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1 | **Long-lasting bradypnea induced by repeated social defeat**

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13  
14  
15 **Running head:** Anxiety and long-lasting respiratory modulation

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18  
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22 **Abstract**

23 Repeated social defeat in the rat induces long-lasting cardiovascular changes associated  
24 with anxiety. In this study, we investigated the effects of repeated social defeat on breathing.  
25 Respiratory rate was extracted from the respiratory sinus arrhythmia (RSA) peak frequency of the  
26 ECG in rats subjected to social defeat for four consecutive days.

27 Respiratory rate was recorded under anesthesia six days (D+10) or 26 days (D+30) after  
28 social defeat. At D+10, defeated (D) rats spent less time in the open arms of the elevated plus  
29 maze test, had heavier adrenal glands, and displayed bradypnea, unlike non-defeated (ND)  
30 animals. At D+30, all signs of anxiety had disappeared. However, half of the rats still displayed  
31 bradypnea (D<sub>L</sub> rats, for low respiratory rate indicated by a lower RSA frequency), while those  
32 with higher respiratory rate (D<sub>H</sub> rats) had recovered. Acute blockade of the dorsomedial  
33 hypothalamus (DMH) or nucleus tractus solitarii (NTS) 5-HT<sub>3</sub> receptors reversed bradypnea in  
34 all D rats at D+10 and in D<sub>L</sub> rats at D+30.

35 Respiratory rate was also recorded in conscious animals implanted with radiotelemetric  
36 ECG probes. D<sub>H</sub> rats recovered between D+10 and D+18, while D<sub>L</sub> rats remained bradypneic  
37 until D+30.

38 In conclusion, social stress induces sustained chronic bradypnea mediated by DMH  
39 neurons and NTS 5-HT<sub>3</sub> receptors. These changes are associated with an anxiety-like state that  
40 persists until D+10, followed by recovery. However, bradypnea may persist in half of the  
41 population up until D+30 despite apparent recovery of the anxiety-like state.

42 **Glossary**

43

44 HF: High-Frequency domain

45 HRV: Heart Rate Variability

46 RSA: Respiratory Sinus Arrhythmia

47 DMH: Dorsomedial Hypothalamus

48 NTS: Nucleus Tractus Solitarii

49 ND: Non-defeated rats

50 D: Defeated rats

51 D<sub>L</sub>: Defeated rats with low RSA peak frequency at D+30

52 D<sub>H</sub>: Defeated rats with high RSA peak frequency at D+30

53

## 54 **Introduction**

55 Breathing and anxiety are intimately related (44). For example, respiratory distress and  
56 asphyxia are associated with dreadful feelings, and fear and anxiety can have profound effects on  
57 breathing. Clinical studies have shown that panic disorder, characterized by acute and unexpected  
58 anxiety attacks and substantial anxiety over the possibility of experiencing further attacks, is  
59 associated with symptoms including palpitations, shortness of breath, sweating and  
60 hyperventilation (1). In addition, high levels of anxiety-related behavior in rats are associated  
61 with elevation of the resting respiratory rate (9). The respiratory rate also decreases during certain  
62 specific responses to stress, for instance during freezing behavior in the rat, when it is associated  
63 with ultrasonic vocalizations (19). However, much less is known about the long-term effects of  
64 emotional stress on breathing. In adult rats, intense neonatal emotional stress, such as maternal  
65 separation, can lead to a decrease in breathing rate during non-REM sleep (24). A lower  
66 respiratory rate was also observed in anesthetized Flinder-Sensitive rats, a well-validated animal  
67 model of depression (33). However, respiratory rate does not appear to be altered in patients with  
68 major depression, although cardiovascular changes are observed (4). Clearly, more work needs to  
69 be done to understand the long-term effects of emotional stress on breathing.

70 Only one study has reported the impact of social challenge on breathing in the rat, but the  
71 results focused on only the first few minutes after the attack (14). The primary objective of this  
72 study was to assess the long-term effects of social defeat on breathing. We analyzed the effect of  
73 an anticipation-based social defeat procedure (37) on respiratory rate, acutely and continuously  
74 up to 25 days after the stress procedure (D+30). Respiratory rate was extracted from ECG  
75 recordings of respiratory sinus arrhythmia (RSA). RSA is a naturally occurring rhythm in the  
76 beat-to-beat heart rate pattern that occurs at the same frequency as respiration (8). It can be  
77 measured by spectral analysis of heart rate variability (HRV) as the highest peak in the high-  
78 frequency band (HF) (11, 43).

79 The secondary objective was to investigate the central mechanisms underlying these  
80 changes and, more specifically, the role of the dorsomedial hypothalamus (DMH) and 5-HT<sub>3</sub>  
81 receptors in the nucleus tractus solitarius (NTS). It has been clearly established that the DMH plays  
82 a critical role in mediating the cardiovascular and neuroendocrine response to acute (15) and  
83 chronic (37) psychological stress. This response may include respiratory effects, as demonstrated  
84 by a recent study showing that the DMH mediates tachypnea associated with acute stress

85 responses (7). Activation of NTS 5-HT<sub>3</sub> receptors is also known to contribute to the expression of  
86 the autonomic and cardiovascular changes evoked by chronic stress or acute stimulation of DMH  
87 (37, 38). These receptors also contribute to breathing control, while activation of 5-HT<sub>3</sub> receptors  
88 has an inhibitory effect on respiration (45).

89 In the first part of this study, we therefore determined the long-term effect on respiratory  
90 rate 10 and 30 days after social defeat, a chronic emotional stress that induces an anxiety-like  
91 state (6, 36, 37). We also tested the involvement of the DMH and 5-HT<sub>3</sub> NTS receptors in this  
92 effect. The second part of the study consisted of a longitudinal study using implanted telemetric  
93 probes in which we evaluated off-line changes in breathing during the 30 days that followed  
94 social defeat.

95

## 96 **Materials and Methods**

### 97 **Animals**

98 Experiments were carried out in male Sprague-Dawley rats (n=212, Centre d'Élevage R.  
99 Janvier, Le Genest-St.- Isle, France), weighing 290-310 g. They were housed in individual cages  
100 (length, 45 cm; width, 25 cm; height, 17 cm) for one week before the beginning of the  
101 experiments. Wild-type Groningen male rats (*Rattus norvegicus*, WTG strain), originally bred at  
102 the University of Groningen (The Netherlands) under conventionally clean conditions (40),  
103 weighing 400–500 g, served as resident rats, in confrontation encounters. The same WTG rats  
104 were used for all successive series of experiments. All animals were kept under controlled  
105 environmental conditions (22 ± 1°C; 60% relative humidity; 12 h light/dark cycle; food and water  
106 *ad libitum*). Procedures involving animals and their care were all performed in conformity with  
107 institutional guidelines, which are in compliance with national and international laws and policies  
108 (Council directive 87-848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service  
109 Vétérinaire de la Santé et de la Protection Animale; permissions 75855 to C. Sévoz-Couche and  
110 6180 to J.-J. Benoliel).

111

### 112 **Experimental overview**

113 The study was organized into two parts (Fig. 1).

114 Study 1 (n=168) was a cross-sectional study that ended either 10 days (group A, D+10, n=95) or  
115 30 days after the first social defeat session (group B, D+30, n=73).

116 Group A was divided into two cohorts. Animals in Cohort A1 (n=68) did not receive any  
117 treatment after social defeat, while those in Cohort A2 (n=27) received continuous infusion of an  
118 anxiolytic or saline from the time of the last social defeat session until D+10. At D+9, Cohorts  
119 A1 and A2 were tested in the elevated plus maze (EPM). At D+10, Cohorts A1 and A2 were  
120 anesthetized to record ECG and extract respiratory rate. Cohort A1 received random  
121 microinjections of saline or active drugs into the DMH or NTS (see below).

122 Group B was tested in the EPM at D+29, and anesthetized at D+30 to record ECG and received  
123 random microinjections saline or active drugs into the DMH or NTS (see below).

124

125 Study 2 (n=44) was a longitudinal study that ended 30 days after the first social defeat session  
126 (D+30). These animals were implanted with telemetric probes for daily recording of their ECG,  
127 which was then used to extract respiratory rate and reconstruct its time-course over the 30-day  
128 period. ECG was also recorded on the last day under anesthesia, as in animals of Study 1.

129

### 130 **Experimental procedures**

131 **Social defeat paradigm.** Social defeat consisted of four daily conditioning sessions (Fig. 1) that  
132 involved the same pairs of residents and intruders (3). Briefly, intruders were placed singly in a  
133 protective cage inside the resident home cage, allowing unrestricted visual, auditory, and  
134 olfactory contacts with the resident, but precluding close physical contact. The protective cage  
135 was then removed with the resident present, allowing physical confrontation with the intruder (D  
136 or defeated intruders) (three to four confrontations each lasting 10 s, during which the intruding  
137 animal was always dominated by the resident rat). For non-defeated intruders (ND, controls), the  
138 intruder had access to the entire resident home cage without the resident. ND rats were  
139 considered to be controls for social defeat due to the presence of the stress of a novel  
140 environment, but without application of a challenge. This chronic stress model induces an  
141 anxiety-like state that can be detected five days later on D+10 (6, 36, 37).

142 **Body weight.** Body weights in defeated and non-defeated rats were recorded daily at 9:00 a.m.,  
143 from 7 days before social defeat to the end of the protocol (Group A: D+10 and Group B: D+30).

144 **Elevated plus-maze test (EPM).** Five or twenty-five days after the last confrontation (D9 or D29,  
145 Fig 1), the elevated plus-maze test was used to evaluate anxiety-related behavior in the animals.  
146 The plus-maze consisted of a weakly illuminated plain wood structure with two open arms

147 (50×10cm) and two enclosed arms (50×10×40cm), placed at a height of 50cm. Rats were placed  
148 at the center of the plus-maze and allowed to freely explore the maze for 8 min. The rats'  
149 behavior was videotaped with an LCD camera connected to control and recording equipment  
150 located in an adjacent room. Animals were tested between 9:00 and 11:00 a.m. The time spent in  
151 the open and closed arms, and the numbers of entries into the open and closed arms of the plus-  
152 maze were recorded during 8 min with custom-made software. The total number (open+closed)  
153 of arm entries was taken as an indicator of general activity. A lower time spent in open arms was  
154 considered to be an indicator of an anxiety-like state.

155 **Adrenal gland weight.** At the end of the physiological recording, the animals were killed and  
156 their adrenal glands were removed and weighed. Data were expressed relative to body weight (in  
157 mg/100 g body weight).

158 **ECG recordings under anesthesia and RSA analysis.** Rats were anesthetized with pentobarbital  
159 sodium (Ceva Santé Animale, Libourne, France; 60 mg.kg<sup>-1</sup>, I.P.(39)) and placed in a stereotaxic  
160 frame, with the head fixed in the flat skull position. ECG was recorded using stainless steel pins  
161 placed subcutaneously into forepaws and hindpaws. These signals were amplified and filtered  
162 (Universal Amplifier; Gould, Courtaboeuf, France). The ECG signal was then relayed to a 1401  
163 interface (1401 Plus; CED, Cambridge, UK) connected to a computer running Spike 2 (version  
164 6.08) software (CED). Waveform data were imported offline into Spike CED (version 6.0). The  
165 RR interval signal was derived from the ECG. Power spectra were derived using fast Fourier  
166 transformation (size 256, Hanning window (33)), giving a final frequency resolution of 0.04 Hz.  
167 The spectra were performed on the time interval between two consecutive beats (RR interval)  
168 derived from the ECG. Low and high frequency (LF and HF, respectively) powers were  
169 calculated within the frequency ranges 0.2– 0.7 Hz and 0.7-2.5 Hz, respectively. RSA peak  
170 frequency is the highest peak in the HF band (37).

