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Running head: Anxiety and long-lasting respiratory modulation

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Abstract

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

Respiratory rate was recorded under anesthesia six days (D+10) or 26 days (D+30) after social defeat. At D+10, defeated (D) rats spent less time in the open arms of the elevated plus maze test, had heavier adrenal glands, and displayed bradypnea, unlike non-defeated (ND) animals. At D+30, all signs of anxiety had disappeared. However, half of the rats still displayed bradypnea (D_L rats, for low respiratory rate indicated by a lower RSA frequency), while those with higher respiratory rate (D_H rats) had recovered. Acute blockade of the dorsomedial hypothalamus (DMH) or nucleus tractus solitarii (NTS) 5-HT_3 receptors reversed bradypnea in all D rats at D+10 and in D_L rats at D+30.

Respiratory rate was also recorded in conscious animals implanted with radiotelemetric ECG probes. D_H rats recovered between D+10 and D+18, while D_L rats remained bradypneic until D+30.

In conclusion, social stress induces sustained chronic bradypnea mediated by DMH neurons and NTS 5-HT_3 receptors. These changes are associated with an anxiety-like state that persists until D+10, followed by recovery. However, bradypnea may persist in half of the population up until D+30 despite apparent recovery of the anxiety-like state.

Glossary

HF: High-Frequency domain

HRV: Heart Rate Variability

RSA: Respiratory Sinus Arrhythmia

DMH: Dorsomedial Hypothalamus

NTS: Nucleus Tractus Solitarii

ND: Non-defeated rats

D: Defeated rats

D_L: Defeated rats with low RSA peak frequency at D+30

D_H: Defeated rats with high RSA peak frequency at D+30

Introduction

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

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

The secondary objective was to investigate the central mechanisms underlying these changes and, more specifically, the role of the dorsomedial hypothalamus (DMH) and 5-HT_3 receptors in the nucleus tractus solitarii (NTS). It has been clearly established that the DMH plays a critical role in mediating the cardiovascular and neuroendocrine response to acute (15) and chronic (37) psychological stress. This response may include respiratory effects, as demonstrated by a recent study showing that the DMH mediates tachypnea associated with acute stress

responses (7). Activation of NTS 5-HT_3 receptors is also known to contribute to the expression of the autonomic and cardiovascular changes evoked by chronic stress or acute stimulation of DMH (37, 38). These receptors also contribute to breathing control, while activation of 5-HT_3 receptors has an inhibitory effect on respiration (45).

In the first part of this study, we therefore determined the long-term effect on respiratory rate 10 and 30 days after social defeat, a chronic emotional stress that induces an anxiety-like state (6, 36, 37). We also tested the involvement of the DMH and 5-HT_3 NTS receptors in this effect. The second part of the study consisted of a longitudinal study using implanted telemetric probes in which we evaluated off-line changes in breathing during the 30 days that followed social defeat.

Materials and Methods

Animals

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

Experimental overview

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

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

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

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

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

Experimental procedures

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

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

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

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

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

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

Specific procedures

Study 1 (Cohort A1 and Group B):

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

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

Pharmacological blockade of NTS 5-HT$_3$ receptors. In other animals, microinjections of either saline or a selective 5-HT$_3$ receptor antagonist, granisetron (SmithKline-Beecham, Harlow, UK, 250 pmol in 0.1 µl) were performed in the NTS at the level of the calamus scriptorius (38) (L 0.5 and V 0.5 mm). Injections were also asymmetric bilaterally to maximize the effect. RSA was measured from 90-s segments 20 min before and 5 min after pharmacological blockade of NTS 5-HT$_3$ receptors.

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

Study 1 (Cohort A2):

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

Study 2 (Group A) ***Radiotelemetric probe implantation and ECG recordings in conscious rats.*** Anesthesia may affect respiratory rate. Therefore, to verify the absence of a confounding effect of anesthesia on changes in respiration, RSA peak frequency was analyzed in conscious rats implanted with radiotelemetric probes. These experiments also allowed us to monitor the changes of this parameter over time. Two weeks before social defeat (D-15), rats were implanted with radiotelemetric probes (Data Sciences International, St. Paul, MN, USA) to enable recording of ECG and locomotor activity (Fig 1). Surgery was performed under aseptic conditions and under anesthesia (Isoflurane). The rats were also pretreated with an analgesic (Xylocaine, 5 mg/kg, s.c.), an anti-inflammatory drug (metacam, 1 mg/kg, s.c.) and received antibiotics (Penicillin, 0.3 ml, i.p.) at the end of surgery. The probes were implanted in the peritoneal cavity and wires were

tunneled subcutaneously along the rib cage. The positive lead was attached to the dorsal side of the xiphoid process and the negative lead was passed between the right sternocleidomastoid and sternohyoid muscles beside the trachea towards the manubrium and thoracic inlet (41).

ECG was recorded every afternoon (1 to 6 pm) during the week from D-3 to D+30. The afternoon was chosen for two main reasons. First, recordings are more stable when animals are less active, even if sniffing and active periods associated with tachypneic episodes occur sporadically. Second, the social defeat procedure took place in the morning (when animals are receptive to stress); ECG was analyzed after the stress procedure and recordings therefore started and were continued in the afternoons. Data were acquired using Dataquest A.R.T. 3.1 Gold software (Data Sciences). ECG waveform data were imported offline into Spike CED (version 6.0) and were then analyzed to extract RSA peak frequency as described above for Study 1. The RSA peak was determined from 2-min segments of ECG data and averaged across four segments. These segments may have contained periods of sniffing (characterized by high respiratory rate, (23)), however, these periods were short (only a few seconds). In any case, we have previously shown that sniffing bouts did not interfere with respiratory rate measurements derived from RSA analysis (8). ECG was also recorded on the last day (D+30), under pentobarbital sodium anesthesia, as mentioned above. Signals were exported to Spike 2 software (version 6.14, CED, UK) for off-line respiratory and ECG analyses.

