons test. (c) GB1107 (10 nM) reversed mechanical hypersensitivity both in male and female WT K/BxN ST mice. Data represent mean ± SEM, n = 8 mice per group. ***$p < 0.001$ GB1107-treated vs DMSO-treated same sex WT K/BxN ST, Two-Way RM ANOVA, Tukey's multiple comparisons test.