171

## 172 *Specific procedures*

### 173 *Study 1 (Cohort A1 and Group B):*

174 **Pharmacological blockade of the DMH.** Microinjections of either saline or muscimol (Sigma  
175 Chemicals, St Louis, USA; 500 pmol in 0.1 µl of saline) into the DMH were performed at the  
176 following coordinates: P 3.0, L 0.5 and V 8 mm from bregma (31). Injections were performed  
177 bilaterally to maximize the effect and because DMH control of HR has been shown to be



178 asymmetric (47). RSA was measured from 90-s segments 20 min before and 5 min after  
179 pharmacological blockade of the DMH.

180 **Pharmacological blockade of NTS 5-HT<sub>3</sub> receptors.** In other animals, microinjections of either  
181 saline or a selective 5-HT<sub>3</sub> receptor antagonist, granisetron (SmithKline-Beecham, Harlow, UK,  
182 250 pmol in 0.1 µl) were performed in the NTS at the level of the calamus scriptorius (38) (L 0.5  
183 and V 0.5 mm). Injections were also asymmetric bilaterally to maximize the effect. RSA was  
184 measured from 90-s segments 20 min before and 5 min after pharmacological blockade of NTS  
185 5-HT<sub>3</sub> receptors.

186 **Histology.** DMH and NTS microinjection sites were identified from the tip of the micropipette  
187 track in 70 µm thick sections of brain tissue previously fixed in 10% formalin solution and  
188 cryoprotected in 20% sucrose solution for 5 days. Only rats with injection sites correctly  
189 positioned in the DMH or NTS were considered for data analysis.

190

191 **Study 1 (Cohort A2):**

192 **Anxiolytic treatment.** ALZET osmotic pumps supplying vehicle (saline) or a benzodiazepine  
193 receptor agonist chlordiazepoxide (10 mg kg<sup>-1</sup> day<sup>-1</sup>, F. Hoffmann-La Roche, Basel,  
194 Switzerland) (36) were implanted in the rats in the morning after completion of the social defeat  
195 paradigm (D5). The pumps (ALZET 2ML1) were implanted subcutaneously on the back under  
196 light isoflurane anesthesia, as previously described (37). Vehicle or chlordiazepoxide was infused  
197 continuously from D5 to D+10 to prevent the development of anxiety-like state (36).

198

199 **Study 2 (Group A) Radiotelemetric probe implantation and ECG recordings in conscious rats.**

200 Anesthesia may affect respiratory rate. Therefore, to verify the absence of a confounding effect of  
201 anesthesia on changes in respiration, RSA peak frequency was analyzed in conscious rats  
202 implanted with radiotelemetric probes. These experiments also allowed us to monitor the changes  
203 of this parameter over time. Two weeks before social defeat (D-15), rats were implanted with  
204 radiotelemetric probes (Data Sciences International, St. Paul, MN, USA) to enable recording of  
205 ECG and locomotor activity (Fig 1). Surgery was performed under aseptic conditions and under  
206 anesthesia (Isoflurane). The rats were also pretreated with an analgesic (Xylocaine, 5 mg/kg,  
207 s.c.), an anti-inflammatory drug (metacam, 1 mg/kg, s.c.) and received antibiotics (Penicillin, 0.3  
208 ml, i.p.) at the end of surgery. The probes were implanted in the peritoneal cavity and wires were

209 tunneled subcutaneously along the rib cage. The positive lead was attached to the dorsal side of  
210 the xiphoid process and the negative lead was passed between the right sternocleidomastoid and  
211 sternohyoid muscles beside the trachea towards the manubrium and thoracic inlet (41).  
212 ECG was recorded every afternoon (1 to 6 pm) during the week from D-3 to D+30. The  
213 afternoon was chosen for two main reasons. First, recordings are more stable when animals are  
214 less active, even if sniffing and active periods associated with tachypneic episodes occur  
215 sporadically. Second, the social defeat procedure took place in the morning (when animals are  
216 receptive to stress); ECG was analyzed after the stress procedure and recordings therefore started  
217 and were continued in the afternoons. Data were acquired using Dataquest A.R.T. 3.1 Gold  
218 software (Data Sciences). ECG waveform data were imported offline into Spike CED (version  
219 6.0) and were then analyzed to extract RSA peak frequency as described above for Study 1. The  
220 RSA peak was determined from 2-min segments of ECG data and averaged across four segments.  
221 These segments may have contained periods of sniffing (characterized by high respiratory rate,  
222 (23)), however, these periods were short (only a few seconds). In any case, we have previously  
223 shown that sniffing bouts did not interfere with respiratory rate measurements derived from RSA  
224 analysis (8). ECG was also recorded on the last day (D+30), under pentobarbital sodium  
225 anesthesia, as mentioned above. Signals were exported to Spike 2 software (version 6.14, CED,  
226 UK) for off-line respiratory and ECG analyses.

227

228

### 229 **Statistical analysis**

230 Differences in behavioral and physiological parameters between defeated and non-defeated  
231 groups were analyzed using an unpaired Student's *t* test. Comparisons of pharmacological  
232 treatments (pumps or microinjections) between defeated and non-defeated groups used a two-way  
233 ANOVA. Changes in body weight and RSA peak frequency between defeated and non-defeated  
234 groups across time were analyzed by two-way repeated-measure ANOVA. Bonferroni *post hoc*  
235 correction was applied after ANOVA when necessary, and the results were considered to be  
236 significant for  $P < 0.05$ . Statistical analysis was performed with Prism 5.04 (GraphPad Software).

237

238 **Results**

239 **Study 1/Group A/Cohort A1. Behavioral and respiratory changes evoked by social defeat at**  
240 **D+10 and contribution of DMH neurons and NTS 5HT<sub>3</sub> receptors.**

241 Cohort A1 comprised a total of 68 animals, which were either defeated intruders (D, n=36) or  
242 non-defeated intruder controls (ND, n=32). The experiment stopped at D+10, 6 days after the last  
243 social defeat session (Fig. 1).

244 *Body weight.* Changes in body weight before, during and after social defeat are shown on Fig.  
245 2A. No significant difference in body weight was observed between ND and D rats during the  
246 days preceding social defeat (416±3 and 415±3 g, respectively, at D-1). However, D rats stopped  
247 gaining weight right from the first social defeat session, while ND rats continued their normal  
248 growth. By the end of social defeat, D rats weighed less than ND rats, (423±4 vs 447±4 g,  
249 respectively at D5). Weight gain in D rats remained decreased over the following days, further  
250 widening the gap with ND rats until the last day (430±6g vs 470±7g, respectively, at D+10). A  
251 repeated-measure ANOVA from D1 to D+10 confirmed a statistically significant difference  
252 between the two groups (p=0.001, Fig 2A) and Bonferroni *post hoc* analysis showed that the  
253 difference was significant from D4 onwards (p<0.05).

254 *Elevated plus-maze test.* This behavioral test was performed at D9 to assess the level of anxiety in  
255 the animals. The percentage of time spent in the open arm was significantly lower (34 %) in D  
256 rats compared to ND rats (137±5 vs 208±6 s, respectively, p<0.001, Fig. 2B). No significant  
257 difference in the total number of arm entries was observed between the two groups (29±2 vs  
258 25±3, respectively, p=0.7), indicating that the reduced time spent in the open arms was due to a  
259 higher level of anxiety rather than a reduced level of general activity.

260 *Adrenal gland weight.* Adrenal gland weight was significantly higher in D rats than in ND rats at  
261 D+10 (13.6±0.4 vs 10.3±0.3 mg/100g, respectively, p<0.001, Fig. 2C), indicating increased  
262 activity in the stress axis. Thus, as previously reported (37) (36), all D rats presented an anxiety-  
263 like state at D+10.

264 *RSA peak frequency analysis.* Power spectral analysis performed on the RR interval signal  
265 extracted from the ECG at D+10 revealed that D rats had a lower RSA peak frequency than ND  
266 rats (1.28±0.02 vs 1.65±0.02 Hz, respectively, p<0.001, Fig. 3A&B), corresponding to  
267 respiratory rates of 76.51±2.10 and 99.88±2.19 cpm, respectively. Remarkably, RSA peak  
268 frequency values in D rats were all lower than in ND rats with no overlap between the two

269 groups. In addition, RSA was negatively correlated with adrenal gland weight (Fig 3 C). Social  
270 defeat therefore induced very marked bradypnea in these animals, associated with anxiety.

271 *Effect of DMH inhibition and NTS 5-HT<sub>3</sub> receptor blockade on RSA peak frequency.*

272 Previous work from our laboratory has shown that DMH inhibition and NTS 5-HT<sub>3</sub> receptor  
273 blockade markedly reduce the long-term cardiovascular effects evoked by social defeat when  
274 recorded at D+10 (37). To determine whether this was also the case for bradypnea, we randomly  
275 selected ND and D of this cohort for microinjection of either saline or muscimol into the DMH,  
276 and either saline or granisetron into the NTS. As shown in Fig. 4A1, the reduction in RSA peak  
277 frequency observed in D rats compared to ND rats was still observed after saline but not after  
278 bilateral muscimol microinjections into the DMH (Fig. 4 A1 & A2), which was confirmed by a  
279 statistically significant interaction between defeat and treatment effects. Similarly, the reduction  
280 in RSA peak frequency in D rats (Fig. 4 B1) was still observed after saline but not after bilateral  
281 injections of granisetron into the NTS (Fig. 4 B1 & B2), which was also confirmed by a  
282 significant interaction between defeat and treatment effects.

283 DMH neurons and 5-HT<sub>3</sub> NTS receptors therefore contribute not only to cardiovascular changes  
284 but also to the bradypnea associated with the anxiety-like state induced by social defeat. We then  
285 investigated whether bradypnea was a result of the anxiety-like state and whether it could be  
286 prevented by anxiolytic treatment.

287

288 **Study 1/Group A/Cohort A2- Behavioral and respiratory changes evoked by social defeat at**  
289 **D+10, after anxiolytic treatment.**

290 ND and D rats (n=14 and 13, respectively) of Cohort A2 were treated with a continuous infusion  
291 of anxiolytic (chlordiazepoxide) from D4 to D+10. Behavioral tests and physiological recordings  
292 were the same as in Cohort A1 (Fig. 1).

293 *Elevated plus-maze test.* Saline-treated D rats still spent less time in open arms than ND rats, as in  
294 Cohort A1. However, this difference was no longer observed after chlordiazepoxide treatment  
295 (Fig. 5A) and this effect was confirmed by a significant interaction between defeat and treatment  
296 effects. In other words, anxiolytic treatment was effective.

297 *Adrenal gland weight.* As with the elevated plus maze test, chlordiazepoxide treatment prevented  
298 the increase in adrenal gland weight induced by social defeat in D rats (Fig. 5B), which was  
299 confirmed by a significant interaction between defeat and treatment effects.

