Title: Sex differences and similarities in the neuroimmune response to central administration of poly I:C

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Keywords: poly I:C; sex differences; neuroimmune; cytokines; hippocampus; chemokines; male; female; interferon; mouse

ABSTRACT

Background: The neuroimmune system is required for normal neural processes, including modulation of cognition, emotion, and adaptive behaviors. Aberrant neuroimmune activation is associated with dysregulation of memory and emotion, though the precise mechanisms at play are complex and highly context dependent. Sex differences neuroimmune activation and function further complicate our understanding of its roles in cognitive and affective regulation.

Methods: Here, we characterized the physiological sickness and inflammatory response of the hippocampus following intracerebroventricular (ICV) administration of a synthetic viral mimic, polyinosinic:polycytidylic acid (poly I:C), in both male and female C57Bl/6 mice.

Results: We observed that poly I:C induced weight loss, fever, and elevations of cytokine and chemokines in the hippocampus of both sexes. Specifically, we found transient increases in gene expression and protein levels of IL-1α, IL-1β, IL-4, IL-6, TNFα, CCL2, and CXCL10, where males showed a greater magnitude of response compared with females. Only males showed increased IFNα and IFNγ in response to poly I:C, whereas both males and females exhibited elevations of IFNβ, demonstrating a specific sex difference in the anti-viral response in the hippocampus.

Conclusion: Our data suggest that type I interferons are one potential node mediating sex-specific cytokine responses and neuroimmune effects on cognition. Together, these findings highlight the importance of using both males and females and analyzing a broad set of inflammatory markers in order to identify the precise, sex-specific roles for neuroimmune dysregulation in neurological diseases and disorders.

52 **1. BACKGROUND**

53 The neuroimmune system is responsible for surveying the microenvironment and
54 responding to illness, injury, and infection. Importantly, it is also required to carry out normal
55 neural processes (1–3), thus making it well-placed to modify or disrupt learning and memory. In
56 fact, dysregulated immune signaling has been implicated in disorders of affect and cognition,
57 many of which show sex-biases in prevalence and outcomes (4–6). There is significant
58 evidence for sex differences in immune responses in the periphery (7,8), but there is limited
59 literature on similar findings in adult brains. Knowing whether such sex differences occur in
60 similar magnitudes and directions in the neuroimmune system is important for understanding
61 exactly how neuroimmune dysregulation impacts cognition, and contributes to psychiatric and
62 neurological disorders, in both sexes.

63 Illness, injury, or aseptic triggers of the innate immune system – either bacterial
64 endotoxins (e.g., lipopolysaccharide, LPS) or viral mimics (e.g., polyinosinic:polycytidylic acid,
65 poly I:C) – cause activation of neuroimmune cells, including microglia and astrocytes, and rapid
66 production of cytokines in the brain (9,10). Due to key roles in peripheral inflammation, the
67 cytokines IL-1β (11–13), IL-6 (14–16), and TNFα (17–19) have been the focus of much of the
68 research of neuroimmune function (20). More recently, other cytokines, including interferons
69 (4,21), CCL2 (22,23), and CXCL10 (21,24,25) also play critical roles in modulation of behavior,
70 cognition, and affective states, suggesting that many cytokines play important roles in these
71 processes.

72 Sex differences in immune and neuroimmune activity have also been reported in various
73 contexts. Females have a greater peripheral immune response compared with males (7). In
74 contrast, neuroimmune cells *in vitro*, including astrocytes derived from male cortical tissue, have
75 a significantly greater reaction to inflammatory insults compared with female-derived cells
76 (26,27). We have identified sex differences in the magnitude, time course, and pattern of
77 cytokines activated in the hippocampus following peripheral LPS (28), and in the long-lasting

78 impact of LPS on hippocampal function (29). Thus, sex differences in neuroimmune responses
79 specifically may be a contributing factor to sex differences in neural and cognitive processes
80 and disorders.

81 Despite incredible advances in psychoneuroimmunology over the past decade, there are
82 critical gaps in our knowledge that preclude a holistic understanding of neuroimmune function
83 and its impacts on cognition and disease. First, with some notable exceptions (22,30,31),
84 studies have typically focused on a few inflammatory cytokines (e.g., IL-1β, IL-6, and TNFα)
85 critical for neuroimmune activation and its effects on cognition. Yet more recently, it has become
86 clear that the massive, coordinated cytokine response observed in the periphery also occurs in
87 the CNS (28,32). The roles played by other cytokines, and the similarities and differences of this
88 response in males versus females are yet to be defined (20). Second, the bulk of studies aimed
89 at understanding neuroimmune activation and its behavioral sequelae have used the gram-
90 negative bacterial shell and toll-like receptor 4 (TLR4) agonist LPS. Nevertheless, viral illnesses
91 also trigger changes in behavior, cognition, and emotional states, and significant sex differences
92 have been observed in the context of viral infections (7,8,33) – an issue that has been propelled
93 to the forefront during the current COVID-19 pandemic (34,35). Given that viruses act through
94 distinct toll-like receptors, their impact is likely mediated by a different, albeit overlapping,
95 pattern of cytokine activation compared with LPS or bacterial triggers. Third, due to its relevance
96 for disease states, many *in vivo* studies of neuroimmune function use a peripheral immune
97 challenge. Here, neuroimmune activation is primarily driven by peripheral immune signals that
98 infiltrate the brain (36). This complicates the interpretation of whether sex differences in cytokine
99 levels observed in the brain are due to indirect effects based on sex differences in peripheral
100 immune response, or to direct effect of sex differences in neuroimmune function.

101 In this study, we aimed to identify a broad set of inflammatory cytokines induced in the
102 hippocampus by direct neuroimmune stimulation *via* central administration of poly I:C in both
103 males and females. Elevation of hippocampal cytokines, in particular, is associated with both

disruption of memory processes (1,11,13,37–40) and increased depression-like behaviors (41,42). Here, we demonstrate that poly I:C induces fever, weight loss, and changes in mRNA expression and protein levels of cytokines, chemokines, and markers of glial activation across a 24-hour period in both sexes. Notably, only IFNα and IFNɣ showed male-specific patterns of activation after central poly I:C administration, and many cytokines and chemokines showed a greater magnitude increase in males compared with females. Whether these sex differences in neuroimmune activation contribute to sex differences in modulation of cognition and affect and subsequent prevalence of memory- and mood-related diseases and disorders is an important area of research for our ongoing studies.

2. METHODS

2.1. Animals

A total of 99 male and female 8-9 week-old C57BL/6N mice from Envigo (Indianapolis, IN) were used in these experiments. For all experiments, mice were individually housed in standard mouse cages with *ad libitum* access to food and water in a room with maintained temperature and pressure under a 12:12-hour light:dark cycle. All mice had at least one week of acclimation to the colony room prior to any manipulations. All protocols were approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC).

2.2. Stereotaxic Surgeries

Bilateral guide cannulae (PlasticsOne, Roanoke, VA) targeting the lateral ventricles were implanted using standard stereotaxic methods (KOPF, Tujunga, CA) at the following coordinates relative to Bregma: medial-lateral: +/- 1.00mm, anterior-posterior: 0.30mm, dorsal-ventral: -2.50mm. Animals were administered a pre-surgical analgesic (5mg/kg Carprofen, subcutaneous) and anesthetized for surgery using an intraperitoneal injection of 250mg/kg of Avertin (2,2,2-tribromoethanol) which maintained a surgical plane of anesthesia for the duration

130 of the craniotomy. Bilateral holes were drilled into the skull at the above coordinates, and guide
131 cannulae were implanted using dental cement. Animals were given a second dose of Carprofen
132 (5mg/kg, subcutaneous) 24 hours after surgery to maintain a total of 48 hours of analgesia.
133 Mice were monitored daily for 10 days post-operative and were given at least 2 weeks to
134 recover from surgery prior to use in experiments.
135

136 **2.3. Poly I:C Administration**

137 Polyinosinic:polycytidylic acid (poly I:C; Cat. No. P1530; Sigma-Aldrich, St. Louis, MO)
138 was prepared according to the manufacturer's instructions and sterile-filtered using a 0.22μm
139 filter prior to administration. For intracerebroventricular (ICV) administration, we infused 20μg of
140 poly I:C (2μL of 10μg/μL poly I:C) (43) or an equal volume of 0.9% sterile saline *via* the
141 implanted guide cannula under brief isoflurane anesthesia.
142

143 **2.4. Sickness Behavior Assessment**

144 To determine whether poly I:C increased physiological measures of sickness in males
145 and females, body weights and rectal temperatures (RET-3; Physitemp, Clifton, NJ) were
146 assessed at 2, 4, 6, 12, 24, and 48 hours following ICV administration of poly I:C (n = 10 male; n
147 = 9 female) or sterile saline (n = 10 male; n = 8 female; Figure 2A). Visual and behavioral
148 measures of sickness (piloerections, squinted eyes, hunched posture, and low responsivity)
149 were assessed throughout (44). No changes in overt sickness behaviors were observed for any
150 experiment (data not shown).
151

152 **2.4.1. Statistical Analysis of Sickness Behaviors**

153 Analysis of body weight and temperature changes in response to poly I:C was
154 completed using a mixed repeated-measures ANOVA, using time post-infusion as the within-

155 subjects factor and treatment and sex as the between-subjects factors with Greenhouse-
156 Geisser corrections for sphericity. Significant main effects and interactions ($p < 0.05$) were
157 followed up using post-hoc tests with Bonferroni corrections for multiple comparisons, and effect
158 sizes were calculated using the partial eta squared method. Any outliers were identified as
159 samples outside the range of 2 standard deviations from the group mean.
160

161 **2.5. Characterization of the Acute Neuroimmune Response**

162 We used RNA and protein endpoints to examine induction of cytokines and glial
163 activation markers in the hippocampus. Males and females were treated with either poly I:C (*n* =
164 22 male; *n* = 24 female) or sterile saline (*n* = 8/sex) and brains were collected 0.5 hours (*n* = 5
165 male; *n* = 6 female), 2 hours (*n* = 6/sex), 4 hours (*n* = 5 male; *n* = 6 female), and 24 (*n* = 6/sex)
166 hours later. All animals were transcardially perfused with 0.1M phosphate buffer to remove
167 circulating blood from the brain. Both hemispheres of dorsal hippocampus tissue were collected
168 in separate RNase-/DNase-free, sterile microcentrifuge tubes and immediately flash frozen. All
169 samples were stored at -80°C before tissue processing.
170

171 **2.5.1. Quantitative Real-Time PCR**

172 One hemisphere of dorsal hippocampal tissue per mouse was processed for gene
173 expression analysis using quantitative real-time PCR (qPCR). Frozen samples were
174 homogenized, and messenger RNA (mRNA) was extracted (PureLink RNA Mini Kit; Cat. No.
175 12183020; Invitrogen, Carlsbad, CA) under sterile, RNase-free conditions. RNA quality was
176 assessed using gel electrophoresis, and UV spectroscopy was used to assess RNA purity
177 (A260/280 > 1.80) and quantity (BioSpectrometer Basic; Eppendorf, Hamburg, Germany). Any
178 genomic DNA in the sample was removed using DNase treatment, and 800ng of cDNA was
179 synthesized from each mRNA sample (QuantiTect Reverse Transcriptase Kit; Cat. No. 205314;
180 Qiagen, Hilden, Germany). Any samples that did not have a high enough concentration of RNA

181 to make 800ng of cDNA were removed from further analyses (*n* = 3 male; *n* = 5 female).
182 Relative gene expression was measured using Power SYBR Green PCR Master Mix (Cat. No.
183 4368702; Applied Biosystems, Foster City, CA) in 10μL reactions (ABI 7500 real-time PCR
184 system; Cat. No. 4351105; Applied Biosystems).

185 We measured expression of four commonly-used housekeeping genes: *18s*, *gapdh*,
186 *hprt1*, and *rplp0* (all QuantiTect Primer Assays: *18s* Cat. No. QT02448082, *gapdh* Cat. No.
187 QT01658692, *hprt1* Cat. No. QT00166768, *rplp0* Cat. No. QT00249375; Qiagen). We analyzed
188 the relative expression of the following genes of interest: *ccl2*, *cd11b*, *cxcl10*, *gfap*, *ifnα*, *ifnβ*,
189 *ifnγ*, *il-1α*, *il-1β*, *il-6*, *il-10*, and *tnfα*. The gene primer for *il-1α* was a QuantiTect Primer Assay
190 (Cat. No. QT00113505; Qiagen). The sequences for the remaining gene primers can be found
191 in Table 1 and were ordered through Integrated DNA Technologies and diluted to 0.13 μM to be
192 used for PCR. All Qiagen primers were diluted as per the manufacturer's instruction.

