

The Carotid Body Detects Circulating Tumor Necrosis Factor-Alpha to Activate a Sympathetic Anti-Inflammatory Reflex

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39 **Abstract**

40 Recent evidence has suggested that the carotid bodies might act as immunological
41 sensors, detecting pro-inflammatory mediators and signalling to the central nervous
42 system, which, in turn, orchestrates autonomic responses. Here, we demonstrated
43 that the TNF- α receptor type I is expressed in the carotid bodies of rats. The systemic
44 administration of TNF- α increased carotid body afferent discharge and activated
45 glutamatergic neurons in the nucleus tractus solitarius (NTS) that project to the rostral
46 ventrolateral medulla (RVLM), where the majority of pre-sympathetic neurons reside.
47 The activation of these neurons was accompanied by generalized activation of the
48 sympathetic nervous system. Carotid body ablation blunted the TNF- α -induced
49 activation of RVLM-projecting NTS neurons and the increase in splanchnic
50 sympathetic nerve activity. Finally, plasma and spleen levels of cytokines after TNF- α
51 administration were higher in rats subjected to either carotid body ablation or
52 splanchnic sympathetic denervation. Collectively, our findings indicate that the carotid
53 body detects circulating TNF- α to activate a counteracting sympathetic anti-
54 inflammatory mechanism.

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56 **Keywords:** Carotid Body; Sympathetic Nervous System; Inflammation; Neuroimmune
57 Interactions; Neuroimmunomodulation; Neural Circuits

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72 **Introduction**

73 The existence of neuroimmune interactions and their relevance to the control of
74 inflammation are well-established and have been extensively explored in the last 20
75 years (Abe et al., 2017; Bassi et al., 2020; Filiano et al., 2016; Kressel et al., 2020;
76 Lankadeva et al., 2020; Martelli et al., 2014; Mughrabi et al., 2021; Murray et al., 2021;
77 Steinman, 2004; Tanaka et al., 2021) since the discovery of the “inflammatory reflex”
78 (Borovikova et al., 2000). In general, there is a consensus that this reflex works as a
79 negative-feedback mechanism that comprises: 1) a detection component, which
80 identifies pathogen- or danger-associated molecular patterns, generating an
81 inflammatory response; 2) an afferent arm, which conveys information about the
82 systemic inflammatory status to the central nervous system; 3) integrative centers in
83 the brain, that receive and process signals regarding the systemic inflammatory
84 condition, orchestrating an appropriate counteracting response and; 4) an efferent
85 arm, which are the effectors that exert immunomodulatory functions to promote
86 resolution of infection and inflammation.

87 The vagus nerve is considered an important element in neuroimmune
88 interactions (Borovikova et al., 2000; Kressel et al., 2020; Mughrabi et al., 2021). Its
89 afferent (sensory) and efferent (motor) fibers are involved in the bidirectional
90 communication between the nervous and the immune systems, providing a reflex
91 mechanism known as the “cholinergic anti-inflammatory pathway” (Borovikova et al.,
92 2000; Mughrabi et al., 2021). According this mechanism, vagal sensory neurons detect
93 inflammatory mediators produced in conditions of systemic inflammation and send this
94 information to the central nervous system (Watkins et al., 1995), which, in turn,
95 generates a vagal efferent output that counteracts inflammation mainly through
96 acetylcholine-induced inhibition of cytokine production (Borovikova et al., 2000). The
97 importance of this cholinergic anti-inflammatory mechanism is beyond doubt since its
98 dysfunction is involved in the pathophysiology of several conditions (Bassi et al., 2017;
99 Chang et al., 2019; Kanashiro et al., 2017; Li et al., 2011; van Maanen et al., 2009).
100 However, several studies have shown convincing evidence for the existence of other
101 neural mechanisms that regulate inflammation. For instance, animal and human
102 studies have demonstrated that the efferent sympathetic nervous system can
103 modulate inflammatory conditions through catecholamine-mediated suppression of
104 innate immune responses (Abe et al., 2017; Kox et al., 2014; Lankadeva et al., 2020;

105 Martelli et al., 2014; Tanaka et al., 2021; van Westerloo et al., 2011). Moreover, some
106 studies demonstrated that the sympathetic-mediated anti-inflammatory reflexes do not
107 depend on vagal afferent signalling, suggesting the existence of other peripheral
108 mechanisms able to detect inflammation and communicate with the central nervous
109 system to activate downstream sympathetic anti-inflammatory pathways (Abe et al.,
110 2017; Martelli et al., 2014).

111 In this regard, the carotid body, classically known as the main peripheral
112 monitor of the O₂ levels in the blood, has been considered a polymodal sensor due to
113 its particular ability to detect diverse molecules present in the circulation, such as
114 glucose, sodium chloride, hormones, and also, inflammatory mediators (Allen, 1998;
115 da Silva et al., 2019; Jendzjowsky et al., 2018; Katayama, 2016; Kumar and
116 Prabhakar, 2012; Thompson et al., 2016). In the context of inflammation, several
117 pieces of evidence indicate that the carotid bodies might be involved in the intricate
118 interplay between the immune system and the sympathetic nervous system. First, the
119 carotid body expresses receptors for inflammatory mediators such as
120 lysophosphatidic acid (LPA) and pro-inflammatory cytokines such as IL-1 β , IL-6, and
121 tumor necrosis factor-alpha (TNF- α) (Fernández et al., 2008; Jendzjowsky et al., 2018;
122 Kumar and Prabhakar, 2012; Mkrтчian et al., 2012; Wang et al., 2002). Second, LPA
123 and pro-inflammatory cytokines stimulate the carotid body and increase the carotid
124 sinus nerve (CSN) afferent activity in isolated *in vitro* preparations (Jendzjowsky et al.,
125 2021, 2018). Third, carotid body stimulation by its typical stimulus (hypoxia) activates
126 central autonomic areas that control parasympathetic (Erickson and Millhorn, 1994;
127 Zera et al., 2019) and, also, the sympathetic nervous system (Kline et al., 2010;
128 Koshiya and Guyenet, 1996; Luise King et al., 2012) which, besides vagally-mediated
129 mechanisms, represents an important component in the neural regulation of immunity
130 (Abe et al., 2017; Lankadeva et al., 2020; Martelli et al., 2014). Last, carotid body
131 denervation worsens systemic inflammation and accelerates multiple organ
132 dysfunction and death in rats with lipopolysaccharide (LPS)-induced sepsis (Nardocci
133 et al., 2015), suggesting that the carotid body is a protective factor during acute
134 inflammatory conditions. Altogether, these observations led to the hypothesis that the
135 carotid body plays a role in neuroimmune interactions, but the exact mechanisms
136 underlying this cross-talk are largely unknown.

137 In this study, we focused on investigating the impact of TNF- α (a ubiquitous
138 cytokine that triggers inflammation)(Grieve et al., 2017) on the carotid body-mediated

139 activation of the sympathetic nervous system, as well as the relevance of this
140 interaction in the modulation of TNF- α -induced systemic inflammation. We revealed
141 that the carotid body expresses the TNF- α receptor type I (TNFR1) and detects
142 increased levels of TNF- α in peripheral circulation, transmitting this information to the
143 brain via CSN afferent inputs to commissural nucleus tractus solitarius (cNTS)
144 glutamatergic neurons that project to rostral ventrolateral medulla (RVLM) pre-
145 sympathetic neurons, resulting in activation of the sympathetic nervous system to
146 counteract the TNF- α -induced inflammation. We, therefore, propose the existence of
147 a physiological carotid body-mediated neuroimmune reflex that acutely controls
148 inflammation. The identification of this neuroimmune reflex provides potential
149 mechanistic insights into the pathophysiology of inflammation-mediated diseases as
150 well as into the development of novel therapeutic strategies to treat these conditions.

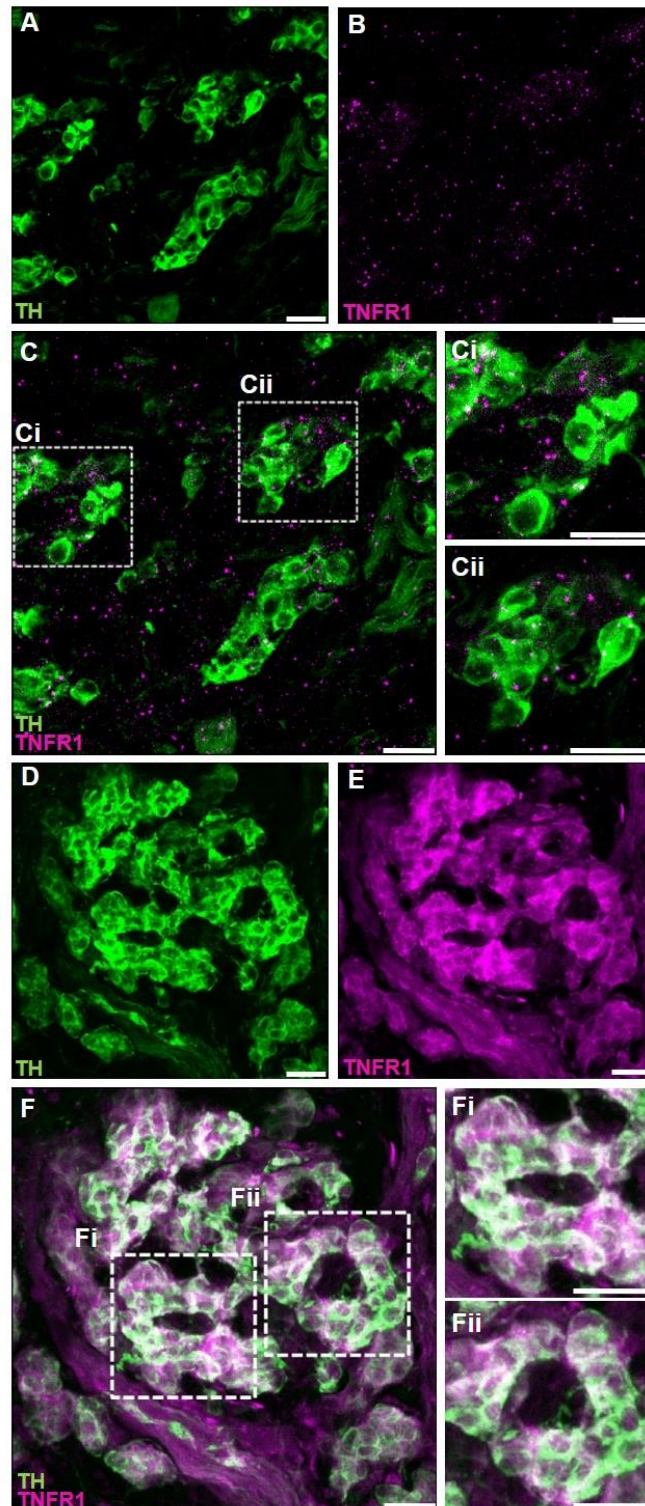
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172 **Results**

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174 **TNFR1 is expressed in the carotid body**

175 The expression of TNF- α receptors type I (TNFR1) in the carotid body was verified
176 using two different methods: 1) RNAscope *in situ* hybridization for labelling TNFR1
177 mRNA molecules combined with immunofluorescence staining for tyrosine
178 hydroxylase (TH) to identify carotid body glomus cells (Fig. 1A – C) and; 2) double
179 immunofluorescence staining for TNFR1 and TH (Fig. 1D – F). We found that the
180 TNFR1 is expressed in the carotid body of rats at both mRNA and protein levels (Fig.
181 1).



182

183 **Figure 1.** TNF- α receptors type I (TNFR1) are expressed in the carotid body of rats. **A – C.** Combined
184 fluorescent *in situ* hybridization (TNFR1, magenta puncta) and immunofluorescence (Tyrosine
185 hydroxylase, TH, green staining). **A.** TH positive cells (glomus cells) in the carotid body. **B.** RNAscope
186 *in situ* hybridization showing TNFR1 mRNA expression in the carotid body. **C.** Overlay of images A and
187 B showing the colocalization of TH and TNFR1. **Ci** and **Cii.** Zoom into selected regions of image C. **D**
188 – **F.** Double immunofluorescence staining (TNFR1, magenta staining; and TH, green staining). **D.** TH
189 positive cells (glomus cells) in the carotid body. **E.** TNFR1 expression in the carotid body. **F.** Overlay of
190 images D and E showing the colocalization of TH and TNFR1. **Fi** and **Fii.** Zoom into selected regions
191 of image F. Scale bars: 20 μ m.

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193 **Circulating TNF- α increases carotid sinus nerve afferent activity**

194 Next, we investigated if elevated TNF- α levels in the blood could activate its receptors
195 in the carotid body and increase CSN activity in vivo. We found that exogenous TNF-
196 α administration increased CSN activity by $34 \pm 5\%$ at 30 minutes after administration
197 compared to baseline (Figure 2B). This TNF- α -induced excitation of CSN was
198 sustained and lasted the whole experiment ($46 \pm 7\%$, $55 \pm 8\%$, $60 \pm 10\%$ at 60, 90,
199 and 120 minutes after TNF- α administration, respectively; Figure 2B). It is important
200 to highlight that throughout the experimental protocol, the animals were artificially
201 ventilated with a slight hyperoxia (50% O₂, balance N₂) avoiding any potential hypoxia
202 episode. We also performed additional experiments which demonstrated that the
203 intravenous TNF- α did not affect the partial pressure of oxygen (PaO₂), the partial
204 pressure of carbon dioxide (PaCO₂), the pH, and the bicarbonate (HCO₃⁻)
205 concentration in the arterial blood of unanesthetized, spontaneously breathing rats,
206 confirming that the treatment does not produce hypoxia, hypercapnia or acidosis.
207 (figure supplement 1). Thus, our data indicate that TNF- α can stimulate the carotid
208 body and increase CSN activity independently of changes in blood gases and pH
209 alterations. We, therefore, hypothesized that this TNF- α -induced increase in CSN
210 afferent activity could activate central pathways similar to those activated by hypoxic
211 stimuli, generating autonomic responses such as the activation of the sympathetic
212 nervous system.