Statistical analysis

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

Results

Study 1/Group A/Cohort A1. Behavioral and respiratory changes evoked by social defeat at D+10 and contribution of DMH neurons and NTS 5HT$_3$ receptors.

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

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

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

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

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

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

Effect of DMH inhibition and NTS 5-HT_3 receptor blockade on RSA peak frequency.

Previous work from our laboratory has shown that DMH inhibition and NTS 5-HT_3 receptor blockade markedly reduce the long-term cardiovascular effects evoked by social defeat when recorded at D+10 (37). To determine whether this was also the case for bradypnea, we randomly selected ND and D of this cohort for microinjection of either saline or muscimol into the DMH, and either saline or granisetron into the NTS. As shown in Fig. 4A1, the reduction in RSA peak frequency observed in D rats compared to ND rats was still observed after saline but not after bilateral muscimol microinjections into the DMH (Fig. 4 A1 & A2), which was confirmed by a statistically significant interaction between defeat and treatment effects. Similarly, the reduction in RSA peak frequency in D rats (Fig. 4 B1) was still observed after saline but not after bilateral injections of granisetron into the NTS (Fig. 4 B1 & B2), which was also confirmed by a significant interaction between defeat and treatment effects.

DMH neurons and 5-HT_3 NTS receptors therefore contribute not only to cardiovascular changes but also to the bradypnea associated with the anxiety-like state induced by social defeat. We then investigated whether bradypnea was a result of the anxiety-like state and whether it could be prevented by anxiolytic treatment.

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

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

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

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

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

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

Study 1/Group B. Behavioral and respiratory changes evoked by social defeat at D+30 and contribution of DMH neurons and NTS $5HT_3$ receptors.

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

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

Elevated plus-maze test. D rats spent the same amount of time in the open arms as ND rats (183±6 vs 194±10 s, respectively, p=0. 4, Fig. 6B). No sign of an anxiety-like state was therefore detected in D rats at D29, in contrast with Cohort A1 rats when they were tested at D9.

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

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

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

Thus, although the anxiety levels of D rats had recovered by D+30, half of the rats (D_L) still presented the same bradypnea as at D+10. We then tested the role of the DMH and $5HT_3$ NTS receptors in this D+30 bradypnea as performed for the D+10 bradypnea in Cohort A1 animals.

Effect of DMH inhibition and NTS 5-HT$_3$ receptor blockade. As shown in Fig. 8A, the D+30 bradypnea was still observed in D_L rats after bilateral saline microinjections into the DMH, but not after bilateral muscimol microinjections. In fact, the RSA peak frequency in muscimol-injected D_L animals was the same as in ND and D_H rats injected with muscimol or saline. Statistical analysis confirmed a significant interaction between defeat and muscimol with pair-wise *post hoc* comparisons showing a significant difference between muscimol- and saline-injected D_L rats (p=0.003) but not between muscimol- and saline-injected ND and D_H rats (p=0.85 and p=0.857, respectively). Similarly, the D+30 bradypnea was still observed in D_L rats after bilateral saline microinjections into the NTS, but not after bilateral granisetron microinjections (Fig. 8B). A significant interaction was observed between defeat and granisetron with significant pair-wise *post hoc* comparisons between granisetron- and saline-injected D_L rats ($p<0.001$) but not ND and D_H rats (p=0.63 and p=0.89, respectively).

DMH neurons and $5HT_3$ NTS receptors therefore contribute to the bradypnea observed at D+30 in D_L rats as at D+10, suggesting that the same mechanism is involved at the two time-points. In other words, social defeat in these D_L rats produced a long-lasting effect on respiration that outlasted the anxiety-like state. We then tried to determine when D_H rats recovered and whether there was any difference between D_L and D_H rats at an earlier stage.

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

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

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

Discussion

This study shows, for the first time, that social defeat induces long-lasting bradypnea that can be detected as a reduction in RSA peak frequency. At D+10, the effect was observed in all defeated animals and was associated with an elevated level of anxiety. No anxiety was detected 20 days later, at D+30. However, bradypnea was still present in approximately half of the defeated animals. Importantly, this long-lasting respiratory change involves the DMH and NTS 5-HT_3 receptors, as we have previously shown for the associated cardiovascular changes (37).

Respiratory changes induced by social defeat

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

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

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

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

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

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

Role of the DMH and NTS 5-HT_3 receptors in the respiratory changes induced by social defeat

Social defeat induces a persistent increase in c-fos protein in the hypothalamus (including the DMH) and the NTS (28). We know from previous work that the DMH plays a key role in autonomic alteration (increase in LF/HF ratio associated with a reduction in RSA amplitude and parasympathetic baroreflex gain) evoked by social defeat at D+10 (37). This inhibition of cardiac parasympathetic activity, which is GABAergic and occurs on vagal preganglionic neurons, is thought to result from activation of presynaptic vagal 5-HT_3 receptors in the NTS, themselves indirectly activated by the DMH via a cascade of activation involving the dorsolateral periaqueductal gray (dlPAG) and serotonergic cells in the raphe magnus (5, 37). This 5-HT_3-mediated inhibition of the cardiac vagal activity induced by chronic stress appears to be mediated via activation of NTS GABAergic interneurons that block the second-order neurons of the baroreceptor arc, as previously observed after acute DMH stimulation (38). To determine whether NTS 5-HT_3 receptors are also involved in the DMH-induced bradypnea evoked by social defeat, we blocked the DMH with bilateral microinjections of muscimol and antagonized 5-HT_3 NTS receptors with bilateral microinjections of granisetron. As with cardiac vagal inhibition, we found that blockade of DMH and 5-HT_3 NTS receptors abolished the bradypnea of defeated rats at both D+10 and D+30 (in D_L rats). Similar results were found when granisetron was administered systemically (data not shown), as 5-HT_3 receptor antagonists can cross the blood-brain barrier (10). In line with these results, systemic administration of ondansetron, another 5-HT_3 receptor antagonist, has been shown to prevent sleep apneas (46). These findings suggest that the same neuronal pathway that mediates cardiac vagal inhibition may also mediate bradypnea. It is noteworthy that cells sensitive to $H+/CO_2$ in the retrotrapezoid/parafacial (RTN-pFRG) region, located in the ventral medulla and linked to the central command of respiration, participate in maintenance of a tonic respiratory drive. The RTN-pFRG region receives direct GABAergic inhibitory inputs from the NTS (30). GABAergic inhibition from the NTS to chemoreceptor RTN/pFRG neurons, following DMH activation, may therefore inhibit the tonic excitatory drive exerted by this region on the central respiratory pattern generator, leading to bradypnea. Further studies are needed to support this hypothesis.