300 *RSA peak frequency analysis.* As in cohort A1, a reduction in RSA peak frequency was observed  
301 in saline-treated D rats. Chlordiazepoxide treatment practically abolished this effect (Fig. 5C),  
302 which was also confirmed by a significant interaction between defeat and treatment effects.

303 These results confirm those observed in Cohort A1 and demonstrate that the bradypnea observed  
304 in D rats at D+10 is due to the state of chronic anxiety induced by social defeat. The next  
305 question was whether this bradypnea would persist at later times, eg, at D+30, 20 days later,  
306 when anxiety levels are known to have returned to normal (6).

307

308 **Study 1/Group B. Behavioral and respiratory changes evoked by social defeat at D+30 and**  
309 **contribution of DMH neurons and NTS 5HT<sub>3</sub> receptors.**

310 Group B comprised a total of 73 rats (51 D and 22 ND rats), which were kept until D+30, 25 days  
311 after the last social defeat session (Fig. 1).

312 *Body weight.* Changes in body weight from D-6 to D+10 were practically the same as in Cohort  
313 A1 (Fig. 6A). D rats did not gain weight during the four days of social defeat, and recovered  
314 slowly over the following days compared to ND rats (D5: 403±3 vs 433±5g, D+10: 424±3 vs  
315 453±5g, respectively). However, D rats gradually increased their weight gain thereafter, slowly  
316 closing the gap with ND rats. A repeated-measure ANOVA from D1 to D+30 confirmed a main  
317 group effect (p=0.019). *Post hoc* analysis showed that the difference between D and ND rats was  
318 statistically significant from D5 to D12, but not thereafter.

319 *Elevated plus-maze test.* D rats spent the same amount of time in the open arms as ND rats  
320 (183±6 vs 194±10 s, respectively, p=0.4, Fig. 6B). No sign of an anxiety-like state was therefore  
321 detected in D rats at D29, in contrast with Cohort A1 rats when they were tested at D9.

322 *Adrenal gland weight.* Similarly, at D+30, adrenal gland weight in D rats was the same as in ND  
323 rats (10.7±0.3 vs 10.0±0.4 mg/100g, p=0.3, Fig. 6C).

324 *RSA peak frequency analysis.* RSA peak frequency at D+30 was still lower in D rats than in ND  
325 rats (1.44±0.04 vs 1.58±0.02 Hz, respectively, p=0.008, Fig. 7A), although the difference was not  
326 as marked as at D+10 (less than half). More detailed analysis of individual data of the D group  
327 suggested that this group was composed of two subgroups. Using the 5% percentile of the RSA  
328 peak frequency of the ND group as the cut-off (1.40 Hz), we divided the D group into two  
329 subgroups: the D<sub>L</sub> subgroup for D rats in which the RSA peak frequency was lower than the cut-  
330 off (RSA: 1.28±0.02 Hz, n=23) and the D<sub>H</sub> subgroup in which the RSA peak frequency was

331 higher than the cut-off ( $1.59 \pm 0.02$  Hz,  $n=28$ ). A main group effect was still observed when  
332 comparing ND, D<sub>H</sub> and D<sub>L</sub> ( $F(2,70)=83.6$ ,  $p<0.001$ ), with a significant difference between ND  
333 and D<sub>L</sub> ( $p<0.001$ ) but not between ND and D<sub>H</sub> ( $p=0.9$ ) (Bonferroni *post hoc* analysis). A similar  
334 analysis was then performed for the other parameters using these three groups (ND, D<sub>H</sub> and D<sub>L</sub>).  
335 No difference in terms of body weight change was detected between D<sub>H</sub> and D<sub>L</sub> over the period  
336 D1 to D+30 ( $p=0.88$ , Fig. 7B) and no main effect was detected for time spent in open arms in the  
337 EPM or adrenal gland weight (Fig. 7C and 7D, respectively  $F(2,70)=2.3$ ,  $p=0.15$  and  
338  $F(2,70)=1.34$ ,  $p=0.26$ ), indicating the absence of difference in anxiety-like state between ND rats  
339 and the two subgroups of D rats.

340 Thus, although the anxiety levels of D rats had recovered by D+30, half of the rats (D<sub>L</sub>) still  
341 presented the same bradypnea as at D+10. We then tested the role of the DMH and 5HT<sub>3</sub> NTS  
342 receptors in this D+30 bradypnea as performed for the D+10 bradypnea in Cohort A1 animals.

343 *Effect of DMH inhibition and NTS 5-HT<sub>3</sub> receptor blockade.* As shown in Fig. 8A, the D+30  
344 bradypnea was still observed in D<sub>L</sub> rats after bilateral saline microinjections into the DMH, but  
345 not after bilateral muscimol microinjections. In fact, the RSA peak frequency in muscimol-  
346 injected D<sub>L</sub> animals was the same as in ND and D<sub>H</sub> rats injected with muscimol or saline.  
347 Statistical analysis confirmed a significant interaction between defeat and muscimol with pair-  
348 wise *post hoc* comparisons showing a significant difference between muscimol- and saline-  
349 injected D<sub>L</sub> rats ( $p=0.003$ ) but not between muscimol- and saline-injected ND and D<sub>H</sub> rats  
350 ( $p=0.85$  and  $p=0.857$ , respectively). Similarly, the D+30 bradypnea was still observed in D<sub>L</sub> rats  
351 after bilateral saline microinjections into the NTS, but not after bilateral granisetron  
352 microinjections (Fig. 8B). A significant interaction was observed between defeat and granisetron  
353 with significant pair-wise *post hoc* comparisons between granisetron- and saline-injected D<sub>L</sub> rats  
354 ( $p<0.001$ ) but not ND and D<sub>H</sub> rats ( $p=0.63$  and  $p=0.89$ , respectively).

355 DMH neurons and 5HT<sub>3</sub> NTS receptors therefore contribute to the bradypnea observed at  
356 D+30 in D<sub>L</sub> rats as at D+10, suggesting that the same mechanism is involved at the two time-  
357 points. In other words, social defeat in these D<sub>L</sub> rats produced a long-lasting effect on respiration  
358 that outlasted the anxiety-like state. We then tried to determine when D<sub>H</sub> rats recovered and  
359 whether there was any difference between D<sub>L</sub> and D<sub>H</sub> rats at an earlier stage.

360

361 **Study 2. Respiratory changes evoked by social defeat at D+30 and time-course of**  
362 **respiratory changes.**

363 This experiment was a longitudinal study conducted on 44 rats implanted with  
364 radiotelemetric probes and kept until D+30. At D+30, all 44 animals were anesthetized and RSA  
365 peak frequency was extracted from the ECG as in Study 1/Group B. As shown on Fig. 9A and as  
366 mentioned above, RSA peak frequency presented a wide distribution in the D group at D+30,  
367 very similar to that observed in Study 1/Group B. Consequently, the D group was divided into D<sub>H</sub>  
368 and D<sub>L</sub> subgroups according to the 5% percentile of RSA peak frequency of the ND group (1.32  
369 Hz). Comparison of these three groups (ND, n=12; D<sub>L</sub>, n=15; D<sub>H</sub>, n=17) revealed a significant  
370 group effect ( $F(2,41)=40.07$ ,  $p<0.001$ ), with a significantly lower RSA peak frequency in D<sub>L</sub>  
371 compared to ND ( $p<0.001$ ) and D<sub>H</sub> ( $p<0.001$ ), while ND and D<sub>H</sub> were similar ( $p<0.05$ ). Adrenal  
372 glands were also weighed at D+30. As in Study 1/Group B, no significant difference was  
373 observed between ND, D<sub>H</sub> and D<sub>L</sub> ( $8.96\pm 0.45$ ,  $9.67\pm 0.37$  and  $8.2\pm 0.3$  mg/100g, respectively,  
374  $F(2,41)=2.5$ ,  $p=0.10$ ).

375 In the same animals, we performed off-line analysis of the changes in RSA peak  
376 frequency extracted from the daily telemetric ECG recording in ND, D<sub>L</sub> and D<sub>H</sub> rats over the  
377 preceding 30 days (Fig. 9B). The three groups of rats had the same average RSA peak frequency  
378 before social defeat on D-3 ( $1.56 \pm 0.05$ ,  $1.59 \pm 0.03$  and  $1.54 \pm 0.04$  Hz, respectively). A marked  
379 drop in RSA peak frequency was then observed in the defeated D<sub>L</sub> and D<sub>H</sub> animals, which lasted  
380 until D+10 as in Study 1/Cohort A1. Thereafter, D<sub>H</sub> rats gradually recovered. Within one week,  
381 by D17, they had fully recovered and presented the same RSA peak frequency as ND rats. In  
382 contrast, D<sub>L</sub> did not recover and had a persistently low RSA peak frequency until the last day. A  
383 repeated-measure ANOVA over the entire 30 days confirmed a significant defeat effect  
384 ( $p<0.001$ ) and a significant interaction between defeat and time ( $p<0.001$ ). Bonferroni *post hoc*  
385 analysis revealed that D<sub>H</sub> and D<sub>L</sub> rats had significantly lower RSA peak frequency than ND rats  
386 by D2 ( $1.35 \pm 0.03$ ,  $1.33 \pm 0.04$  and  $1.58 \pm 0.06$  Hz, respectively) and that, at D17, RSA peak  
387 frequency became significantly higher in D<sub>H</sub> ( $1.48\pm 0.04$ ) than in D<sub>L</sub> ( $1.33\pm 0.03$ ) and equivalent  
388 to ND ( $1.51\pm 0.04$  Hz). These differences persisted at D+30, when they were equivalent to those  
389 recorded under anesthesia.

390

391

392 **Discussion**

393 This study shows, for the first time, that social defeat induces long-lasting bradypnea that  
394 can be detected as a reduction in RSA peak frequency. At D+10, the effect was observed in all  
395 defeated animals and was associated with an elevated level of anxiety. No anxiety was detected  
396 20 days later, at D+30. However, bradypnea was still present in approximately half of the  
397 defeated animals. Importantly, this long-lasting respiratory change involves the DMH and NTS  
398 5-HT<sub>3</sub> receptors, as we have previously shown for the associated cardiovascular changes (37).

399  
400

401 *Respiratory changes induced by social defeat*

402 Respiratory rate was extracted from the RSA peak frequency of the ECG by analysis of  
403 HRV. Peripheral and central respiratory/cardiovascular regulatory mechanisms are tightly  
404 coupled. RSA constitutes an element of this interaction, as reflected by the regular increase in  
405 heart rate during inspiration and its decrease during expiration. RSA is also referred to as high  
406 frequency (HF) HRV, with reference to the relatively high frequency range at which the  
407 parasympathetic but not the sympathetic division of the autonomic nervous system can respond to  
408 respiration and influence heart rate (49). RSA amplitude is consequently an indicator of the  
409 sensitivity of parasympathetic cardiac activity during the breathing cycle (34), and we have  
410 previously found that social defeat reduces this gain (37). On the other hand, RSA peak  
411 frequency is a reliable method for extracting respiratory rate in both anesthetized and conscious  
412 animals, as it provides respiratory rates comparable to tracheal and pleural respiratory rates under  
413 conditions of both low activity and high activity (8). Long-term changes in respiratory rate after a  
414 social challenge had not been previously studied. We therefore investigated long-term changes in  
415 RSA peak frequency (and therefore changes in basal respiratory rate) after the social defeat  
416 procedure used in a previous study (37).