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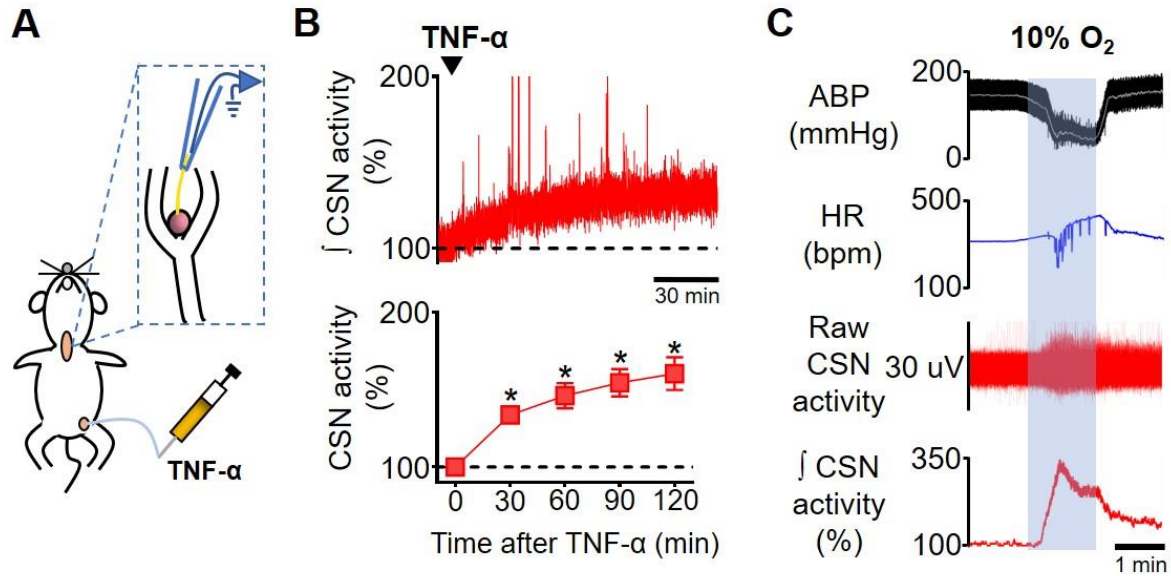
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221 **Figure 2.** Carotid sinus nerve afferent activity (CSN activity) in response to intravenous TNF- α . **A.**
222 Schematic illustration of the experimental protocol. **B.** Representative trace of the integrated CSN
223 activity (\int CSN activity; time constant = 1 s) from one rat during baseline and after TNF- α (500 ng, IV,
224 black arrowhead) administration (top; scale bar = 30 minutes) and summary data showing CSN activity
225 at baseline and 30, 60, 90 and 120 minutes after TNF- α administration (bottom; n = 6). Baseline CSN
226 activity was normalized to 100% after noise subtraction. A one-way repeated measures ANOVA
227 detected statistically significant differences in CSN activity over time, $F_{(4, 20)} = 21,282$, $p < 0.001$.
228 Subsequent post hoc analysis with a Bonferroni adjustment revealed that, as compared to time 0
229 (baseline), CSN activity was statistically significantly higher at 30 minutes (34%, 95% CI [9, 59], $p =$
230 0.014); at 60 minutes (46%, 95% CI [8, 85], $p = 0.023$); at 90 minutes (55%, 95% CI [13, 96], $p = 0.016$);
231 and at 120 minutes (60%, 95% CI [10, 111], $p = 0.023$) after TNF- α administration. * $p < 0.05$. Data are
232 means \pm SEM. **C.** Representative traces showing the viability of CSN activity recordings assessed by
233 a brief exposure to hypoxia (10% O₂, balance N₂; grey shaded area). The typical acute response of
234 urethane-anesthetized rats to hypoxia includes hypotension, bradycardia, and a robust increase in CSN
235 activity. ABP, arterial blood pressure; HR, heart rate.

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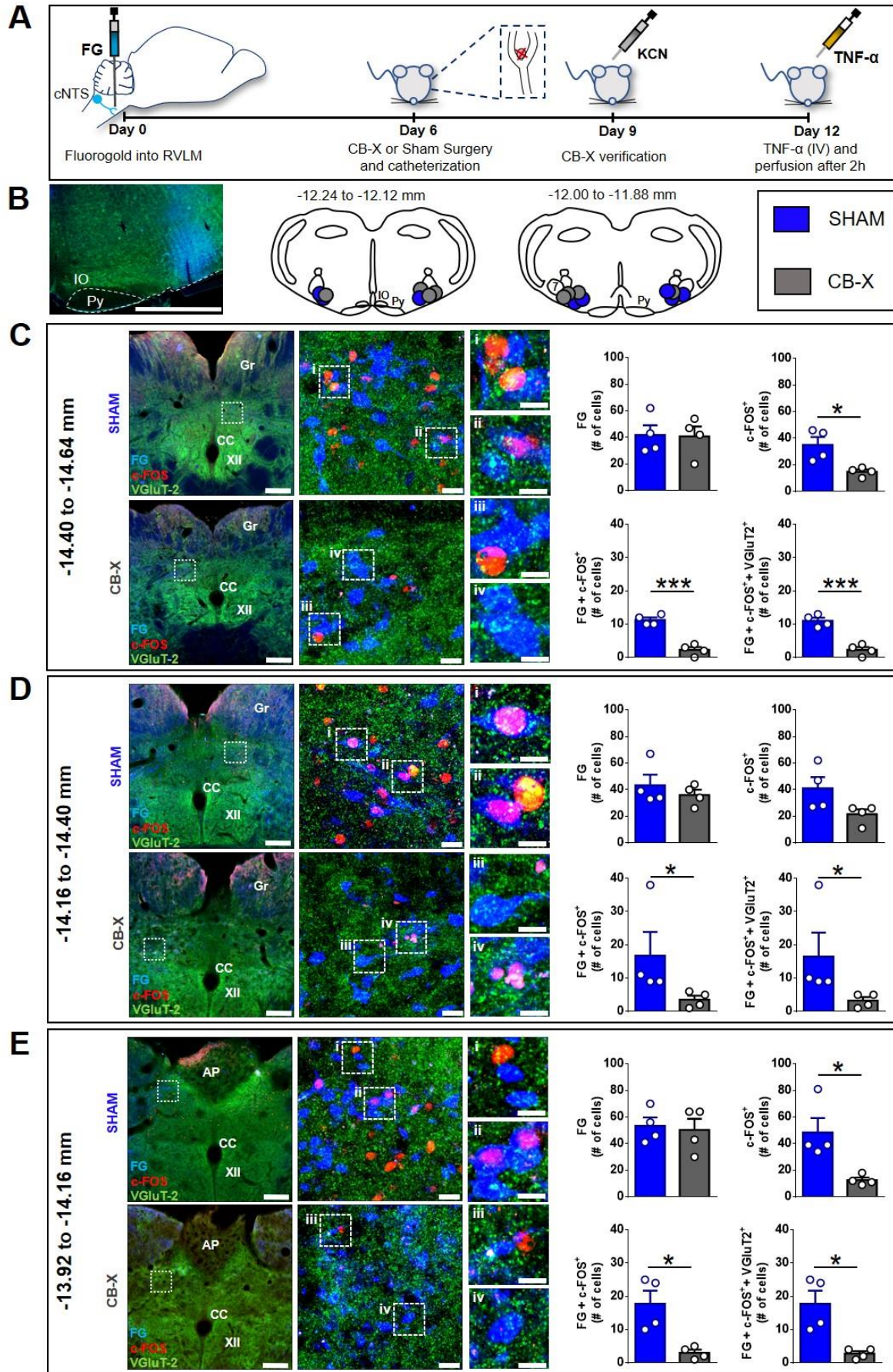
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250 **RVLM-projecting cNTS glutamatergic neurons are activated by TNF- α**

251 The first synapse of carotid body afferents within the central nervous system occurs in
252 the cNTS, as extensively described in the literature (Colombari et al., 1996; Cruz et
253 al., 2010; Kline et al., 2010; Malheiros-Lima et al., 2020). The cNTS sends excitatory
254 glutamatergic projections to several areas, being implicated in diverse physiological
255 functions. In the context of the carotid body-related functions, the cNTS neurons
256 project to important autonomic areas involved in the neural control of cardiovascular
257 and respiratory functions (Kline et al., 2010; Zera et al., 2019). For example, a previous
258 report demonstrated direct monosynaptic projections from cNTS to RVLM, where the
259 majority of pre-sympathetic neurons are located (Kline et al., 2010). It was also shown
260 that most of these RVLM-projecting cNTS neurons are activated by hypoxia and
261 constitute the major neural pathway of hypoxia-induced sympathetic activation (Kline
262 et al., 2010; Koshiya and Guyenet, 1996). Thus, we sought to investigate if this
263 sympathoexcitatory pathway is activated by circulating TNF- α since this cytokine
264 increased the discharge of carotid body afferents, as shown in Figure 2B. Our results
265 demonstrated massive monosynaptic projections from cNTS to RVLM (FG-labeled
266 cells, blue staining, Figure 3C – E) in both SHAM and CB-X rats at all evaluated rostro-
267 caudal levels: -14.40 mm to -14.64 mm (SHAM, 42 ± 7 cells; CB-X, 41 ± 7 cells), -
268 14.16 mm to -14.40 mm (SHAM, 43 ± 8 cells; CB-X, 36 ± 4 cells), and -13.92 mm to -
269 14.16 mm. (SHAM, 53 ± 6 cells; CB-X, 50 ± 8 cells). Most of these projections are
270 excitatory (VGLuT2⁺ cells, green staining, Figure 3C – E). Circulating TNF- α activated
271 a considerable proportion of these RVLM-projecting glutamatergic cNTS neurons in
272 SHAM rats, as indicated by c-FOS expression (red staining) in FG⁺/VGLuT2⁺ cells
273 (Figure 3C – E); Importantly, the number of activated RVLM-projecting glutamatergic
274 cNTS neurons was dramatically reduced by carotid body ablation: -14.40 mm to -14.64
275 mm (SHAM, 11 ± 1 cells; CB-X, 2 ± 1 cells), -14.16 mm to -14.40 mm (SHAM, 17 ± 7
276 cells; CB-X, 3 ± 1 cells), and -13.92 mm to -14.16 mm. (SHAM, 18 ± 4 cells; CB-X, 3
277 ± 1 cells). The efficacy of the bilateral carotid body ablation procedure was confirmed
278 by the lack of cardiovascular responses to KCN (figure supplement 2A – B). Together
279 with our previous findings (Figures 1 and 2), these results suggest that the carotid
280 body detects the circulating TNF- α through TNFR1 and transmits this information to
281 the central nervous system via carotid sinus nerve afferents, resulting in the activation
282 of a sympathoexcitatory pathway.

283



286 **Figure 3.** Activation of RVLM-projecting cNTS glutamatergic neurons by circulating TNF- α in SHAM
287 and CB-X rats. **A.** Schematic illustration of the experimental protocol. **B.** Representative image from a
288 typical retrograde tracer (Fluorogold; FG) injection-site into RVLM and schematic pictures of RVLM
289 injections-sites of all bilaterally FG-injected animals (n=4 per group). IO, inferior olive; Py, pyramidal
290 tract; 7, facial motor nucleus. Scale bar is 1000 μ m. **C, D** and **E.** Images are representative pictures of
291 cNTS sections at three different rostro-caudal levels, processed for c-FOS (red) and VGluT2 (green)
292 immunofluorescence, and containing FG-positive cells retrogradely labeled from the RVLM (blue). Gr,
293 gracile nucleus; CC, central canal; XII, hypoglossal nucleus; AP, area postrema. Scale bars are 200
294 μ m for 5x magnification pictures (left), 20 μ m for 40x magnification pictures (middle) and 10 μ m for
295 zoom pictures (right). **i, ii, iii,** and **iv.** Digital zoom into selected regions. Bar graphs show the
296 quantification of retrogradely labeled FG neurons, c-FOS⁺ neurons, double stained (FG/c-FOS⁺)
297 neurons and triple stained (FG/c-FOS⁺/VGluT2⁺) neurons in the cNTS 2 hours after TNF- α
298 administration (500 ng, IV) in SHAM (n=4) and CB-X (n=4) rats. The number of RVLM-projecting
299 neurons (FG-labeled cells) was not different between SHAM and CB-X rats in all evaluated cNTS levels:
300 -14.40 to -14.64 mm (C), $t(6) = 0.096$, $p = 0.926$ (Student's t -test); -14.16 to -14.40 mm (D), $U = 7.5$, z
301 $= -0.145$, $p = 0.886$ (Mann-Whitney U -test); and -13.92 to -14.16 mm (E), $t(6) = 0.285$, $p = 0.785$
302 (Student's t -test). General neuronal activation (i.e., both RVLM-projecting and RVLM- non-projecting;
303 c-FOS⁺ cells) was higher in SHAM as compared to CB-X rats at -14.40 to -14.64 mm (C), $t(3.505) =$
304 3.326 , $p = 0.036$ (Welch's t -test) and at -13.92 to -14.16 mm (E), $U = 0$, $z = -2.323$, $p = 0.029$ (Mann-
305 Whitney U -test); but not at -14.16 to -14.40 mm (D), $t(6) = 2.141$, $p = 0.076$ (Student's t -test). The
306 specific activation of RVLM-projecting neurons (c-FOS⁺/FG⁺ cells) was higher in SHAM as compared to
307 CB-X rats in all 3 cNTS levels: -14.40 to -14.64 mm (C), $t(6) = 7.919$, $p < 0.001$ (Student's t -test); -14.16
308 to -14.40 mm (D), $U = 0$, $z = -2.323$, $p = 0.029$ (Mann-Whitney U -test); and -13.92 to -14.16 mm (E),
309 $t(3.324) = 3.661$, $p = 0.030$ (Welch's t -test). Virtually all activated RVLM-projecting cNTS neurons are
310 glutamatergic (FOS⁺/FG/VGluT2⁺ cells). The number of activated RVLM-projecting cNTS glutamatergic
311 neurons was higher in SHAM as compared to CB-X rats in all 3 cNTS levels: -14.40 to -14.64 mm (C),
312 $t(6) = 7.000$, $p < 0.001$ (Student's t -test); -14.16 to -14.40 mm (D), $U = 0$, $z = -2.337$, $p = 0.029$ (Mann-
313 Whitney U -test); and -13.92 to -14.16 mm (E), $t(3.219) = 3.755$, $p = 0.029$ (Welch's t -test). * $p < 0.05$
314 and *** $p < 0.001$. Data are means \pm SEM.