It has been known for a long time that the DMH is a key structure involved in the physiological response to acute stress, i.e. the defense reaction (32), and that its activation induces an array of physiological responses including arousal, and increased blood pressure, heart rate and regional vascular resistance (12, 13). While the pathway involved in the tachycardic response remains controversial, it appears that the hypertensive response is due to activation of the rostroventrolateral part of the medulla (16). Classically, an increase in respiratory activity is also the characteristic feature of the physiological response to psychological stress. Some studies investigating the effects of disinhibition of neurons within the DMH on respiratory rate (35) or phrenic nerve activity (29) concluded that acute DMH activation induces increased respiratory activity. Conversely, muscimol blockade of the DMH prevents the tachypnea evoked by acute stress, such as a novel environment or restraint (7), or activation of the dlPAG (20). The descending pathways mediating the DMH-evoked increase in respiratory activity may involve chemosensitive orexin neurons. In support of this hypothesis, microdialysis of CO_2-enriched fluid (25% CO_2) into the orexin neuron-rich DMH/perifornical region of the hypothalamus increases resting activity (26), and activation of hypothalamic orexin neurons produces defense-like or panic-like cardiorespiratory responses (17, 21, 22). Descending orexin projections to the retrotrapezoid/parafacial (RTN-pFRG) region, located in the ventral medulla and intimately related to the central command of respiration, have also been identified (25). Given that DMH is almost certainly activated during exposure to the aggressive resident and that the breathing response almost certainly corresponds to tachypnea, defeated rats would have been expected to present long-term hyperventilation. However, we found that the DMH in defeated rats was the origin of the bradypnea mediated by NTS 5-HT_3 receptors. One explanation for this effect may be provided by a study by Lutter et al. (27) that showed decreased hypothalamic prepro-orexin mRNA and orexin cell count and activation after social defeat. The hypothalamic area sampled included the DMH. If orexin expression was reduced in our defeated rats, then the orexinergic drive on breathing would also have been reduced. This reduction could have left the NTS 5-HT_3 and $GABA_A$ receptor inhibitory pathway to the RTN unopposed, tilting the balance in favor of a bradypnea instead of tachypnea, in response to chronic activation of the DMH. Further studies are needed to evaluate the level of orexin expression in the DMH after application of our stress procedure. In addition, long-term changes may have occurred in the DMH and/or its targets that would have modified the way they modulate breathing. For example, the DMH could have

remained active in the days following stress exposure, but at a subthreshold level, nevertheless sufficient to activate NTS 5-HT_3-mediated bradypnea, but too low to increase respiratory rate. This mechanism needs to be further investigated, but we have observed that subthreshold electrical or chemical DMH stimulation, that does not increase basal respiratory rate, inhibits RTN/pFRG chemoreceptor-induced increases in ventilation via activation of a NTS 5-HT_3/$GABA_A$ receptor mechanism (48). The fact that the DMH may remain activated at a sub-threshold state until at least D+30 in D_L rats may also explain why these rats still presented bradypnea after anxiety behavior had resolved.

Perspectives and Significance

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

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

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

underlying this long-lasting effect may include sub-threshold activation of the DMH that reduced respiratory rate via excitation of NTS 5-HT_3 receptors.

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Figure Captions

Figure 1

Protocol of social defeat.

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

Figure 2

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

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

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

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

Figure 3

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

A. Spectral analysis of RR intervals in two representative animals under anesthesia: the RSA peak shifted to a lower frequency in D rats (1.3Hz) compared to ND rats (1.7 Hz), indicating long-lasting bradypnea in stressed animals.

B & C. RSA peak frequency was lower in D rats than in anesthetized ND rats (B), and was correlated with adrenal gland weight in D but not in ND rats (C). Values are the mean±SEM of data obtained in ND and D animals. ***p<0.001 versus ND rats.

Figure 4

Effect of blockade of DMH and NTS 5-HT_3 receptors on bradypnea induced by social defeat at D+10, in study 1 group A cohort A1.

In anesthetized rats, at D+10, local microinjections of muscimol (musc, 5 mM) into the DMH (A) or granisetron (grani, 2.5 mM) into the NTS (B) reversed the decrease in RSA peak frequency normally observed in defeated rats. Values are the mean±SEM of data obtained in D and ND rats. ***p<0.001 versus ND. ## p<0.01 and ###p<0.001 versus saline

A2 and B2: Photomicrographs showing representative examples of microinjection sites (arrows) in the DMH (A2) and NTS (B2). AP: area postrema, cc: central canal, Cu: cuneate nucleus, DMH: dorsomedial nucleus of the hypothalamus, Gr: gracilis nucleus, VMH: ventromedial nucleus of the hypothalamus, NTS: nucleus tractus solitarii, mt: mammillothalamic tract, 12: hypoglossal nucleus.

Figure 5

Effects of anxiolytic treatment on behavioral and respiratory changes induced by social defeat at D+10, in study 1 group A cohort A2.