417 As expected, all defeated rats lost weight following the social defeat paradigm, indicating  
418 that this paradigm constitutes a major stress. At D+10, defeated rats spent less time in the open  
419 arm of the EPM and adrenal gland weight was higher in defeated rats compared to non-defeated  
420 rats. All defeated animals presented bradypnea, which was observed under anesthesia (Study 1)  
421 an in the conscious state when animals were at rest (Study 2 with telemetry). More importantly,  
422 bradypnea was related to the state of stress, as it was i/ correlated with adrenal gland weight, and



423 ii/ prevented by anxiolytic treatment from D5 to D+10. Bradypnea was therefore associated with  
424 onset of an anxiety-like state until D+10.

425 RSA was highly variable in D animals at D+30 in contrast with D+10. One half of the  
426 population presented an RSA at D+30 comparable to that observed in ND animals. Consequently,  
427 the D group was subdivided using the 5% percentile of the ND group distribution as the cut-off.  
428 When this cut-off was used, bradypnea was still observed in one half of the defeated animals at  
429 D+30 ( $D_L$ ), although the anxiety-like state had resolved at that time, as no signs of anxiety or  
430 stress were observed in these bradypneic animals, as measured by time spent in the open arms of  
431 the EPM or adrenal gland weight. In this respect, these animals did not differ from the other  
432 group of defeated rats, in which respiratory rate returned to normal ( $D_H$ ), or from the non-  
433 defeated animals.

434 Off-line examination of changes in respiratory rate in longitudinal Study 2 (telemetry)  
435 revealed that  $D_L$  and  $D_H$  both had low RSA peak frequencies after social defeat. ECG segments  
436 used for extraction of RSA peak frequency presumably contained periods of sniffing  
437 (characterized by high respiratory rate (23)), as they represented 4 hours of recording. Periods of  
438 sniffing would have been short (only a few seconds) and, as it has been shown that frequencies  
439 above heart rate do not modify RSA frequency, it is unlikely that they would have affected  
440 calculation of this parameter (8). However, sniffing periods were not taken into account in this  
441 analysis, which constitutes a limitation of this study.

442  
443  $D_H$  animals started recovering after D+10 and the group had completely recovered one  
444 week later, at D17. Interestingly, our previous work has shown that the anxiety-like state persists  
445 until D15 in defeated animals (36). It is therefore likely that, as expected, bradypnea recovered in  
446 parallel with the anxiety state in  $D_H$  rats. So, why did the respiratory rate of  $D_L$  rats not recover?  
447 What made these animals different? ,  $D_L$  rats were not significantly different from  $D_H$  rats or non-  
448 defeated rats (ND) in terms of respiratory rate prior to social defeat. However, a trend towards a  
449 more marked reduction of respiratory rate was observed in  $D_L$  rats during the social defeat period.  
450 This difference was not statistically significant, but it may be a sign that these animals were either  
451 more sensitive or had experienced more intense distress during social defeat or were less resilient.  
452 Examination of the weight curve of  $D_L$  and  $D_H$  rats in Study 1 did not reveal any notable  
453 difference between the two groups of rats before, during or in the 10 days following social defeat.

454

455 ***Role of the DMH and NTS 5-HT<sub>3</sub> receptors in the respiratory changes induced by social defeat***

456 Social defeat induces a persistent increase in c-fos protein in the hypothalamus (including  
457 the DMH) and the NTS (28). We know from previous work that the DMH plays a key role in  
458 autonomic alteration (increase in LF/HF ratio associated with a reduction in RSA amplitude and  
459 parasympathetic baroreflex gain) evoked by social defeat at D+10 (37). This inhibition of cardiac  
460 parasympathetic activity, which is GABAergic and occurs on vagal preganglionic neurons, is  
461 thought to result from activation of presynaptic vagal 5-HT<sub>3</sub> receptors in the NTS, themselves  
462 indirectly activated by the DMH via a cascade of activation involving the dorsolateral  
463 periaqueductal gray (dIPAG) and serotonergic cells in the raphe magnus (5, 37). This 5-HT<sub>3</sub>-  
464 mediated inhibition of the cardiac vagal activity induced by chronic stress appears to be mediated  
465 via activation of NTS GABAergic interneurons that block the second-order neurons of the  
466 baroreceptor arc, as previously observed after acute DMH stimulation (38). To determine  
467 whether NTS 5-HT<sub>3</sub> receptors are also involved in the DMH-induced bradypnea evoked by social  
468 defeat, we blocked the DMH with bilateral microinjections of muscimol and antagonized 5-HT<sub>3</sub>  
469 NTS receptors with bilateral microinjections of granisetron. As with cardiac vagal inhibition, we  
470 found that blockade of DMH and 5-HT<sub>3</sub> NTS receptors abolished the bradypnea of defeated rats  
471 at both D+10 and D+30 (in D<sub>L</sub> rats). Similar results were found when granisetron was  
472 administered systemically (data not shown), as 5-HT<sub>3</sub> receptor antagonists can cross the blood-  
473 brain barrier (10). In line with these results, systemic administration of ondansetron, another 5-  
474 HT<sub>3</sub> receptor antagonist, has been shown to prevent sleep apneas (46). These findings suggest  
475 that the same neuronal pathway that mediates cardiac vagal inhibition may also mediate  
476 bradypnea. It is noteworthy that cells sensitive to H<sup>+</sup>/CO<sub>2</sub> in the retrotrapezoid/parafacial (RTN-  
477 pFRG) region, located in the ventral medulla and linked to the central command of respiration,  
478 participate in maintenance of a tonic respiratory drive. The RTN-pFRG region receives direct  
479 GABAergic inhibitory inputs from the NTS (30). GABAergic inhibition from the NTS to  
480 chemoreceptor RTN/pFRG neurons, following DMH activation, may therefore inhibit the tonic  
481 excitatory drive exerted by this region on the central respiratory pattern generator, leading to  
482 bradypnea. Further studies are needed to support this hypothesis.

483

484 It has been known for a long time that the DMH is a key structure involved in the  
485 physiological response to acute stress, i.e. the defense reaction (32), and that its activation  
486 induces an array of physiological responses including arousal, and increased blood pressure, heart  
487 rate and regional vascular resistance (12, 13). While the pathway involved in the tachycardic  
488 response remains controversial, it appears that the hypertensive response is due to activation of  
489 the rostroventrolateral part of the medulla (16). Classically, an increase in respiratory activity is  
490 also the characteristic feature of the physiological response to psychological stress. Some studies  
491 investigating the effects of disinhibition of neurons within the DMH on respiratory rate (35) or  
492 phrenic nerve activity (29) concluded that acute DMH activation induces increased respiratory  
493 activity. Conversely, muscimol blockade of the DMH prevents the tachypnea evoked by acute  
494 stress, such as a novel environment or restraint (7), or activation of the dIPAG (20). The  
495 descending pathways mediating the DMH-evoked increase in respiratory activity may involve  
496 chemosensitive orexin neurons. In support of this hypothesis, microdialysis of CO<sub>2</sub>-enriched fluid  
497 (25% CO<sub>2</sub>) into the orexin neuron-rich DMH/perifornical region of the hypothalamus increases  
498 resting activity (26), and activation of hypothalamic orexin neurons produces defense-like or  
499 panic-like cardiorespiratory responses (17, 21, 22). Descending orexin projections to the  
500 retrotrapezoid/parafacial (RTN-pFRG) region, located in the ventral medulla and intimately  
501 related to the central command of respiration, have also been identified (25). Given that DMH is  
502 almost certainly activated during exposure to the aggressive resident and that the breathing  
503 response almost certainly corresponds to tachypnea, defeated rats would have been expected to  
504 present long-term hyperventilation. However, we found that the DMH in defeated rats was the  
505 origin of the bradypnea mediated by NTS 5-HT<sub>3</sub> receptors. One explanation for this effect may  
506 be provided by a study by Lutter et al. (27) that showed decreased hypothalamic prepro-orexin  
507 mRNA and orexin cell count and activation after social defeat. The hypothalamic area sampled  
508 included the DMH. If orexin expression was reduced in our defeated rats, then the orexinergic  
509 drive on breathing would also have been reduced. This reduction could have left the NTS 5-HT<sub>3</sub>  
510 and GABA<sub>A</sub> receptor inhibitory pathway to the RTN unopposed, tilting the balance in favor of a  
511 bradypnea instead of tachypnea, in response to chronic activation of the DMH. Further studies  
512 are needed to evaluate the level of orexin expression in the DMH after application of our stress  
513 procedure. In addition, long-term changes may have occurred in the DMH and/or its targets that  
514 would have modified the way they modulate breathing. For example, the DMH could have

515 remained active in the days following stress exposure, but at a subthreshold level, nevertheless  
516 sufficient to activate NTS 5-HT<sub>3</sub>-mediated bradypnea, but too low to increase respiratory rate.  
517 This mechanism needs to be further investigated, but we have observed that subthreshold  
518 electrical or chemical DMH stimulation, that does not increase basal respiratory rate, inhibits  
519 RTN/pFRG chemoreceptor-induced increases in ventilation via activation of a NTS 5-  
520 HT<sub>3</sub>/GABA<sub>A</sub> receptor mechanism (48). The fact that the DMH may remain activated at a sub-  
521 threshold state until at least D+30 in D<sub>L</sub> rats may also explain why these rats still presented  
522 bradypnea after anxiety behavior had resolved.

523

524

525

## 526 **Perspectives and Significance**

527 To the best of our knowledge, there is no published report on the long-term changes in  
528 respiration associated with post-traumatic stress disorder (PTSD). Anxiety and panic disorder are  
529 classically associated with hyperventilation (31); Carnevali et al. 2013; Grassi et al. 2014 (9).  
530 However, our results show an opposite effect. This difference is perhaps due to the form of stress  
531 applied in our study, i.e. social defeat, or to the fact that this stress was repeated and not just a  
532 single traumatic event, and therefore associated with anticipation and inescapability.  
533 Nevertheless, the findings of this study are consistent with those described by Kinkead et al  
534 (2009) in adult rats after neonatal separation or those reported by Padley et al (2005) in Flinder-  
535 Sensitive rats.

536 The long-term consequences of bradypnea are unknown, but Anderson et al suggested the  
537 possibility that chronic anticipation of an avoidance task may lead to hypertension as a  
538 consequence of a decrease in respiratory rate and therefore renal excretory function (2). A daily  
539 correlation between the development of hypertension and hypoventilation would help to support  
540 this hypothesis. Further work is needed, especially in PTSD patients.

541

542 In conclusion, our social defeat procedure induced long-lasting changes in breathing,  
543 leading to chronic bradypnea. All defeated rats were initially affected. Recovery occurred in  
544 some animals, but bradypnea persisted in a group of sensitive rats. The central mechanisms

545 underlying this long-lasting effect may include sub-threshold activation of the DMH that reduced  
546 respiratory rate via excitation of NTS 5-HT<sub>3</sub> receptors.

