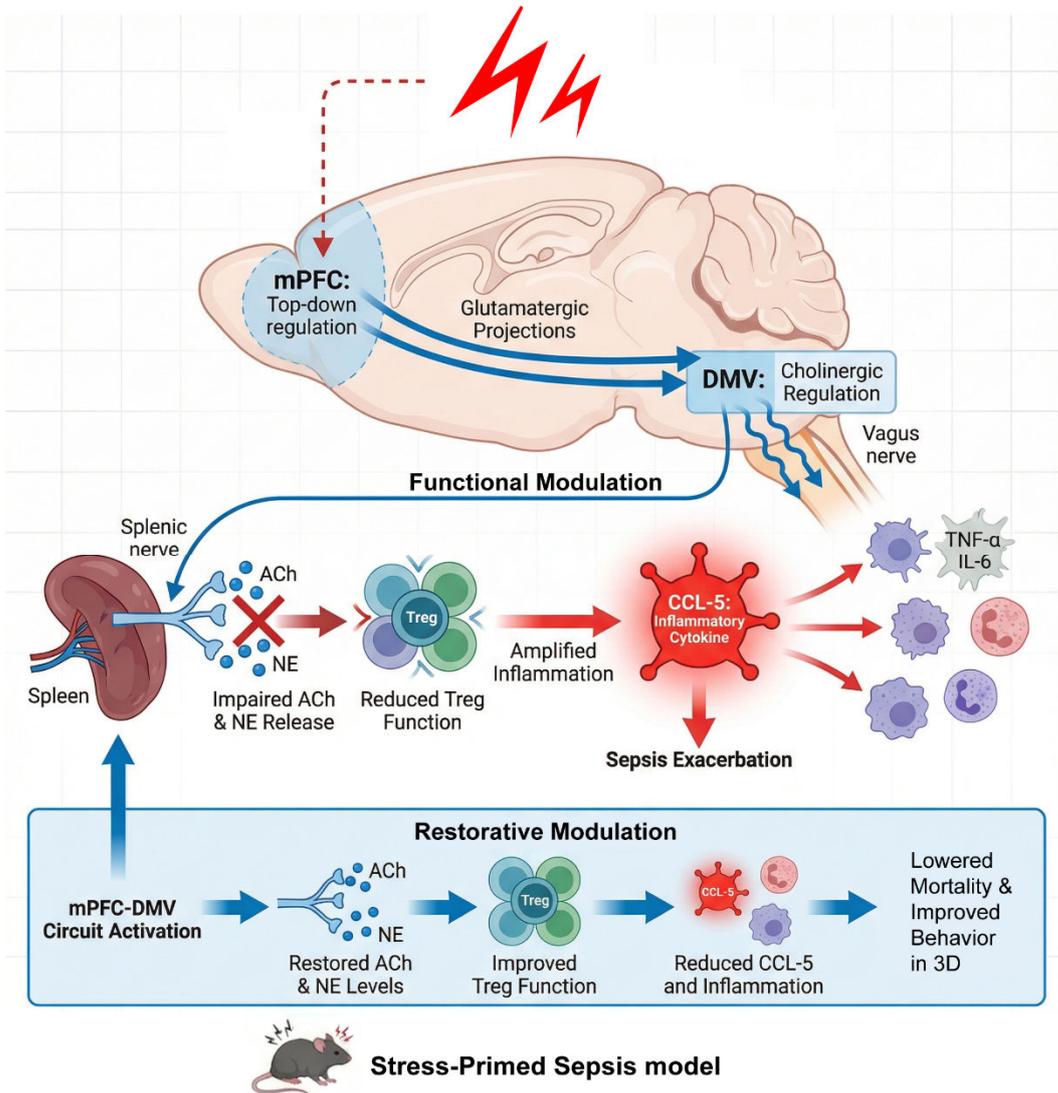


1 **Supplementary conceptual model:**



2

3 *Schematic summary of the revised functional framework illustrating how stress-*
4 *induced cortical dysfunction alters central autonomic modulation and downstream*
5 *immune outcomes in sepsis.*

6 **Supplementary Methods**

7 This appendix provides detailed experimental procedures corresponding to the
8 Methods section in the main manuscript.

9 **Animals**

10 Adult male ChAT-Cre transgenic mice (6–8 weeks old) were purchased from Jackson
11 Laboratory (Bar Harbor, ME, USA), and C57BL/6J mice were obtained from Charles
12 River Co., Ltd. (Beijing, China). Only male mice were used to eliminate variability
13 related to the estrous cycles. The animals were randomly assigned to the experimental

14 groups using a random number generator. The sample size was determined based on a
15 previous study ¹. Mice showing no detectable fluorescence after viral transfection or
16 unsuccessful injections were excluded from the analysis. Animal experiments were
17 performed according to the procedures approved by the Animal Care and Use
18 Committee of the Fourth Military Medical University (Xi'an, China; study number
19 KY20233257-1; approved May 3, 2023) and conducted in accordance with the
20 institutional Guidelines for Animal Experimentation and the Animal Research:
21 Reporting of In Vivo Experiments (ARRIVE) guidelines.

22 **Statistical Analysis**

23 For time-course analyses (e.g., body weight and serum cytokine levels), data were
24 analyzed using two-way repeated-measures ANOVA with group and time as factors,
25 followed by Bonferroni-corrected post hoc comparisons when significant main effects
26 or interactions were detected. Detailed procedures for c-Fos immunohistochemistry
27 and quantification are provided in the SI Appendix.

28 **Stereotaxic Surgery and Viral Delivery**

29 The mice were anesthetized with pentobarbital sodium (100 mg kg⁻¹,
30 intraperitoneally) and positioned in a stereotaxic frame for the experiment. The scalp
31 was shaved, and the skin was locally anesthetized with 2.0% lidocaine. A midline
32 sagittal incision was made, and small craniotomies were drilled at the following
33 coordinates relative to the bregma: for the dorsal motor nucleus of the vagus (DMV),
34 anteroposterior (AP) -6.75 mm, mediolateral (ML) ±0.65 mm, and dorsoventral (DV)
35 -3.6 mm; and for the medial prefrontal cortex (mPFC), AP +1.9 mm, ML ±0.3 mm,
36 and DV -1.4 mm. Viral solutions were delivered at a rate of 50 nl min⁻¹ (200 nl per
37 side) using a glass micropipette connected to a micro-syringe pump. The pipette was
38 left in place for 10 min before a slow withdrawal to minimize backflow. Postsurgical
39 care included placement on a warming pad until full recovery from anesthesia and
40 administration of meloxicam (0.03 mg kg⁻¹, subcutaneously) for three days after the
41 surgery. All in vivo electrophysiological, splenic nerve activity, and behavioral
42 experiments were conducted at least three weeks after surgery to ensure recovery and
43 robust viral expression. For all in vivo circuit-manipulation experiments involving

44 DREADDs, control groups received clozapine-N-oxide (CNO i.p., 5mg/kg)
45 administration following injection of control AAV-DIO-mCherry viruses (CNO-only
46 controls). Detailed information on all viral vectors used for chemogenetic
47 manipulation and circuit tracing is provided in the SI Appendix.