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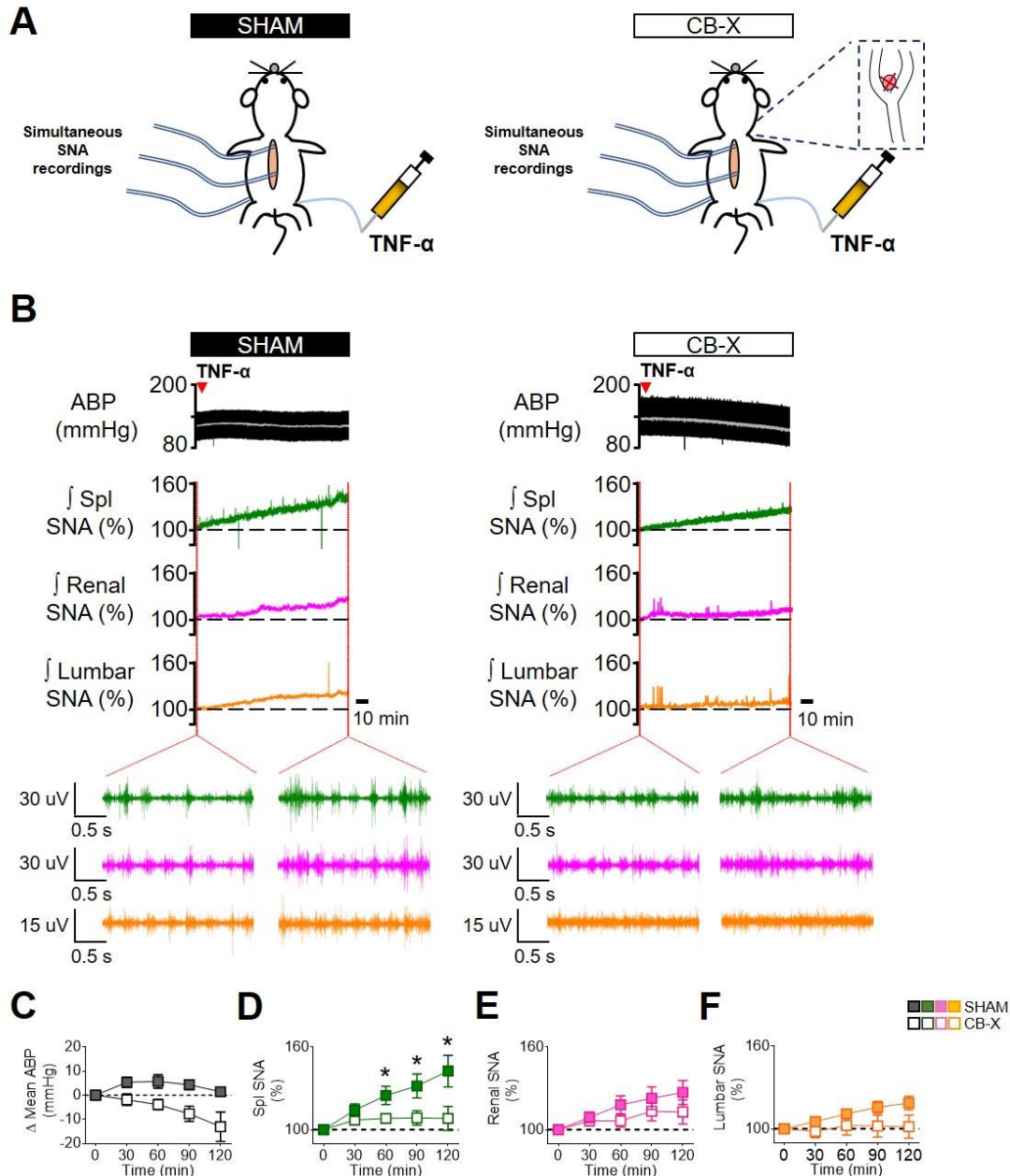
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332 **TNF- α promotes a carotid-body mediated increase in splanchnic SNA**

333 Because circulating TNF- α activated a well-known sympathoexcitatory central
334 pathway, we next performed experiments to investigate the effect of this cytokine on
335 sympathetic activity directly recorded from multiple sympathetic nerves in vivo. Our
336 results showed that intravenously administered TNF- α promotes a generalized
337 sympathoexcitation in SHAM rats (Figure 4B – F), consistent with the activation of the
338 RVLN-projecting cNTS glutamatergic neurons demonstrated in Figure 3: Δ Splanchnic
339 SNA ($14 \pm 4\%$, $25 \pm 7\%$, $32 \pm 9\%$ and $42 \pm 11\%$ respectively at 30, 60, 90, and 120
340 minutes after TNF- α administration); Δ Renal SNA ($9 \pm 4\%$, $18 \pm 6\%$, $22 \pm 8\%$, $27 \pm$
341 9% respectively at 30, 60, 90, and 120 minutes after TNF- α administration) and Δ
342 lumbar SNA ($5 \pm 1\%$, $11 \pm 3\%$, $16 \pm 4\%$, $19 \pm 5\%$ respectively at 30, 60, 90, and 120
343 minutes after TNF- α administration). Interestingly, despite the generalized
344 sympathetic activation, mean arterial blood pressure (ABP) only slightly increased
345 (Figure 4B – C).

346 Since carotid body ablation almost abolished the TNF- α -induced activation of
347 RLVM-projecting cNTS glutamatergic neurons (Figure 3), we tested whether the
348 carotid bodies would be necessary to the observed sympathoexcitation in response to
349 TNF- α administration. To accomplish that, we administered TNF- α to rats subjected to
350 bilateral carotid body ablation (Figure 4B – F). CB-X rats displayed an attenuated
351 increase in SNA in response to TNF- α : Δ Splanchnic SNA ($7 \pm 1\%$, $8 \pm 2\%$, $8 \pm 5\%$
352 and $8 \pm 9\%$ respectively at 30, 60, 90, and 120 minutes after TNF- α administration), Δ
353 renal SNA ($6 \pm 3\%$, $6 \pm 5\%$, $13 \pm 7\%$ and $13 \pm 9\%$ respectively at 30, 60, 90, and 120
354 minutes after TNF- α administration), and Δ lumbar SNA ($-2 \pm 5\%$, $2 \pm 7\%$, $2 \pm 8\%$ and
355 $1 \pm 8\%$ respectively at 30, 60, 90, and 120 minutes after TNF- α administration). These
356 SNA responses were diminished compared to those displayed by SHAM rats,
357 especially on splanchnic SNA at 60, 90, and 120 minutes after TNF- α administration,
358 suggesting that the carotid bodies contribute to this specific response (Figure 4D).
359 Unlike SHAM rats, mean ABP in CB-X rats tended to decrease even without
360 reductions in the activity of any of the recorded sympathetic nerves (Figure 4B – C).
361 At the end of the experiments, bilateral carotid body ablation was confirmed by the
362 lack of sympathetic and blood pressure responses to KCN (figure supplement 3A –
363 B).

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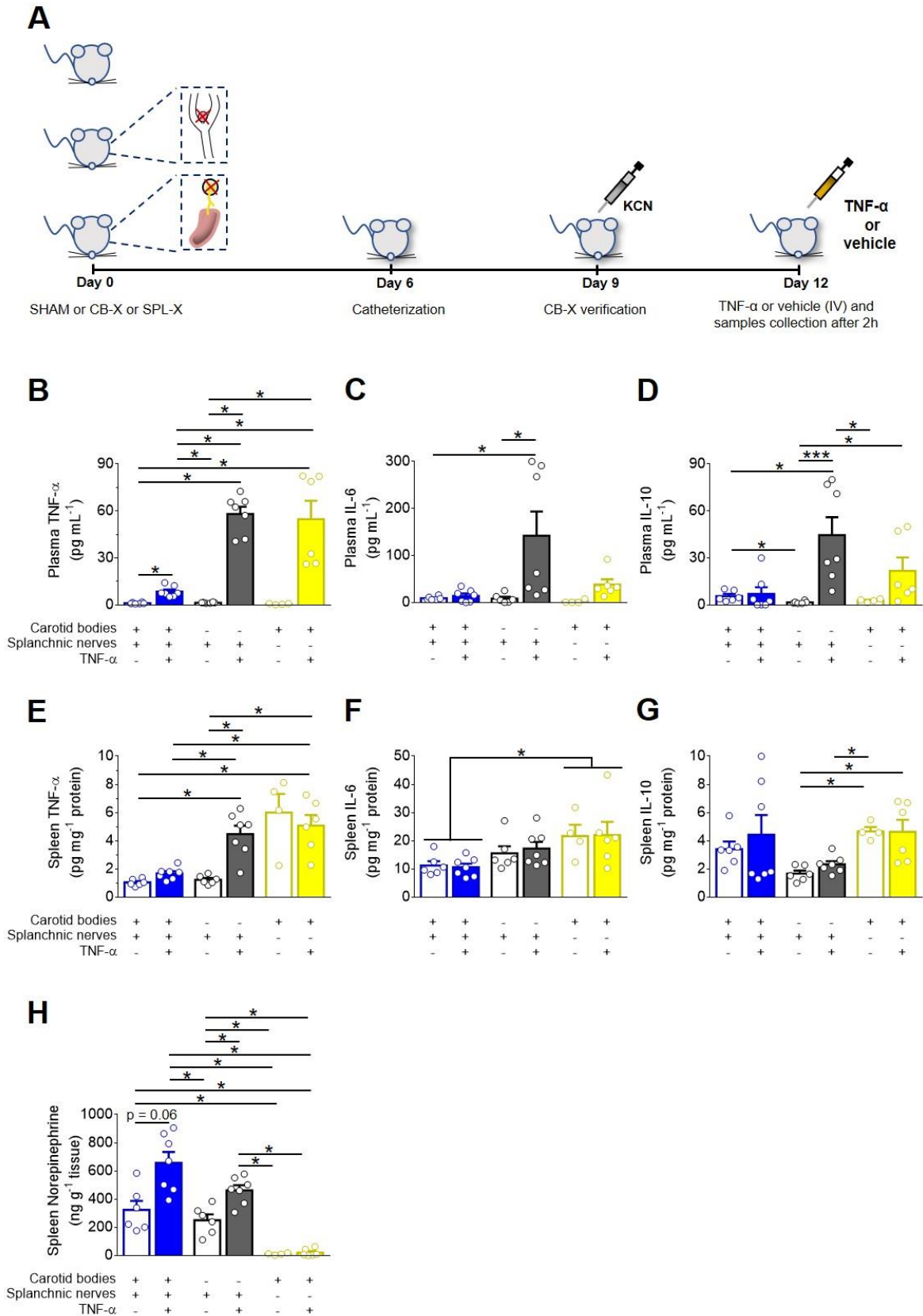
366 **Figure 4.** Carotid body ablation attenuates the TNF- α -induced splanchnic sympathetic activation. **A.**
 367 Schematic illustration of the experimental protocol. **B.** Representative traces of arterial blood pressure
 368 (pulsatile ABP, black; mean ABP, white), splanchnic (Spl; green), renal (magenta) and lumbar (orange)
 369 integrated (\int ; time constant = 1s) sympathetic nerve activity (SNA) in sham-operated rats (SHAM) and
 370 carotid body-ablated rats (CB-X) during baseline conditions and in the next 2 hours after TNF- α
 371 administration (500 ng, IV, red arrowhead). For each sympathetic nerve, raw SNA signals at baseline
 372 and 2 hours after TNF- α administration are also presented (as indicated by the red dotted lines). **C, D,**
 373 **E and F.** Summary data showing the changes in mean ABP (**C**), Spl SNA (**D**), Renal SNA (**E**)
 374 and Lumbar SNA (**F**) in response to TNF- α in SHAM (filled symbols, n = 6) and CB-X (open symbols, n = 6)
 375 rats. For each rat, baseline integrated SNA was normalized to 100%, and the relative changes were
 376 calculated at four different time points (30, 60, 90 and 120 minutes after TNF- α administration). A
 377 statistically significant group x time interaction on spl SNA was detected by two-way repeated-measures
 378 ANOVA, $F_{(3,15)} = 11.119$, $p < 0.001$. Subsequent simple main effects analyses revealed that spl SNA
 379 changes were significantly greater in SHAM as compared to CB-X rats at 60 minutes, $F_{(1,5)} = 7.042$, p
 380 $= 0.045$; at 90 minutes, $F_{(1,5)} = 10.224$, $p = 0.024$; and at 120 minutes, $F_{(1,5)} = 16.515$, $p = 0.010$ after
 381 TNF- α administration. * $p < 0.05$. There were no statistically significant group x time interactions on
 382 Mean ABP, $F_{(1,113, 5.567)} = 0.807$, $p = 0.420$, $\epsilon = 0.371$; Renal SNA, $F_{(3,15)} = 0.805$, $p = 0.510$; and Lumbar
 383 SNA, $F_{(3,15)} = 1.685$, $p = 0.213$ (two-way repeated measures ANOVA). Data are means \pm SEM.

384 **Carotid body ablation or splanchnic sympathetic denervation exacerbates TNF-**
385 **α -induced inflammation**

386 Considering that the exogenous TNF- α activated a carotid body-cNTS-RVLM circuitry
387 to excite a specific sympathetic nerve (splanchnic), and because the splanchnic
388 sympathetic nerves have been considered essential components of sympathetic-
389 mediated mechanisms to control inflammation (Lankadeva et al., 2020; Martelli et al.,
390 2014), we next investigated if the activation of this newly described circuit could play
391 an anti-inflammatory role in the TNF- α -induced inflammation. We found that, in SHAM
392 rats that received TNF- α , the plasma levels of this cytokine (8.5 ± 1.3 pg mL⁻¹) were
393 found significantly higher in comparison to SHAM rats that received vehicle (1.1 ± 0.2
394 pg mL⁻¹) (Figure 5B). It is important to mention that, the half-life of TNF- α is very short
395 (few minutes) (Ma et al., 2015; Simó et al., 2012), and, hence, it is very likely that the
396 measured levels of this cytokine in the plasma (2 hours after TNF- α or vehicle
397 administrations) reflect endogenously produced TNF- α . In rats subjected to either
398 carotid body ablation (CB-X) or splanchnic sympathetic denervation (SPL-X), the
399 administration of TNF- α resulted in significant higher plasma levels of this cytokine
400 compared to SHAM rats injected with TNF- α (CB-X + TNF- α = 58.1 ± 4.7 pg mL⁻¹,
401 SPL-X + TNF- α = 54.8 ± 11.8 pg mL⁻¹) (Figure 5B), suggesting that the absence of the
402 carotid bodies or the splanchnic sympathetic nerves exacerbated the systemic
403 inflammatory status triggered by the exogenous TNF- α . In the same direction, the
404 levels of TNF- α in the spleen were found higher in CB-X + TNF- α (4.5 ± 0.6 pg mg⁻¹)
405 and in SPL-X + TNF- α (5.1 ± 0.8 pg mg⁻¹) groups compared to SHAM + TNF- α ($1.7 \pm$
406 0.2 pg mg⁻¹) group (Figure 5E). These results support the idea that the exogenously
407 administered TNF- α induced the endogenous production of additional TNF- α likely via
408 stimulation of splenic macrophages and, that, the removal of the carotid bodies (a
409 potential sensor of TNF- α) or of the splanchnic sympathetic nerves (a potential
410 suppressor of spleen-derived TNF- α production), significantly increased TNF- α levels
411 in the spleen. It is important to highlight that in SPL-X + vehicle animals, the levels of
412 TNF- α in the spleen were also elevated (6.0 ± 1.3 pg mg⁻¹) (Figure 5E), reinforcing the
413 notion that the splanchnic sympathetic innervation of the spleen (via celiac ganglion),
414 exerts a kind of inhibitory tonus over splenic production of TNF- α . By way of
415 comparison, in rats with intact splanchnic nerves (SHAM and CB-X) injected with
416 vehicle, the levels of TNF- α in the spleen were low: (SHAM + vehicle = 1.0 ± 0.1 pg
417 mg⁻¹, CB-X + vehicle = 1.2 ± 0.1 pg mg⁻¹) (Figure 5E).