547

548

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557

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698

699

700 **Figure Captions**

701  
702

703 **Figure 1**

704 **Protocol of social defeat.**

705 The experimental procedure consisted of four daily conditioning sessions (*D1–D4*) involving the  
706 same pairs of residents and intruders. Two main studies were conducted, during which RSA peak  
707 was extracted from ECG. In Study 1, RSA peak was extracted under anesthesia at i) D+10 (group  
708 A) with microinjections into the DMH and NTS (cohort A1) or with anxiolytic treatment (cohort  
709 A2), and ii) at D+30 (group B) with microinjections into the DMH and NTS. The elevated plus  
710 maze (EPM) test was performed the day before ECG recordings. In Study 2, RSA peak only was  
711 extracted daily in conscious rats implanted with radiotelemetric probes, and finally under  
712 anesthesia at D+30.

713

714 **Figure 2**

715 **Long-term effects (D+10) of social defeat on behavioral parameters in non-defeated (ND)**  
716 **and defeated (D) rats, in study 1 group A cohort A1.**

717 A. Daily body weight measured before, during, and after the four days of social defeat (SD).  
718 After the last social defeat session, body weights of D animals were lower than those of ND rats,  
719 and this difference persisted until D+10. Each point is the mean±SEM of data obtained in D and  
720 ND rats. \* over bar indicates period when D and ND were significantly different ( $p<0.05$ ,  
721 Bonferroni *post hoc* analysis).

722 B. Evaluation of the anxious profile in the elevated plus maze test at D9: D animals spent less  
723 time in the open arms than ND rats. Values are the mean±SEM of data obtained in D and ND  
724 rats. \*\*\* $p<0.001$  versus ND rats.

725 C. Evaluation of the hypothalamic-pituitary-adrenal axis: adrenal gland weight calculated relative  
726 to body weight was higher in D rats than in ND rats. Values are the mean±SEM of data obtained  
727 in D and ND rats. \*\*\* $p<0.001$  versus ND rats.

728

729 **Figure 3**

730 **Long-term effects (D+10) of social defeat on respiratory parameters in non-defeated (ND)**  
731 **and defeated (D) rats, in study 1 group A cohort A1.**

732  
733 A. Spectral analysis of RR intervals in two representative animals under anesthesia: the RSA  
734 peak shifted to a lower frequency in D rats (1.3Hz) compared to ND rats (1.7 Hz), indicating  
735 long-lasting bradypnea in stressed animals.  
736 B & C. RSA peak frequency was lower in D rats than in anesthetized ND rats (B), and was  
737 correlated with adrenal gland weight in D but not in ND rats (C). Values are the mean±SEM of  
738 data obtained in ND and D animals. \*\*\* $p < 0.001$  versus ND rats.

739  
740 **Figure 4**  
741 **Effect of blockade of DMH and NTS 5-HT<sub>3</sub> receptors on bradypnea induced by social**  
742 **defeat at D+10, in study 1 group A cohort A1.**

743 In anesthetized rats, at D+10, local microinjections of muscimol (musc, 5 mM) into the DMH (A)  
744 or granisetron (grani, 2.5 mM) into the NTS (B) reversed the decrease in RSA peak frequency  
745 normally observed in defeated rats. Values are the mean±SEM of data obtained in D and ND rats.  
746 \*\*\* $p < 0.001$  versus ND. ##  $p < 0.01$  and ### $p < 0.001$  versus saline  
747 A2 and B2: Photomicrographs showing representative examples of microinjection sites (arrows)  
748 in the DMH (A2) and NTS (B2). AP: area postrema, cc: central canal, Cu: cuneate nucleus,  
749 DMH: dorsomedial nucleus of the hypothalamus, Gr: gracilis nucleus, VMH: ventromedial  
750 nucleus of the hypothalamus, NTS: nucleus tractus solitarii, mt: mammillothalamic tract, 12:  
751 hypoglossal nucleus.

752  
753  
754 **Figure 5**  
755 **Effects of anxiolytic treatment on behavioral and respiratory changes induced by social**  
756 **defeat at D+10, in study 1 group A cohort A2.**

757 Compared to vehicle (saline), chlordiazepoxide (CDP) treatment prevented the decrease in time  
758 spent in open arms of the elevated plus maze (A), the increase in adrenal gland weight (B) and  
759 the bradypnea (C) normally observed in defeated rats at D+10. Values are the mean±SEM of data  
760 obtained in D and ND rats. \* $p < 0.05$  and \*\* $p < 0.01$  versus ND, # $p < 0.05$ , ## $p < 0.01$  and ### $p < 0.001$   
761 versus saline.

762

763 **Figure 6**

764 **Long-term effects (D+30) of social defeat on behavioral parameters in non-defeated (ND)**  
765 **and defeated (D) rats, in study 1 group B.**

766 A. As previously observed, body weights of D rats were lower than those of ND rats during the  
767 first days of conditioning sessions (i.e. social defeat, SD) and at least until D+10. However, these  
768 differences were no longer observed at D+30. Each point is the mean±SEM of data obtained in D  
769 and ND rats. \* over horizontal bar indicates period when D and ND were significantly different  
770 ( $p<0.05$ , Bonferroni *post hoc* analysis).

771 B and C. Evaluation of the anxious profile and the hypothalamic-pituitary-adrenal axis of D and  
772 ND rats in the elevated plus maze test at D29. No difference was observed in time spent in the  
773 open arms of the elevated plus maze (B) and adrenal gland weight relative to body weight (C)  
774 between ND and D rats. Values are the mean±SEM of data obtained in D and ND rats.

775

776 **Figure 7**

777 **Long-term effect (D+30) of social defeat on respiration and physiological parameters in**  
778 **non-defeated (ND) and defeated (D) rats in study 1 group B.**

779 A. At D+30, RSA was still lower in D rats than in ND rats. However, D rats could be subdivided  
780 relative to a 5% percentile of RSA peak frequency at D+30 of the ND group (1.40 Hz), resulting  
781 in two subgroups, D<sub>H</sub> (RSA above 1.40 Hz) and D<sub>L</sub> (RSA below 1.40 Hz). RSA was similar in  
782 D<sub>H</sub> rats and ND rats, while RSA was lower in D<sub>L</sub> rats than in ND and D<sub>H</sub> rats.

783 B. Body weights of D<sub>H</sub> (RSA above 1.40 Hz) and D<sub>L</sub> (RSA below 1.40 Hz) rats were similar  
784 throughout the entire protocol. Each point is the mean±SEM of data obtained in D<sub>H</sub> and D<sub>L</sub> rats.  
785 SD: social defeat.

786 C and D. Evaluation of the anxious profile and the hypothalamic-pituitary-adrenal axis of D<sub>H</sub> and  
787 D<sub>L</sub> rats in the elevated plus maze test at D9. No difference was observed in time spent in the open  
788 arms (B) and adrenal gland weight relative to body weight (C) between D<sub>H</sub> and D<sub>L</sub> rats. Values  
789 are the mean±SEM obtained in D<sub>H</sub> and D<sub>L</sub> rats.

790 \*\* $p<0.01$  and \*\*\* $p<0.001$  versus ND, <sup>SSS</sup> $p<0.001$  versus DL rats.

791

792 **Figure 8**

793 **The role of DMH and NTS 5-HT<sub>3</sub> receptors in the long-term effect (D+30) of social defeat**  
794 **on respiration in non-defeated (ND) and both groups of defeated (D<sub>L</sub> and D<sub>H</sub>) rats in study**  
795 **1 group B.**

796 A and B. At D+30, local microinjections of muscimol (musc, 5 mM) into the DMH (A) or  
797 granisetron (grani, 2.5 mM) into the NTS (B) reversed the decrease of RSA peak frequency  
798 normally observed in defeated rats. Values are the mean±SEM of data obtained in D<sub>L</sub>, D<sub>H</sub> and  
799 ND rats. \*\*\**p*<0.001 versus ND, <sup>ss</sup>*p*<0.01 versus DL rats, <sup>##</sup>*p*<0.01 versus saline.

800

801 **Figure 9**

802 **Long-term effects (D+30) of social defeat on respiration in non-defeated (ND) and defeated**  
803 **(D<sub>L</sub> and D<sub>H</sub>) rats, in study 2.**

804 A. RSA peak frequency in ND and D rats at D+30 under anesthesia. D rats (All D) were  
805 subdivided relative to the 5% percentile RSA peak frequency of the ND group (1.32 Hz),  
806 resulting in two subgroups D<sub>L</sub> and D<sub>H</sub> similar to those of Study I, group B (D+30). Values are the  
807 mean±SEM of data obtained in ND, D<sub>L</sub> and D<sub>H</sub> animals. \*\*\**p*<0.001 versus ND rats and  
808 <sup>sss</sup>*p*<0.001 versus D<sub>L</sub> rats.

809 B. Time-course of the changes in RSA peak frequency before, during and after social defeat up  
810 until D+30. The RSA peak frequency in D<sub>H</sub> and D<sub>L</sub> rats after social defeat was significantly lower  
811 than in ND rats as early as D2. RSA peak frequency became significantly higher in D<sub>H</sub> rats  
812 compared to D<sub>L</sub> rats and equivalent to ND rats from D17 to D+30. D<sub>L</sub> rats remained bradypneic  
813 until the end of the experiment at D+30. Values are the mean±SEM of data obtained in D<sub>L</sub>, D<sub>H</sub>  
814 and ND rats.

815 Symbols over horizontal bars indicate when ND and D<sub>L</sub> (\*) ND and D<sub>H</sub> (#) and D<sub>L</sub> and D<sub>H</sub> (\$) *p*<0.05, Bonferroni *post hoc* analysis).

816

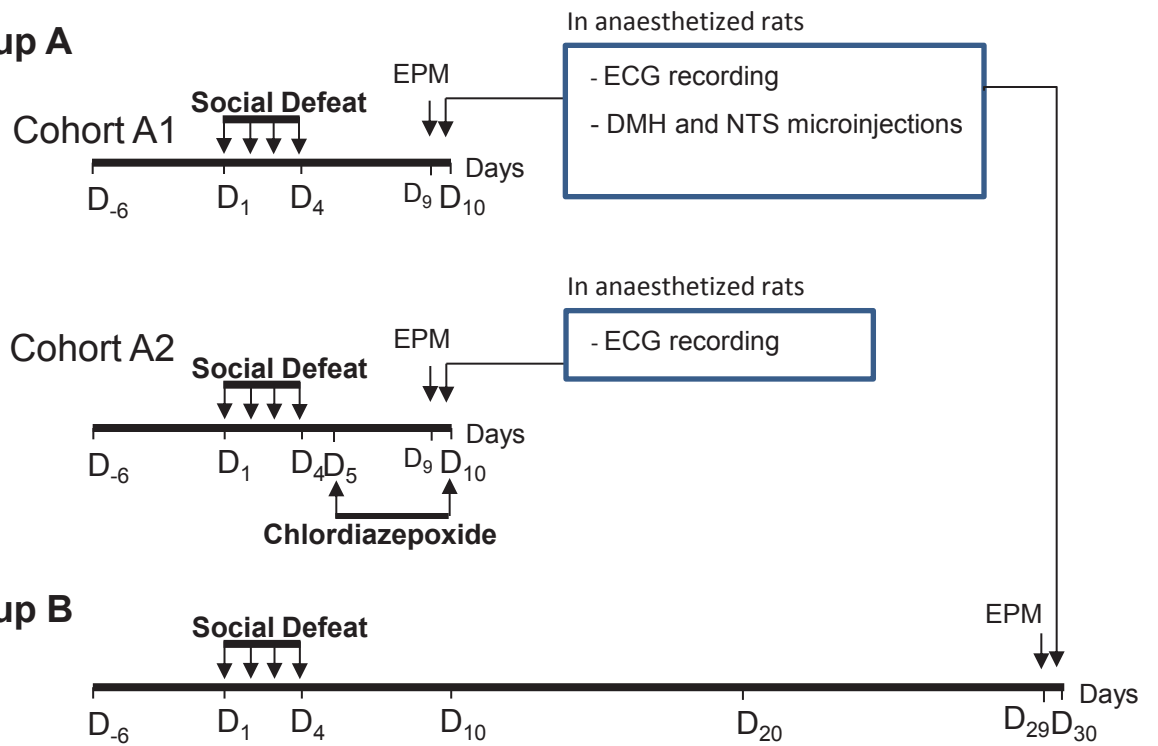
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818