Compared to vehicle (saline), chlordiazepoxide (CDP) treatment prevented the decrease in time spent in open arms of the elevated plus maze (A), the increase in adrenal gland weight (B) and the bradypnea (C) normally observed in defeated rats at D+10. Values are the mean±SEM of data 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 versus saline.

Figure 6

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

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

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

Figure 7

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

A. At D+30, RSA was still lower in D rats than in ND rats. However, D rats could be subdivided relative to a 5% percentile of RSA peak frequency at D+30 of the ND group (1.40 Hz), resulting in two subgroups, D_H (RSA above 1.40 Hz) and D_L (RSA below 1.40 Hz). RSA was similar in D_H rats and ND rats, while RSA was lower in D_L rats than in ND and D_H rats.

B. Body weights of D_H (RSA above 1.40 Hz) and D_L (RSA below 1.40 Hz) rats were similar throughout the entire protocol. Each point is the mean±SEM of data obtained in D_H and D_L rats. SD: social defeat.

C and D. Evaluation of the anxious profile and the hypothalamic-pituitary-adrenal axis of D_H and D_L rats in the elevated plus maze test at D9. No difference was observed in time spent in the open arms (B) and adrenal gland weight relative to body weight (C) between D_H and D_L rats. Values are the mean±SEM obtained in D_H and D_L rats.

**p<0.01 and ***p<0.001 versus ND, $^{\$\$\$}$p<0.001 versus DL rats.

Figure 8

The role of DMH and NTS 5-HT_3 receptors in the long-term effect (D+30) of social defeat on respiration in non-defeated (ND) and both groups of defeated (D_L and D_H) rats in study 1 group B.

A and B. At D+30, local microinjections of muscimol (musc, 5 mM) into the DMH (A) or granisetron (grani, 2.5 mM) into the NTS (B) reversed the decrease of RSA peak frequency normally observed in defeated rats. Values are the mean±SEM of data obtained in D_L, D_H and ND rats. ***p<0.001 versus ND, $^{\$\$}$p<0.01 versus DL rats, $^{\#\#}$p<0.01 versus saline.

Figure 9

Long-term effects (D+30) of social defeat on respiration in non-defeated (ND) and defeated (D_L and D_H) rats, in study 2.

A. RSA peak frequency in ND and D rats at D+30 under anesthesia. D rats (All D) were subdivided relative to the 5% percentile RSA peak frequency of the ND group (1.32 Hz), resulting in two subgroups D_L and D_H similar to those of Study I, group B (D+30). Values are the mean±SEM of data obtained in ND, D_L and D_H animals. ***p<0.001 versus ND rats and $^{\$\$\$}$p<0.001 versus D_L rats.

B. Time-course of the changes in RSA peak frequency before, during and after social defeat up until D+30. The RSA peak frequency in D_H and D_L rats after social defeat was significantly lower than in ND rats as early as D2. RSA peak frequency became significantly higher in D_H rats compared to D_L rats and equivalent to ND rats from D17 to D+30. D_L rats remained bradypneic until the end of the experiment at D+30. Values are the mean±SEM of data obtained in D_L, D_H and ND rats.

Symbols over horizontal bars indicate when ND and D_L (*) ND and D_H (#) and D_L and D_H ($) rats were significantly different (p<0.05, Bonferroni *post hoc* analysis).