418 Regarding plasma IL-6 levels, CB-X + TNF- α animals displayed higher levels
419 (142.2 \pm 51.0 pg mL⁻¹) than SHAM + vehicle (9.1 \pm 1.6 pg mL⁻¹) and CB-X + vehicle
420 (8.2 \pm 3.8 pg mL⁻¹) (Figure 5C). Although not statistically significant, the levels of IL-6
421 in the plasma tended to be higher in CB-X + TNF- α and SPL-X + TNF- α (38.1 \pm 11.2
422 pg mL⁻¹) compared to all other groups: (SHAM + vehicle = 9.1 \pm 1.6 pg mL⁻¹, SHAM +
423 TNF- α = 14.1 \pm 5.0 pg mL⁻¹, CB-X + vehicle = 8.2 \pm 3.8 pg mL⁻¹, SPL-X + vehicle =
424 2.2 \pm 1.9 pg mL⁻¹) (Figure 5C). Concerning the spleen levels of IL-6, no interactions
425 between group x treatment were detected by two-way ANOVA. However, a statistically
426 main effect of group indicated that the spleen levels of IL-6 were higher in SPL-X +
427 vehicle (21.7 \pm 4.0 pg mg⁻¹) and SPL-X + TNF- α (22.0 \pm 4.7 pg mg⁻¹) groups compared
428 to SHAM + vehicle (11.2 \pm 1.5 pg mg⁻¹) and SHAM + TNF- α (10.6 \pm 1.3 pg mg⁻¹)
429 groups (Figure 5F), suggesting that splanchnic sympathetic denervation was
430 permissive to IL-6 production in the spleen, even in the absence of the TNF- α stimulus.
431 With regard to plasma IL-10, the levels of this anti-inflammatory cytokine tended to be
432 higher in CB-X + TNF- α (44.7 \pm 11.4 pg mL⁻¹) and SPL-X + TNF- α (21.7 \pm 8.6 pg mL⁻¹)
433 ¹) as compared to SHAM + TNF- α (7.1 \pm 4.2 pg mL⁻¹) and to every other group that
434 received vehicle (SHAM + vehicle = 5.6 \pm 1.4 pg mL⁻¹, CB-X + vehicle = 1.7 \pm 0.3 pg
435 mL⁻¹, SPL-X + vehicle = 2.8 \pm 0.5 pg mL⁻¹) (Figure 5D). These results match with the
436 increased levels of TNF- α in the plasma and the spleen of CB-X and SPL-X rats that
437 received TNF- α , indicating a worse systemic inflammatory status in these animals.
438 Finally, spleen levels of IL-10 tended to be lower in both CB-X groups, but was only
439 statistically different between: CB-X + vehicle (1.7 \pm 0.2 pg mg⁻¹) compared to SPL-X
440 + vehicle (4.7 \pm 0.3 pg mg⁻¹); CB-X + vehicle group compared to SPL-X + TNF- α (4.6
441 \pm 0.9 pg mg⁻¹); and between CB-X + TNF- α (2.3 \pm 0.2 pg mg⁻¹) compared to SPL-X +
442 vehicle (Figure 5G). In regard to norepinephrine levels in the spleen, SHAM rats
443 injected with TNF- α displayed the highest mean levels (656.2 \pm 77.6 pg mg⁻¹), followed
444 by CB-X + TNF- α (462.3 \pm 36.4 pg mg⁻¹), SHAM + vehicle (322.9 \pm 64.4 pg mg⁻¹), CB-
445 X+ vehicle (249.9 \pm 40.9 pg mg⁻¹), SPL-X + TNF- α (20.6 \pm 10.7 pg mg⁻¹), and SPL-X
446 + vehicle (10.3 \pm 3.5 pg mg⁻¹) groups (Figure 5H). Note that splanchnic sympathetic
447 denervation almost depleted the norepinephrine content in the spleen, confirming the
448 efficacy of the denervation procedure. In addition, the efficacy of splanchnic
449 sympathetic denervation was also verified by the less pronounced TH staining in the
450 spleen (figure supplement 4C). The efficacy of the bilateral carotid body ablation
451 procedure was confirmed by the lack of cardiovascular responses to KCN (figure

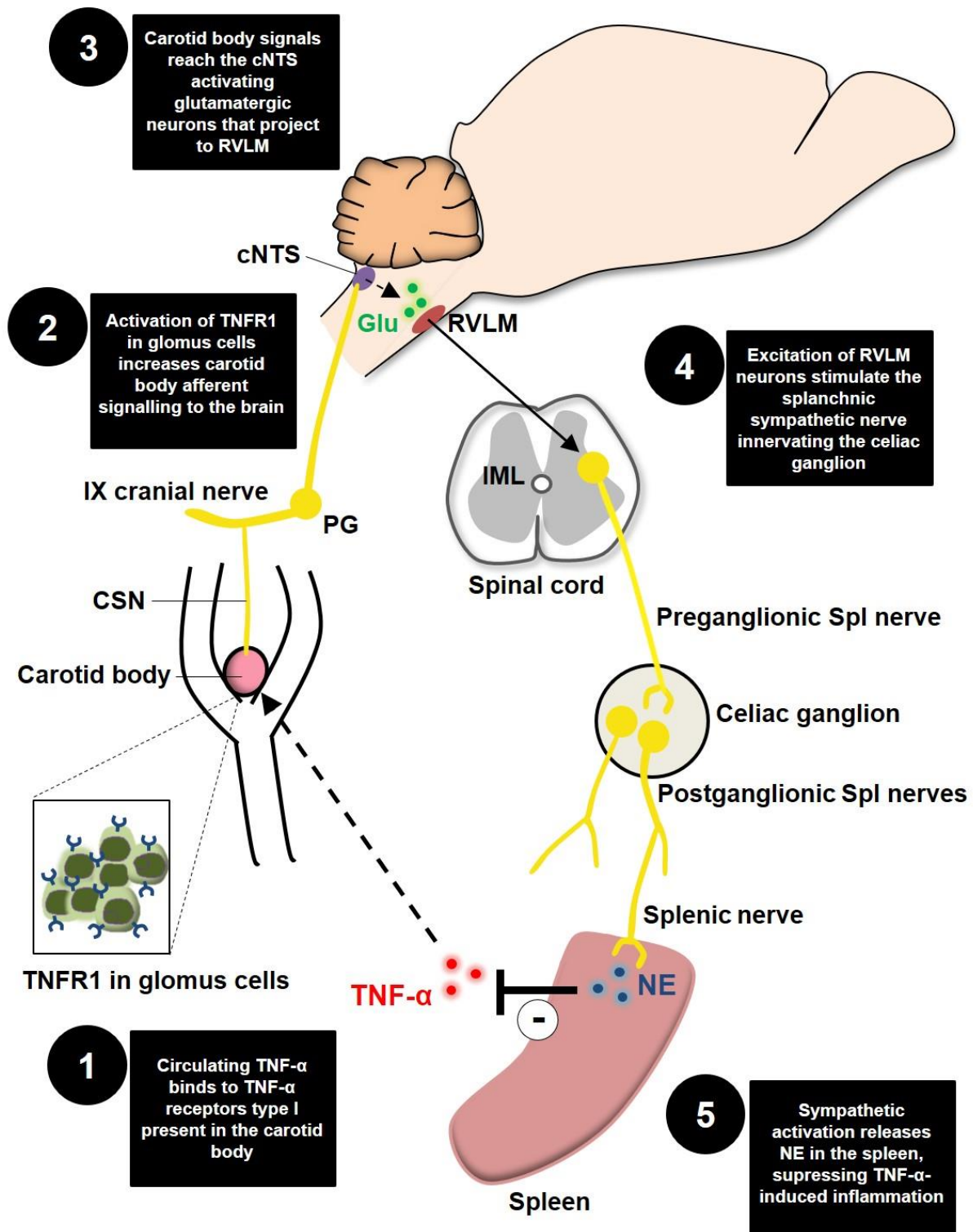
452 supplement 4A – B). Collectively, our data suggest that elevated circulating levels of
453 TNF- α activates a neural mechanism (carotid body-cNTS-RVLM-splanchnic
454 sympathetic nerves) that controls the ongoing inflammation by inhibiting the synthesis
455 of additional TNF- α in the spleen likely via direct norepinephrine-mediated
456 suppression of splenic macrophage TNF- α production.



457

458 **Figure 5.** Carotid body ablation (CB-X) or splanchnic sympathetic denervation (SPL-X) intensify the
 459 TNF- α -induced inflammation. **A.** Schematic illustration of the experimental protocol. **B, C and D.**
 460 Plasma levels of TNF- α , IL-6 and IL-10 in SHAM (blue bars), CB-X (gray bars), and SPL-X (yellow bars)
 461 rats, measured 2 hours after vehicle (empty bars) or TNF- α (filled bars) intravenous administration (n =
 462 4 - 7 per group). **B.** Statistically significant differences in the plasma levels of TNF- α across groups were