48 **Ex vivo Patch-Clamp Electrophysiology**

49 The incubated brain slices were transferred to a recording chamber and continuously
50 superfused with oxygenated artificial cerebrospinal fluid (aCSF) for the duration of
51 the experiment. The dorsal motor nuclei of the vagus (DMV) and medial prefrontal
52 cortex (mPFC) regions were identified in each slice according to the mouse brain
53 atlas, and DMV^{ChAT} or mPFC^{Glut} neurons were visualized under a DM LFSA
54 microscope (Leica, Wetzlar, Germany). Recording electrodes (3–6 M Ω) were filled
55 with an intracellular solution. Signals were amplified using a Multiclamp 700B patch-
56 clamp amplifier, digitized using a Digidata 1550B interface, and acquired using the
57 Clampex 10.5 software (Molecular Devices). The data were sampled at 10 kHz and
58 low-pass filtered at 2 kHz, respectively. All recordings were conducted at room
59 temperature (25–30 °C).

60 **Spontaneous Excitatory Postsynaptic Currents (sEPSCs)**

61 For sEPSC recordings, picrotoxin (PTX; 100 μ M) was added to the perfusate to block
62 GABAA receptor-mediated currents. The pipette solution contained (in mM) 120
63 potassium gluconate, 20 KCl, 2 MgCl₂, 0.2 EGTA, 10 HEPES, 4 Na₂-ATP, and 0.3
64 GTP (pH 7.3, adjusted with KOH). Neurons were voltage-clamped at –60 mV in gap-
65 free mode, and sEPSCs were recorded for 10 min per cell. For the analysis, a stable 3-
66 min segment was selected, and the event amplitudes and frequencies were quantified.

67 **Intrinsic Excitability**

68 Intrinsic excitability was assessed in the presence of PTX (100 μ M), CNQX (20 μ M),
69 and AP-5 (50 μ M) to block the synaptic transmission. Using the same intracellular
70 solution, a whole-cell configuration was established, and the neurons were allowed to
71 stabilize in the I = 0 mode. If necessary, a holding current of < 50 pA was applied to
72 adjust the membrane potential to the resting level. For DMV^{ChAT} neurons, step

73 currents from 0 to 150 pA (10 pA increments, 500 ms duration, 15 s intersweep
74 interval) were injected into the cells. For mPFC^{Glut} neurons, step currents from 0 to
75 300 pA (50 pA increments, 2000 ms duration, and 15 s intersweep interval) were
76 applied. Before each depolarizing step, a hyperpolarizing pulse (-40 pA, 400 ms) was
77 delivered to measure the voltage responses, and the input resistance was calculated
78 using Ohm's law. Cells with >20% change in input resistance during recording were
79 excluded from the analysis.

80 For electrophysiological recordings, individual cells were treated as independent
81 observations, with 2–3 cells recorded per animal; animals were sampled across
82 experimental groups.

83 **Splenic Nerve Activity Recording and Circuit Intervention**

84 The mice were anesthetized with isoflurane and implanted with wireless neural
85 recording electrodes, according to the manufacturer's instructions (Shanghai QuanLan
86 Technology Co., Ltd.). After the fur was shaved, the head-mounted connector of the
87 wireless signal acquisition device was fixed to the skull using stainless-steel screws
88 and dental cement. A cuff electrode preconnected to the recording interface was
89 tunneled subcutaneously from the skull to the abdominal region through the cervical
90 route.

91 Following skin preparation and disinfection of the abdominal area, a small midline
92 laparotomy was performed to gently expose the splenic hilum for cannulation of the
93 splenic veins. The splenic nerve was bluntly dissected from the surrounding tissue,
94 and a cuff electrode was placed longitudinally along the nerve's surface. The spleen
95 was carefully returned to its anatomical position, and the abdominal musculature and
96 skin were sutured in the layers. The second disinfection was performed before the
97 recovery process.

98 Postoperative care included placement on a warming pad until full recovery from
99 anesthesia and administration of meloxicam (0.03 mg kg⁻¹, subcutaneously) once
100 daily for three days after surgery. Circuit manipulations were conducted after full
101 recovery and paired with simultaneous splenic nerve activity recordings to assess the
102 effects of DMV or mPFC-DMV intervention.

103 **Recording Data Analysis**

104 The splenic nerve activity signals were sampled at 500 Hz. The raw recordings were
105 digitally bandpass-filtered between 10 and 60 Hz to minimize the baseline drift and
106 high-frequency noise. Spike events were detected in MATLAB (R2024b,
107 MathWorks) using the findpeaks function, with the amplitude threshold set to the
108 mean \pm 1 standard deviation of the signal amplitude at each recording point. The
109 mean spike count per 1-s bin was calculated over a continuous 3,600-s recording
110 period for each mouse in its home cage. Group differences in mean firing rates were
111 analyzed using one-way ANOVA, followed by appropriate post hoc multiple
112 comparison tests.

113 **Flow Cytometry**

114 *Splenocyte Isolation*

115 Spleens were harvested from the mice immediately after euthanasia and placed in ice-
116 cold sterile PBS. Each spleen was gently dissociated using a sterile syringe plunger
117 until no visible red tissue fragments were present. The cell suspension was passed
118 through a 70- μ m nylon mesh filter and rinsed with 2% RPMI-1640 medium. The
119 filtrate was collected in 15-mL conical tubes and centrifuged at 1,600 rpm for 5 min at
120 4 °C. The supernatant was discarded, and the cell pellet was resuspended in 10 mL
121 PBS and centrifuged under the same conditions. Red blood cells were lysed by
122 resuspending the pellet in 2–3 mL of 1 \times RBC lysis buffer and incubating on ice for 5–
123 10 min. Lysis was stopped by adding 10 mL of PBS, and the cells were centrifuged.
124 The pellet was finally resuspended in 2% RPMI-1640 medium. Large tissue clumps
125 were removed, and the viable cells were counted.