193 **Table 1.**

194 *Primer sequences used for real-time PCR.*

Gene Target	Forward Primer Sequence (5' to 3')	Reverse Primer Sequence (5' to 3')	NCBI Reference Sequence
ccl2	CCACAACCACCTCAAGCACT	AAGGCATCACAGTCCGAGT	NM_011333.3
cd11b	CGTGAATGGGGACAAACTGAC	GCACTGAGGCTGGCTATTGA	NM_008401.2
cxcl10	TCCATCACTCCCCTTTACCCA	TGGCTTGACCATCATCCTGC	NM_021274.2
gfap	AAACCGCATCACCATTCCTG	CCCGCATCTCCACAGTCTTTA	NM_010277.3
ifnα	AGAGAAGAAACACAGCCCCT	AGCACATTGGCAGAGGAAGA	NM_010502.2
ifnβ	GCTCCAAGAAAGGACGAACAT	GGATGGCAAAGGCAGTGTAA	NM_010510.1
ifnγ	GTCAACAACCCACAGGTCCA	CGACTCCTTTTCCGCTTCCT	NM_008337.4
il-1β	TGCCACCTTTTGACAGTGATG	GCTCTTGTTGATGTGCTGCT	NM_008361.4
il-6	GAGACTTCCATCCAGTTGCCT	TCATTTCCACGATTTCCCAGAG	NM_001314054.1
il-10	CTGGACAACATACTGCTAACCG	AATGCTCCTTGATTTCTGGGC	NM_010548.2
tnfα	ACCCCTTTACTCTGACCCCTT	ACTGTCCCAGCATCTTGTGT	NM_001278601.1

195

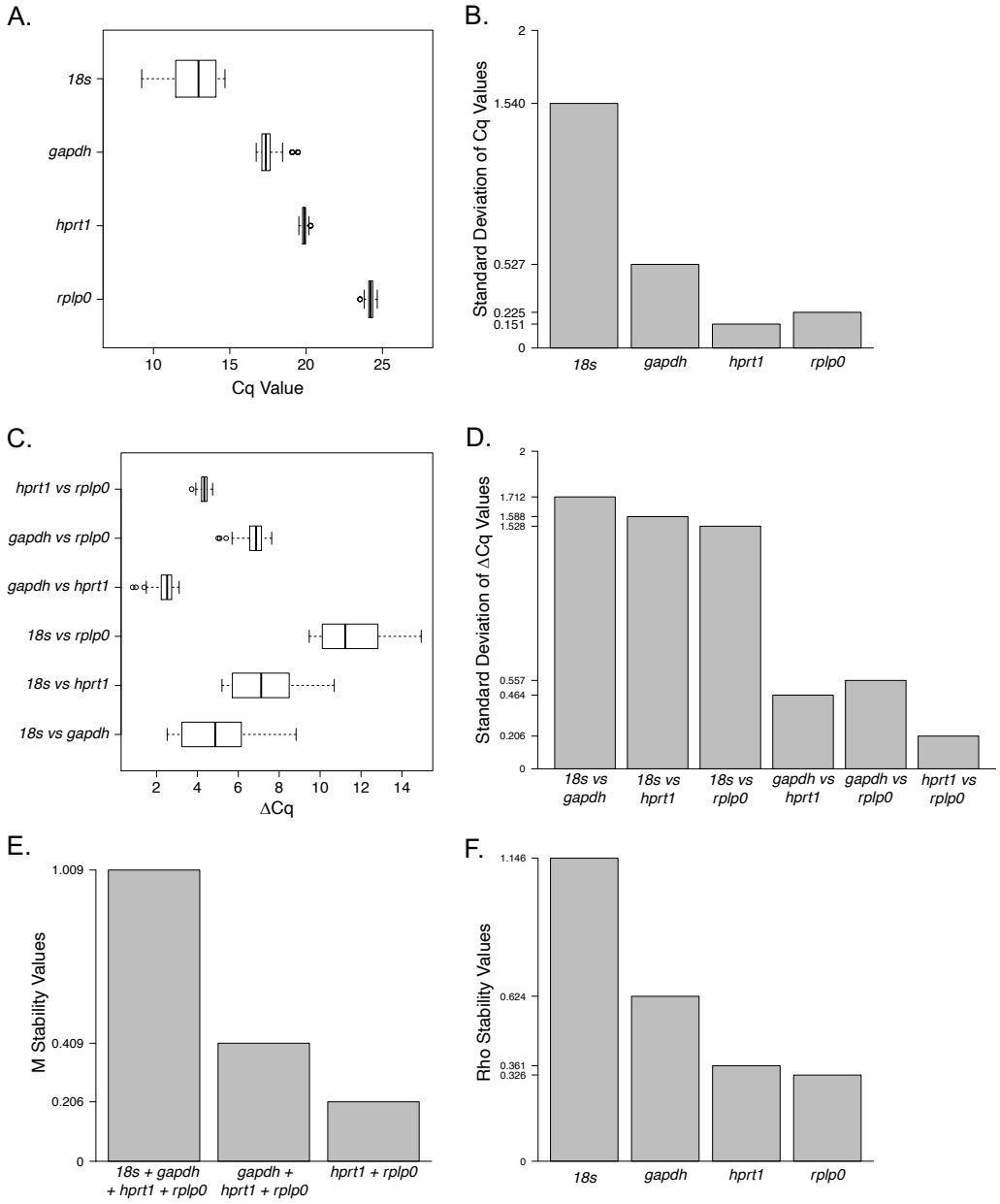
196 **2.5.1.1. Housekeeping Gene Stability Analysis**

197 To control for the transcriptional activity of the samples being analyzed, we confirmed
198 the stability of four housekeeping genes (*18s*, *gapdh*, *hprt1*, and *rplp0*). While many studies use
199 common housekeeping genes such as GAPDH or HPRT1, it is less common for authors to
200 report that their chosen housekeeping gene is indeed stable across experimental groups or
201 tissues prior to use in analyses. Thus, we confirmed the stability of our housekeeping genes
202 using a combination of four techniques to ensure the most reliable quantification of gene
203 expression in our studies. First, we assessed the variability of the candidate genes by
204 measuring the standard deviation of the raw quantification cycle (Cq) values from all samples
205 (Figure 1A). We found that *18s* had the largest standard deviation of Cq values (1.540), followed
206 by *gapdh* (0.527), *rplp0* (0.225), and *hprt1* (0.151; Figure 1B). By this approach, *rplp0* and *hprt1*
207 showed the greatest stability compared to *18s* and *gapdh*, with *hprt1* exhibiting the lowest
208 variability.

209 Second, we employed a comparative ΔCq approach in which the standard deviations of
210 the differences in Cq values (ΔCqs) between all possible pairs of candidate genes were
211 compared (45) (Figure 1C). From highest to lowest variability, the genes ranked as follows: *18s*
212 (1.609 average standard deviation), *gapdh* (0.911), *rplp0* (0.764), and *hprt1* (0.753). Again, this
213 method indicated that the most variable genes were *18s* and *gapdh* while the most stable genes
214 were *rplp0* and *hprt1*, and this is most apparent when considering the lowest ΔCq standard
215 deviation from this method was from the *rplp0* and *hprt1* comparison at 0.206 (Figure 1D).

216 The third method we employed was that developed by Vandesompele and colleagues,
217 which calculated the average pairwise variation of one candidate gene with all other candidate
218 genes (46). We used R packages ReadqPCR and NormqPCR (47) to calculate *M* stability

219 values, as depicted in Figure 1E. Consistent with the previous methods, *hprt1* and *rplp0* were
220 the most stable of the candidate genes, with the lowest pairwise variability, *M* value, of 0.206.
221 Fourth, and last, we used a model-based stability analysis approach developed by
222 Andersen et al., an algorithm called NormFinder (v5) (48). This method protects against
223 identifying two genes via the pairwise approach that might be misinterpreted as being the most
224 stable if they are coregulated. Using this method, again, *hprt1* and *rplp0* were found to be the
225 most stable genes with the lowest expression stability values (Figure 1F). However, NormFinder
226 resulted in *rplp0* having the lowest stability value of 0.326, indicating that the model-based
227 approach identified *rplp0* as the most stable gene.
228 Together, these methods identified the two most stable candidate housekeeping genes
229 as *hprt1* and *rplp0*. Vandesompele et al. (46) posits that using the geometric mean of multiple
230 housekeeping genes results in more accurate expression levels of genes of interest. We
231 calculated the geometric mean of the Cq values from *hprt1* and *rplp0* to be used in the $2^{-\Delta\Delta Cq}$
232 method for calculations of relative expression for our target genes.
233



G.

	Cq Value Standard Deviations	Avg Standard Deviations of ΔCq Values	M Stability Values	Rho Stability Values
18s	1.5402440	1.609	1.609	1.1459049
gapdh	0.5266292	0.911	0.510	0.6235678
hprt1	0.1507111	0.753	0.206	0.3610396
rplp0	0.2245596	0.764	0.206	0.3256827

Figure 1. Housekeeping gene stability analysis. (A) Distribution of the quantification cycles (Cq) for housekeeping genes *18s*, *gapdh*, *hprt1*, and *rplp0***,** with **(B)** associated standard deviations. **(C)** Distribution of the difference of Cq values (ΔCq) between pairs of housekeeping genes, and **(D)** the associated standard deviations. **(E)** Stability values calculated using gene ratio method by Vandesompele et al., 2002, which uses stepwise elimination of lowest stability

240 (highest *M* value) to rank gene stability. **(F)** Stability values calculated using a model-based
241 approach by Andersen et al., 2004 which measures expression variation such that highest
242 stability results in the lowest Rho value. **(G)** Summary of results from each of the four methods
243 of housekeeping gene stability are shown.
244

245 **2.5.1.2. Statistical Analysis of mRNA Gene Expression**

246 For each PCR reaction, the quantification cycle (Cq) was determined, and the $2^{-\Delta\Delta Cq}$
247 method was used to calculate the relative gene expression of each gene. Any samples with
248 abnormal amplification curves, melt curves, and/or melt peaks across replicates were removed
249 from analyses (n = 1/sex). Any outliers were identified as samples outside the range of 2
250 standard deviations from the group mean and excluded from analyses.

251 Baseline sex differences in relative gene expression (qPCR) were assessed by
252 evaluating the male and female saline-treated groups. To directly and meaningfully compare
253 these two groups in the PCR analysis, the male saline-treated group was normalized to the
254 female saline-treated group and analyzed using independent, two-sample t-tests.

255 To appropriately analyze sex differences in relative gene expression (qPCR) across the
256 24-hour time course, we normalized each group to its respective same-sex saline-treated group
257 to control for any sex differences in gene expression at baseline and used two-way ANOVA
258 tests using treatment and sex as factors. Significant main effects and interactions ($p < 0.05$)
259 were followed up using post-hoc tests with Bonferroni corrections for multiple comparisons, and
260 effect sizes were calculated using the partial eta squared method.
261

262 **2.5.2. Multiplex Assays**

263 The second hemispheres of dorsal hippocampal tissue were processed as previously
264 described using low-detergent RIPA buffer sonication (28). Milliplex magnetic bead panel
265 assays (CCL2, CXCL10, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-6, and IL-10; Millipore Sigma,
266 Burlington, MA) were used as per manufacturer's instructions. Cytokine concentrations were

calculated as pg/mg of hippocampal tissue via Luminex software. Only samples that showed readable bead counts according to the Luminex software were included in the analyses.

2.5.2.1. Statistical Analysis of Protein Levels

Baseline sex differences in protein levels from multiplex assays were analyzed with independent, two-sample t-tests comparing the saline-treated groups. To analyze changes in protein levels from poly I:C across the 24-hour time frame, we used two-way ANOVA tests using treatment and sex as factors. Significant main effects and interactions ($p < 0.05$) were followed up using post-hoc tests with Bonferroni corrections for multiple comparisons, and effect sizes were calculated using the partial eta squared method. Any outliers were identified as samples outside the range of 2 standard deviations from the group mean and excluded from analyses.

2.6. Data Visualization and Statistical Software

Data visualization and statistical analyses were completed using R 3.6.2 (R Core Team, 2019) with the following packages: dplyr (v0.8.5; (49)), tidyr (v1.0.2; (50)), rstatix (v0.5.0; (51)), DescTools (v0.99.34; (52)), sjstats (v0.17.9; (53)), ReadqPCR and NormqPCR (47), ggplot2 (54), gridExtra (v2.3; (55)), pheatmap (v1.0.12; (56)), and viridis (v0.5.1; (57)).

3. RESULTS

3.1. Central administration of poly I:C induces physiological sickness responses

Both females and males showed physiological responses to poly I:C. Whereas both saline- and poly I:C-treated animals showed changes in weight across the 48-hour period (Figure 2B, main effect of Time: $F(3.13, 96.92) = 28.899$, $p < 0.001$, $\eta^2_p = 0.482$), poly I:C caused weight loss in both sexes (main effect of Treatment: $F(1, 31) = 8.781$, $p = 0.006$, $\eta^2_p = 0.221$; trend towards a Time x Treatment interaction: $F(3.13, 96.92) = 2.476$, $p = 0.064$, $\eta^2_p = 0.074$). Specifically, males and females treated with poly I:C lost significantly more weight than

the saline-treated animals at the 12- (p = 0.004) and 24-hour (p = 0.022) time points. By 48 hours post-treatment, the weights of poly I:C-treated animals had recovered and were no longer different from those of saline-treated animals (p = 1.00).