Figure 1

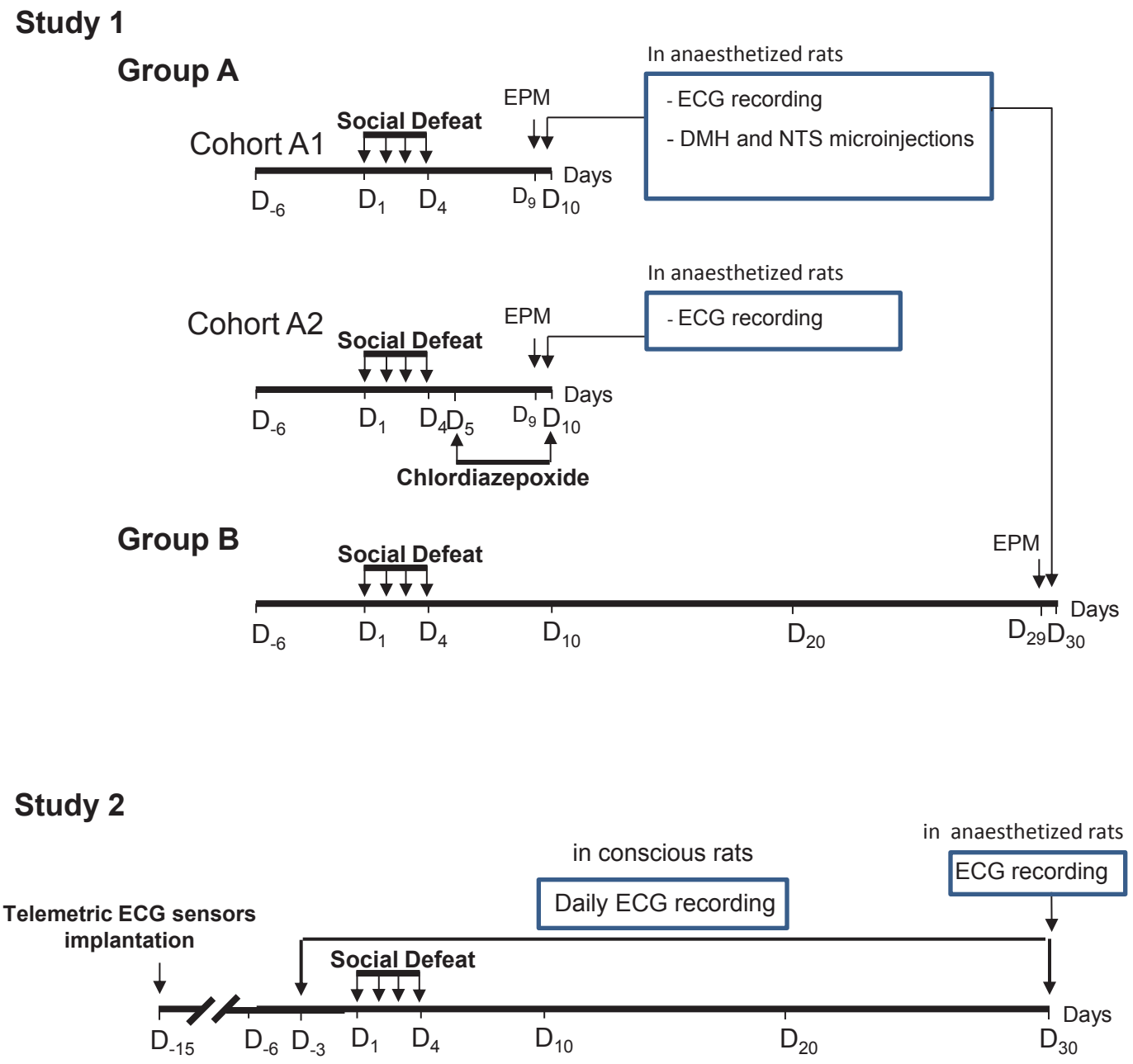
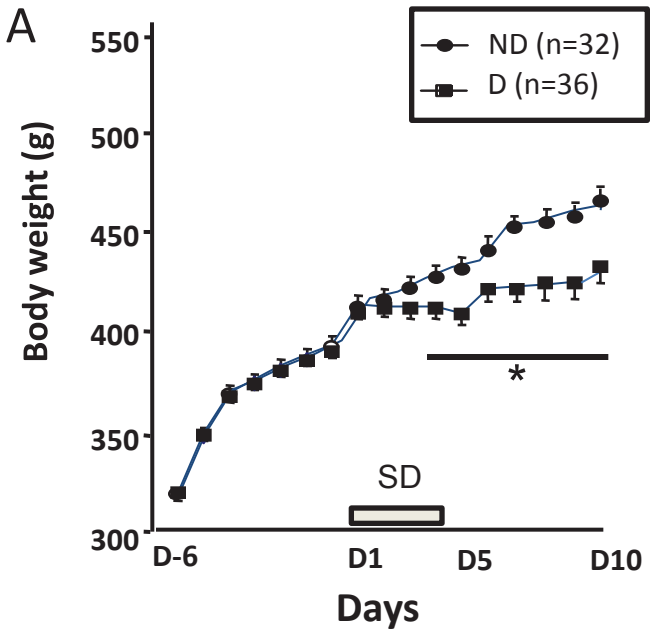
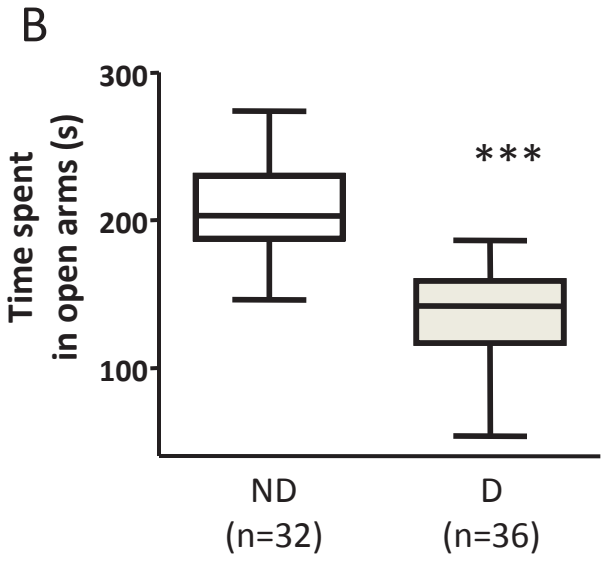

Study 1
Group A
In anaesthetized rats
- ECG recording
- DMH and NTS microinjections
EPM
Cohort A1
Social Defeat
Days
D-6 D1 D4 D9 D10
In anaesthetized rats
- ECG recording
Cohort A2
EPM
Social Defeat
Days
D-6 D1 D4D5 D9 D10
Chlordiazepoxide
Group B
Social Defeat
EPM
Days
D-6 D1 D4 D10 D20 D29D30
Study 2
in anaesthetized rats
ECG recording
in conscious rats
Daily ECG recording
Telemetric ECG sensors implantation
Social Defeat
Days
D-15 D-6 D-3 D1 D4 D10 D20 D30

Figure 2



Defeat	$F(1.66)=11.6$, $p=0,0011$
Time	$F(9.594)=103.4$, $p<0.001$
Defeat X Time	$F(9.594)=16.1$, $p<0.001$