126 *Cell Surface Staining*

127 Splenocytes were transferred into 1.5-mL tubes, centrifuged at 1,600 rpm for 5 min,
128 and washed once with 500 μ L of PBS. The cell pellet was resuspended in 50 μ L of
129 pre-prepared antibody staining buffer for surface markers (antibody panels prepared
130 according to the manufacturer's instructions) and incubated at room temperature for

131 30 min in the dark. After incubation, the cells were washed with 500 μ L PBS and
132 centrifuged at 1,600 rpm for 5 min.

133 *Fixation, Permeabilization, and Intracellular Staining*

134 Cells were fixed in 250 μ L of fixation buffer for 20 min at room temperature in the
135 dark, followed by incubation with 250 μ L of 1 \times permeabilization buffer for 10 min.
136 After permeabilization, the cells were centrifuged (2,200 rpm, 5 min), the supernatant
137 was discarded, and 500 μ L of 1 \times permeabilization buffer was added for a single wash.
138 The cell pellet was then resuspended in 50 μ L of pre-prepared intracellular antibody
139 staining solution and incubated for 30–40 min at room temperature in the dark,
140 according to the manufacturer's instructions. Following incubation, the cells were
141 washed twice with 500 μ L PBS (2,200 rpm, 5 min each) and resuspended in 200–300
142 μ L PBS for flow cytometric acquisition. If immediate analysis was not performed, the
143 samples were stored at 4 $^{\circ}$ C in the dark until further processing.

144 **Neurotransmitter Extraction and LC–MS/MS Analysis**

145 Following euthanasia, splenorenal tissue was rapidly isolated on ice and immediately
146 snap-frozen at -80° C, as described previously². Benzoyl chloride derivatization was
147 performed before the analysis. Neurotransmitter quantification was performed using
148 an ACQUITY Premier UPLC system (Waters) equipped with an ACQUITY UPLC
149 HSS T3 column (100 \times 2.1 mm, 1.8 μ m). Multiple reaction monitoring (MRM) data
150 were acquired and processed using SCIEX Analyst Work Station Software (version
151 1.6.3) and Biobud-DDF (version 2.0.3.11).

152 **Metabolomic Data Acquisition and Analysis**

153 Following sample pretreatment, metabolite profiling was performed using an AB
154 SCIEX 5500 QQQ-MS mass spectrometer, and biochemical analyses were performed.
155 Quality control (QC) samples were included to assess data stability, as recommended
156 for mass-spectrometry-based metabolomics. Although QC samples are theoretically
157 identical, systematic errors during sample extraction, detection, and analysis can
158 introduce variations. The data quality was evaluated by calculating the relative

159 standard deviation (RSD) of each targeted metabolite within the QC samples, with an
160 RSD of < 10% indicating high-quality data.

161 For statistical analysis, Student's *t-test* was applied to identify metabolites showing
162 significant differences between the comparison groups, with $p < 0.05$ considered to
163 indicate statistical significance. The abundances of differentially abundant metabolites
164 were normalized using the *z-score* method and visualized via hierarchical clustering
165 in R (pheatmap package) to illustrate the differences in accumulation between groups.

166 **3D-AI Animal Behavior Analysis System**

167 3D Behavioral Analysis

168 A multimodal behavioral analysis pipeline was established to convert 2D video
169 recordings into 3D behavioral representations, as previously described³. During
170 testing, a synchronized multicamera video acquisition system (resolution 960×540 ,
171 frame rate 30 fps) was positioned around a circular open-field arena (diameter: 40
172 cm). The inner wall of the arena was thoroughly cleaned with 75% ethanol before
173 each trial to eliminate any residual odors. Each mouse was recorded for 10 min of
174 continuous, free exploration. From each recording, 800 frames were extracted for the
175 manual annotation of 16 anatomical landmarks (nose, left ear, right ear, neck, left
176 forelimb, right forelimb, left hind limb, right hind limb, left forepaw, right forepaw,
177 left hind paw, right hind paw, back, tail base, tail middle, and tail tip). The
178 annotations were verified in a double-blind manner to ensure accuracy.

179 The annotated dataset was used for transfer learning of a deep convolutional neural
180 network with spatiotemporal feature extraction capability to enable automated pose
181 estimation across all the video frames. Keypoint positions from four synchronized 2D
182 projections were geometrically corrected and reconstructed into a 3D skeletal model
183 using multi-view triangulation. For the reconstructed spatiotemporal sequences,
184 hierarchical clustering analysis was applied to segment the behavior into 40 distinct
185 action units with precise timestamps.

186 The resulting multidimensional behavioral representation matrix integrates
187 millisecond-level temporal features, 3D kinematic parameters (joint angle and limb

188 length variation rates), and action classification labels. Nonlinear dimensionality
189 reduction was performed using the uniform manifold approximation and projection
190 (UMAP) algorithm for behavioral state visualization.

191 To assign an ethical meaning, each of the 40 behavior units was manually labeled
192 according to the Mouse Ethogram database and the literature, yielding a standardized
193 set of named behavioral subtypes for subsequent statistical analyses.

194

195 **Viral vectors and chemogenetic tools**

196 The following viral vectors were used in this study. For chemogenetic manipulation
197 of cholinergic neurons, AAV2/9-ChAT-DIO-hM3Dq-mCherry and AAV2/9-ChAT-
198 DIO-hM4Di-mCherry (BrainVTA, Wuhan, China) were used. For projection-defined
199 circuit targeting, AAV2/1-hSyn-Cre (BrainVTA) and AAV2/Retro-CaMKII α -Cre
200 (BrainVTA) were employed as indicated.

201 For retrograde and transsynaptic tracing, PRV-EGFP (200 nL per injection;
202 BrainVTA) was used for splenic nerve-associated tracing. Monosynaptic rabies-based
203 tracing was performed using a combination of AAV vectors encoding TVA and rabies
204 glycoprotein (RG) together with RV Δ G-EGFP, as described in the experimental
205 procedures. All rabies-related viral components were obtained from BrainVTA. All
206 viral aliquots were stored at -80°C and thawed immediately prior to use. Injection
207 volumes and stereotaxic coordinates for each experiment are described in the
208 corresponding sections below.