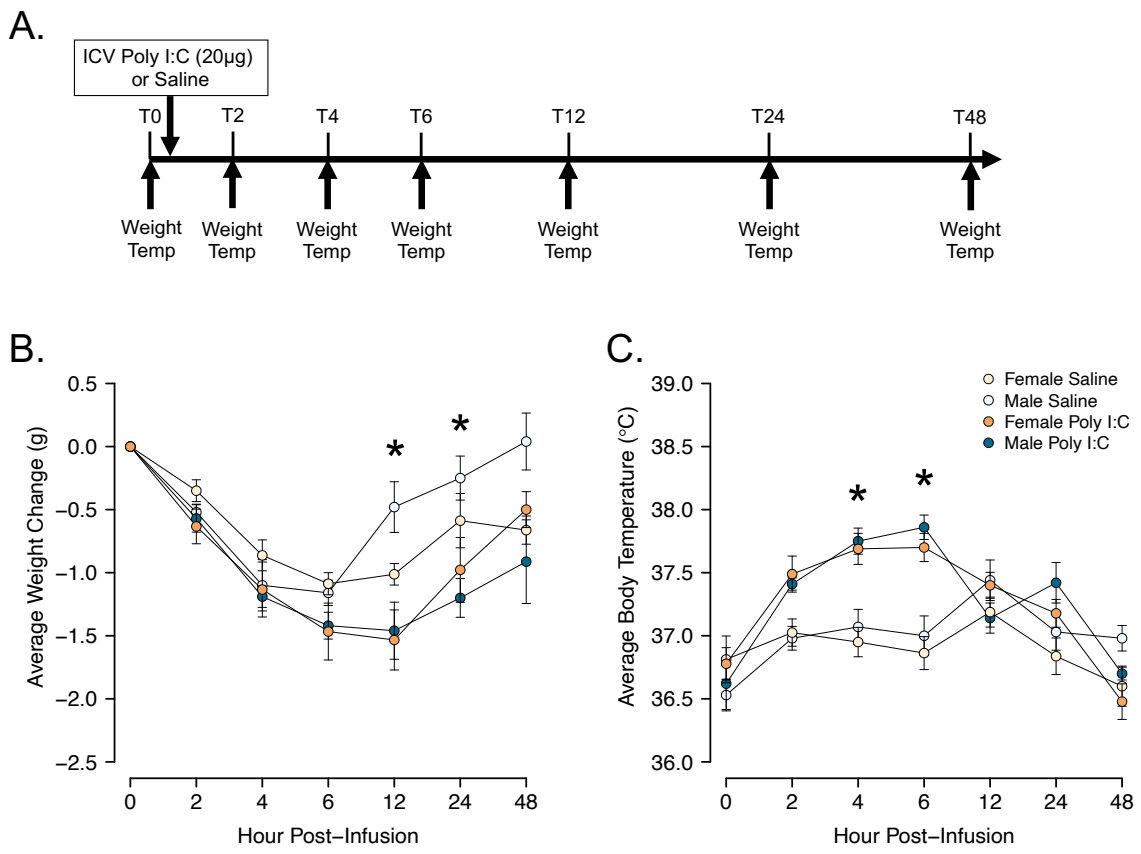


Figure 2. Analysis of sickness behaviors following poly I:C administration. (A) Timeline of body weight and temperature measurements following poly I:C or sterile saline administration. **(B)** Average weight change from baseline (Time = 0 hour) prior to treatment. **(C)** Average body temperature as measured via rectal thermometer. Analyzed using mixed repeated-measures ANOVA. *: $p < 0.05$ poly I:C- vs saline-treated groups.

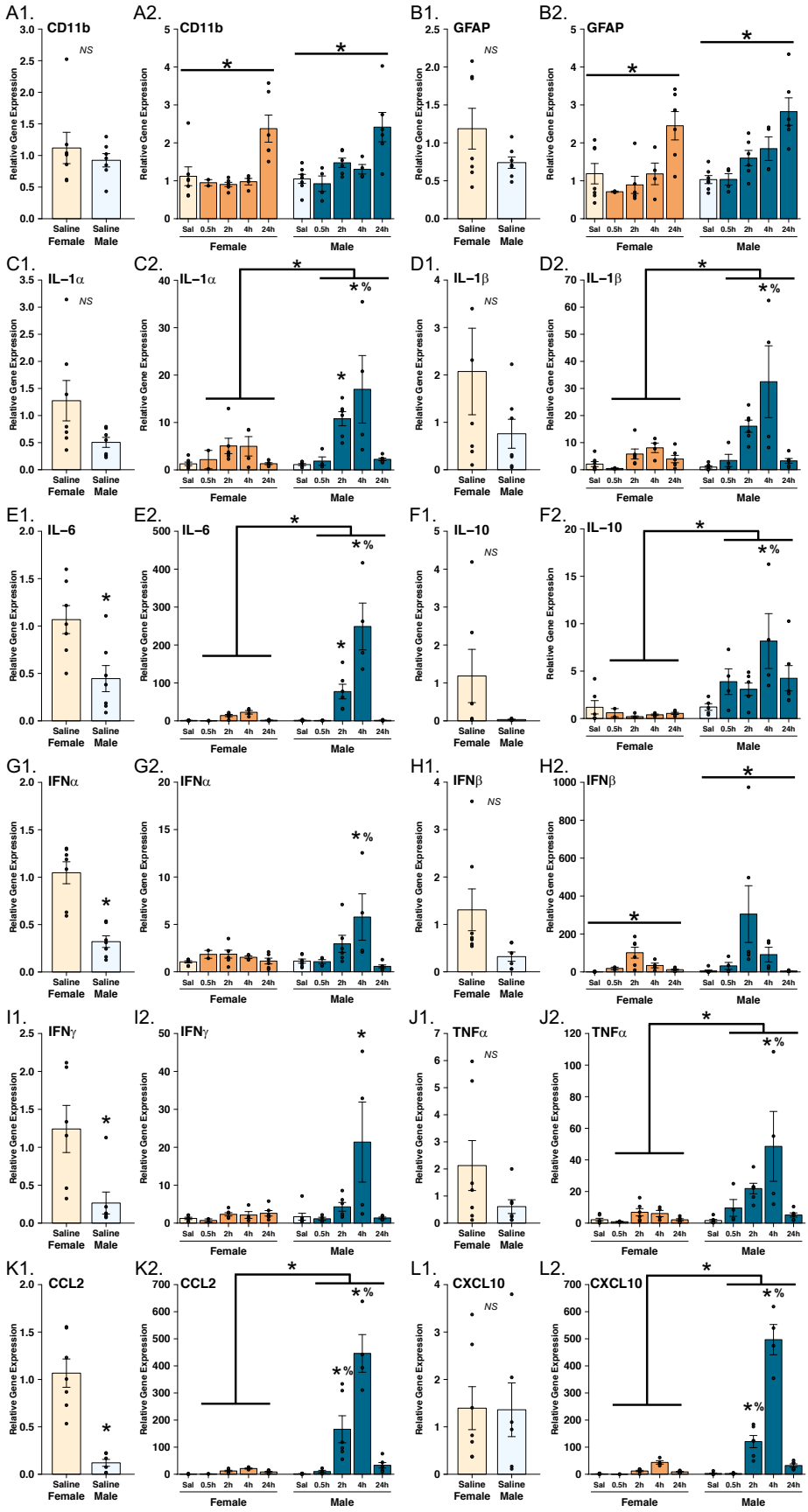
In both males and females, poly I:C caused significant increases in body temperature relative to the saline-treated group (Figure 2C; main effect of Treatment: $F(1, 31) = 23.759$, $p < 0.001$, $\eta^2_p = 0.434$; Time x Treatment interaction: $F(4.6, 142.62) = 11.635$, $p < 0.001$, $\eta^2_p = 0.273$). Post-hoc tests revealed that body temperature began to increase 2 hours following poly

307 I:C (p = 0.068), remained elevated at the 4- (p < 0.001) and 6-hour (p < 0.001) time points, and
308 recovered to saline-treated body temperatures by 12 hours post treatment (all p = 1.00).
309
310 **3.2. Baseline sex differences in mRNA expression and protein levels of select**
311 **hippocampal immune molecules**
312 **3.2.1. Interleukins**
313 Select immune signaling markers showed baseline sex differences. mRNA expression of
314 interleukin *(il)-1α* exhibited a trend towards a sex difference in baseline expression (Figure 3C1;
315 $t(12) = 2.006$, $p = 0.068$), and *il-6* showed a significant sex difference (Figure 3E1; $t(11) = 3.079$,
316 $p = 0.01$, 95% CI [0.182, 1.062]) where females showed greater mRNA expression than males.
317 These results did not hold true for protein levels of IL-1α or IL-6. Rather, multiplex data showed
318 that protein levels of IL-1β and IL-10 were significantly different between saline-treated males
319 and females (Figures 4D and 4F, respectively; IL-1β: $t(13) = 4.275$, $p = 0.001$, 95% CI [5.682,
320 17.291]; IL-10: $t(13) = 2.236$, $p = 0.044$, 95% CI [0.672, 39.314]). Again, for these cytokines,
321 females showed greater protein levels at baseline compared with males.
322 **3.2.2. Interferons**
323 Two interferons (IFN), *ifnα* and *ifnγ*, also showed baseline sex differences in relative
324 mRNA expression (Figures 3G1 and 3I1: IFNα: $t(12) = 5.546$, $p = 0.0001$, 95% CI [0.441, 1.01];
325 IFNγ: $t(11) = 2.995$, $p = 0.012$, 95% CI [0.259, 1.694]). Likewise, protein levels of IFNγ exhibited
326 a sex difference in saline-treated groups (Figure 4G; $t(14) = 6.475$, $p < 0.001$, 95% CI [6.534,
327 13.006]). For both mRNA expression and protein levels, female levels were greater than males.
328 **3.2.3. Chemokines**
329 Expression of CC chemokine *ccl2* similarly followed this pattern at baseline (Figure 3K1;
330 $t(12) = 3.287$, $p = 0.006$, 95% CI [0.259, 1.279]), and this held true for protein levels of CCL2 as
331 well (Figure 4H; $t(12) = 2.751$, $p = 0.018$, 95% CI [12.798, 110.318]). Interestingly, all genes with

332 sex differences in saline-treated animals showed that both female baseline mRNA expression

333 and protein levels were at least twice as high as that of males.

334


A1. CD11b
A2. CD11b
B1. GFAP
B2. GFAP
C1. IL−1α
C2. IL−1α
D1. IL−1β
D2. IL−1β
E1. IL−6
E2. IL−6
F1. IL−10
F2. IL−10
G1. IFNα
G2. IFNα
H1. IFNβ
H2. IFNβ
I1. IFNγ
I2. IFNγ
J1. TNFα
J2. TNFα
K1. CCL2
K2. CCL2
L1. CXCL10
L2. CXCL10
Relative Gene Expression
NS
Saline
Female
Male
Sal
0.5h
2h
4h
24h

335

Figure 3. mRNA gene expression of cytokines, chemokines, and markers of glial activation in the hippocampus. Baseline gene expression was measured by normalizing the male saline-treated group to the female saline-treated group and analyzed using independent, two-sample t-tests. Baseline expression of **(A1)** CD11b, **(B1)** GFAP, **(C1)** IL-1α, **(D1)** IL-1β, **(E1)** IL-6, **(F1)** IL-10, **(G1)** IFNα, **(H1)** IFNβ, **(I1)** IFNγ, **(J1)** TNFα, **(K1)** CCL2, and **(L1)** CXCL10 are shown. Gene expression changes following poly I:C treatment were calculated by normalizing time points after poly I:C treatment to the saline-treated groups within sex to eliminate confounding variables of baseline sex differences. Gene expression was analyzed using two-way ANOVA tests for **(A2)** CD11b, **(B2)** GFAP, **(C2)** IL-1α, **(D2)** IL-1β, **(E2)** IL-6, **(F2)** IL-10, **(G2)** IFNα, **(H2)** IFNβ, **(I2)** IFNγ, **(J2)** TNFα, **(K2)** CCL2, and **(L2)** CXCL10. ***** above a bracket covering both sexes indicates a significant main effect of sex ($p < 0.05$); * above a horizontal line covering just one sex indicates a significant main effect of treatment ($p < 0.0.5$); ***** above a single bar indicates a significant post-hoc test ($p < 0.05$) vs the saline-treated group within sex; **%** above a single bar indicates a significant post-hoc test ($p < 0.05$) vs females at the same time point.