463 detected: $H(5) = 31.454$, $p < 0.001$ (Kruskal-Wallis). In SHAM + TNF- α , the plasma levels of this cytokine
464 were found significantly higher in comparison to SHAM + vehicle ($U = 0$, $z = -3.000$, $p = 0.001$, Mann-
465 Whitney U -test). In CB-X and SPL-X rats, TNF- α administration resulted in significant higher plasma
466 levels of this cytokine as compared to SHAM + TNF- α : SHAM + TNF- α vs. CB-X + TNF- α ($U = 0$, $z = -$
467 3.130 , $p = 0.001$, Mann-Whitney U -test); SHAM + TNF- α vs. SPL-X + TNF- α ($U = 0$, $z = -3.000$, $p =$
468 0.001 , Mann-Whitney U -test). Between vehicle-treated groups, the plasma levels of TNF- α were not
469 different ($p > 0.003$). Regarding plasma IL-6 levels, significant differences between groups were
470 detected: $H(5) = 22.024$, $p = 0.001$ (Kruskal-Wallis). **C.** The plasma levels of IL-6 were higher in CB-X
471 + TNF- α as compared to SHAM + vehicle ($U = 1$, $z = -2.857$, $p = 0.002$, Mann-Whitney U -test) and to
472 CB-X + vehicle ($U = 1$, $z = -2.857$, $p = 0.002$, Mann-Whitney U -test). No statistical differences were
473 found in plasma levels of IL-6 in the other pairwise comparisons ($p > 0.003$). **D.** Finally, the plasma
474 levels of IL-10 were found significantly different across groups: $F_{(5, 13.522)} = 14.524$, $p < 0.001$ (Welch
475 ANOVA). Games-Howell post hoc test revealed that the plasma levels of IL-10 were significantly higher
476 in CB-X + TNF- α as compared to all groups that received vehicle: CB-X + TNF- α vs. SHAM + vehicle
477 (Mean difference = 2.0 pg mL^{-1} , 95% CI [0.6, 3.3], $p = 0.005$); CB-X + TNF- α vs. CB-X + vehicle (Mean
478 difference = 3.1 pg mL^{-1} , 95% CI [1.8, 4.4], $p < 0.001$); CB-X + TNF- α vs. SPL-X + vehicle (Mean
479 difference = 2.6 pg mL^{-1} , 95% CI [1.2, 3.9], $p = 0.001$). In SPL-X + TNF- α , the plasma levels of IL-10
480 were higher as compared to CB-X + vehicle (Mean difference = 2.2 pg mL^{-1} , 95% CI [0.2, 4.1], $p =$
481 0.032). Between vehicle-administered groups, SHAM rats displayed higher plasma levels of IL10 as
482 compared to CB-X rats (Mean difference = 1.2 pg mL^{-1} , 95% CI [0.1, 2.2], $p = 0.033$). **E, F and G.** Spleen
483 levels of TNF- α , IL-6 and IL-10 in SHAM (blue bars), CB-X (gray bars), and SPL-X (yellow bars) rats, 2
484 hours after vehicle (empty bars) or TNF- α (filled bars) intravenous administration ($n = 4 - 7$ per group).
485 **E.** Statistically significant differences in the spleen levels of TNF- α between groups were found: $F_{(5,$
486 $12.262)} = 12.957$, $p < 0.001$ (Welch ANOVA). Games-Howell post hoc test revealed that the spleen levels
487 of TNF- α were significantly higher in CB-X and SPL-X that received TNF- α as compared to SHAM rats
488 that received TNF- α : CB-X + TNF- α vs. SHAM + TNF- α (Mean difference = 2.8 pg mg^{-1} protein, 95%
489 CI [0.5, 5.1], $p = 0.021$); SPL-X + TNF- α vs. SHAM + TNF- α (Mean difference = 3.4 pg mg^{-1} protein,
490 95% CI [0.2, 6.6], $p = 0.039$). Within vehicle-treated groups, the spleen levels of TNF- α were not different
491 ($p > 0.05$). No statistical differences were found when comparing SHAM + vehicle vs. SHAM + TNF- α
492 (Mean difference = -0.6 pg mg^{-1} protein, 95% CI [-1.3, 0.0], $p = 0.064$). **F.** Regarding the spleen levels
493 of IL-6, no interactions between group x treatment were detected: $F_{(2,30)} = 0.092$, $p = 0.912$, partial $\eta^2 =$
494 0.006 . However, a statistically significant main effect of group was found: $F_{(2,30)} = 7.130$, $p = 0.003$,
495 partial $\eta^2 = 0.322$. A Bonferroni post hoc analysis indicated that the spleen levels of IL-6 were significant
496 higher in SPL-X groups as compared to SHAM groups: (Mean difference = $10.9 \text{ pg mg protein}$, 95% CI
497 [3.5, 18.3], $p = 0.002$). **G.** Concerning the spleen levels of IL-10, statistically significant differences
498 between groups were found: $F_{(5, 13.792)} = 12.491$, $p < 0.001$ (Welch ANOVA). Games-Howell post hoc
499 test revealed that the spleen levels of IL-10 were significantly lower in CB-X groups as compared to
500 SPL-X groups: CB-X + vehicle vs. SPL-X + vehicle (Mean difference = -1.1 pg mg^{-1} protein, 95% CI [-
501 $1.6, -0.5$], $p = 0.002$); CB-X + vehicle vs. SPL-X + TNF- α (Mean difference = -1.0 pg mg^{-1} protein, 95%
502 CI [-1.8, -0.1], $p = 0.026$); CB-X + TNF- α vs. SPL-X + vehicle (Mean difference = -0.7 pg mg^{-1} protein,
503 95% CI [-1.1, -0.3], $p = 0.002$). **H.** Spleen levels of norepinephrine in SHAM (blue bars), CB-X (gray
504 bars), and SPL-X (yellow bars) rats, 2 hours after vehicle (empty bars) or TNF- α (filled bars) intravenous
505 administration ($n = 4 - 7$ per group). Statistically significant differences in the spleen levels of
506 norepinephrine between groups were found: $F_{(5, 13.050)} = 45.864$, $p < 0.001$ (Welch ANOVA). * $p < 0.05$
507 and *** $p < 0.001$. Data are means \pm SEM. Games-Howell post hoc test revealed that the administration
508 of TNF- α in SHAM rats, resulted in a trend to increase the spleen norepinephrine levels compared to
509 SHAM animals receiving vehicle (Mean difference = 333.3 pg mg^{-1} tissue, 95% CI [-11.2, 677.8], $p =$
510 0.060) and in significant increases as compared to CB-X + vehicle (Mean difference = 406.3 pg mg^{-1}
511 tissue, 95% CI [94.4, 718.2], $p = 0.011$), to SPL-X + vehicle (Mean difference = 645.9 pg mg^{-1} tissue,
512 95% CI [337.1, 954.7], $p = 0.001$), and to SPL-X + TNF- α (Mean difference = 635.5 pg mg^{-1} tissue, 95%
513 CI [327.6, 943.5], $p = 0.001$). In CB-X rats, TNF- α administration led to higher levels of norepinephrine
514 in the spleen as compared to CB-X + vehicle (Mean difference = 212.4 pg mg^{-1} tissue, 95% CI [24.4,
515 400.4], $p = 0.025$), to SPL-X + vehicle (Mean difference = 452.0 pg mg^{-1} tissue, 95% CI [307.5, 596.6],
516 $p < 0.001$), and to SPL-X + TNF- α (Mean difference = 441.7 pg mg^{-1} tissue, 95% CI [298.1, 585.3], $p <$
517 0.001). SPL-X + vehicle animals also displayed lower levels of norepinephrine in the spleen compared
518 to SHAM + vehicle (Mean difference = $-312.6 \text{ pg mg}^{-1}$ tissue, 95% CI [-587.0, -38.1], $p = 0.030$) and
519 CB-X + vehicle (Mean difference = $-239.6 \text{ pg mg}^{-1}$ tissue, 95% CI [-413.5, -65.7], $p = 0.013$). Similarly,
520 the levels of norepinephrine in the spleen were also lower in SPL-X + TNF- α compared to SHAM +
521 vehicle (Mean difference = $-302.2 \text{ pg mg}^{-1}$ tissue, 95% CI [-574.7, -29.8], $p = 0.033$) and CB-X + vehicle
522 (Mean difference = $-229.3 \text{ pg mg}^{-1}$ tissue, 95% CI [-400.7, -57.9], $p = 0.014$).



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Figure 6. Schematic model of the novel proposed neuroimmune mechanism. TNFR1, TNF- α receptors type I; CSN, carotid sinus nerve; IX cranial nerve, glossopharyngeal nerve; PG, petrosal ganglion; cNTS, commissural nucleus tractus solitarius; Glu, glutamate; RLVM, rostral ventrolateral medulla; IML, intermediolateral nucleus; Spl, splanchnic; NE, norepinephrine.

532 **Discussion**

533 In the present study, we provide a series of anatomical and functional evidence for the
534 existence of a previously unrecognized mechanism of neuroimmune interaction. The
535 main finding is that the carotid body is able to detect elevated levels of the pro-
536 inflammatory cytokine TNF- α in the blood and communicate with the central nervous
537 system via carotid sinus nerve afferents, activating RVLM-projecting cNTS excitatory
538 neurons that contribute to a counteracting sympathetic-mediated anti-inflammatory
539 response. These results advance our understanding of the complex mechanisms
540 underlying the bidirectional connection between the nervous and the immune systems.

541 Recently, the carotid bodies emerged as potential candidates for peripheral
542 detectors of inflammation. This possibility is supported by a growing number of studies
543 indicating that they are polymodal sensors, able to monitor the chemical composition
544 of the arterial blood. More specifically, these studies have shown that besides
545 promoting autonomic and respiratory adjustments in response to arterial hypoxemia
546 (i.e., peripheral chemoreflex), the carotid bodies can respond to several other
547 circulating stimuli such as leptin, angiotensin II, glucose, sodium chloride, insulin,
548 adrenaline, and, also, inflammatory mediators (Allen, 1998; da Silva et al., 2019;
549 Jendzjowsky et al., 2021, 2018; Katayama, 2016; Kumar and Prabhakar, 2012; Shin
550 et al., 2019; Thompson et al., 2016). Regarding inflammatory mediators, studies
551 reported that the carotid body of many species, including rats, cats and humans,
552 expresses receptors for lysophosphatidic acid (LPA), IL-1 β , IL-6, and TNF- α
553 (Fernández et al., 2008; Jendzjowsky et al., 2018; Mkrtchian et al., 2012; Wang et al.,
554 2002). Accordingly, in the present study, we combined immunofluorescence and
555 RNAscope FISH protocols to confirm that TNFR1 is expressed in the carotid body of
556 rats at both mRNA and protein levels. Moreover, in addition to the anatomical
557 evidence, previous functional studies demonstrated that inflammation-related factors
558 can impact carotid body activity (Jendzjowsky et al., 2021, 2018; Shu et al., 2007),
559 opening a wide range of possibilities regarding the role of the carotid body in the
560 context of neuroimmune interactions. For instance, a recent study showed that LPA
561 potently increased CSN activity in an isolated perfused carotid body/carotid sinus
562 nerve preparation (Jendzjowsky et al., 2018). Furthermore, the same research group
563 showed that the perfusion of the isolated carotid body/carotid sinus nerve preparation
564 with diverse pro-inflammatory cytokines (IL-4, IL-5, IL-13, IL-1, IL-6, and TNF- α), one

565 at a time or in combination, also increased CSN activity (Jendzjowsky et al., 2021),
566 confirming the unique ability of the carotid body to sense and respond to inflammatory
567 mediators. In our study, CSN activity was recorded in vivo and TNF- α was given
568 systemically (IV). We chose the IV administration route because it better mimics a real
569 scenario of systemic inflammation. We observed a progressive and significant
570 increase in CSN activity, indicating that the carotid body could detect the elevated
571 levels of TNF- α in the blood and alert the central nervous system via afferent signals.
572 The reasons by which TNF- α increased CSN activity in a sustained manner (for at
573 least 2 hours) are not clear, especially because the half-life of TNF- α in the plasma is
574 reported to be very short (few minutes) (Ma et al., 2015; Simó et al., 2012). We
575 hypothesize that the exogenous administered TNF- α stimulated the synthesis and
576 release of additional TNF- α , probably via direct activation of splenic macrophages as
577 suggested by our data (Figure 5) and/or by indirect activation of liver Kupffer cells as
578 observed during endotoxemia in rats (Fonseca et al., 2021). This endogenously
579 produced TNF- α could either sustain the carotid body activation and, also, stimulate
580 the synthesis of further TNF- α .

581 We found that besides increasing CSN activity, the intravenous administration
582 of TNF- α promoted the activation of cNTS neurons, the first relay site for carotid body
583 afferents. Notably, the systemic administration of TNF- α resulted in activation of the
584 cNTS neurons at the same rostro-caudal levels reported to be activated after carotid
585 body stimulation by hypoxia or intravenous KCN (Cruz et al., 2010; Kline et al., 2010;
586 Malheiros-Lima et al., 2020). These cNTS neurons, activated by carotid body
587 stimulation, project to several brain areas, including the RVLM, to control the
588 sympathetic nervous system (Kline et al., 2010; Koshiya and Guyenet, 1996). A
589 previous study observed that after 3 hours of hypoxia (10% O₂) exposure, a high
590 proportion of RVLM-projecting cNTS neurons were activated (Kline et al., 2010).
591 Furthermore, the authors injected anterograde tracers into the carotid body and
592 observed that carotid body afferents terminate in close apposition to the RVLM-
593 projecting cNTS neurons. Thus, this neural circuitry elegantly revealed by Kline et al.
594 (2010), along with previous data (Aicher et al., 1996; Koshiya and Guyenet, 1996),
595 provides a major neural pathway for hypoxia-induced sympathoexcitation. Of note, the
596 blockade of glutamatergic receptors in the NTS was shown to strongly reduce the
597 sympathetic responses to chemical stimulation of the carotid body (Ferreira et al.,
598 2018). Since in the present study, circulating TNF- α induced the activation of RLVM-

599 projecting cNTS glutamatergic neurons at the same rostro-caudal levels reported in
600 the literature (Cruz et al., 2010; Kline et al., 2010; Malheiros-Lima et al., 2020) and,
601 because carotid body ablation almost abolished the activation of these neurons, we
602 believe that TNF- α might be stimulating a similar neural pathway (carotid body-cNTS-
603 RVLM) activated by hypoxia to increase sympathetic activity. It is important to highlight
604 that more than a half of c-FOS positive neurons observed in SHAM rats treated with
605 TNF- α were not co-localized with FG (non-RVLM-projecting). We hypothesize that
606 these neurons project to other nuclei involved in sympathetic modulation, such as the
607 PVN, regions involved in respiratory control, and vagal nuclei (Luise King et al., 2012;
608 Malheiros-Lima et al., 2020; Neff et al., 1998; Willis et al., 1996; Zera et al., 2019). In
609 fact, recent studies suggested that inflammation-induced carotid body stimulation
610 could also activate brainstem vagal nuclei (nucleus ambiguus and dorsal motor
611 nucleus of the vagus) to increase parasympathetic activity (Jendzjowsky et al., 2021,
612 2018). Therefore, the results of the present and previous studies suggest that the
613 carotid body detects circulating inflammatory mediators and activates central
614 autonomic areas to modulate sympathetic and/or parasympathetic functions.

615 Our study shows that the TNF- α -induced activation of a sympathoexcitatory
616 circuit (carotid body-cNTS-RVLM) resulted in increased SNA as revealed by
617 simultaneous recordings of splanchnic, renal and lumbar SNA. To the best of our
618 knowledge, this is the first study describing the effects of circulating TNF- α , an
619 important inflammatory mediator, on the activity of three different sympathetic nerves
620 recorded simultaneously in vivo. Previous studies have already demonstrated that
621 circulating TNF- α increases renal SNA in rats (Wei et al., 2013; Zhang et al., 2003).
622 However, since sympathetic outflows to other tissues/organs have distinct functions
623 and can be differentially regulated (Morrison, 2001; Tromp et al., 2018), it becomes
624 relevant to study the effects of TNF- α on sympathetic outflows directed to other targets
625 besides the kidneys. Here, we found that TNF- α promoted a generalized activation of
626 the sympathetic nervous system, increasing splanchnic, renal, and lumbar SNA in
627 carotid body-intact rats. The removal of the afferent inputs from the carotid bodies (by
628 bilateral carotid body ablation) blunted, in part, this TNF- α -induced sympathetic
629 activation, consistent with the attenuated activation of RLVM-projecting cNTS neurons
630 observed in CB-X rats (Figure 3C – E). Interestingly, the blunting effect of carotid body
631 ablation was significant only on splanchnic SNA. Therefore, our data indicate that
632 increased circulating TNF- α activates a carotid body-cNTS-RVLM neural circuit that

633 selectively controls splanchnic SNA in this condition. It is noteworthy that a previous
634 study reported that the increase in renal SNA following the systemic administration of
635 TNF- α was largely attenuated in rats with lesions of the subfornical organ (Wei et al.,
636 2013). It suggests that splanchnic, renal, and lumbar SNA might be under the control
637 of different neural routes and might have different functions in the course of TNF- α -
638 driven inflammation.