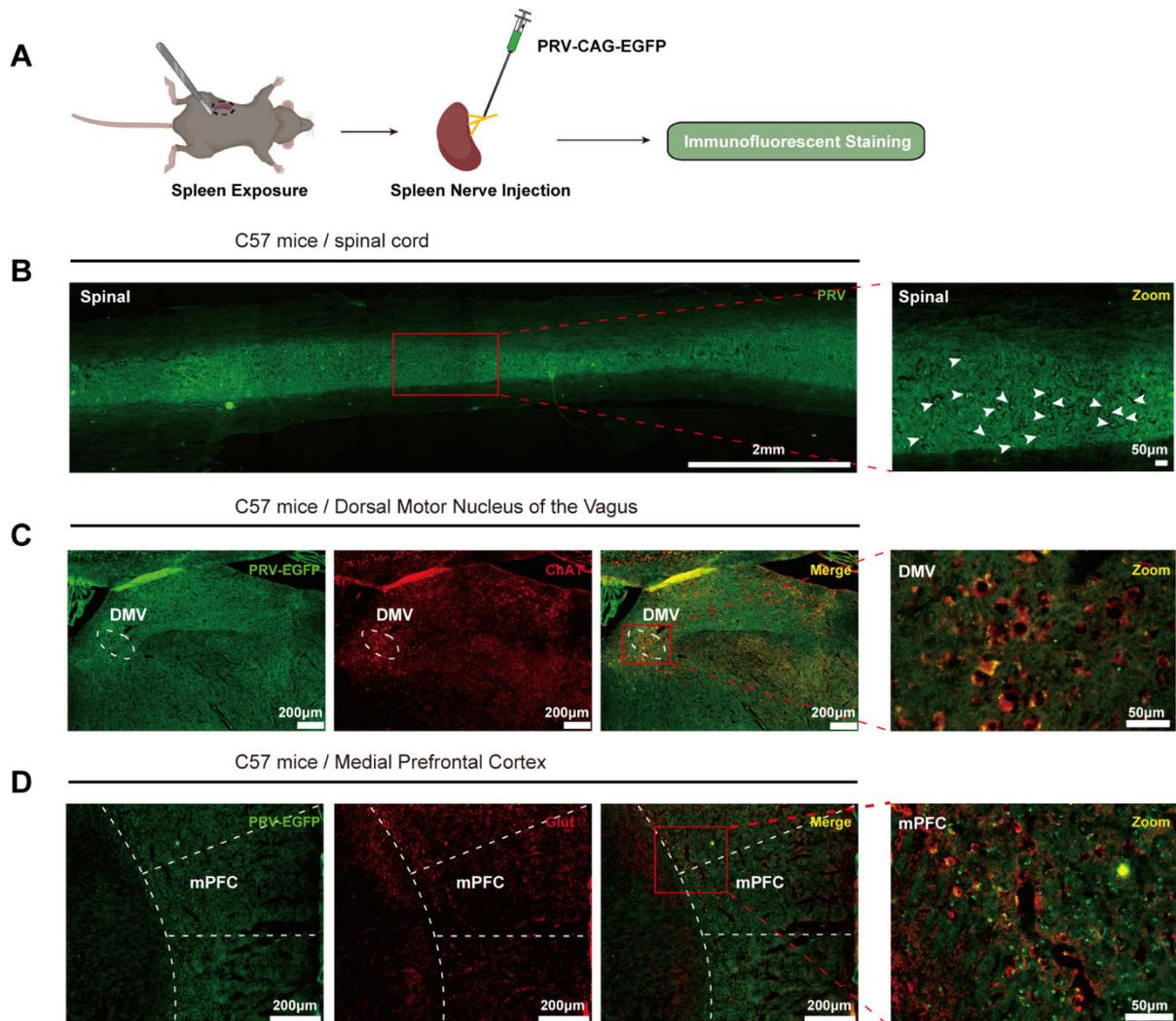
209 **c-Fos immunohistochemistry and quantification**

210 Neuronal activity-associated signaling was assessed using c-Fos
211 immunohistochemistry. Twenty-four hours after experimental initiation, mice were
212 deeply anesthetized and transcardially perfused with phosphate-buffered saline
213 followed by 4% paraformaldehyde. Brains were removed, post-fixed overnight,
214 cryoprotected in 30% sucrose, and sectioned coronally at 30 μm thickness using a
215 cryostat.

216 Free-floating sections containing the medial prefrontal cortex (mPFC) or dorsal motor
217 nucleus of the vagus (DMV) were processed for immunostaining. Sections were

218 blocked and incubated with a rabbit anti-c-Fos primary antibody (1:500; Sigma-
219 Aldrich), followed by species-appropriate fluorescent secondary antibodies. All
220 staining procedures were performed in parallel across experimental groups to
221 minimize batch effects.
222 For each animal, three anatomically matched sections per region were selected for
223 analysis. c-Fos-positive cells were quantified using ImageJ/FIJI software with
224 consistent thresholding parameters applied across all images. Cell counting was
225 performed by an experimenter blinded to experimental group assignment. c-Fos
226 expression was interpreted as a surrogate marker of neuronal activity-associated
227 signaling rather than a direct measure of neuronal firing.