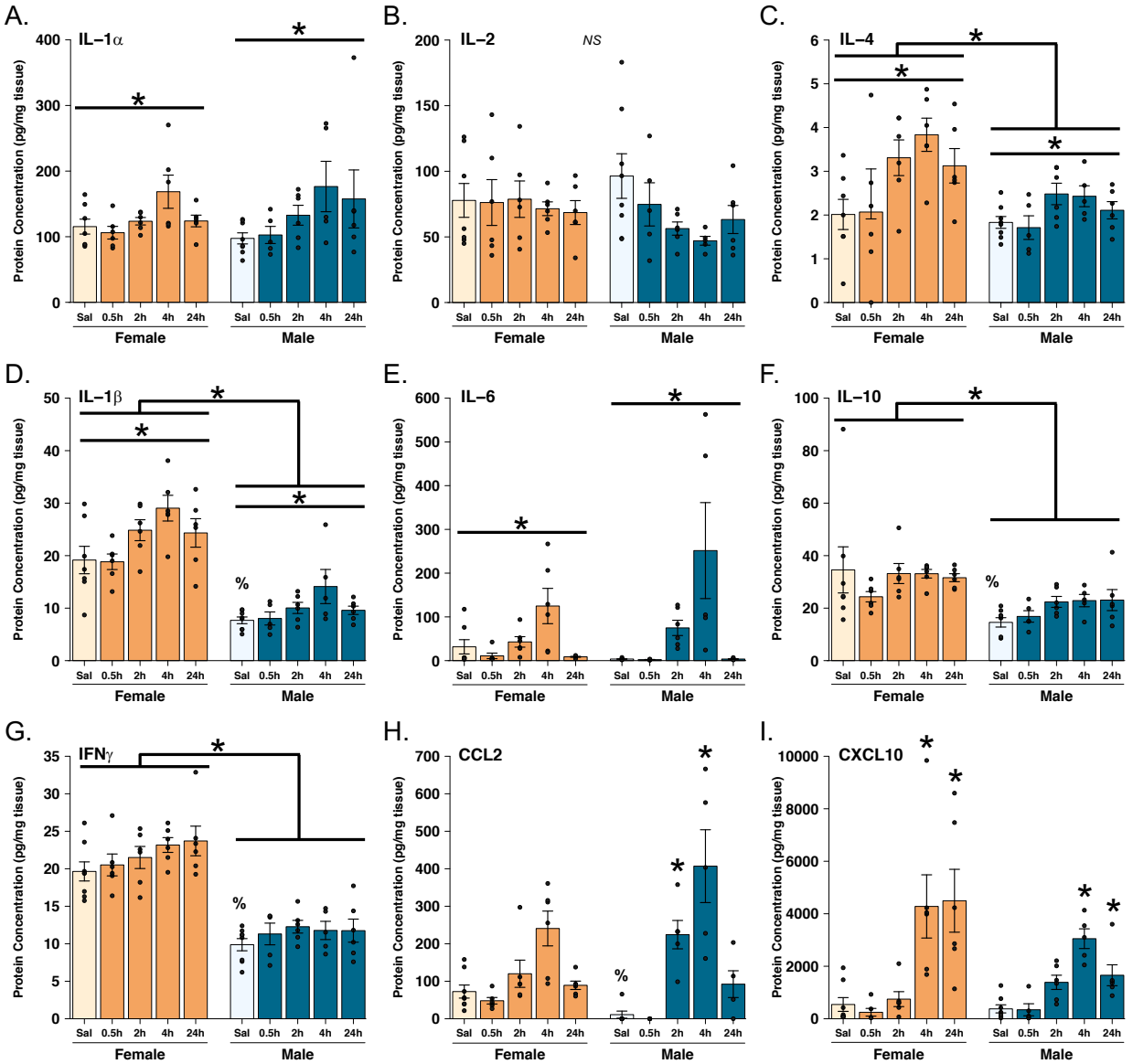


Figure 4. Protein levels of cytokines and chemokines in the hippocampus. Protein concentration (pg/mg) of **(A)** IL-1α, **(B)** IL-2, **(C)** IL-4, **(D)** IL-1β, **(E)** IL-6, **(F)** IL-10, **(G)** IFNγ, **(H)** CCL2, and **(I)** CXCL10 are shown. Two-way ANOVA tests were used to analyze these data. * above a bracket covering both sexes indicates a significant main effect of sex ($p < 0.05$); * above a horizontal line covering just one sex indicates a significant main effect of treatment ($p < 0.0.5$); * above a single bar indicates a significant post-hoc test ($p < 0.05$) vs the saline-treated group within sex; **%** above a single bar indicates a significant post-hoc test ($p < 0.05$) vs females at the same time point.

3.2.4. Glial activation markers and other cytokines did not show baseline sex differences

It is equally important to note the markers that did not show sex differences at baseline. Both the microglial activation marker *cd11b* and the astrocyte activation marker *gfap* showed

364 equivalent levels of gene expression in the saline-treated groups in both sexes (Figures 3A1
365 and 3B1, respectively; *cd11b*: $t(12) = 0.723$, $p = 0.483$; *gfap*: $t(12) = 1.603$, $p = 0.135$). Other
366 commonly examined inflammatory cytokines, including IL-2, IL-4, and tumor necrosis factor
367 (TNF)α similarly showed no sex difference at baseline (IL-2 p = 0.420; IL-4 p = 0.633; TNFα =
368 0.139). No other sex differences in mRNA gene expression or protein levels were observed
369 under baseline conditions.
370

371 **3.3. Central poly I:C causes greater increases in male mRNA expression of hippocampal**
372 **cytokines compared with females**

373 **3.3.1. Glial Activation Markers**

374 Poly I:C treatment significantly increased expression of both *cd11b* and *gfap*, although
375 this appeared to be true only at the 24-hour time point (Figures 3A2 and 3B2; *cd11b* main effect
376 of Treatment: $F(4, 42) = 12.96$, $p < 0.001$, $\eta^2_p = 0.552$; *gfap* main effect of Treatment: $F(4, 42) =$
377 12.992, $p < 0.001$, $\eta^2_p = 0.553$). Sex did not affect the response of either *cd11b* or *gfap* to poly
378 I:C (insignificant Sex x Treatment interactions: *cd11b*: $F(4, 42) = 0.684$, $p = 0.607$; *gfap*: $F(4, 42)$
379 $= 0.923$, $p = 0.460$).

380 **3.3.2. Interleukins**

381 Poly I:C caused increased expression of *il-1α*, *il-1β*, and *il-6* in both males and females
382 (Figures 3C2, 3D2, and 3E2; main effects of Treatment: *il-1α*: $F(4, 42) = 9.784$, $p < 0.001$, $\eta^2_p =$
383 0.482; *il-1β*: $F(4, 42) = 9.512$, $p < 0.001$, $\eta^2_p = 0.475$; *il-6*: $F(4, 42) = 22.28$, $p < 0.001$, $\eta^2_p =$
384 0.680). In males, expression began to increase at the 2-hour time point following poly I:C
385 treatment for *il-1α* ($p = 0.015$), *il-1β* ($p = 0.057$), and *il-6* (p = 0.029), showed peaks at the 4-
386 hour time point ($p < 0.001$ for all), and decreased to saline-treated levels by 24 hours ($p = 1.00$
387 for all). Each of these genes also showed an overall greater expression in males than females
388 (main effects of Sex: *il-1α*: $F(1, 42) = 6.398$, $p = 0.015$, $\eta^2_p = 0.132$; *il-1β*: $F(1,42) = 6.695$, $p =$

389 0.013, $\eta^2_p = 0.137$; *il-6*: $F(1, 42) = 21.1$, $p < 0.001$, $\eta^2_p = 0.334$), and a significantly greater
390 magnitude of response in males compared with females (Sex x Treatment interactions: *il-1α*:
391 $F(4, 42) = 3.103$, $p = 0.025$, $\eta^2_p = 0.228$; *il-1β*: $F(4, 42) = 4.288$, $p = 0.005$, $\eta^2_p = 0.290$; *il-6*: $F(4,$
392 $42) = 15$, $p < 0.001$, $\eta^2_p = 0.588$). Post-hoc tests revealed for all three genes, males exhibited an
393 even greater response at only the 4-hour time point compared with females ($p < 0.05$ for all).
394 Notably, the peak *il-1α* and *il-1β* expression in males was roughly 3-fold higher than that of the
395 peak female expression for these cytokines, and the *il-6* peak expression in males was more
396 than 10-fold higher than that of females (Figures 3C2, 3D2, and 3E2).
397 Males showed greater *il-10* gene expression across all time points compared with
398 females (Figure 3F2; main effect of Sex: $F(1, 39) = 25.642$, $p < 0.001$, $\eta^2_p = 0.397$). Additionally,
399 poly I:C significantly increased gene expression of *il-10* in males, but not females (Sex x
400 Treatment interaction: $F(4, 39) = 3.304$, $p = 0.02$, $\eta^2_p = 0.253$). Specifically, male expression of
401 *il-10* at the 4-hour time point following poly I:C was significantly greater than that of saline-
402 treated controls ($p = 0.001$), and this was also greater than the 4-hour expression in females (p
403 $= 0.001$).
404 **3.3.3. Tumor Necrosis Factor Alpha**
405 Gene expression of *tnfα* increased in response to poly I:C, males had significantly higher
406 expression than females overall, and males showed a greater magnitude of response compared
407 with females (Figure 3J2; main effect of Treatment: $F(4, 42) = 6.407$, $p = 0.0004$, $\eta^2_p = 0.379$;
408 main effect of Sex: $F(1, 42) = 10.1$, $p = 0.003$, $\eta^2_p = 0.194$; Sex x Treatment interaction: $F(4, 42)$
409 $= 4.117$, $p = 0.007$, $\eta^2_p = 0.282$). Post-hoc tests showed that males 4 hours post-treatment had
410 significantly greater expression than those treated with saline ($p < 0.001$), and this was again
411 greater than the 4-hour peak expression in females ($p = 0.001$).
412 **3.3.4. Interferons**

413 Both *ifnα* and *ifnγ* showed a similar response pattern to poly I:C, whereby males treated
414 with poly I:C exhibited a significant acute increase in gene expression of both cytokines, but
415 females did not show the same response (Figures 3G2 and 3I2; *ifnα*: main effect of Treatment:
416 $F(4, 42) = 5.007$, $p = 0.002$, $\eta^2_p = 0.323$; Sex x Treatment interaction: $F(4, 42) = 3.35$, $p = 0.018$,
417 $\eta^2_p = 0.242$; *ifnγ*: main effect of Treatment: $F(4, 40) = 4.698$, $p = 0.003$, $\eta^2_p = 0.32$; Sex x
418 Treatment interaction: $F(4, 40) = 4.178$, $p = 0.006$, $\eta^2_p = 0.295$). Specifically, 4 hours after poly
419 I:C treatment, males showed significantly elevated expression compared to the saline-treated
420 controls ($p = 0.0001$), and this was greater in magnitude than the 4-hour time point in females (p
421 $= 0.001$). Female *ifnα* and *ifnγ* did not seem to respond to poly I:C treatment at any time point.
422 In contrast, poly I:C transiently increased expression of *ifnβ* in both males and females,
423 and there were no sex differences seen in magnitude of expression increase (Figure 3H2; main
424 effect of Treatment: $F(4, 42) = 4.855$, $p = 0.003$, $\eta^2_p = 0.316$; insignificant Sex x Treatment
425 interaction: $F(4, 42) = 1.297$, $p = 0.287$). Unlike all other cytokines examined in this study, peak
426 expression appeared to be at the 2-hour time point, and expression began decreasing again by
427 4 hours post-treatment. The magnitude increase was also notable, with a 100-fold increase in
428 females and a 300-fold increase in males.

429 **3.3.5. Chemokines**

430 Poly I:C significantly increased the expression of both *ccl2* and *cxcl10* in males and
431 females, with a peak increase in expression at 4-hours post-infusion (Figures 3K2 and 3L2;
432 main effects of Treatment: *ccl2*: $F(4, 41) = 25.47$, $p < 0.001$, $\eta^2_p = 0.713$; *cxcl10*: $F(4, 42) =$
433 87.37, $p < 0.001$, $\eta^2_p = 0.893$). Also consistent with many other cytokines, expression of both
434 *ccl2* and *cxcl10* was greater overall in males compared with females (main effects of Sex: *ccl2*:
435 $F(1, 41) = 44.55$, $p < 0.001$, $\eta^2_p = 0.521$; *cxcl10*: $F(1, 42) = 92.79$, $p < 0.001$, $\eta^2_p = 0.688$). Finally,
436 males showed a markedly greater magnitude of response than did females for both chemokines
437 (Sex x Treatment interactions: *ccl2*: $F(4, 41) = 20.96$, $p < 0.001$, $\eta^2_p = 0.672$; *cxcl10*: $F(4, 42) =$

438 60.51, $p < 0.001$, $\eta^2_p = 0.852$). Remarkably, male *ccl2* expression peaked at nearly 450-fold
439 greater than the expression of saline-treated males compared to a roughly 20-fold increased
440 peak in females (Figure 3K2). Similarly, *cxcl10* expression in males peaked at nearly 500-times
441 that of saline-treated males while female *cxcl10* expression peaked at just over 40-times greater
442 than saline-treated females (Figure 3L2). These massive increases in gene expression are
443 reflected in the strong effect sizes noted for the interaction effect above. Post-hoc tests
444 confirmed that the male 2- and 4-hour time points post-treatment showed significantly greater
445 gene expression of both *ccl2* and *cxcl10* than saline-treated males ($p < 0.001$). Additionally,
446 both the male 2- and 4-hour time points of both genes proved to be significantly greater than the
447 2- and 4-hour time points in females, respectively ($p < 0.01$).
448

449 **3.4. Central poly I:C increases select hippocampal cytokine protein levels in males and**
450 **females**

451 **3.4.1. Interleukins**

452 We analyzed several members of the interleukin family of cytokines, including classically
453 inflammatory IL-1α, IL-1β, IL-2, IL-4, and IL-6, and classically anti-inflammatory IL-10. Of these,
454 IL-1α, IL-1β, IL-4, and IL-6 significantly increased following ICV poly I:C administration in both
455 males and females (Figures 4A, 4C, 4D, 4E; main effects of Treatment: IL-1α: $F(4, 51) = 3.523$,
456 $p = 0.013$, $\eta^2_p = 0.216$; IL-1β: $F(4, 51) = 5.721$, $p = 0.001$, $\eta^2_p = 0.31$; IL-4: $F(4, 51) = 5.146$, $p =$
457 0.001, $\eta^2_p = 0.288$; IL-6: $F(4,51) = 10.298$, $p < 0.001$, $\eta^2_p = 0.447$). In all cases, protein levels
458 appear to increase to a peak 4 hours following poly I:C, similar to the effects seen in mRNA
459 expression. In addition, both IL-1β and IL-4 also exhibited a main effect of Sex such that protein
460 levels of these cytokines, regardless of time point, were significantly higher in females
461 compared with males (Figures 4C and 4D; IL-1β: $F(1, 51) = 114.226$, $p < 0.001$, $\eta^2_p = 0.691$; IL-
462 4: $F(1, 51) = 11.03$, $p = 0.002$, $\eta^2_p = 0.178$). No interactions of sex and treatment were found for

463 any of the interleukin cytokines examined here (IL-1α: $F(4, 51) = 0.446$, $p = 0.775$; IL-1β: $F(4,$
464 $51) = 0.513$, $p = 0.726$; IL-4: $F(4, 51) = 0.982$, $p = 0.426$; IL-6: $F(4, 51) = 1.779$, $p = 0.148$).
465 Neither IL-2 nor IL-10 showed any effects of poly I:C treatment in either sex (Figures 4B and 4F;
466 insignificant main effects of Treatment: IL-2: $F(4, 51) = 1.498$, $p = 0.217$; IL-10: $F(4, 51) = 1.122$,
467 $p = 0.357$). Similar to IL-1β and IL-4, though, we found that females had overall higher levels of
468 IL-10 than did males (main effect of Sex; $F(1, 51) = 20.27$, $p < 0.001$, $\eta^2_p = 0.284$).