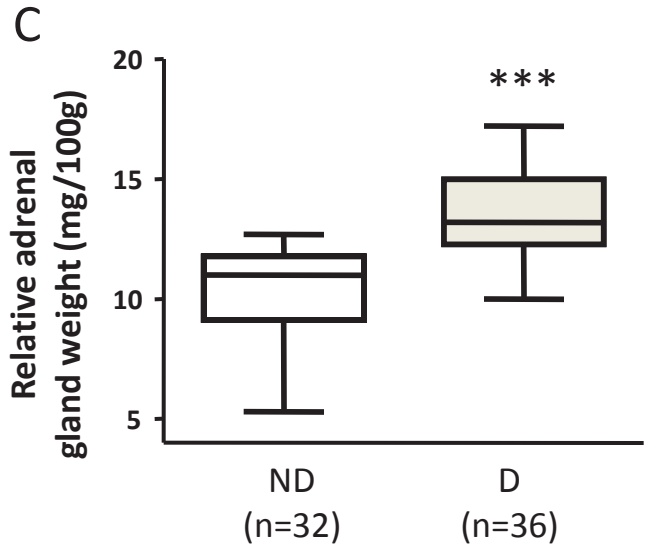


Figure 3

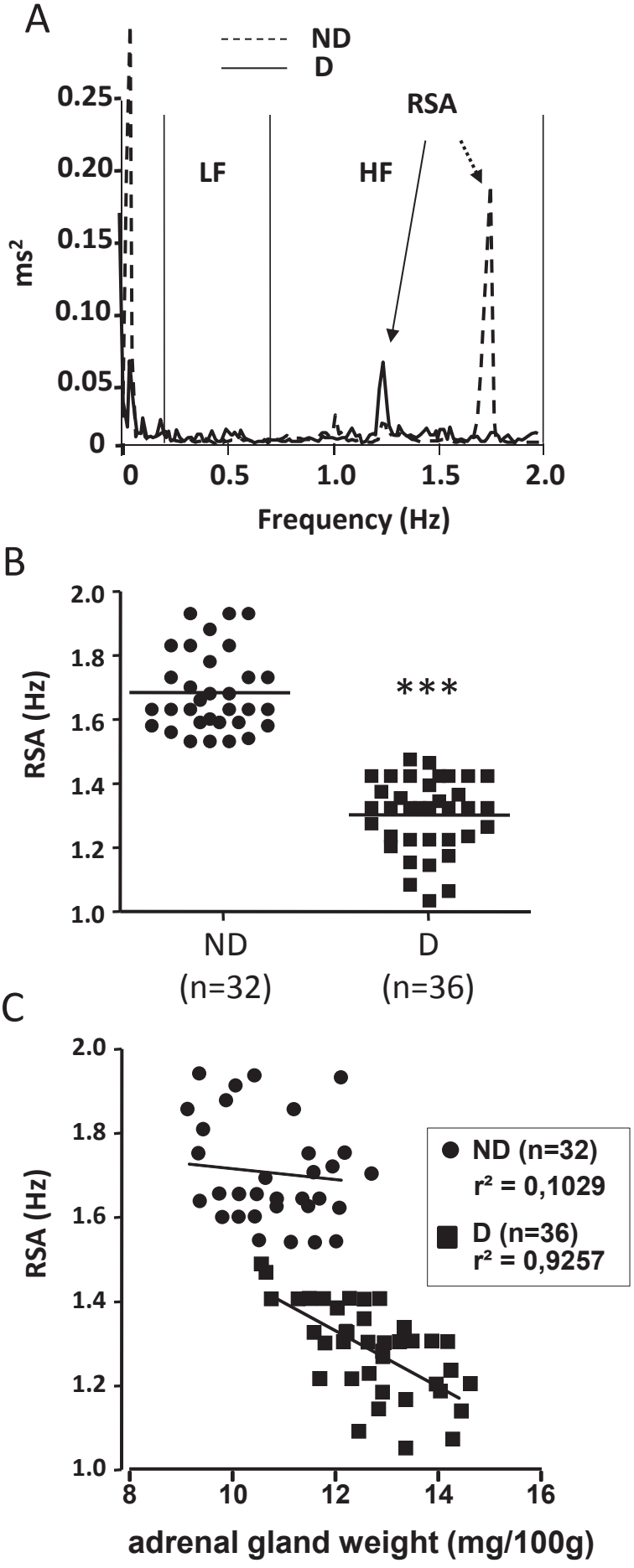
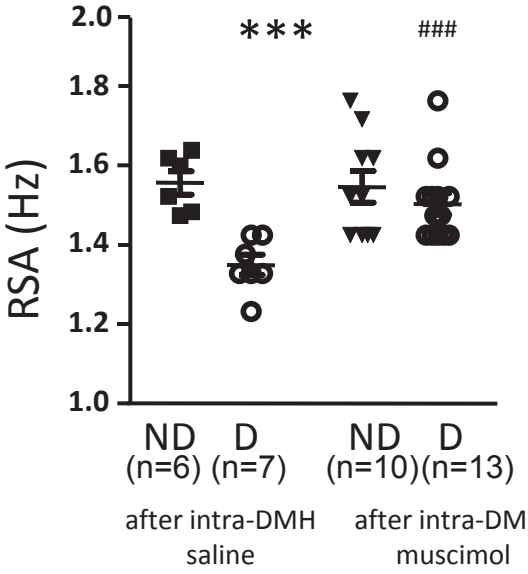

A
ND
D
RSA
LF
HF
0.25
0.20
0.15
0.10
0.05
0
ms²
0
0.5
1.0
1.5
2.0
Frequency (Hz)
B
2.0
1.8
1.6
1.4
1.2
1.0
RSA (Hz)
***
ND
(n=32)
D
(n=36)
C
2.0
1.8
1.6
1.4
1.2
1.0
RSA (Hz)
ND (n=32)
r² = 0,1029
D (n=36)
r² = 0,9257
8
10
12
14
16
adrenal gland weight (mg/100g)

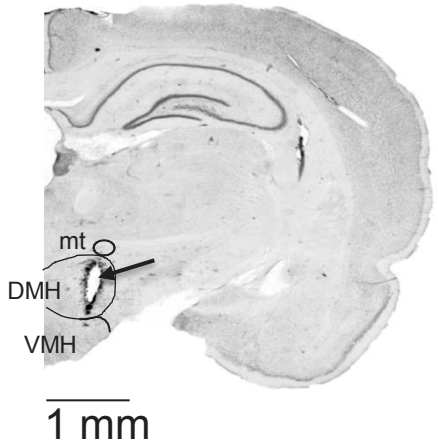
Figure 4

A1

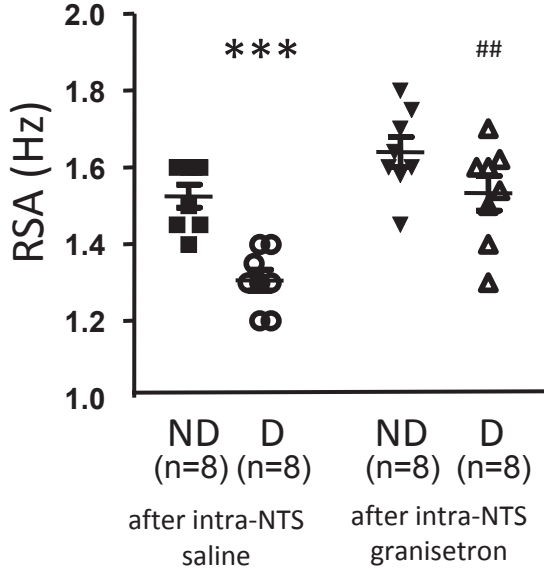


Defeat (ND or D)	$F(1,32)=13.8$, $p<0.001$
Treatment (muscimol or saline)	$F(1,32)=1.7$, $p=0.238$
Defeat X Treatment	$F(1,32)=14.3$, $p<0.001$