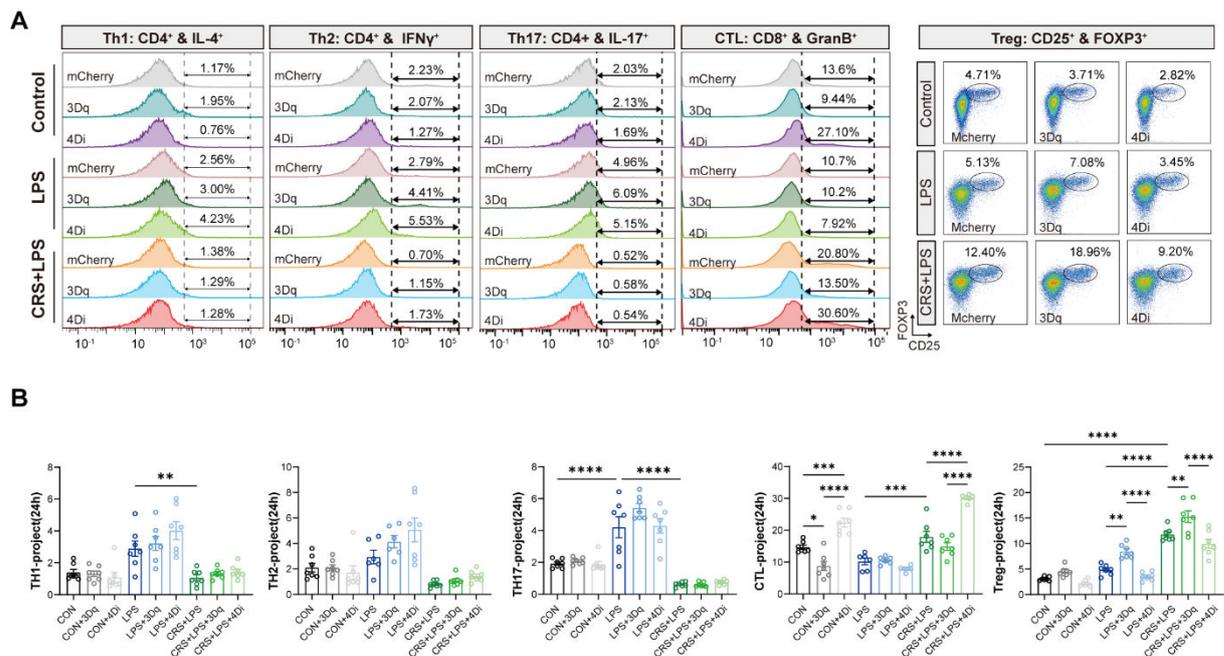
228 **Supplementary Figures:**



229

230 **Fig. S1. Central DMV cholinergic and mPFC glutamatergic neurons are linked**
 231 **to splenic regulation through autonomic pathways.**

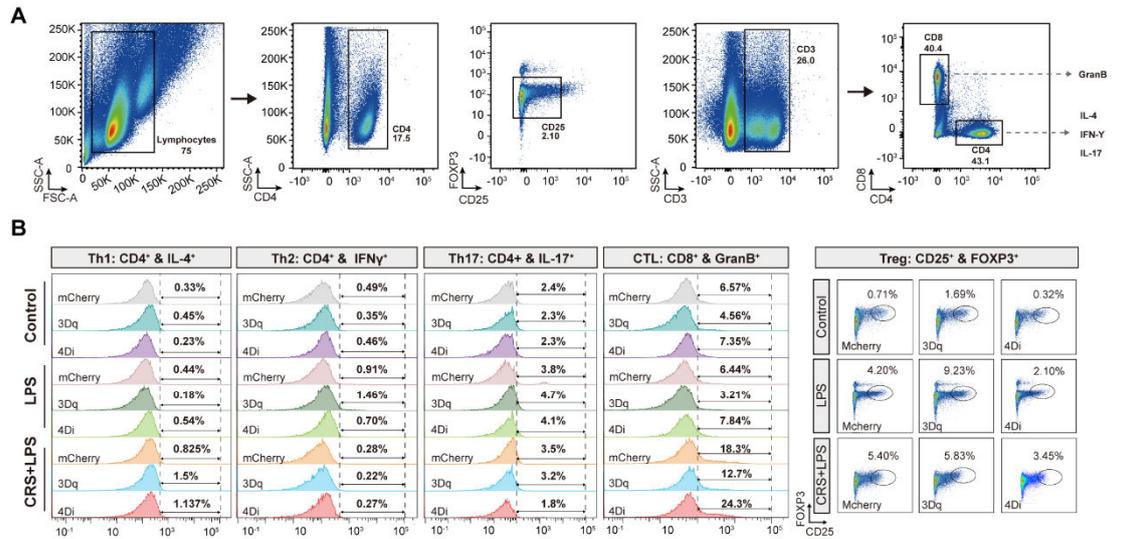
232 (A) Experimental timeline and schematic representation of this study.
 233 (B) Left: Expression of green fluorescent protein-labeled polysynaptic retrograde
 234 tracer PRV-EGFP in the spinal cord following injection into the splenic nerve; right:
 235 enlarged view of the indicated region and the white arrow indicate the infected
 236 neurons.
 237 (C) PRV-EGFP labeling in the dorsal motor nucleus of the vagus (DMV) showing
 238 colocalization with cholinergic neurons (ChAT⁺, red); right: magnified regional view
 239 of the DMV.
 240 (D) PRV-EGFP labeling in the medial prefrontal cortex (mPFC) with colocalization
 241 to Glutamatergic neurons (red); right panel shows a magnified regional view.



242 **Fig. S2. Activation of mPFC-DMV glutamatergic projections selectively rescue**
 243 **Treg deficits in stress-primed sepsis.**

244 (A) Representative flow cytometry plots showing Th1, Th2, Th17, CTLs, and Tregs
 245 in the control, LPS, and CRS + LPS groups and in each group following bidirectional
 246 chemogenetic modulation of mPFC glutamatergic projections to the DMV.
 247 (B) Quantification of splenic T-cell subset frequencies (Th1, Th2, Th17, CTLs, and
 248 Tregs) in the experimental group. Data are presented as the mean \pm SEM and were
 249

250 analyzed using one-way ANOVA with Bonferroni post hoc correction for multiple
 251 comparisons.