639 In this context, some studies have suggested that the splanchnic sympathetic
640 nerves play an important immunomodulatory role during endotoxemia-induced
641 systemic inflammation (Lankadeva et al., 2020; Martelli et al., 2014). For instance, it
642 was demonstrated that acute endotoxemia induced by intravenous administration of
643 lipopolysaccharide (LPS) significantly increased plasma levels of TNF- α after 90
644 minutes in rats (Martelli et al., 2014). In parallel, this LPS administration potently
645 increased splanchnic SNA. Notably, when LPS was given to rats subjected to the
646 bilateral section of the splanchnic sympathetic nerves, the plasma TNF- α levels
647 increased 5 times more than those of intact rats (Martelli et al., 2014). Together, these
648 results indicate that during LPS-induced systemic inflammation, the splanchnic SNA
649 increases to counteract the ongoing inflammation in a kind of negative feedback reflex.
650 Since, in the present study, the elevated circulating TNF- α activated a carotid body-
651 cNTS-RVLM neural circuit to increase splanchnic SNA, we hypothesized that this
652 mechanism could be a neuroimmune reflex to counteract the TNF- α -induced
653 inflammation. To test this hypothesis, we removed either the detection/afferent arm
654 (i.e., the carotid bodies) or the efferent arm (i.e., the splanchnic sympathetic nerves)
655 of this potential neuroimmune reflex and subjected these animals (and SHAM control
656 animals) to systemic injections of TNF- α or vehicle. After 2 hours, we quantified the
657 levels of TNF- α , IL-6, and IL-10 in the blood and in the spleen as well as the levels of
658 norepinephrine in the spleen. We found that in SHAM rats, the administration of TNF-
659 α significantly increased the plasma levels of TNF- α and slightly increased the spleen
660 levels of TNF- α compared to vehicle-injected SHAM rats. In addition, TNF- α
661 administration tended to increase spleen norepinephrine levels in SHAM animals as
662 compared to its vehicle-treated counterparts (Figure 5H, $p = 0.06$), consistent with our
663 data showing a TNF- α induced splanchnic SNA activation. Interestingly, in rats
664 subjected to either carotid body ablation or splanchnic sympathetic denervation, the
665 administration of TNF- α resulted in exacerbated levels of pro-inflammatory cytokines
666 in the plasma and the spleen, supporting the idea that both the detection/afferent arm

667 and the efferent arm are important components of a neuroimmune regulatory
668 mechanism that detects and modulates acute inflammation through sympathetic
669 activation towards the spleen. Disrupting the afferent/detection component (carotid
670 body ablation) resulted in a peculiar elevation of all quantified cytokines, including IL-
671 10 (an anti-inflammatory cytokine). The reason for this elevation in plasma IL-10 in
672 CB-X rats treated with TNF- α is not clear. This could result from the fact that carotid
673 body ablation eliminated only part of the autonomic circuits toward the spleen, possible
674 preserving and/or amplifying other counter-inflammatory mechanisms. In fact, the
675 administration of TNF- α in CB-X rats, still activated splanchnic SNA and resulted in a
676 significant increase in splenic levels of norepinephrine compared to vehicle-injected
677 CB-X rats. However, the TNF- α -induced splanchnic SNA activation and
678 norepinephrine release in the spleen were attenuated in CB-X rats compared to SHAM
679 rats, which could explain, at least in part, the exacerbated inflammatory status
680 observed in the animals lacking the carotid bodies. Differently, the interruption of the
681 efferent component (splanchnic sympathetic denervation) completely blocked the
682 sympathetic signalling to the spleen, removing the norepinephrine “inhibitory tonus”
683 on cytokine production by splenic macrophages, resulting in elevated splenic cytokine
684 levels even in those animals administered with saline. Collectively, our data suggest
685 the existence of an intrinsic and physiological anti-inflammatory reflex that depends
686 on a detection/afferent arm (i.e., the carotid bodies and the carotid sinus nerve), on a
687 central integrative pathway (i.e., RVLM-projecting cNTS neurons), and on an
688 effector/efferent arm (i.e., splanchnic sympathetic nerves) that modulates the splenic
689 production of cytokines through norepinephrine release.

690 The findings of the present study are novel and place the carotid body as a
691 critical player in the context of neuroimmune interactions. In the last years, the
692 contribution of the carotid bodies to sympathetic overactivity has been implicated in
693 the pathophysiology of several diseases such as sleep apnoea, hypertension, and
694 heart failure (Marcus et al., 2014; McBryde et al., 2013; Melo et al., 2019; Narkiewicz
695 et al., 2016; Niewinski et al., 2017; Yuan et al., 2016). In these conditions, exaggerated
696 tonic CSN activity leads to chronic activation of the sympathetic nervous system, often
697 associated with a poor prognosis. Here, we found that the acute carotid body-mediated
698 sympathetic activation induced by intravenous TNF- α is likely to be beneficial because
699 it exerted a counteracting anti-inflammatory reflex. However, it is possible that in
700 chronic pathological inflammatory conditions, the long-term activation of this carotid

701 body-dependent neuroimmune circuit leads to side effects because it generates an
702 aberrant tonic CSN input to central sympathetic networks, leading to sustained
703 sympathetic overactivity to multiple target organs. This possibility raises an intriguing
704 question on whether circulating inflammatory factors could trigger the carotid body-
705 mediated sympathetic overactivity observed in diseases such as hypertension
706 (McBryde et al., 2013; Narkiewicz et al., 2016) and heart failure (Marcus et al., 2014;
707 Niewinski et al., 2017) since these conditions are associated with increased systemic
708 inflammation (Bautista et al., 2005; Norlander et al., 2018; Rauchhaus et al., 2000;
709 Sesso et al., 2015). On the other hand, defects in the carotid body-mediated
710 neuroimmune reflex described here, could impair the ability to regulate the levels of
711 inflammatory mediators in the bloodstream, amplifying systemic inflammation.
712 Nevertheless, further investigations are needed to clarify the beneficial or detrimental
713 effects following the activation/inactivation of the neuroimmune mechanism described
714 in the present study under different conditions and to explore its therapeutic potential
715 in the treatment of inflammatory diseases.

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718 **Methods**

719 **Animals and ethical approval**

720 All experimental procedures were reviewed and approved by the Ethical Committee in
721 Animal Experimentation of the Araraquara School of Dentistry, São Paulo State
722 University (protocol nº 17/2019) and conducted following the Guide for the Care and
723 Use of Laboratory Animals from the Brazilian National Council for Animal
724 Experimentation Control. Experiments were performed on adult male *Holtzman* rats
725 (320 - 400 g) obtained from the Animal Care Unit of the São Paulo State University
726 (Araraquara, SP, Brazil). The animals were housed in collective cages (2 - 4
727 animals/cage), provided with chow and water *ad libitum*, and maintained under
728 controlled conditions of temperature ($22 \pm 1^\circ\text{C}$), humidity (50 - 60%) in a 12:12 hours
729 light/dark cycle.

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734 **General procedures**

735 All surgical procedures were performed under aseptic conditions. The appropriate
736 depth of anesthesia was confirmed by the absence of withdrawal reflex and corneal
737 reflexes in response to pinching the toe. Throughout the surgical procedures and the
738 experimental protocols performed under anesthesia (described below), the body
739 temperature was measured by a rectal probe and maintained at $37 \pm 0.5^{\circ}\text{C}$ with a
740 water-circulating heating pad.

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743 **Experiment 1: Expression of TNF- α receptor type I in carotid body glomus cells**

744 Rats were deeply anesthetized with isoflurane (5% in 100 O₂) and subjected to
745 transcardial perfusion with cold phosphate-buffered saline (PBS, 10 mM, pH 7.4, 100
746 mL/100 g BW) followed by paraformaldehyde (PFA, 4% in PBS, 100 mL/100 g BW).
747 Whole carotid bifurcations containing the carotid bodies were collected as previously
748 described (Pijacka et al., 2018) and fixed in PFA for 24 hours at 4^o C. Next, carotid
749 bifurcations were transferred to 10% sucrose solution and kept at 4^o C until the tissue
750 sinks. This procedure was repeated with 20% and 30% sucrose solutions. Carotid
751 bifurcations were frozen in Tissue Freezing Medium (Triangle Biomedical Sciences,
752 Durham, NC, USA) using dry ice, sectioned at 10 μm in a cryostat and mounted on
753 microscope slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA, USA). To
754 evaluate the expression of TNF- α receptor type I (TNFR1) in the carotid bodies, we
755 employed two different approaches: 1) a fluorescent *in situ hybridization* (FISH) assay
756 (RNAscope, Advanced Cell Diagnostics, Newark, CA, USA) for TNFR1 mRNA
757 detection combined with immunofluorescence staining for TH (a marker of carotid
758 body glomus cells) and; 2) a double immunofluorescence staining for TNFR1 and TH.
759 In the first approach, the FISH assay was performed according to the manufacturer
760 instructions (document #323100-USM, available at
761 <https://acdbio.com/documents/product-documents>) and the following materials were
762 used: RNAscope Multiplex Fluorescent Detection Reagents v2 (product #323110), the
763 kit RNAscope H₂O₂ and Protease Reagents (product #322381), the RNAscope probe
764 for TNFR1 (product #408111) and the TSA Cyanine 3 Plus Evaluation kit (product
765 #NEL744001KT, Akoya Biosciences, Boston, MA, USA). After completing the FISH
766 protocol, an immunofluorescence protocol for TH was performed to identify carotid
767 body glomus cells. Briefly, the slides were incubated in a blocking solution (0.1 M PBS,

768 10% normal horse serum, and 0.3% Triton X-100) for 20 min and subsequently rinsed
769 3 x 10 minutes in 0.1 M PBS at room temperature. Then, the slides were incubated in
770 primary antibody (Mouse anti-TH antibody, 1:1000, product #MAB5280, Millipore,
771 Billerica, MA, USA) for 1 hour at room temperature and 36 hours at 4° C. After rinsing
772 in PBS, the slides were incubated in secondary antibody (Alexa Fluor 488 donkey anti-
773 mouse antibody, 1:200, product #R37114, Molecular Probes-Life Technologies,
774 Eugene, OR, USA) for 4 hours at room temperature. The slides were rinsed in PBS,
775 the excess liquid was drained, mounting medium (Fluoromount) was dropped on the
776 tissue and slides were covered with glass coverslips (Fisherfinest). In the second
777 approach, the immunofluorescence staining as carried out as described above but
778 adding also a primary antibody for TNFR1 (Rabbit anti-TNFR1, 1:500, product
779 #ab19139, Abcam, Cambridge, MA, USA) and a secondary antibody (Alexa Fluor 594
780 donkey anti-rabbit antibody, 1:200, product #A21207, Molecular Probes-Life
781 Technologies). Images were acquired using a laser scanning confocal microscope
782 (LSM800, Zeiss). For presentation purposes (color-blind safe) images were pseudo-
783 colored and representative figures were prepared using the Zen 2 software (Blue
784 edition, Zeiss).

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787 **Experiment 2: Effects of circulating TNF- α on carotid sinus nerve afferent** 788 **activity**

789 Animals were anesthetized with isoflurane (Induction 5% and maintenance 2.5% in
790 100% O₂) and subjected to femoral artery and vein catheterizations for arterial blood
791 pressure (ABP) monitoring and drug administration, respectively, using polyethylene
792 catheters (PE-50 attached to PE-10, Becton Dickinson, Sparks, MD, USA). Next,
793 through a midline neck incision, the trachea was cannulated, and animals were
794 artificially ventilated with a rodent ventilator (model 7025, Ugo Basile, Gemonio, VA,
795 Italy). End-tidal CO₂ was maintained between 4 - 5% (Capstar-100 carbon dioxide
796 analyzer, CWE, Ardmore, PA, USA) by adjusting tidal volume (0.7 - 0.8 mL/100 g of
797 body weight) and respiratory rate (60 - 80 bpm). Isoflurane was slowly replaced with
798 urethane anesthesia (1.2 - 1.4 g/kg of body weight, IV) given over 20 - 25 minutes.
799 Then, O₂ concentration in ventilated air was switched to 50% O₂ (balance N₂) and this
800 condition was kept until the end of the experiments. This slightly hyperoxic

801 concentration was chosen because it ensures a stable preparation without silencing
802 carotid body activity as 100% O₂ would do (Kim et al., 2018; Schultz et al., 2007) and
803 to avoid any period of hypoxia during the experimental protocol.

804 Then, animals were prepared for recordings of CSN afferent activity. The left
805 carotid sinus nerve was identified, carefully isolated, and cut centrally at its junction to
806 the glossopharyngeal nerve. CSN activity was recorded using bipolar suction
807 electrodes and signals were filtered (100 - 3000 Hz), amplified (10,000 X) and digitally
808 sampled (10 kHz). After baseline recordings, TNF- α (500 ng in 0.5 mL sterile saline,
809 IV; PeproTech, Rocky Hill, NJ, USA) was administered and CSN activity was recorded
810 for additional 2 hours. This dose was chosen based on previous works studying the
811 effects of TNF- α on renal SNA in vivo (Zhang et al. 2003, Wei et al. 2015). Reliability
812 of CSN activity was confirmed at the end of experiments by a robust increase in
813 electrical activity during the exposure to hypoxia (10% O₂) for 60 - 90 seconds.

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816 **Experiment 3: Neuroanatomical identification of carotid body-related central** 817 **sympathoexcitatory pathways activated by circulating TNF- α**

818 First, the animals were anesthetized with a mixture of ketamine (80 mg kg⁻¹, IP; União
819 Química Farmacêutica Nacional S/A, Embu-Guaçu, SP, Brazil) and xylazine (8 mg kg⁻¹,
820 IP; Hertape Calier Saúde animal S/A, Juatuba, MG, Brazil), and placed in a
821 stereotaxic frame (David Kopf instruments, Tujunga, CA, USA). The retrograde tracer
822 FluoroGold (FG, 2%, Fluorochrome, Denver, CO, USA) diluted in artificial
823 cerebrospinal fluid (aCSF) was then bilaterally injected (40 nL) into the RVLM.
824 Microinjections were performed with a pressure microinjector (Picospritzer III, Parker
825 Hannifin, Hollis, NH, USA) using glass micropipettes. After each injection, the
826 micropipette was kept in place for 2 minutes to prevent FG reflux. The coordinates
827 used to target the RVLM were: 3.5 mm caudal from Lambda, 1.8 - 2.0 mm lateral from
828 the midline, and 9.4 mm ventral from the skull surface. After injections, the skin
829 incisions were sutured and the animals received anti-inflammatory ketoprofen (3 mg
830 kg⁻¹, SC) and antibiotics penicillin (50,000 IU, IM). This treatment was repeated every
831 24 hours for 3 days.

832 After 6 days recovery, animals were subjected to bilateral carotid body ablation
833 (CB-X group) or Sham procedure (Sham group) and femoral artery/vein
834 catheterizations. Carotid body ablation was performed by combining two previously

835 described methods (Katayama et al., 2015; Pijacka et al., 2018). Briefly, animals were
836 anesthetized with ketamine/xylazine as previously described. The carotid body
837 arteries were ligated and cut, followed by surgical removal of the carotid bodies on
838 both sides. In this procedure, the carotid sinus nerve is maintained intact, preserving
839 carotid baroreflex function (Pijacka et al., 2018). Sham procedure consisted in isolation
840 of carotid body arteries and carotid bodies, but these structures were kept intact. Neck
841 incisions were closed with sutures. Femoral artery/vein catheters were tunneled
842 subcutaneously, exteriorized and fixed in the interscapular region as previously
843 described (Katayama et al., 2019). After surgeries, animals were treated with
844 antibiotics and anti-inflammatory for 3 days as described before. To maintain catheters
845 patency, arterial and venous catheters were flushed every day with heparinized saline
846 (arterial: 500 U/mL, venous: 40 U/mL). Three days after surgery, ABP was recorded
847 in unanesthetized rats under baseline conditions and in response to potassium
848 cyanide (KCN; 40 ug/animal, IV) to verify the efficacy of carotid body ablation in CB-X
849 group and the integrity of carotid bodies in SHAM group. Successful bilateral carotid
850 body ablation was confirmed by the lack of cardiovascular responses to KCN (figure
851 supplement 2A – B). Rats were allowed to recover for 3 days before the next
852 experimental protocol.