Figure 1

**Study 1**

**Group A**



**Study 2**

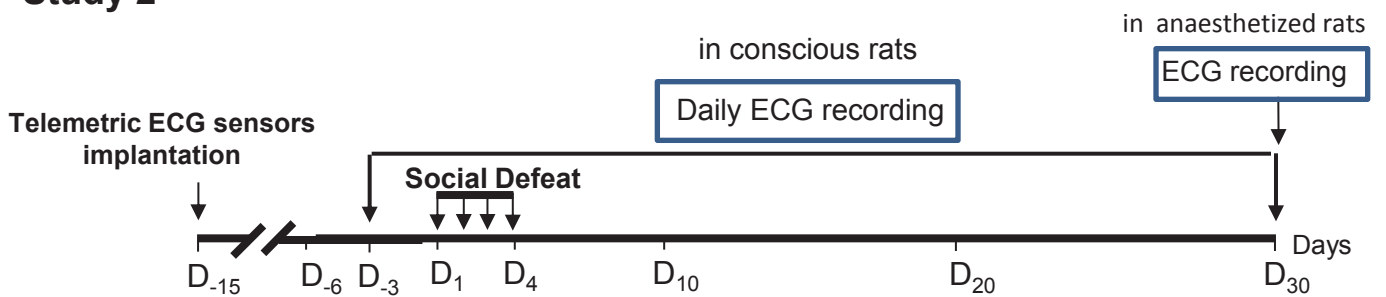
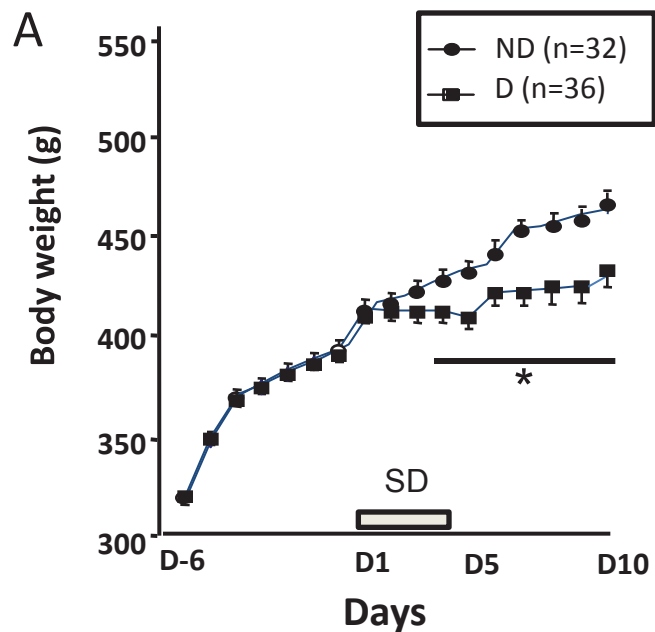


Figure 2



Defeat	$F(1.66)=11.6, p=0.0011$
Time	$F(9.594)=103.4, p<0.001$
Defeat X Time	$F(9.594)=16.1, p<0.001$

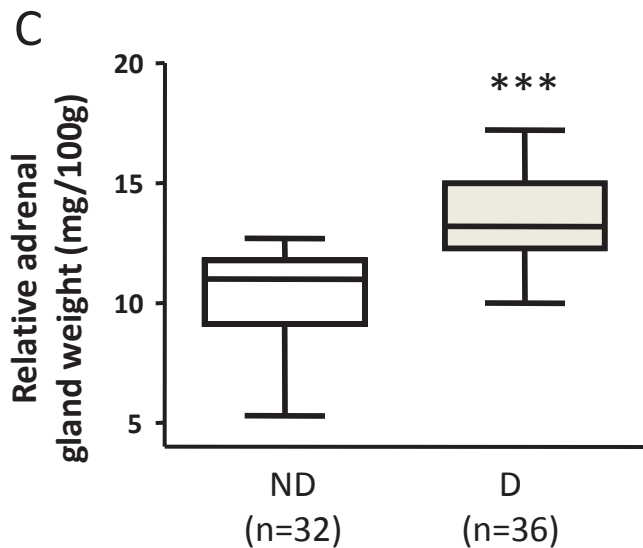
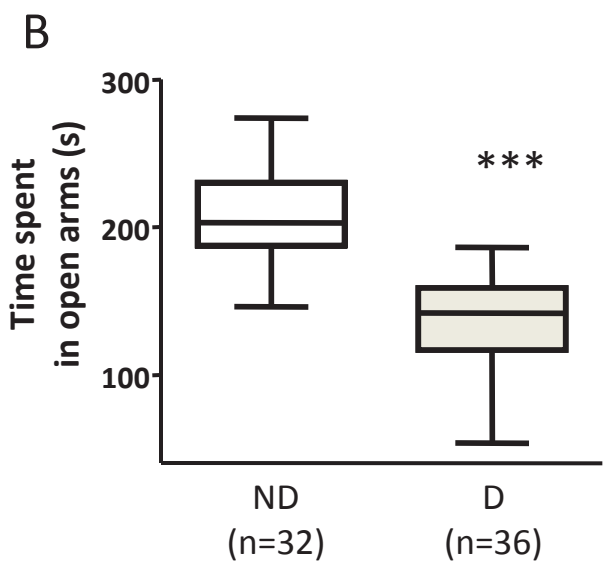




Figure 3

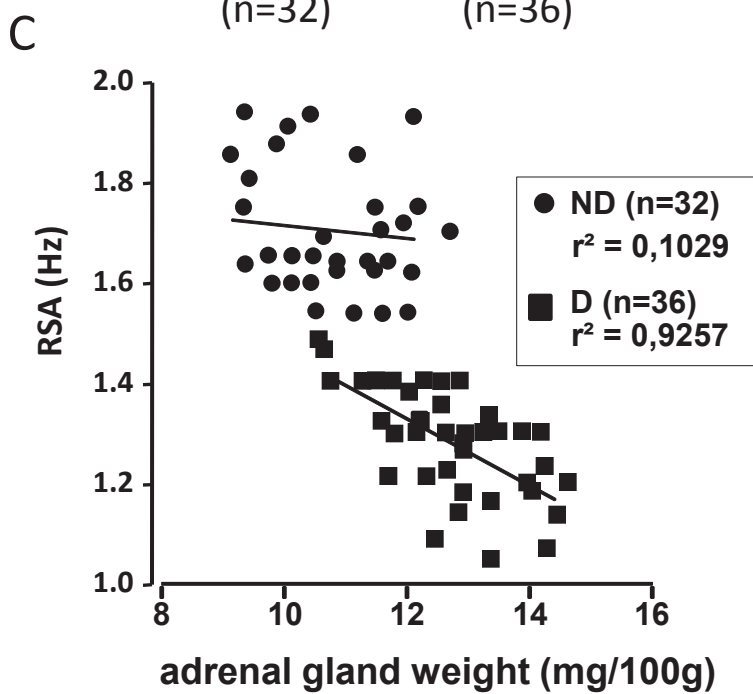
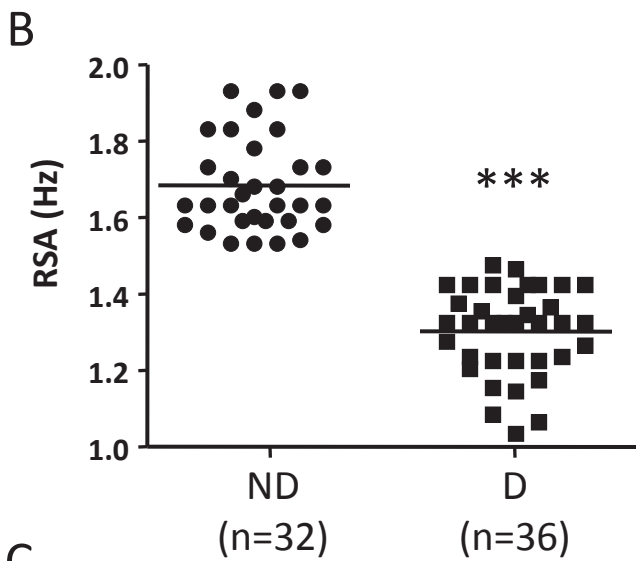
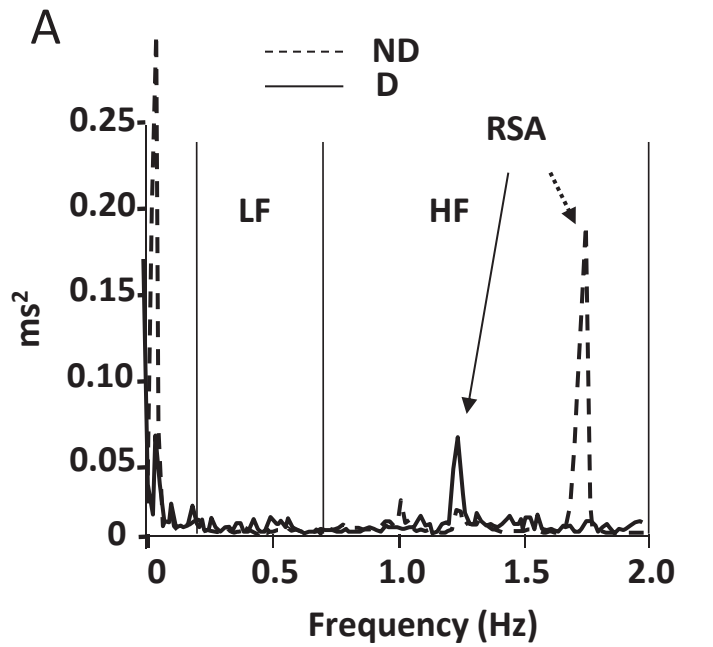
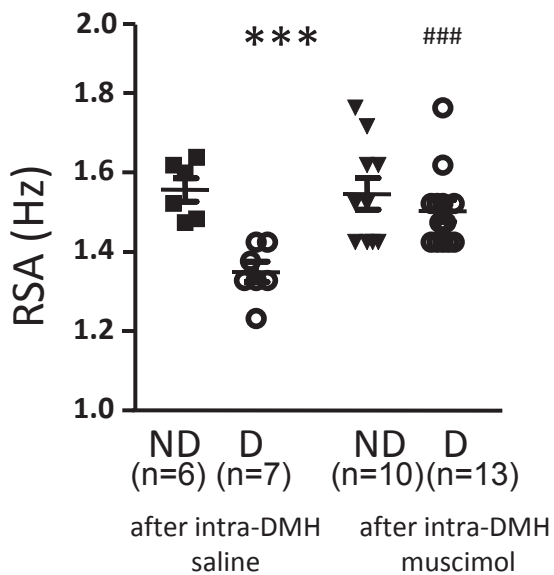