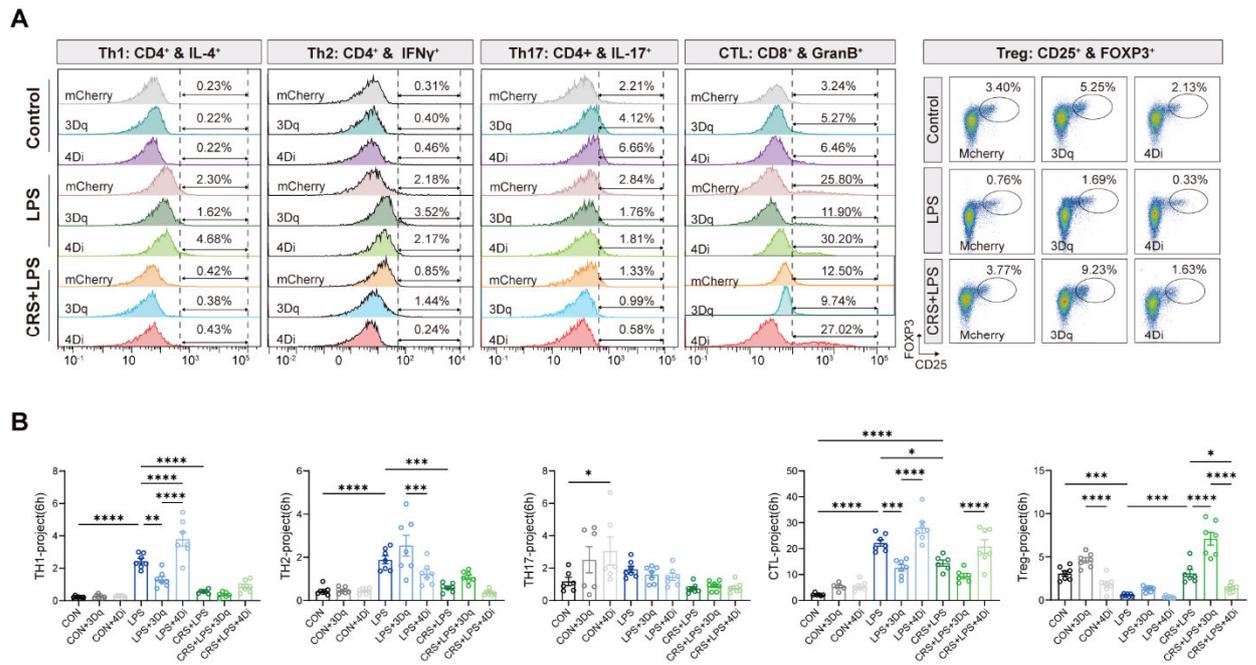


252
 253 **Fig. S3. Early activation of DMV cholinergic neurons fails to alter all splenic T-**
 254 **cell responses during the acute phase of stress-primed sepsis.**

255 (A) Schematic representation of immune cell classification and gating markers used in
 256 flow cytometry.

257 (B) Representative flow cytometry plots showing the activity of Th1 cells, Th2 cells,
 258 Th17 cells, CTLs, and Tregs in the control, LPS, and CRS + LPS groups and in each
 259 group after bidirectional chemogenetic modulation of the DMV cholinergic neurons.

260 (C) Quantification of splenic T-cell subset frequencies (Th1, Th2, Th17, CTLs, and
 261 Tregs) in the experimental groups. Data are presented as the mean \pm SEM and were
 262 analyzed using one-way ANOVA with Bonferroni post hoc correction for multiple
 263 comparisons.

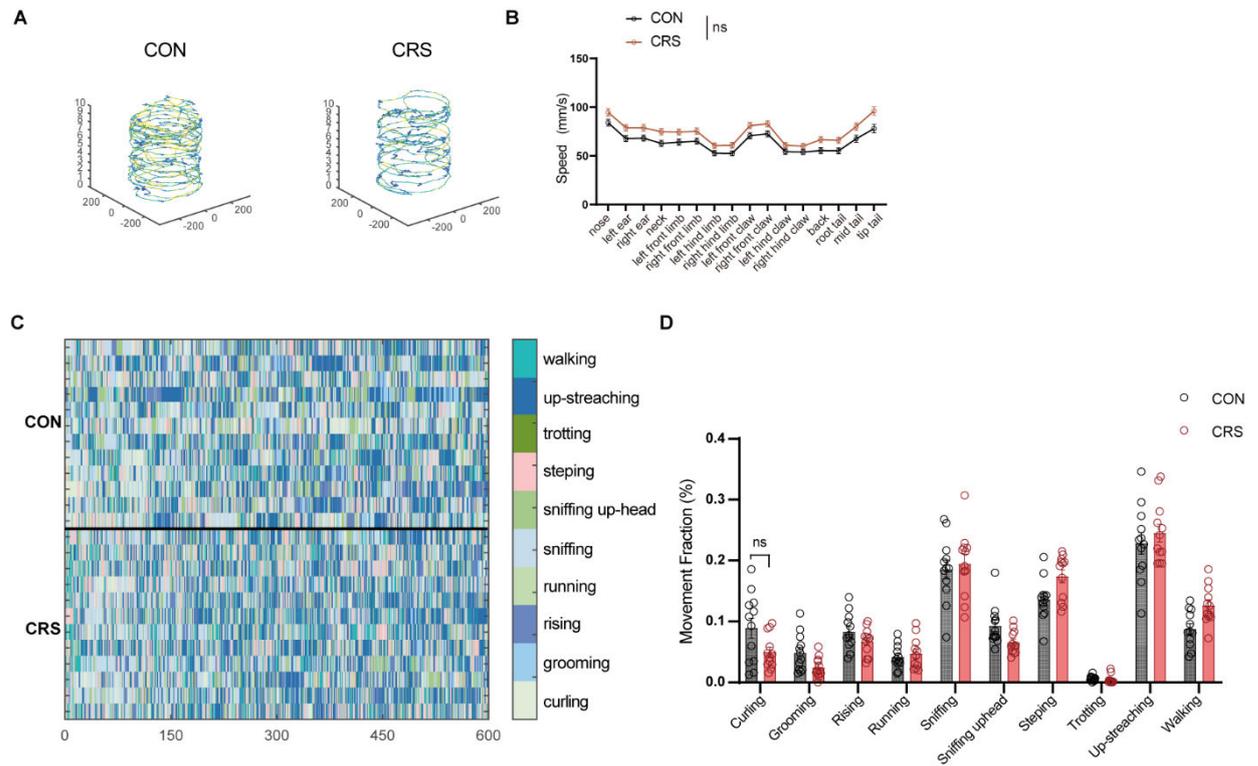


264

265 **Fig. S4. mPFC–DMV circuit modulation does not affect all splenic T-cell activity**
 266 **during the early phase of stress-primed sepsis.**

267 (A) Representative flow cytometry plots showing the activity of Th1, Th2, Th17,
 268 CTLs, and Tregs in the control, LPS, and CRS + LPS groups and in each group after
 269 bidirectional chemogenetic modulation of mPFC glutamatergic projections to the
 270 DMV.

271 (B) Quantification of splenic T-cell subset frequencies (Th1, Th2, Th17, CTLs, and
 272 Tregs) in the experimental groups. Data are presented as the mean \pm SEM and were
 273 analyzed using one-way ANOVA with Bonferroni post hoc correction for multiple
 274 comparisons.