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

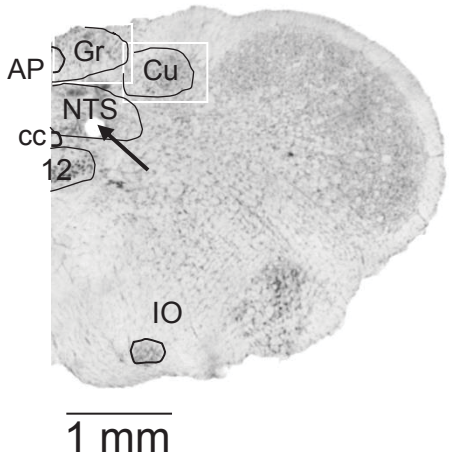
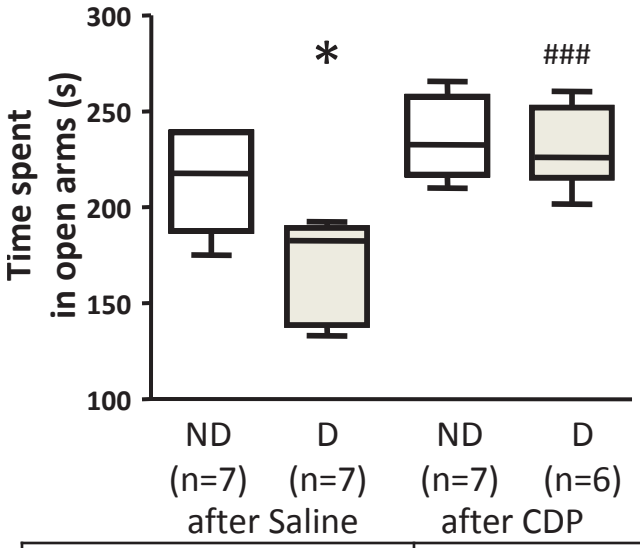


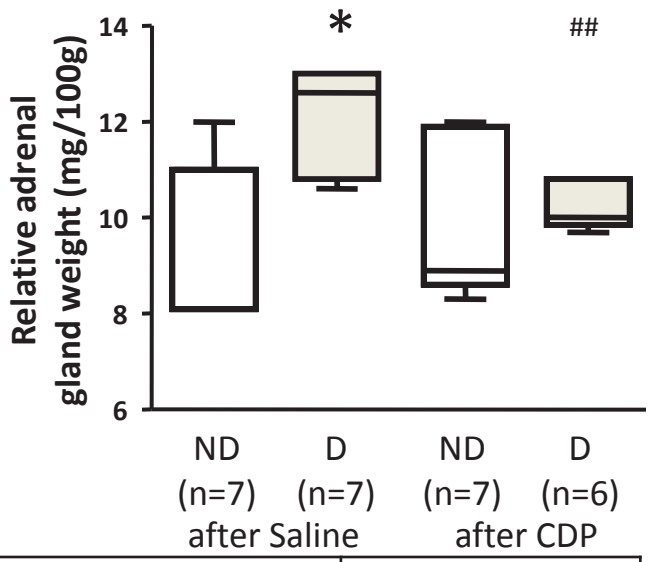
Figure 5

A



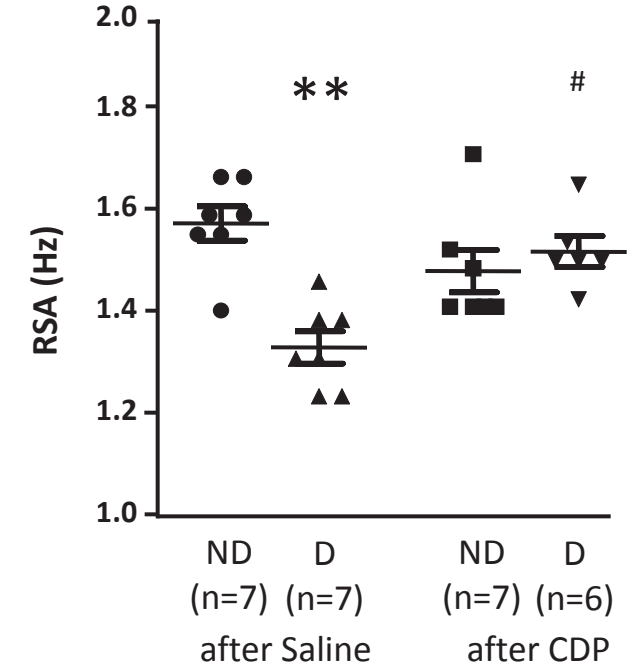
Defeat (ND or D)	$F(1,23)=5.8$; $p=0.024$
Treatment (CDP or saline)	$F(1,23)=19.3$; $p<0.001$
Defeat X Treatment	$F(1,23)=8.4$; $p=0.008$

B



Defeat (ND or D)	$F(1,23)=3.8$; $p=0.051$
Treatment (CDP or saline)	$F(1,23)=0.6$; $p=0.450$
Defeat X Treatment	$F(1,23)=5.4$; $p=0.029$