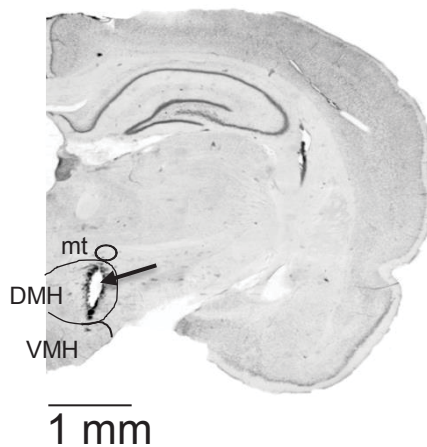
Figure 4

A1

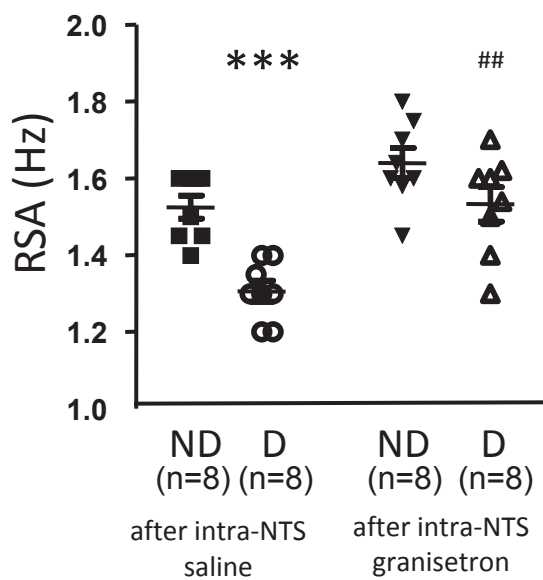


Defeat (ND or D)	$F(1,32)=13.8, p<0.001$
Treatment (muscimol or saline)	$F(1,32)=1.7, p=0.238$
Defeat X Treatment	$F(1,32)=14.3, p<0.001$

A2



B1



Defeat (ND or D)	$F(1,28)=2.9, p=0.098$
Treatment (granisetron or saline)	$F(1,28)=4.5, p=0.042$
Defeat X Treatment	$F(1,28)=4.5, p=0.043$

B2

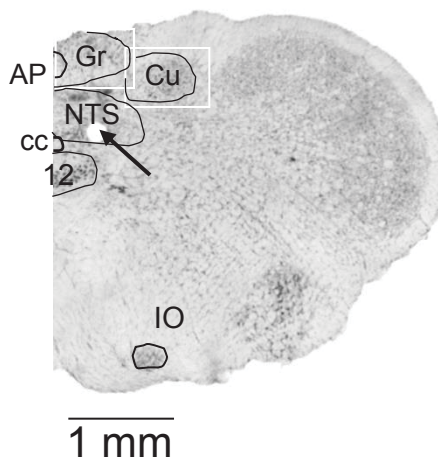


Figure 5

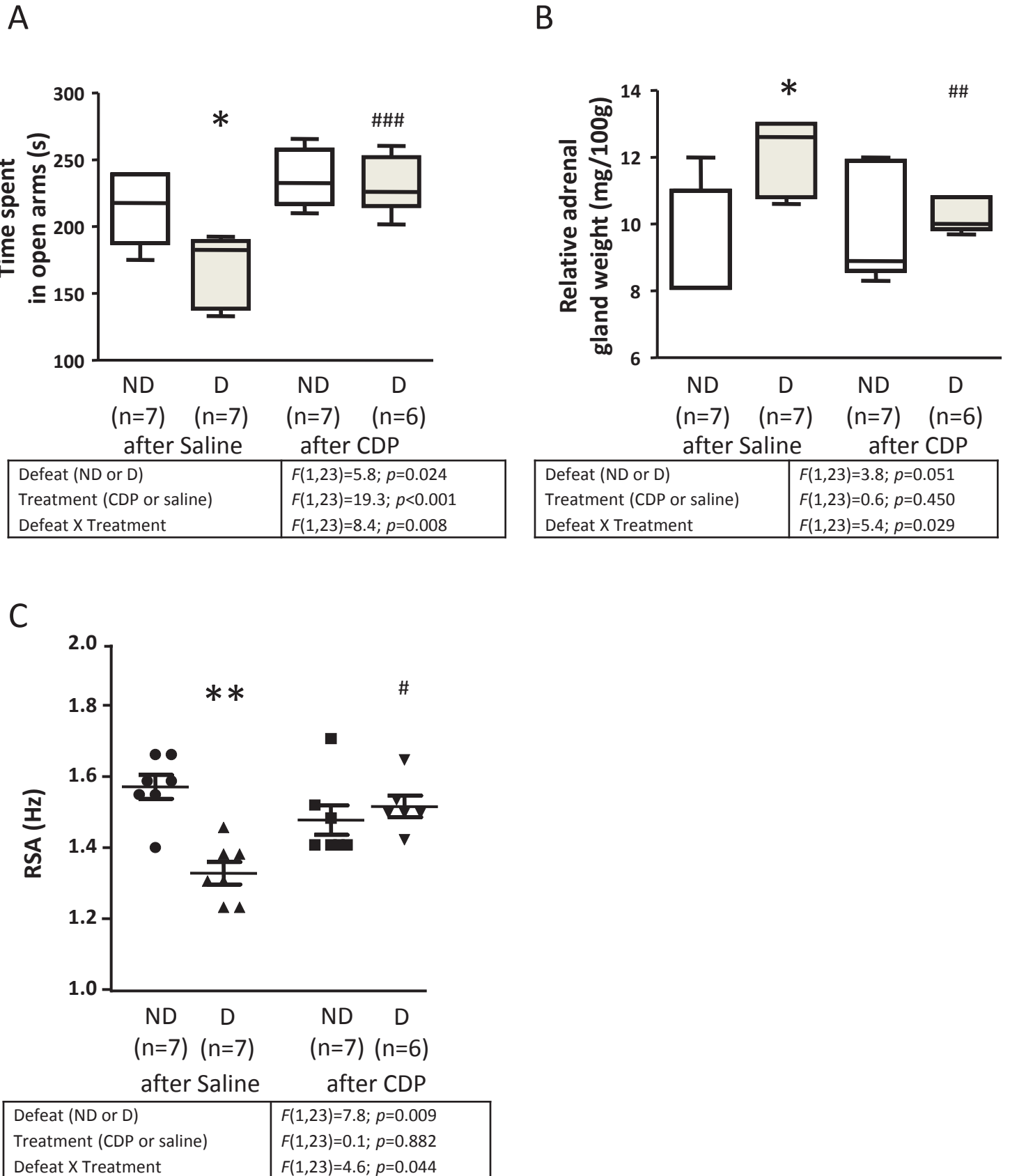
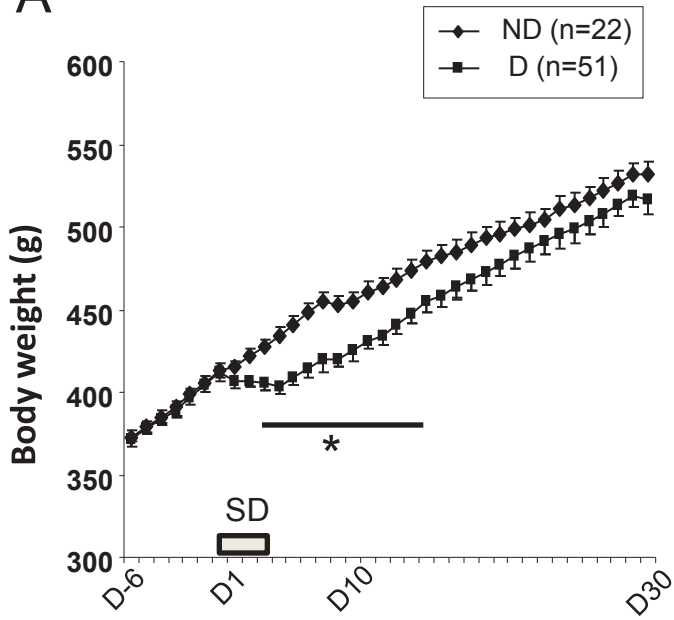


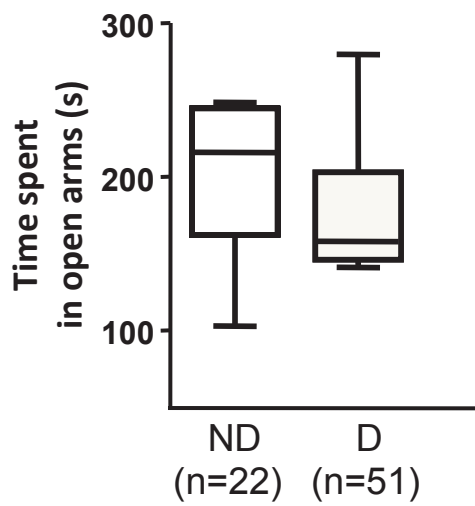
Figure 6

A



Defeat	$F(1,71)=5.76, p=0,0190$
Time	$F(35,2485)=922.23, p<0.001$
Defeat X Time	$F(35, 2485)=12.313, p<0.001$