275

276 **Fig. S5. Chronic stress alone does not alter baseline locomotor or behavioral**
 277 **states.**

278 **(A)** Representative movement trajectories of the CON and CRS groups.

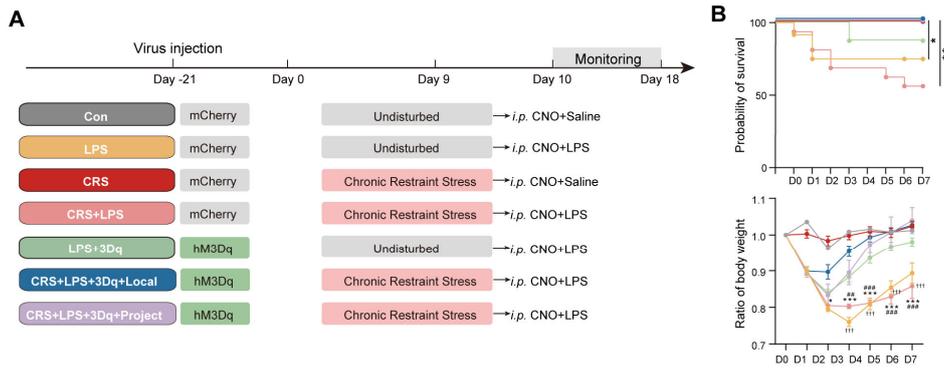
279 **(B)** Statistical comparison of body anchor points velocities across the groups.

280 **(C)** Behavioral ethogram heatmaps showing the temporal distribution of the identified
 281 modes across the recording period.

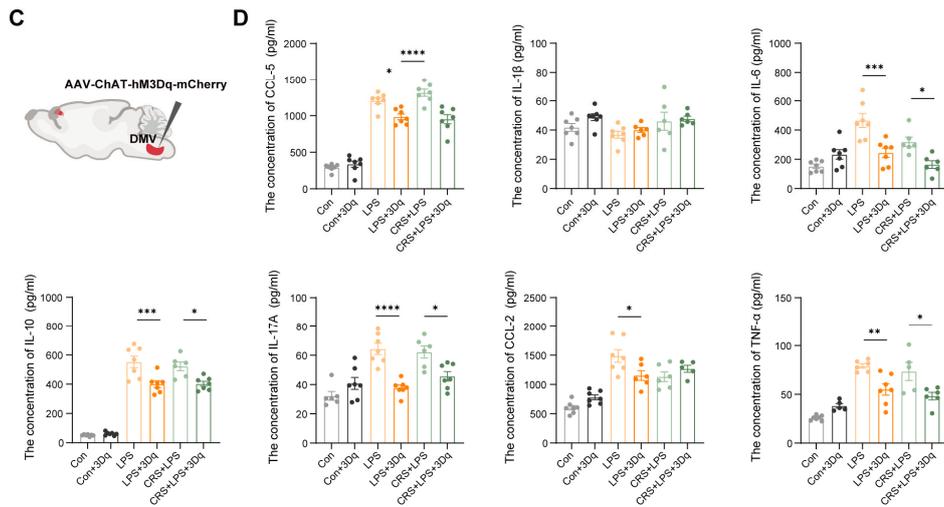
282 **(D)** Quantification of the relative proportions of the ten behavioral modes in each

283 group. Data are presented as the mean \pm SEM; * $P < 0.05$, ** $P < 0.01$ (one-way

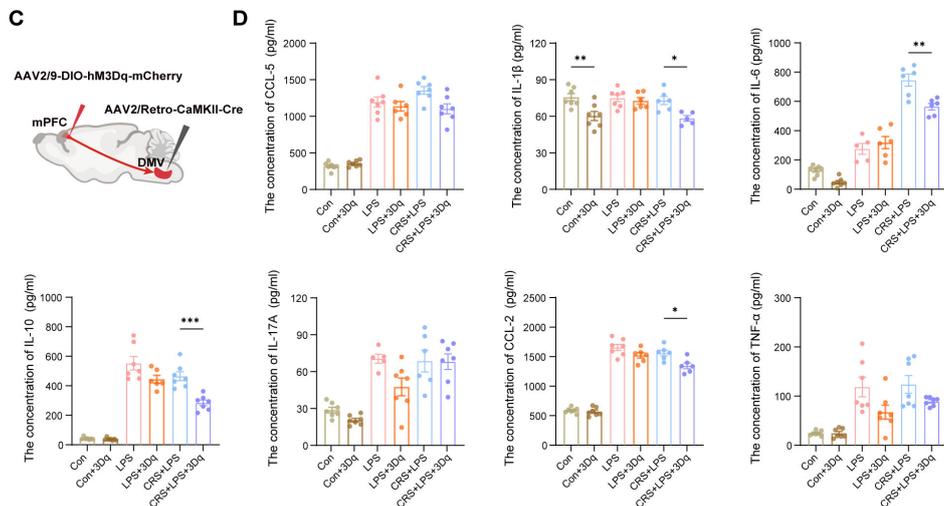
284 ANOVA with Tukey's test).



Serum Inflammatory Factors of Local intervention on 24 h



Serum Inflammatory Factors of Local intervention on 24 h



285

286 **Fig. S6. Reactivation of the mPFC–DMV pathway reduces systemic**
 287 **inflammation, improves recovery, and lowers mortality in stress-primed sepsis.**

288 (A) Experimental timeline and schematic representation of this study.