469 **3.4.2. Interferon**

470 We also measured IFNγ levels in response to poly I:C. Unlike mRNA expression, IFNγ
471 protein levels did not change following poly I:C administration in either sex (Figure 4G;
472 insignificant main effect of Treatment: $F(4, 52) = 1.93$, $p = 0.119$). However, IFNγ levels were
473 higher in females relative to males (main effect of Sex: $F(1, 52) = 150.64$, $p < 0.001$; $\eta^2_p =$
474 0.743). This was consistent with mRNA expression data where saline-treated females also
475 showed significantly higher expression of *ifnγ* at baseline than did males (see Figure 3Q).

476 **3.4.3. Chemokines**

477 Finally, we measured protein levels of the chemokines CCL2 and CXCL10 in both
478 sexes. Both chemokines were significantly affected by central administration of poly I:C and in
479 different ways in males and females (Figures 4H and 4I; CCL2: main effect of Treatment: $F(4,$
480 $46) = 18.517$, $p < 0.001$, $\eta^2_p = 0.617$; Sex x Treatment interaction: $F(4, 46) = 3.381$, $p = 0.017$,
481 $\eta^2_p = 0.227$; CXCL10: main effect of Treatment $F(4, 52) = 14.54$, $p < 0.001$, $\eta^2_p = 0.528$; Sex x
482 Treatment interaction: $F(4, 52) = 2.796$, $p = 0.035$, $\eta^2_p = 0.177$). In males, CCL2 levels increased
483 earlier (at 2 hours) post-infusion relative to females. For CXCL10, females took longer for
484 protein levels to begin to decrease as compared to the time course in males, with females still
485 showing the massive elevation at 24 hours post-infusion as they did at 4 hours.

486 Notably, both CCL2 and CXCL10 levels showed the most substantial increases out of all
487 cytokines measured in protein analysis in the hippocampus. Specifically, female CCL2 levels

488 induced by poly I:C peaked at approximately 4 times that of the saline-treated animals, and
489 male CCL2 levels peaked nearly 8 times that of saline-treated males (Figure 4H). More
490 remarkably, female CXCL10 levels rose roughly 16-fold by 4 hours after poly I:C administration,
491 and males showed a 12-fold increase by 4-hours (Figure 4I).
492
493 **3.5. Summary of mRNA and protein data**
494 Overall, hippocampal mRNA expression and protein levels of most of the cytokines and
495 chemokines examined in this experiment responded to central administration of poly I:C in both
496 males and females. We found significant sex differences in baseline mRNA expression and
497 protein levels of several cytokines, where females showed greater basal levels than males. In
498 addition, we found the magnitude of mRNA expression increases was greater in males than
499 females. Protein data showed this to be true only for 2 chemokines, CCL2 and CXCL10.
500 The heatmaps shown in Figure 5 indicate that most of the immune signaling molecules
501 affected in the immediate phase following poly I:C treatment peaked at 4 hours for both mRNA
502 expression (Figure 5A) and protein levels (Figure 5B) and returned to levels of saline-treated
503 animals by 24 hours post-infusion.

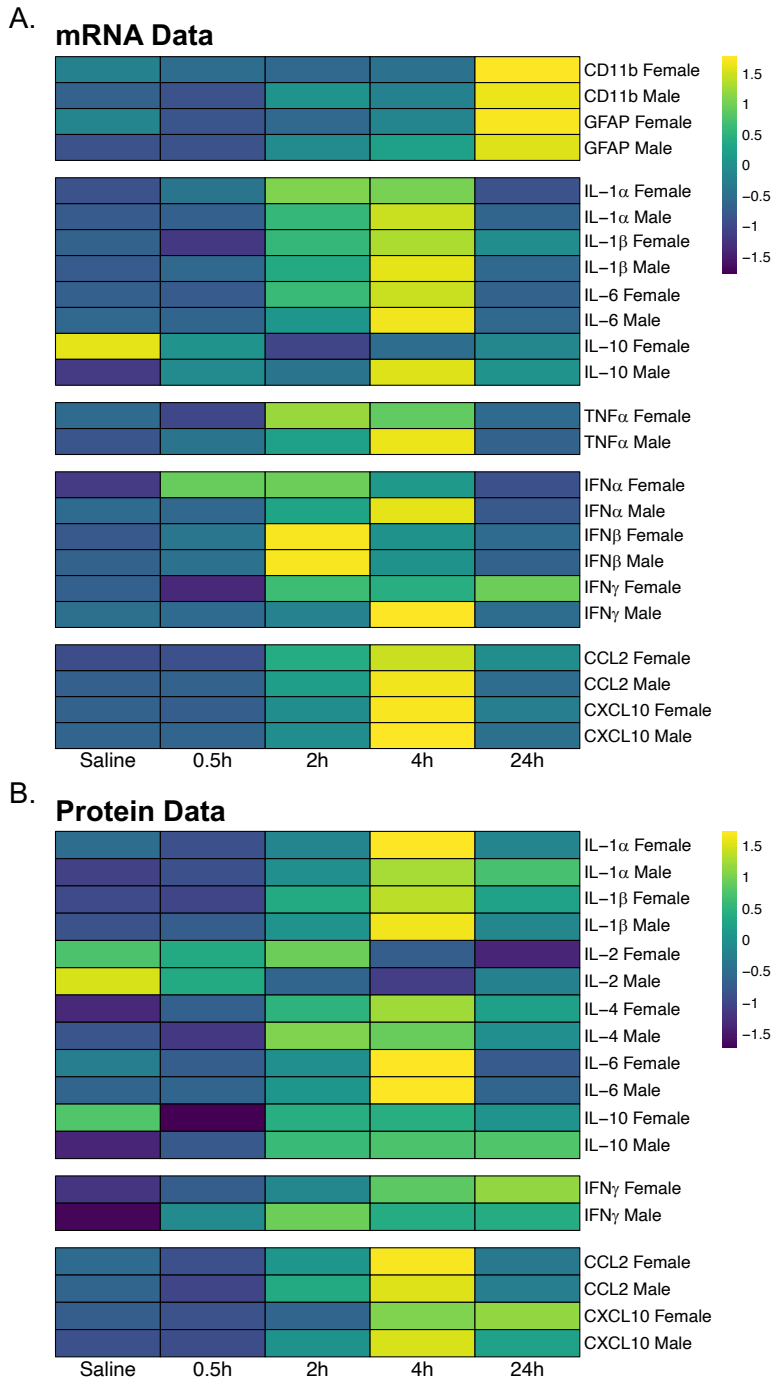


Figure 5. Heatmaps of gene expression and protein levels in the hippocampus. Changes in **(A)** mRNA gene expression and **(B)** protein levels for cytokines, chemokines, and markers of glial activation are shown. Values are centered and scaled across rows to highlight changes across the time course for each gene of interest; thus, differences in magnitude between genes are not depicted.

512 **4. DISCUSSION**

513 Here we demonstrated that central administration of poly I:C induced both physiological
514 measures of sickness and concomitant neuroinflammatory activation in both male and female
515 mice. Both males and females treated with poly I:C mounted an acute fever response and
516 showed weight loss that recovered to saline-treated levels 48 hours later. Additionally, both
517 sexes showed acute cytokine and chemokine responses in the hippocampus, as measured by
518 both mRNA expression and protein levels, that followed the time course of fever. Interestingly,
519 mRNA gene expression of *il-1α*, *il-1β*, *il-6*, *il-10*, *ifnα*, *tnfα*, *ccl2*, and *cxcl10* and protein levels of
520 CCL2 and CXCL10 showed a stronger response in males compared with females. Further,
521 gene expression of *il-10*, *ifnα*, and *ifnγ* increased in males only. Given the specific role of type I
522 interferons in viral response (58–60), this sex difference may have important implications for
523 how neuroimmune activation differentially disrupts cognition in males and females.

524 Poly I:C treatment in both sexes resulted in a significant and transient increase in gene
525 expression and protein levels of most, but not all, cytokines and chemokines in the
526 hippocampus, including IFNβ, IL-1α, IL-1β, IL-6, TNFα, CCL2, and CXCL10. That administration
527 of an immune stimulant, including viral mimics such as poly I:C, induces a neuroimmune
528 response is not new; however, most of the previous studies on poly I:C used peripheral
529 administration (61–64). As such, multiple, indirect mechanisms are likely involved in causing
530 inflammation in the brain (36). Peripheral administration of poly I:C, specifically, was found to
531 induce neuroinflammation through a separate and distinct pathway than central administration
532 (43). Additionally, these studies examined a subset of cytokines or chemokines, including the
533 most commonly examined IL-1β, IL-6, and TNFα, and type I interferons that typically respond to
534 viruses. Given evidence of mechanistic complexities governing neuroinflammation, particularly
535 from stimulants such as poly I:C, and given that there are over 300 cytokines with important
536 roles in the immune system and neural function, it is critical to begin looking beyond IL-1β, IL-6,

537 and TNFα and more strongly consider implications of such limits in experimental design for the
538 field of psychoneuroimmunology.
539 In addition to the intricacies of how immune stimulants such as poly I:C activate the
540 neuroimmune system, males and females differ in immune and neuroimmune responses. The
541 direction of these differences depends on whether you are looking in the periphery (7) or the
542 brain (26,27). We found that mRNA gene expression of *il-1α*, *il-1β*, *il-6*, *il-10*, *ifnα*, *tnfα*, *ccl2*, and
543 *cxcl10* and protein levels of CCL2 and CXCL10 in the hippocampus showed a stronger
544 response in males compared with females. A greater magnitude of cytokine and chemokine
545 response in males is consistent with previous findings that male-derived astrocytes have a
546 greater reaction to inflammatory insults compared with females (26,27,65,66). Poly I:C is
547 recognized by microglia, astrocytes, and neurons via toll-like receptor 3 (TLR3) (67–69). The
548 interaction of these 3 cell types is crucial in mediating inflammatory responses (70,71). Given
549 that TLR3 shows much greater expression in astrocytes relative to microglia (72), the reaction of
550 astrocytes in males may be driving the sex differences in magnitude gene expression response
551 of cytokines following poly I:C. The astrocyte activation marker, GFAP, and the microglial
552 activation marker, CD11b, did not increase until 24 hours after poly I:C treatment and did not
553 show sex differences. However, this does not absolve astrocytes or microglia from the acute
554 response to poly I:C. Specifically, Norden and colleagues found that cytokine gene expression
555 from both astrocytes and microglia preceded increases in astrocyte and microglial activation
556 markers, (GFAP and Iba1, respectively), and that these activation markers similarly did not
557 show reliable increases until the 24-hour time point (73). It is likely, therefore, that a greater
558 astrocytic reaction in males is driving the greater magnitude of response to poly I:C by males.
559 Viral infections are not immune to observations of sex differences. Specifically, males
560 and females show sex differences in prevalence, intensity, and survival outcomes of viruses
561 (7,8,33). Many report that when females show better survival outcomes or seem more protected

562 against certain viruses, it is due to the female immune system having a greater anti-viral
563 response that allows the virus to be cleared faster (33,74–76). Data on SARS-CoV-2, the virus
564 that causes the coronavirus disease 2019 (COVID-19), has shown a similar sex difference in
565 intensity of infection, with more males having worse symptoms, including neurological
566 complications, and survival outcomes relative to females (77–81). However, in contrast to the
567 data that suggests a greater immune response results in faster recovery in females, recent
568 studies indicate that higher levels of inflammatory cytokines including IL-6, IL-1β, CXCL10, and
569 CCL2 are actually correlated with worse COVID-19 outcomes (35,82). Unequivocally, sex as a
570 biological factor matters in the context of immune and neuroimmune responses as well as in the
571 acute and chronic implications of neuroimmune modulation of neural function, cognition, and
572 emotion in males and females. Limiting studies by only using one sex and extrapolating the data
573 to the other is not only inappropriate given the intricacies of sex differences in the literature
574 discussed here, but it is also potentially dangerous for both development and administration of
575 treatments in inflammatory diseases and disorders.

576 We observed that most cytokines examined here showed a sex difference in response to
577 poly I:C. Whereas others have reported increases in select inflammatory markers following poly
578 I:C treatment, these studies used either only used male (62,63) or female rodents (61,64). To
579 the best of our knowledge, this is the first direct comparison of hippocampal cytokines in males
580 and females as a consequence of poly I:C. Whether the greater magnitude in male response to
581 poly I:C indicates greater neuroprotection or vulnerability is yet to be determined. This is an
582 especially intriguing question given that we also directly compared mRNA expression and
583 protein levels of cytokines and chemokines at baseline and found that females had greater
584 basal immune activity relative to males.