B



C

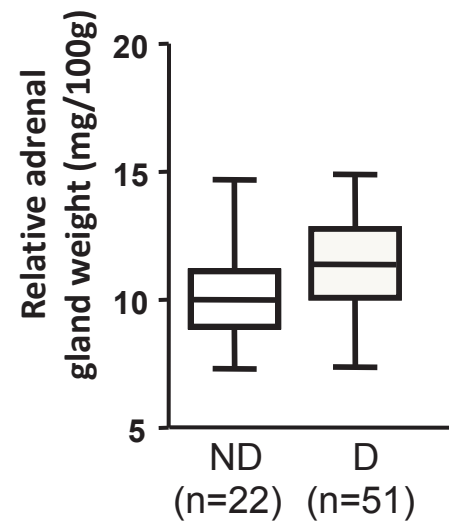


Figure 7

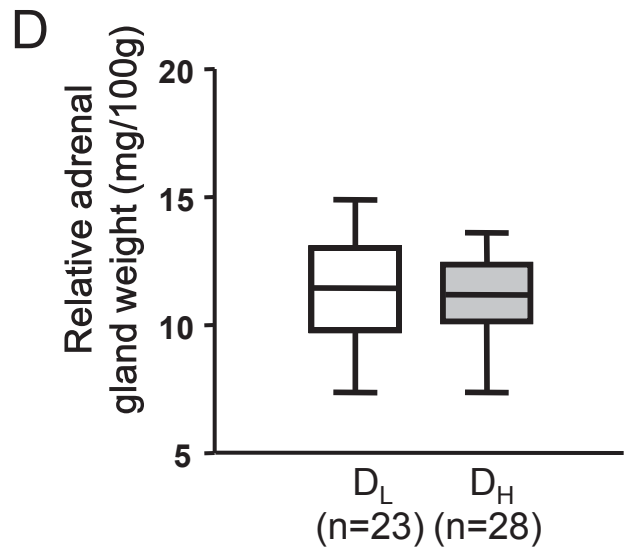
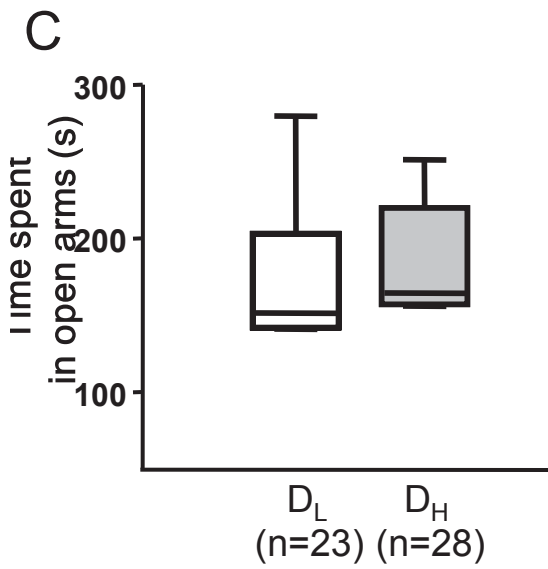
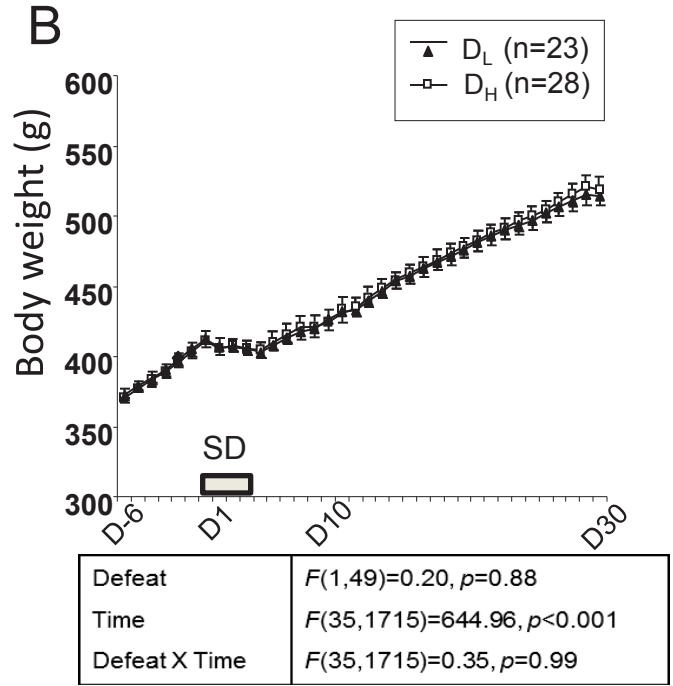
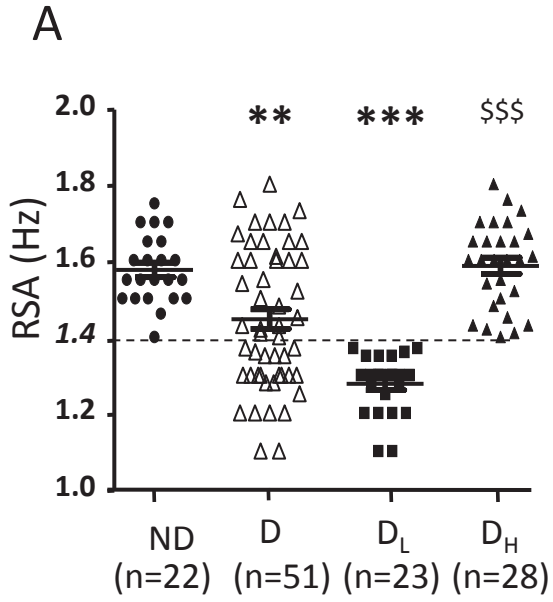
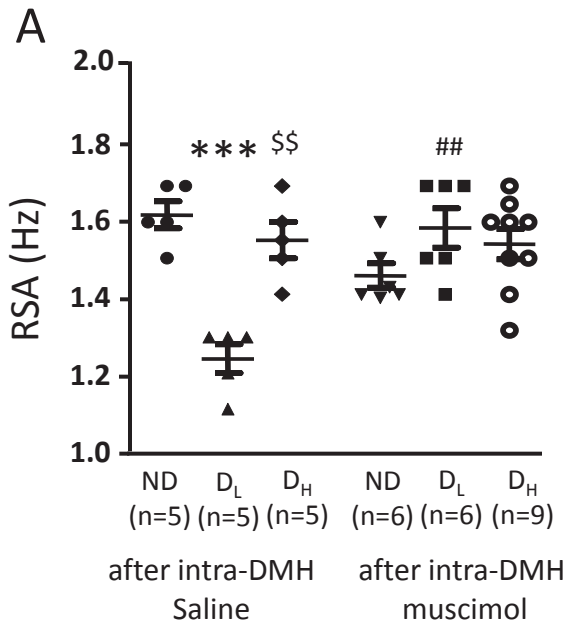
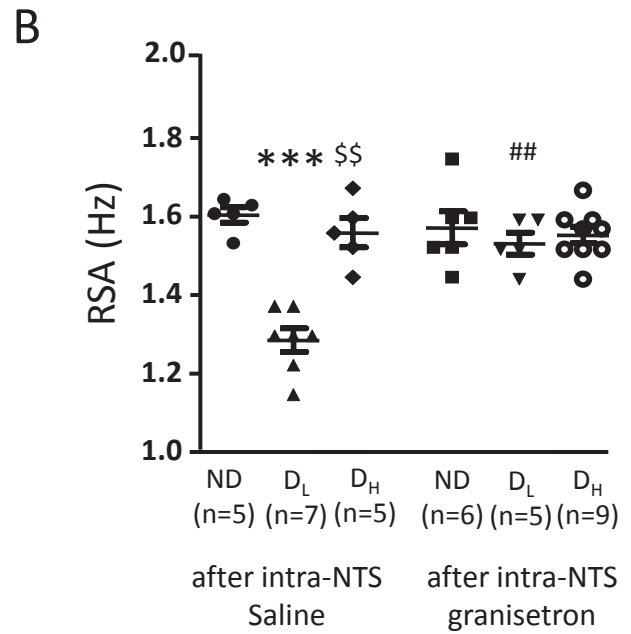


Figure 8



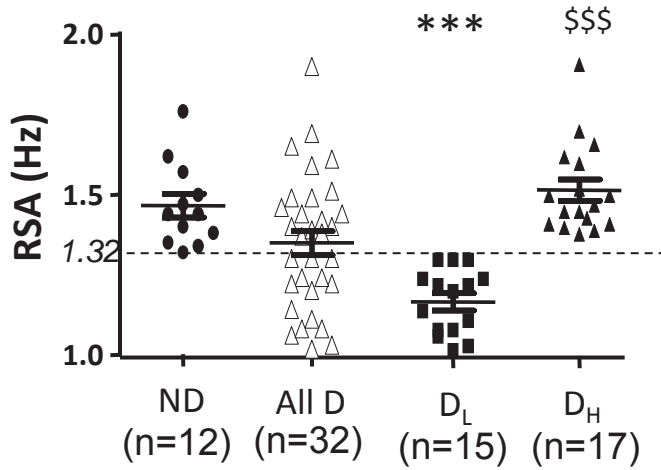
Defeat (ND or D <sub>H</sub> or D <sub>L</sub> )	$F(2,30)=16.5, p<0.001$
Treatment (muscimol or saline)	$F(2,30)=2.1, p=0.15$
Defeat X Treatment	$F(2,30)=4.3, p=0.02$



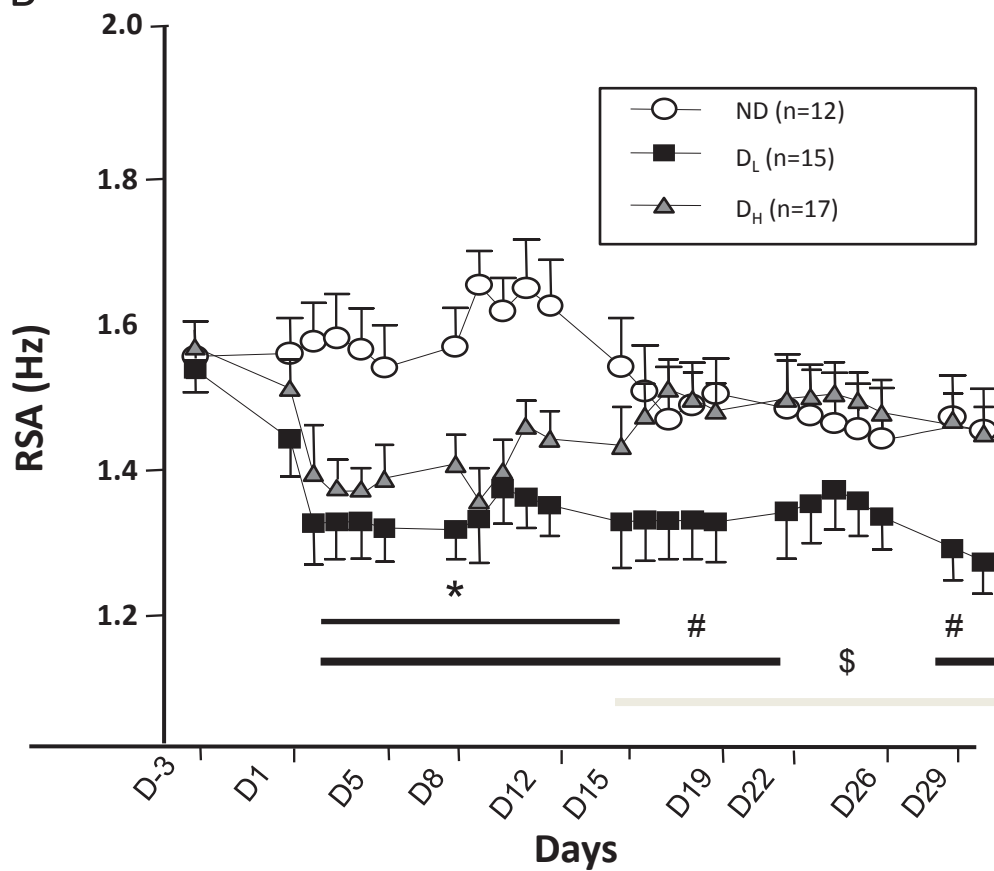
Defeat (ND or D <sub>H</sub> or D <sub>L</sub> )	$F(2,31)=10.4, p<0.001$
Treatment (granisetron or saline)	$F(2,31)=4.1, p=0.050$
Defeat X Treatment	$F(2,31)=6.0, p=0.006$

Figure 9

A



B



Defeat (ND or DH or DL)	$F(2,41)=8.3, p<0.001$
Time	$F(22,902)=7.5, p<0.001$
Defeat X Treatment	$F(44,902)=7.7, p<0.001$