853 On the day of the experiment (12 days after FG microinjections), rats were
854 administered with TNF- α (500 ng in 0.5 mL sterile saline, IV) and left undisturbed for
855 2 hours. Next, rats were deeply anesthetized with urethane (IV) and transcardially
856 perfused with PBS followed by PFA. Brains were collected and fixed in PFA for 12
857 hours at 4^o C. Brains were then transferred to 20% sucrose solution and maintained
858 at 4^o C until the tissue sinks. Finally, brains were frozen in Tissue Freezing Medium
859 (Triangle Biomedical Sciences, Durham, NC, USA) and coronal brain slices (30 μ m)
860 containing the cNTS and the RVLM were obtained on a cryostat. The RVLM sections
861 were mounted on microscope slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA,
862 USA) and used to confirm the location of FluoroGold microinjections within RVLM
863 region (From 12.48 mm to 12.00 mm caudal to bregma, ventral to the compact
864 formation of the Nucleus Ambiguus) accordingly to the rat brain in stereotaxic
865 coordinates atlas (Paxinos and Charles Watson, 2007). The cNTS sections were
866 stored in cryoprotectant solution at -20^o C until processing for c-FOS and VGluT2
867 immunofluorescence as described below.

868 Briefly, sections were first rinsed in 0.1 M PBS for 10 minutes followed by
869 incubation in blocking solution (0.1 M PBS, 10% normal horse serum, and 0.3% Triton
870 X-100) for 20 min at room temperature. After rinsing 3 x 10 minutes in 0.1 M PBS at
871 room temperature, slides were incubated in primary antibodies for c-FOS (1:1000,
872 rabbit anti-c-FOS polyclonal antibody, product #sc-52, Santa Cruz Biotechnology,
873 Santa Cruz, CA, USA) and for VGluT2 (1:2000, guinea pig anti-VGluT2 polyclonal
874 antibody, product #AB2251-I, Millipore, Temecula, CA, USA) for 1 hour at room
875 temperature plus 36 hours at 4^o C. After rinsing in PBS, slides were incubated in
876 secondary antibodies against rabbit (1:400, donkey anti-rabbit Alexa Fluor 594,
877 product #A-21207, Molecular Probes-Life Technologies, Eugene, OR, USA) and
878 against anti-guinea pig (1:400, donkey anti-guinea pig Alexa Fluor 488, product #706-
879 545-148, Jackson ImmunoResearch Inc, West Grove, PA, USA) for 4 hours at room
880 temperature. Slides were rinsed in PBS, the excess liquid was drained, mounting
881 medium (Fluoromount, product # F4680, Sigma, St. Louis, MO, USA) was dropped on
882 the tissue and slides were covered with glass coverslips (Fisherfinest, product
883 #125485M, Fisher Scientific).

884 Images were acquired using a laser scanning confocal microscope (LSM800
885 with airyscan, Zeiss, Jena, TH, Germany). Quantification of retrograde labeled FG
886 cells, c-FOS positive cells, FG/c-FOS cells, and FG/c-FOS/VGluT2 cells within the
887 cNTS were performed on brainstem sections from three different rostro-caudal levels
888 (between 14.76 mm to 14.04 mm caudal to bregma). These levels were chosen based
889 on studies demonstrating NTS regions that are activated after carotid body stimulation
890 (Cruz et al., 2010; Kline et al., 2010; Malheiros-Lima et al., 2020). As anatomical
891 landmarks to identify the cNTS levels, we used: the area postrema, the central canal,
892 the gracile nucleus, and the hypoglossal nucleus. Cell counting was performed on
893 ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA) and
894 representative figures were prepared using the Zen 2 software (Blue edition, Zeiss).

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897 **Experiment 4: Sympathetic responses to circulating TNF- α in Sham and carotid** 898 **body-ablated (CB-X) rats**

899 Animals were anesthetized, subjected to femoral artery/vein catheterizations,
900 tracheotomized and continuously ventilated with 50% O₂ (balance N₂) as described in

901 *Experiment 2.* Next, animals were subjected to bilateral carotid body ablation or sham
902 surgery as described in *Experiment 3*. All animals were then prepared for
903 simultaneous recordings of lumbar, renal, and splanchnic sympathetic nerve activity
904 (SNA). Lumbar sympathetic nerve was isolated through a midline laparotomy and
905 retraction of vena cava, while renal and splanchnic sympathetic nerves were isolated
906 through a retroperitoneal incision and careful retraction of the left kidney. Each
907 sympathetic nerve was placed on a bipolar stainless-steel electrode and insulated with
908 KWIK-SIL (World Precision Instruments, Sarasota, FL, USA). The raw SNA signals
909 were filtered (100 - 1000 Hz), amplified (10,000 X) using biological amplifiers (P511
910 AC Amplifier, Grass Technologies, Warwick, RI, USA), displayed on oscilloscopes
911 (TDS 2022, Tektronix, Beaverton, OR, USA) and digitally sampled (2 kHz) by a data
912 acquisition system (Micro 1401, Cambridge Electronic Design Limited). All incisions
913 were closed with surgical clips (Fine Science Tools, Foster City, CA, USA).

914 After stabilization (~30 minutes after the end of surgical procedures), baseline
915 recordings of ABP, lumbar, renal and splanchnic SNA were performed. Next, TNF- α
916 (500 ng in 0.5 mL sterile saline, IV) was administered and ABP and SNA were
917 recorded for additional 2 hours. At the end of the experiments, carotid body ablation
918 was confirmed by the absence of blood pressure and SNA responses to KCN (40
919 ug/animal, IV) and these results are presented in figure supplement 3A – B.

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922 **Experiment 5: Plasma and spleen levels of pro-inflammatory cytokines following** 923 **intravenous TNF- α in SHAM, CB-X and SPL-X rats**

924 Rats were anesthetized with ketamine/xylazine and prepared accordingly one of the
925 following experimental groups: 1) CB-X: Animals were subjected to bilateral ablation
926 of the carotid bodies; 2) SPL-X: Animals were subjected to splanchnic denervation
927 through celiac ganglionectomy as previously reported in the literature (Asirvatham-
928 Jeyaraj et al., 2021; Li et al., 2010). Briefly, after a midline laparotomy, the visceral
929 organs were gently retracted, and the celiac ganglion was localized and surgically
930 removed using blunt forceps. 3) SHAM: The carotid bodies and the celiac ganglion
931 were localized and manipulated, but these structures were kept intact. All animals
932 were allowed to recover for 6 days. Next, animals were subjected to femoral
933 artery/vein catheterizations. The efficacy of carotid body ablation in CB-X group and
934 the integrity of carotid bodies in SHAM and SPL-X groups was verified three days after

935 catheterizations and these results are presented in figure supplement 4A – B. Then,
936 rats were allowed to recover for additional 3 days before the experimental protocol.

937 The experimental protocol consisted in the administration of TNF- α (500 ng, IV)
938 or vehicle (sterile saline, IV) in SHAM, CB-X and SPL-X rats. After 2 hours, rats were
939 deeply anesthetized for tissue collection. Blood was collected into EDTA-containing
940 tubes, centrifuged (1500 rpm for 10 min) at 4° C and the plasma was aliquoted and
941 stored at -80° C. Spleen was harvested, flash frozen using liquid nitrogen and stored
942 at -80° C.

943 The spleen samples were homogenized in PBS using a Polytron tissue
944 homogenizer, and then centrifuged at 10,000 rpm for 2 min at 4 °C. The plasma and
945 splenic levels of cytokines were quantified by enzyme-linked immunosorbent assay
946 (ELISA) using commercial kits DuoSet ELISA Development Systems (R&D Systems,
947 Minneapolis, MN, USA) for TNF- α (catalog #DY510), IL-6 (catalog #DY506), and IL-
948 10 (catalog #DY522) and following the user manual instructions. The results were
949 expressed as cytokine concentration in pg/mL and pg/mg of protein, based on
950 standard curves, respectively. Spleen norepinephrine was measured as previously
951 described (Garofalo et al., 1996) using HPLC (LC20AT-Shimadzu Proeminence)
952 coupled to an electrochemical detector (Decade Lite-Antec Scientific) with a 5- μ m
953 Spherisorb ODS-2 reversed-phase column (Sigma-Aldrich) and the results were
954 expressed as norepinephrine concentration in ng/g of tissue.

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957 **Statistical analysis**

958 All statistical analyses were performed using IBM SPSS Statistics (version 25, IBM
959 corporation). Data are reported as means \pm SEM. The significance level was set at p
960 < 0.05, unless otherwise stated. No outliers were found as assessed by boxplot
961 analyses. *Experiment 2:* To examine differences between means within the same
962 group over time, the one-way repeated measures analysis of variance (ANOVA)
963 followed by post hoc analysis with Bonferroni adjustment was performed. The normal
964 distribution of the data was verified and confirmed by the Shapiro-Wilk test, and the
965 Mauchly's test indicated that the assumption of sphericity has not been violated.
966 *Experiment 3:* To determine differences between two groups at a single time-point, we
967 first assessed the distribution of the data using the Shapiro-Wilk test and the
968 homogeneity of variances using the Levene's test. For normally distributed data with

969 homogenous variances, an unpaired two-tailed Student's *t*-test was performed. In
970 cases in which the data was normally distributed but the assumption of homogeneity
971 of variances was violated, an unpaired two-tailed Welch's *t*-test was used. When data
972 was not normally distributed, an unpaired two-tailed Mann-Whitney *U*-test was applied.
973 *Experiment 4:* To determine group x time interactions, a two-way repeated measures
974 ANOVA was conducted. In this case, the normal distribution of the studentized
975 residuals was verified and confirmed by the Shapiro-Wilk test. The sphericity for the
976 interaction term was assessed by the Mauchly's test. When the assumption of
977 sphericity was violated, the Greenhouse-Geisser correction was used and the
978 estimated epsilon (ϵ) value was reported. Once statistically significant group x time
979 interactions were detected, simple main effects of group were analyzed using repeated
980 measures general linear models with Bonferroni adjustment. *Experiment 5:* To
981 examine group x treatment interactions and main effects of group, a two-way ANOVA
982 with Bonferroni post hoc was performed. The distribution of the residuals was
983 examined by the Shapiro-Wilk test. The homoscedasticity was analyzed by the
984 Levene's test and by plotting the residuals against the predicted values in a simple
985 scatterplot. If the assumptions of normal distribution and/or homoscedasticity were
986 violated, the dependent variable was log-transformed when appropriate. When both
987 the assumptions of normality and homoscedasticity (requirements for two-way
988 ANOVA) were not satisfied even after transformation, a Kruskal-Wallis followed by
989 Mann-Whitney *U*-tests for pairwise comparisons between groups were performed. In
990 these cases, a Bonferroni adjustment to alpha values was applied and the statistical
991 significance was accepted at the $p < 0.003$ level. In cases in which only the assumption
992 of homoscedasticity was violated, a Welch's ANOVA followed by a Games-Howell
993 post hoc was used to compare groups.

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1007

1008 **Author contributions**

1009 P.L.K. and E.C. conceived and designed research. P.L.K. performed all in vivo
1010 experiments. P.L.K. and I.P.L. performed immunofluorescence and in situ
1011 hybridization. A.K. and J.P.M.L. performed ELISA. L.C.C.N. performed HPLC
1012 measurements. P.L.K., I.P.L., A.K., and J.P.M.L. analyzed data. P.L.K., I.P.L., A.K.,
1013 D.B.Z, and E.C. interpreted data. P.L.K. and A.K. drafted the manuscript. P.L.K., I.P.L.,
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1016 D.S.A.C., and E.C. read and approved the final version of the manuscript.

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1018 **Competing interests**

1019 The authors declare no competing interests.

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1021 **Materials & correspondence**

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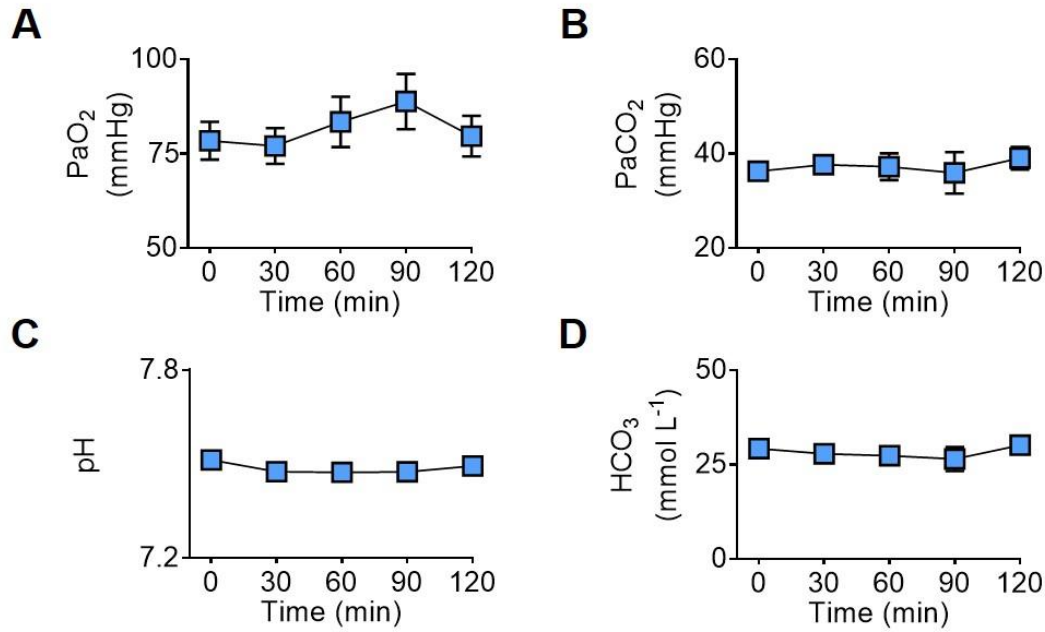
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Supplementary Information