585 Perhaps it is not only that females are able to mount a greater immune response to help
586 clear viral loads faster and aid in a faster recovery as previously discussed (33,74–76), but also
587 that females start out with greater immune activity that allows them to reach necessary

588 activation states faster than males. Specifically, maybe females do not need to have as great of
589 a magnitude increase in cytokines and chemokines when the neuroimmune system is activated
590 because they already have “more players in the game”. Future work will need to address
591 whether and how sex differences in the cytokine and chemokine basal levels or activation in
592 response to immune challenge result in functional differences in modulation of neural function
593 and contribute to sex-biases in neurological and psychiatric disease.

594 Of particular note, we observed a sex-specific pattern of expression of the interferon
595 family of cytokines in the hippocampus. Specifically, males showed increases in IFNα, IFNβ,
596 and IFNγ, but females only showed a significant response in IFNβ. This is consistent with
597 previous findings that showed increased gene expression of IFNβ, but not IFNα, in females in
598 response to peripheral poly I:C, though this study did not measure these effects in males for
599 comparison (64). Type I interferons, IFNα and IFNβ, are key to the anti-viral response of the
600 immune system and, as such, are known to respond to viral stimulants including poly I:C (58–
601 60,83,84). Consistent with our data, in which IFNβ showed an early peak expression levels, type
602 I interferon activity is responsible for inducing inflammatory cytokines such as IL-6 and TNFα
603 (62,64). Additionally, interferon signaling from poly I:C treatment also results in altered
604 glutamatergic signaling (83,84), which is known to be critical for hippocampal memory formation
605 (85). Given that males induce both IFNα and IFNβ in the hippocampus following poly I:C
606 whereas females only induce IFNβ, interferons have important implications for sex differences in
607 neuroimmune disruption of cognition from virus or viral-like stimulants.

608

609 **5. CONCLUSION**

610 This study characterizes the sickness and neuroimmune responses to central
611 administration of poly I:C, and we observed sex-specific patterns of hippocampal cytokine
612 responses. Specifically, we identified type I interferons as one potential node mediating sex-

613 specific cytokine responses and neuroimmune effects on synaptic plasticity and cognition.
614 Additionally, the magnitude of response of cytokines such as CCL2 and CXCL10 highlight the
615 importance of future work incorporating a more comprehensive set of inflammatory markers
616 using multiple endpoints. Neuroimmune activation is known to play a role in cognitive deficits
617 and affective dysregulation in diseases such as Alzheimer's Disease and other dementias (86),
618 Post-Traumatic Stress Disorder (87,88), depression (4,89), and now also COVID-19 (90). Given
619 the sex/gender biases in prevalence, severity, and/or survival outcomes, identifying sex-specific
620 neuroimmune responses will provide novel targets for personalized prevention and treatment of
621 these diseases.
622
623 **LIST OF ABBREVIATIONS**
624 ANOVA = analysis of variance
625 CCL2 = C-C motif chemokine ligand 2
626 Cd11b = cluster of differentiation molecule 11B
627 cDNA = complementary deoxyribonucleic acid
628 COVID-19 = coronavirus disease-19
629 CXCL10 = C-X-C motif chemokine ligand 10
630 DNA = deoxyribonucleic acid
631 GAPDH = glyceraldehyde-3-phosphate dehydrogenase
632 GFAP = glial fibrillary acidic protein
633 HPRT1 = hypoxanthine phosphoribosyltransferase 1
634 Iba1 = ionized calcium-binding adaptor molecule 1
635 ICV = intracerebroventricular
636 IFNα = interferon α
637 IFNβ = interferon β

638 IFNγ = interferon γ

639 IL-1α = interleukin-1α

640 IL-1β = interleukin-1β

641 IL-2 = interleukin-2

642 IL-4 = interleukin-4

643 IL-6 = interleukin-6

644 IL-10 = interleukin-10

645 kg = kilogram

646 LPS = lipopolysaccharide

647 mg = milligram

648 mm = millimeter

649 mRNA = messenger ribonucleic acid

650 PCR = polymerase chain reaction

651 Poly I:C = polyinosinic:polycytidylic acid

652 qPCR = quantitative real-time polymerase chain reaction

653 RNA = ribonucleic acid

654 RPLP0 = ribosomal protein lateral stalk subunit P0

655 SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2

656 TLR = toll-like receptor

657 TNFα = tumor necrosis factor α

658 μg = microgram

659 μL = microliter

660 μm = micrometer

661

662

663 **DECLARATIONS**

664 **Ethics Approval**

665 All animal protocols used in these experiments were approved by the Institutional Animal
666 Care and Use Committee (IACUC) at the University of Michigan.

667 **Consent for Publication**

668 Not applicable.

669 **Availability of Data and Materials**

670 The data used and analyzed for the current study are available from the corresponding
671 author upon reasonable request.

672 **Competing Interests**

673 The authors have no competing interests to declare.

674 **Funding**

675 These experiments were supported by a University of Michigan Office of Research
676 Award to NCT. This funding body did not provide input for the design of the study, collection,
677 analysis, data interpretation, or writing of this manuscript. The award was used solely to fund
678 the cost of animals, reagents, and equipment used in these experiments.

679 **Author's Contributions**

680 CKP designed the experiments, performed all animal surgeries and treatments, collected
681 physiological measurements (body temperature and weights) and tissue, processed tissue
682 samples and conducted all molecular biology assays (qPCR and multiplex assays), ran
683 statistical analyses, and wrote the manuscript. RGH aided in the collection of physiological
684 measurements (body temperature and weights) and monitored animals for sickness behaviors
685 and surgical recovery throughout the experiments. NCT provided intellectual contributions
686 regarding experimental design and data interpretation, supplied financial support and laboratory
687 space and equipment to complete the experiments, and contributed significantly to the editing of
688 this manuscript. All authors read and approved the final version of this manuscript.

Acknowledgements

We would like to thank Dr. Ashley Keiser, Dr. Daria Tchessalova, Brynne Raines, and Sarah Jacob for assistance with the techniques, reagents, and data collection for these experiments.

Author's Information

CKP is trained in behavioral neuroscience with specific interests in behavioral neuroimmunology. Since 2013, CKP has worked on models of neuroimmune activation in both rats and mice with the goal of understanding how the neuroimmune system functions to modulate cognition and affect and both whether and how biological sex interacts with these processes.

NCT studies the mechanisms of learning and memory in males and females, the modulation of memory and affective processes by neuroimmune activation, and long-term impact of illness on the hippocampus, cognitive decline, Alzheimer's Disease, and neuropsychiatric disorders.

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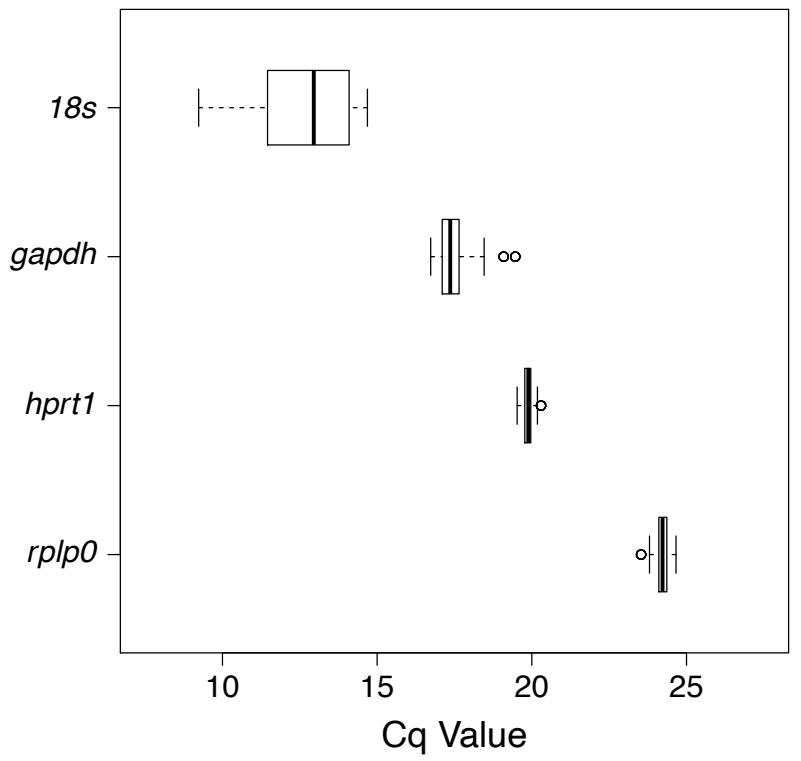
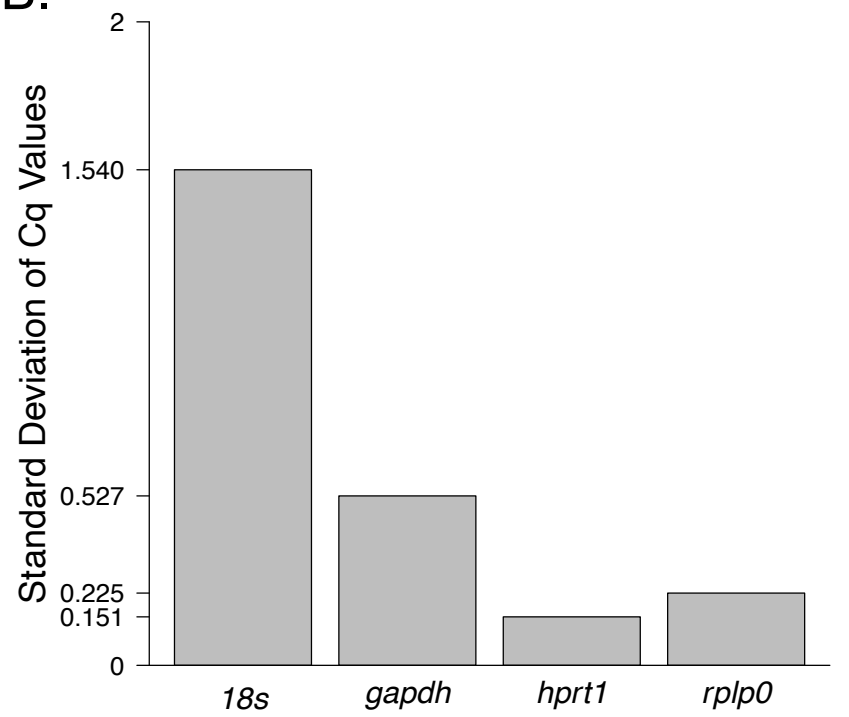
962

A.



B.