289 (B) Kaplan–Meier survival curves depicting the specific survival rates in the LPS,

290 CRS, CRS + LPS, LPS+3Dq, CRS + LPS+3Dq (projection) and CRS + LPS+3Dq

291 (local) groups (upper). And Seven-day profile of body weight changes in the CON,

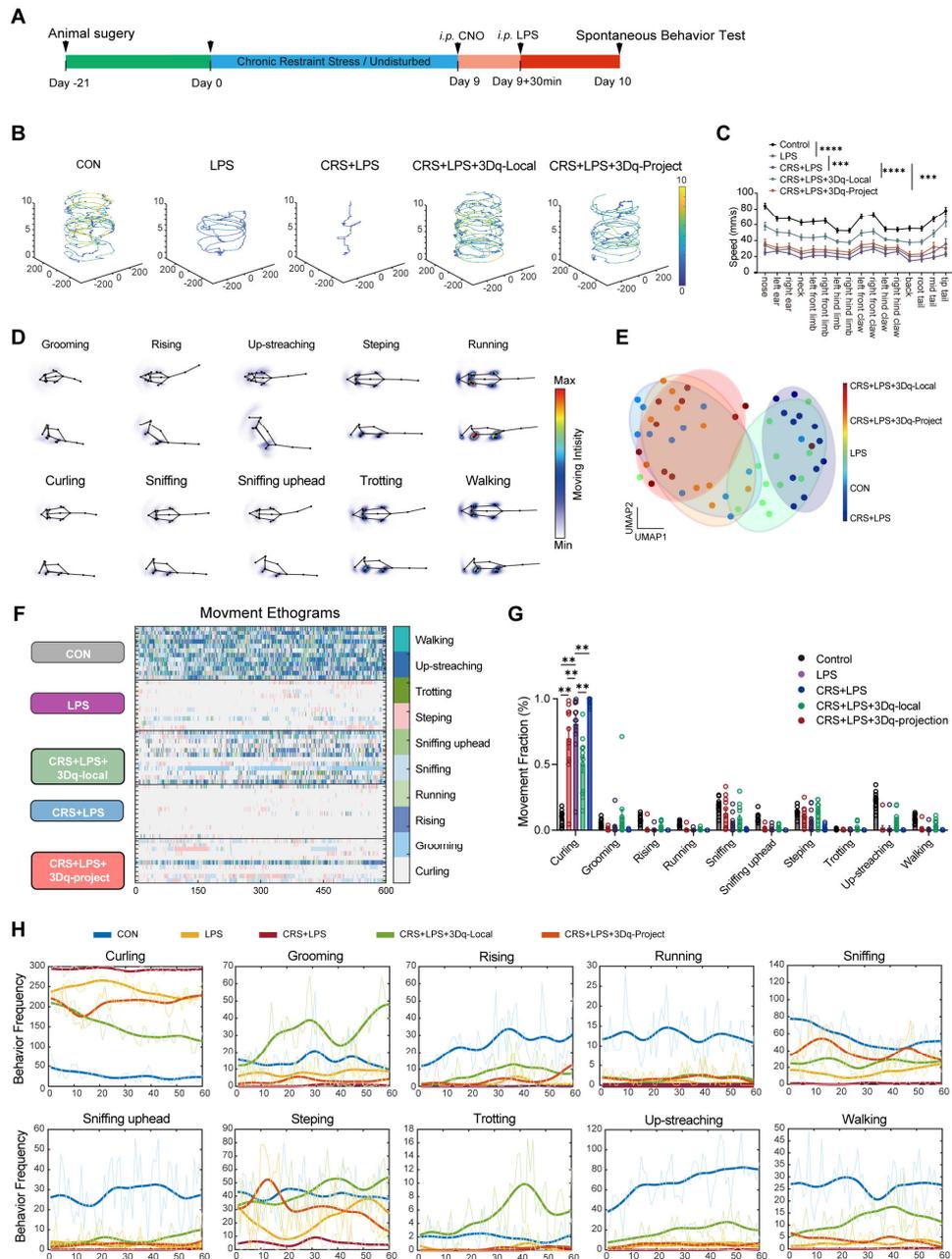
292 LPS, CRS, CRS + LPS, LPS+3Dq, CRS + LPS+3Dq (projection), and CRS +
293 LPS+3Dq (local) groups (bottom)(Two-way ANOVA: *** $p < 0.001$ for CRS + LPS
294 vs. CRS + LPS+3Dq+Local; ### $p < 0.001$ for CRS + LPS vs. CRS +
295 LPS+3Dq+Project; ††† $p < 0.001$ for LPS vs. LPS+3Dq+Local).

296 (Ci) Schematic representation of the viral injection sites and local DMV^{ChAT}
297 modulation.

298 (Di) Quantification of serum levels of the inflammatory factors IL-1 β , IL-6, IL-10,
299 IL-17A, CCL-2, CCL-5 and TNF- α following chemogenetic activation of DMV^{ChAT}
300 neurons 24 h after sepsis induction.

301 (Cii) Schematic representation of the viral injection sites and projection modulation
302 from mPFC^{glu} to DMV.

303 (Dii) Quantification of serum levels of the inflammatory factors IL-1 β , IL-6, IL-10,
304 IL-17A, CCL-2, CCL-5 and TNF- α following chemogenetic activation of mPFC^{glu}-
305 DMV circuit 24 h after sepsis induction.



306

307 **Fig. S7. mPFC-DMV circuit activation restores sepsis-altered behavioral states**
 308 **and improves functional outcomes after stress priming.**

309 (A) Experimental workflow and schematic of behavioral tracking.

310 (B) Representative movement trajectories of each group.

311 (C) Statistical comparison of body anchor point velocities across the groups.

312 (D) Nomenclature and skeletal representations of the ten identified behavioral modes.

313 (E) UMAP dimensionality reduction plots for the control, LPS, CRS + LPS, CRS +

314 LPS + DMV cholinergic neuron activation, and CRS + LPS + mPFC glutamatergic

315 projection-to-DMV activation groups.

316 **(F)** Behavioral ethogram heatmaps showing the temporal distribution of the identified
317 modes across the recording period.

318 **(G)** Quantification of the relative proportions of the ten behavioral modes in each
319 group.

320 **(H)** Fraction of five behavioral categories. Data are presented as the mean \pm SEM; * P
321 < 0.05 , ** $P < 0.01$ vs. control (two-way ANOVA with Tukey's test).

322 **Referance:**

- 323 1. Zhai Q, Lai D, Cui P, et al. Selective Activation of Basal Forebrain Cholinergic Neurons
324 Attenuates Polymicrobial Sepsis-Induced Inflammation via the Cholinergic Anti-Inflammatory
325 Pathway. *Crit Care Med* 2017; **45**(10): e1075–e82.
- 326 2. Wang Y, Zhang Y, Wang K, et al. Esketamine increases neurotransmitter releases but simplifies
327 neurotransmitter networks in mouse prefrontal cortex. *J Neurophysiol* 2022; **127**(2): 586–95.
- 328 3. Huang K, Han Y, Chen K, et al. A hierarchical 3D-motion learning framework for animal
329 spontaneous behavior mapping. *Nat Commun* 2021; **12**(1): 2784.

330