The Carotid Body Detects Circulating Tumor Necrosis Factor-Alpha to Activate a Sympathetic Anti-Inflammatory Reflex

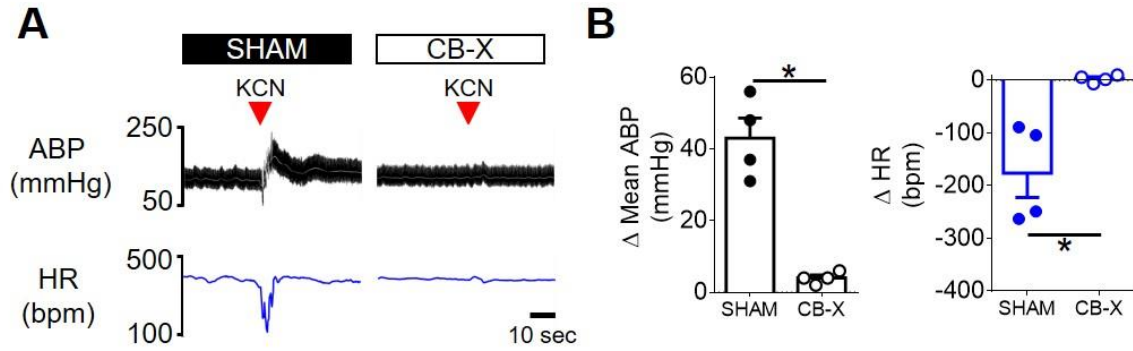
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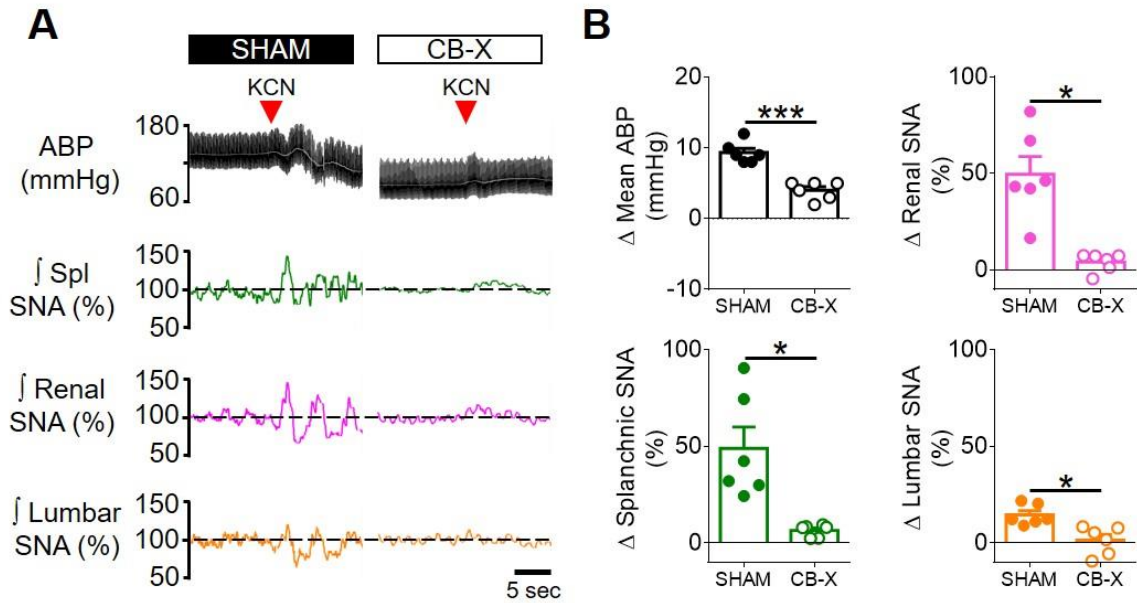
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Figure supplement 1. Acute intravenous TNF- α does not affect arterial blood gases, pH, and bicarbonate. All measures were performed using a I-STAT device with CG4+ cartridges (Abbott, Abbott Park, IL, USA). **A – D.** Summary data (n = 5) showing that the intravenous administration of TNF- α (500 ng) did not affect the partial pressure of oxygen (PaO₂), the partial pressure of carbon dioxide (PaCO₂), the pH, and the bicarbonate (HCO₃⁻) concentration in the arterial blood of unanesthetized, spontaneously breathing rats. One-way repeated measures ANOVA: PaO₂, $F_{(1.525, 6.102)} = 1.659$, $p = 0.259$, $\epsilon = 0.381$; PaCO₂, $F_{(4,16)} = 0.370$, $p = 0.826$; pH, $F_{(4,16)} = 2.838$, $p = 0.059$; HCO₃⁻, $F_{(1.879, 7.515)} = 1.207$, $p = 0.347$, $\epsilon = 0.470$. Data are means \pm SEM.



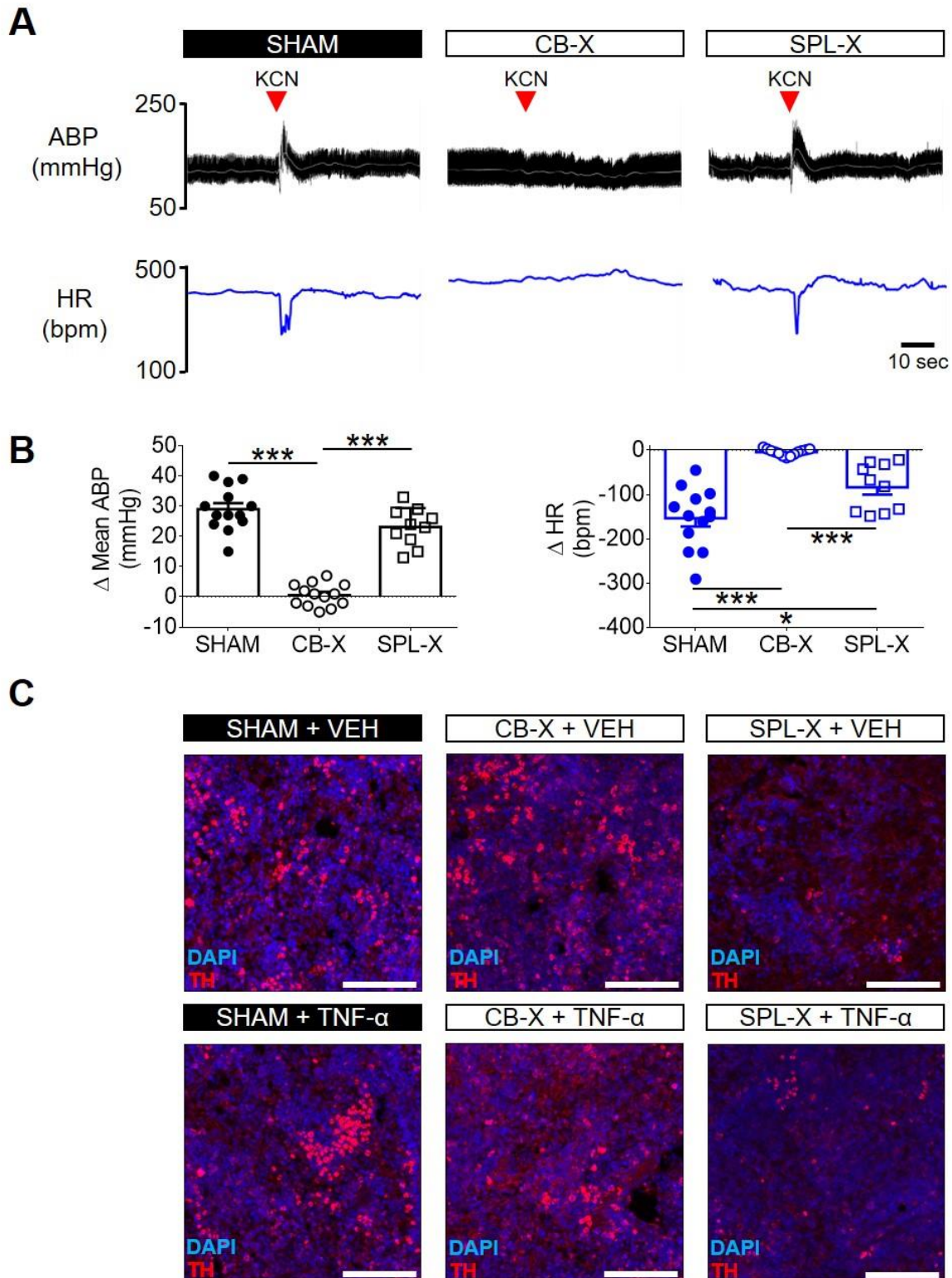
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Figure supplement 2. Verification of carotid body ablation in *experiment 3*. **A.** Representative tracings of arterial blood pressure (pulsatile ABP, black; mean ABP, white) and heart rate (HR; blue) of a SHAM rat (left) and of a CB-X rat (right) in response to KCN (red arrowhead, 40 μ g, IV) under unanesthetized conditions. **B.** Summary data showing the peak changes in mean ABP and HR in response to KCN from SHAM (filled symbols, n = 4) and CB-X (open symbols, n = 4) rats. The cardiovascular responses to carotid body stimulation by intravenous KCN were abolished in CB-X rats, confirming the efficacy of bilateral carotid body ablation: Δ mean ABP (SHAM, 43 ± 6 mmHg; CB-X, 4 ± 1 mmHg; $t(3.128) = 6.912$, $p = 0.005$, Welch's t -test), Δ HR (SHAM, -176 ± 46 bpm; CB-X, 3 ± 3 bpm; $t(3.033) = -3.866$, $p = 0.03$, Welch's t -test). * $p < 0.05$. Data are means \pm SEM.



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Figure supplement 3. Verification of carotid body ablation at the end of *experiment 4*. **A.** Representative tracings of arterial blood pressure (pulsatile ABP, black; mean ABP, white), splanchnic SNA (Spl; green), renal SNA (magenta) and lumbar SNA (orange) of a SHAM rat (left) and of a CB-X rat (right) in response to KCN (red arrowhead, 40 μ g, IV) under anesthetized conditions. **B.** Summary data showing the changes in mean ABP, Splanchnic SNA, Renal SNA and Lumbar SNA in response to KCN from SHAM (filled symbols, n = 6) and CB-X (open symbols, n = 6) rats. For each rat, baseline rectified and integrated SNA was normalized to 100% and the peak changes in response to KCN were calculated. The sympathetic and blood pressure responses to KCN were significantly attenuated in CB-X rats, confirming the efficacy of bilateral carotid body ablation: Δ Spl SNA (SHAM, 48 ± 11 %; CB-X, 7 ± 1 %; $U = 0$, $z = -2.882$, $p = 0.002$, Mann-Whitney U -test), Δ Renal SNA (SHAM, 50 ± 9 %; CB-X, 4 ± 2 %; $t(5.452) = 4.815$, $p = 0.004$, Welch's t -test), Δ Lumbar SNA (SHAM, 15 ± 2 %; CB-X, 1 ± 3 %; $t(10) = 3.547$, $p = 0.005$, Student's t -test), and Δ Mean ABP (SHAM, 9 ± 1 mmHg; CB-X, 4 ± 1 %; $t(10) = 6.644$, $p < 0.001$, Student's t -test). * $p < 0.05$ and *** $p < 0.001$. Data are means \pm SEM.



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Figure supplement 4. Verification of carotid body ablation and splanchnic sympathetic denervation in *experiment 5*. **A.** Representative tracings of arterial blood pressure (pulsatile ABP, black; mean ABP, white) and heart rate (HR; blue) of a SHAM rat (left), of a CB-X rat (middle), and of a SPL-X rat (right) in response to KCN (red arrowhead, 40 μ g, IV) under unanesthetized conditions. **B.** Summary data showing the peak changes in mean ABP (black graphs, left) and HR (blue graphs, right) in response to KCN from SHAM (filled circles, $n = 13$), CB-X (open circles, $n = 13$), and SPL-X (open squares, $n = 10$) rats. The cardiovascular (mean ABP and HR) responses to carotid body stimulation by intravenous KCN were abolished in CB-X rats, confirming the efficacy of bilateral carotid body ablation: Δ mean ABP (SHAM, 29 ± 2 mmHg; CB-X, 1 ± 1 mmHg; SPL-X, 23 ± 2) and Δ mean HR (SHAM, -154 ± 19 bpm;

1529 CB-X, -4 ± 2 bpm; SPL-X, -84 ± 17). Regarding Δ mean ABP, a one-way ANOVA detected statistically
1530 significant differences between groups, $F_{(2, 33)} = 83.134$, $p < 0.001$. Subsequent post hoc analysis with
1531 a Bonferroni adjustment revealed that the mean difference in Δ mean ABP between CB-X and SHAM
1532 rats was statistically significant (-28 mmHg, 95% CI $[-34, -23]$, $p < 0.001$) as well as the mean difference
1533 in Δ mean ABP between CB-X and SPL-X rats (-22 mmHg, 95% CI $[-29, -16]$, $p < 0.001$). Regarding Δ
1534 HR, a Welch ANOVA detected statistically significant differences between groups, $F_{(2, 14.078)} = 40.040$,
1535 $p < 0.001$. Games-Howell post hoc analysis revealed that the mean difference in Δ HR between CB-X
1536 and SHAM rats was statistically significant (149 bpm, 95% CI $[99, 200]$, $p < 0.001$) as well as the mean
1537 difference in Δ mean HR between CB-X and SPL-X rats (79 bpm, 95% CI $[33, 126]$, $p = 0.003$). In
1538 addition, the mean difference in Δ mean HR between SHAM and SPL-X rats was also statistically
1539 significant (-70 bpm, 95% CI $[-133, -7]$, $p = 0.029$). * $p < 0.05$ and *** $p < 0.001$. Data are means \pm SEM.
1540 **C.** Representative images of spleen sections from one animal of each group obtained at the end of
1541 *experiment 5* and processed for nuclear staining (DAPI, blue) and tyrosine hydroxylase (TH, red). Note
1542 that TH staining is substantially less pronounced in the animals subjected to splanchnic sympathetic
1543 denervation (SPL-X + VEH, upper right panel; and SPL-X + TNF- α , bottom right panel) as compared to
1544 SHAM (SHAM + VEH, upper left panel; and SHAM + TNF- α , bottom left panel) and CB-X (CB-X + VEH,
1545 upper middle panel; and CB-X + TNF- α , bottom middle panel). VEH, vehicle. Scale bars: 100 μ m.