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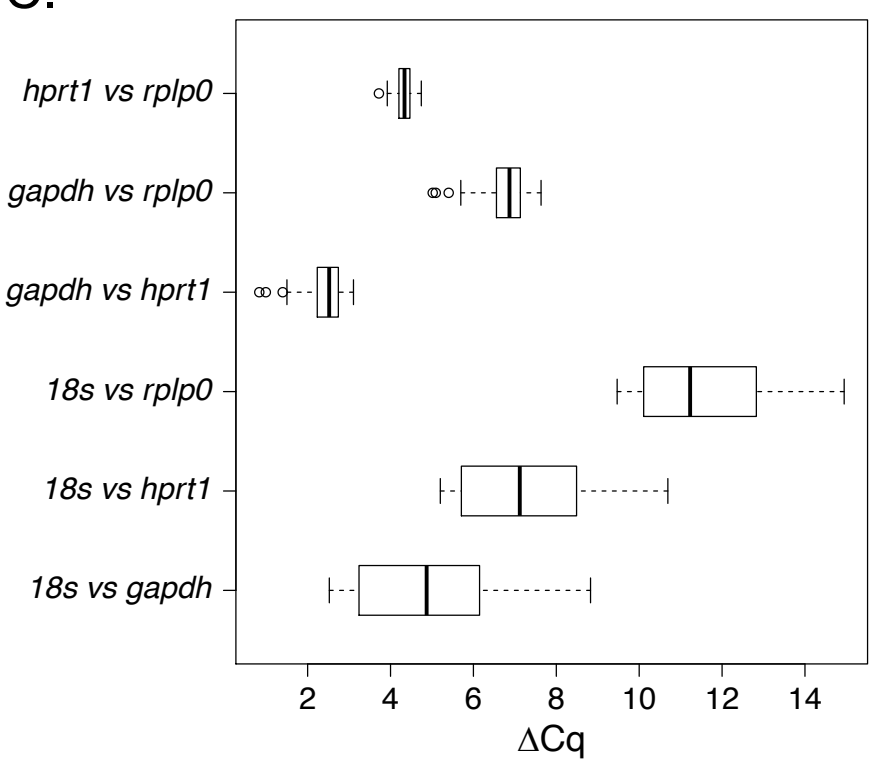
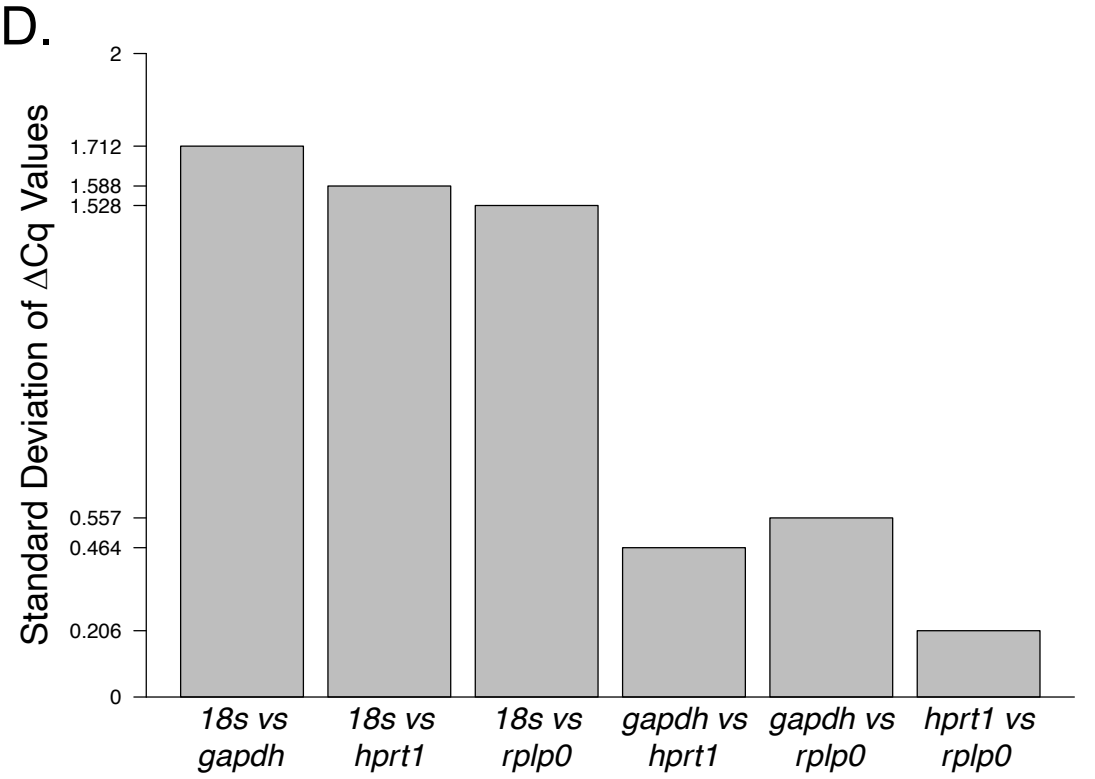
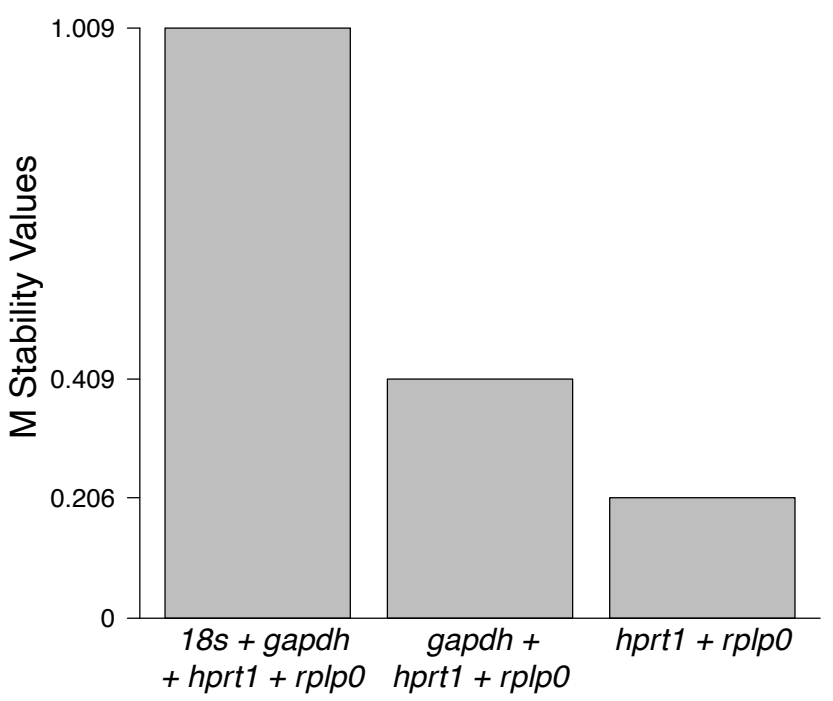


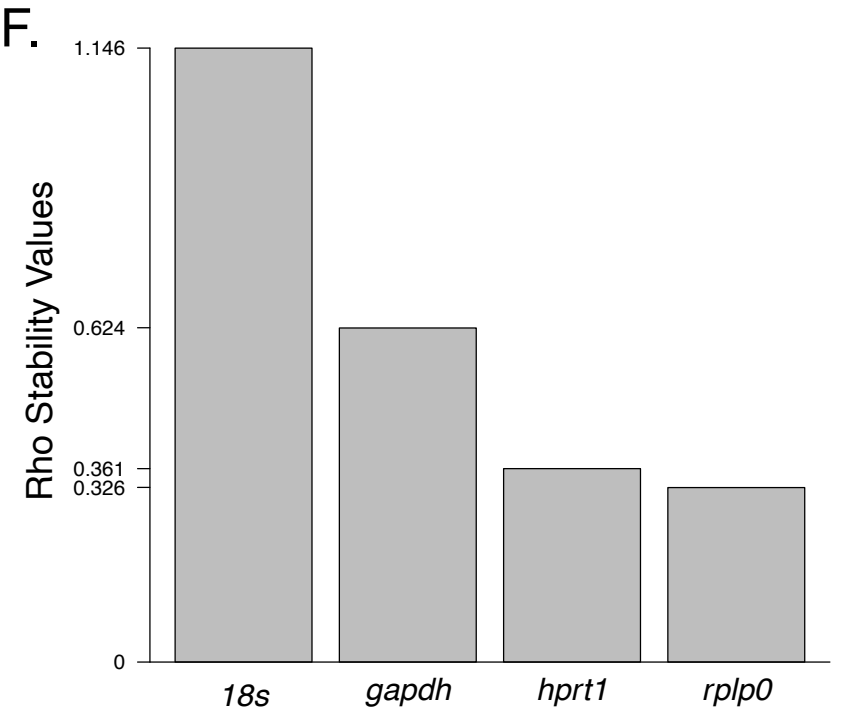
D.



E.

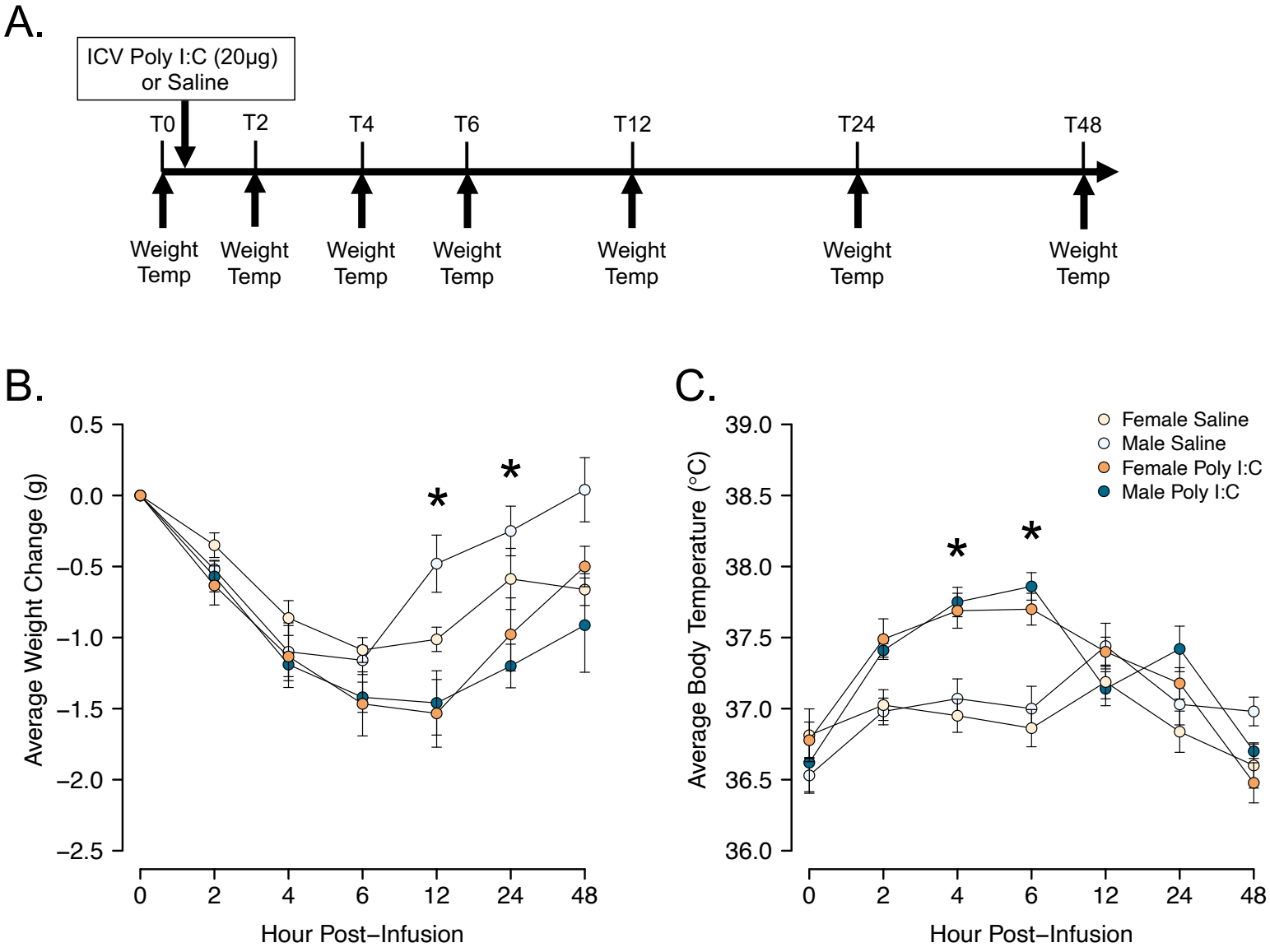


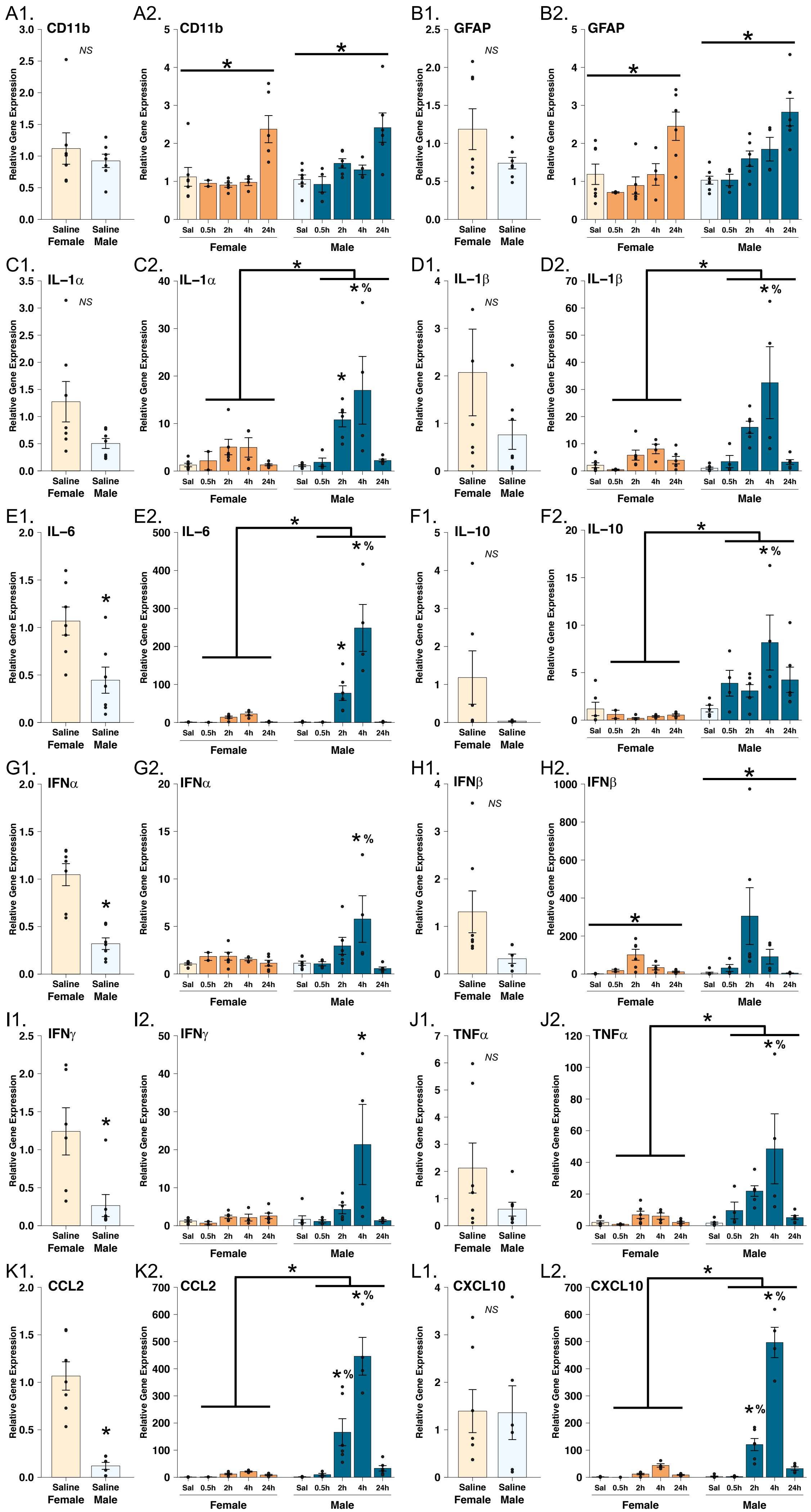
F.

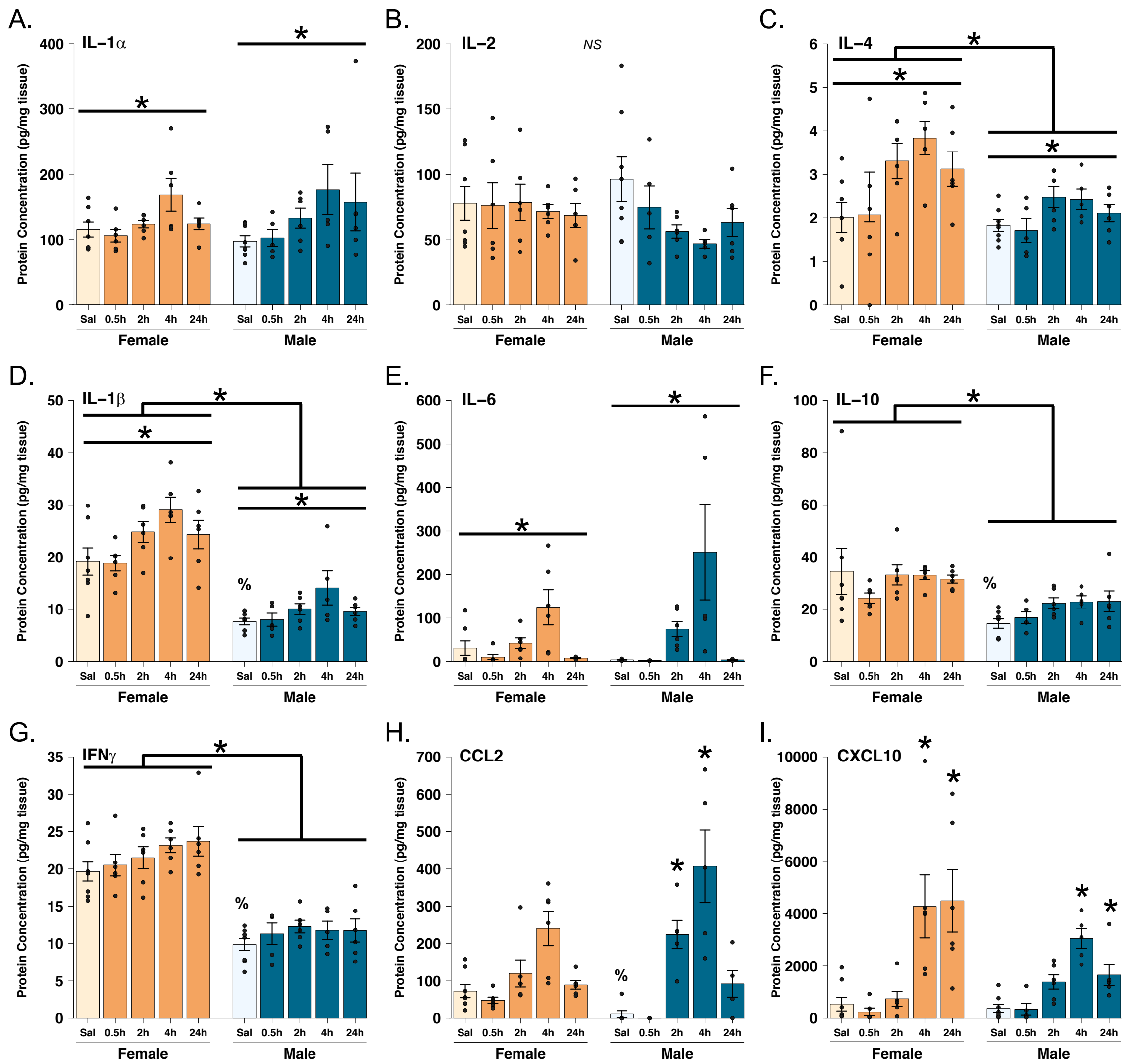


G.

	Cq Value Standard Deviations	Avg Standard Deviations of ΔCq Values	M Stability Values	Rho Stability Values
18s	1.5402440	1.609	1.609	1.1459049
gapdh	0.5266292	0.911	0.510	0.6235678
hprt1	0.1507111	0.753	0.206	0.3610396
rplp0	0.2245596	0.764	0.206	0.3256827

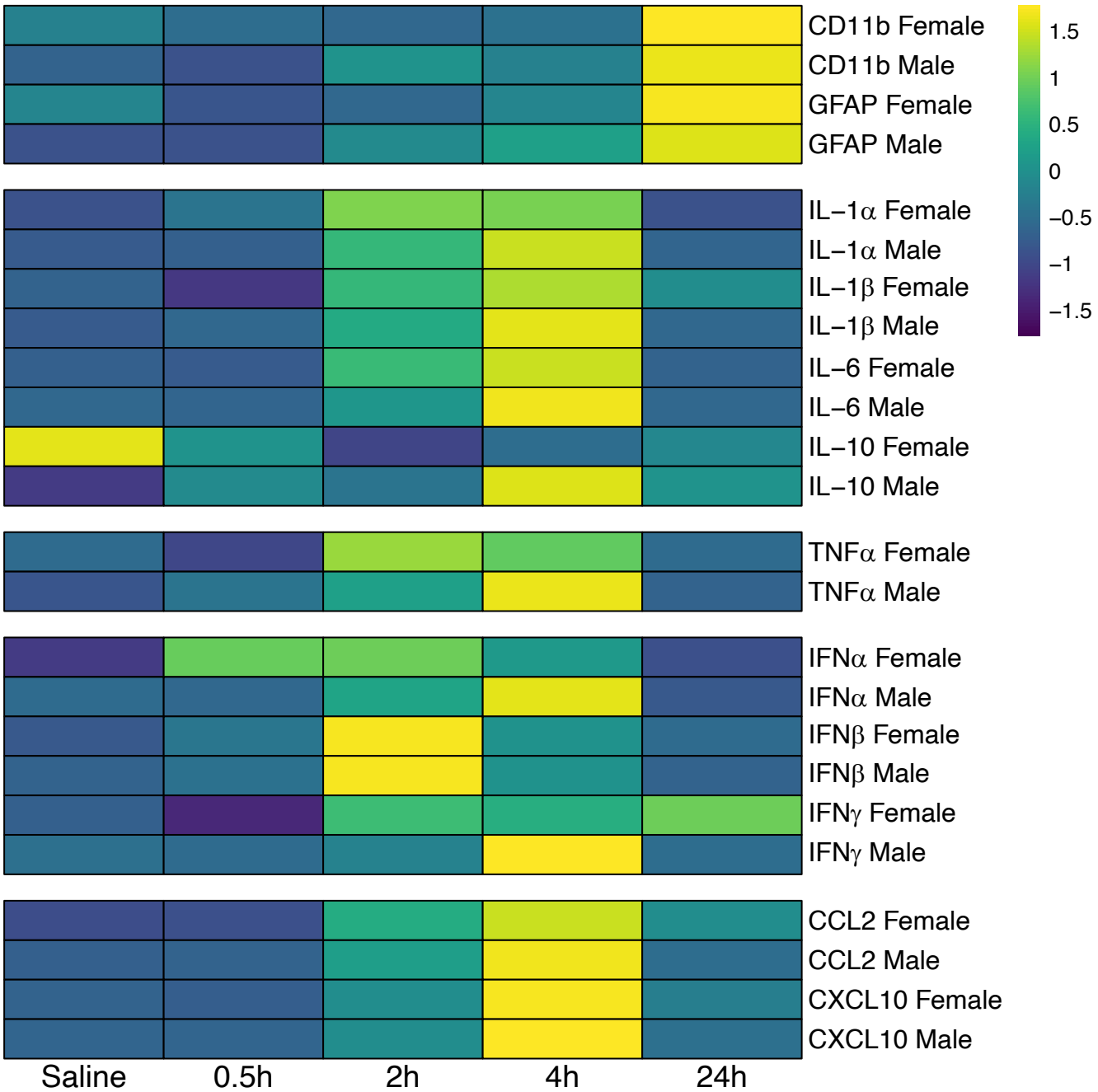

A.
ICV Poly I:C (20μg)
or Saline
T0
T2
T4
T6
T12
T24
T48
Weight
Temp
B.
Average Weight Change (g)
0.5
0.0
−0.5
−1.0
−1.5
−2.0
−2.5
0
2
4
6
12
24
48
Hour Post−Infusion
C.
Average Body Temperature (°C)
39.0
38.5
38.0
37.5
37.0
36.5
36.0
Female Saline
Male Saline
Female Poly I:C
Male Poly I:C

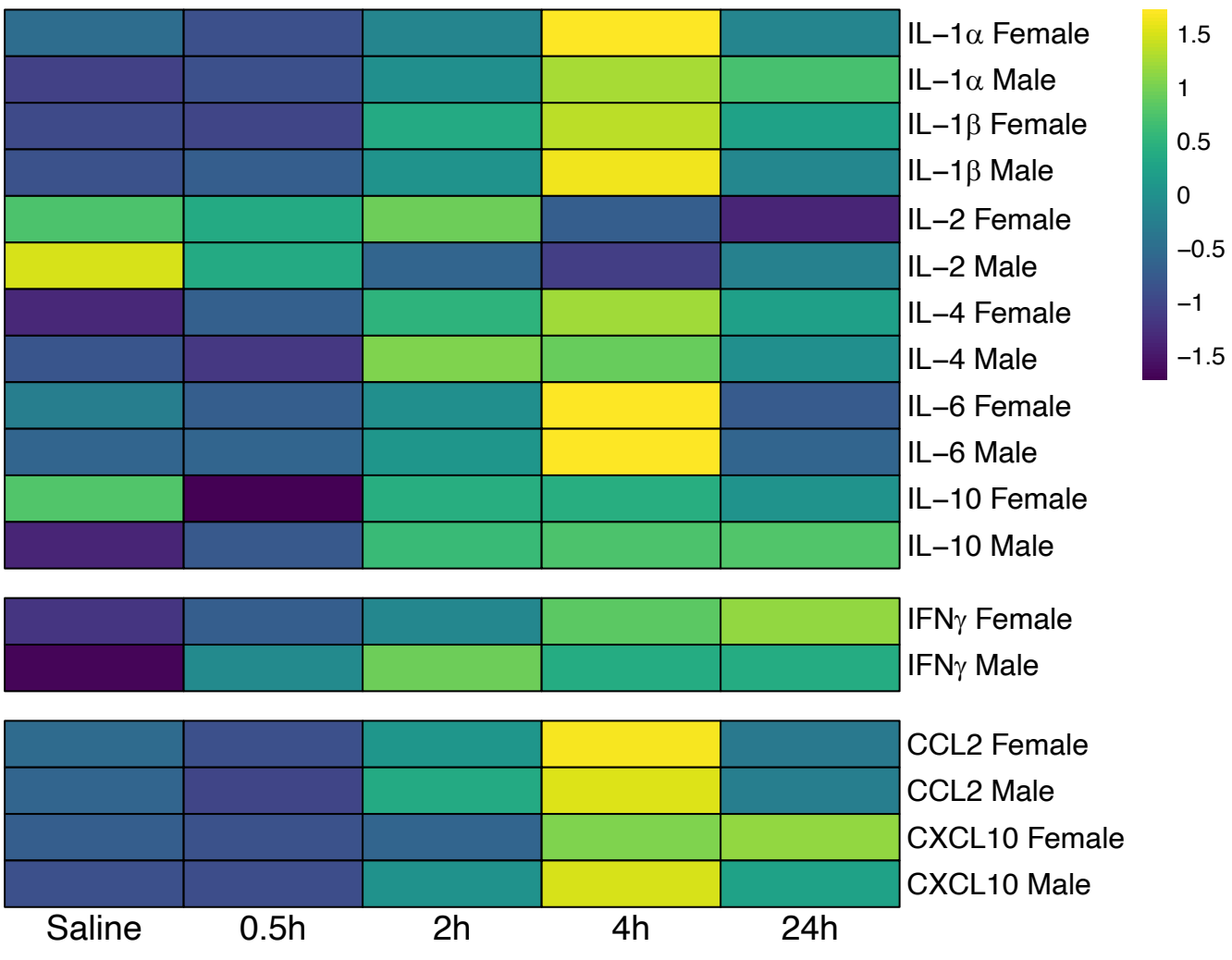

A1.
CD11b
NS
A2.
CD11b
B1.
GFAP
NS
B2.
GFAP
C1.
IL−1α
NS
C2.
IL−1α
D1.
IL−1β
NS
D2.
IL−1β
E1.
IL−6
E2.
IL−6
F1.
IL−10
NS
F2.
IL−10
G1.
IFNα
G2.
IFNα
H1.
IFNβ
NS
H2.
IFNβ
I1.
IFNγ
I2.
IFNγ
J1.
TNFα
NS
J2.
TNFα
K1.
CCL2
K2.
CCL2
L1.
CXCL10
NS
L2.
CXCL10
Relative Gene Expression
Saline Female
Saline Male
Sal
0.5h
2h
4h
24h
Female
Male
*
*%


A.
IL–1α
B.
IL–2
NS
C.
IL–4
D.
IL–1β
E.
IL–6
F.
IL–10
G.
IFNγ
H.
CCL2
I.
CXCL10
Protein Concentration (pg/mg tissue)
Sal
0.5h
2h
4h
24h
Female
Male
*
%

A.

mRNA Data



B.

Protein Data