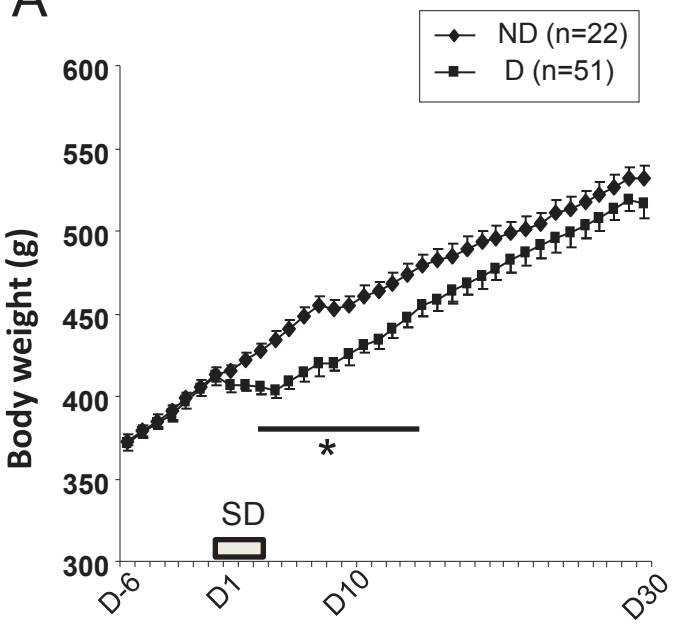
C



Defeat (ND or D)	$F(1,23)=7.8$; $p=0.009$
Treatment (CDP or saline)	$F(1,23)=0.1$; $p=0.882$
Defeat X Treatment	$F(1,23)=4.6$; $p=0.044$

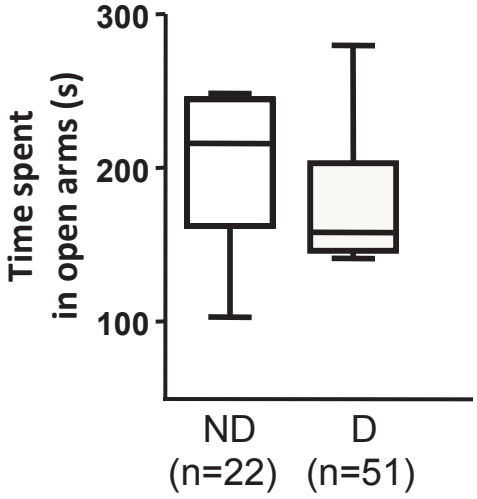
Figure 6

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

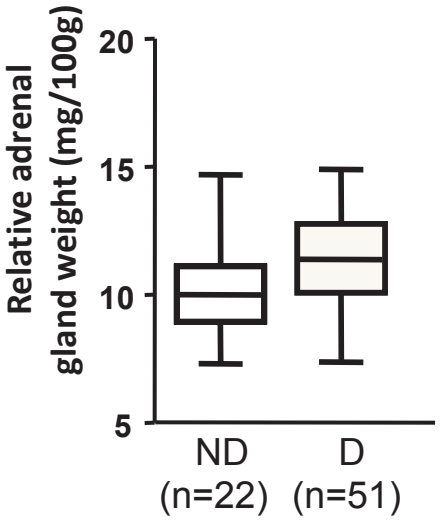
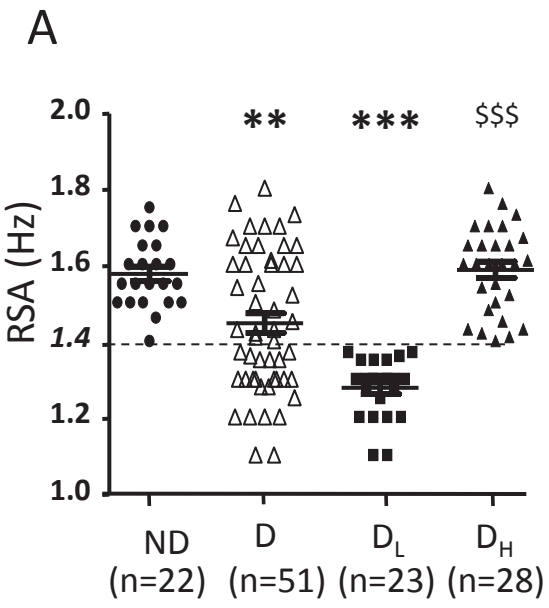
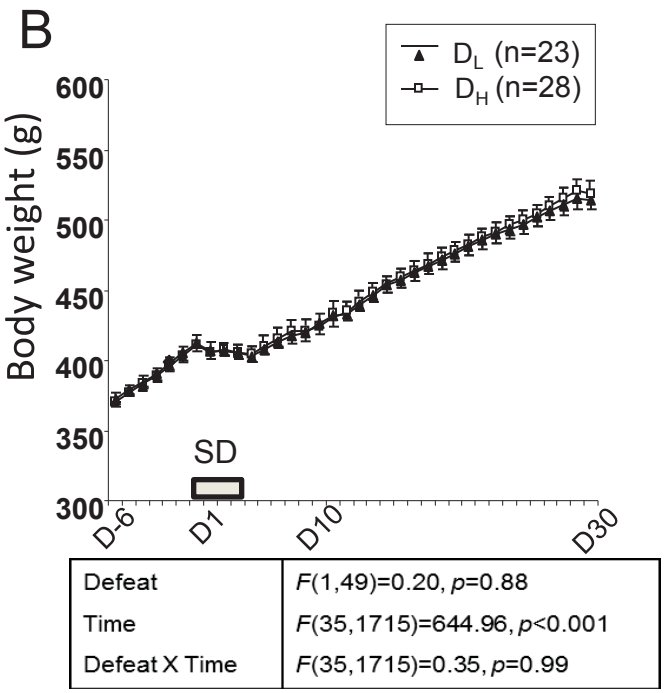
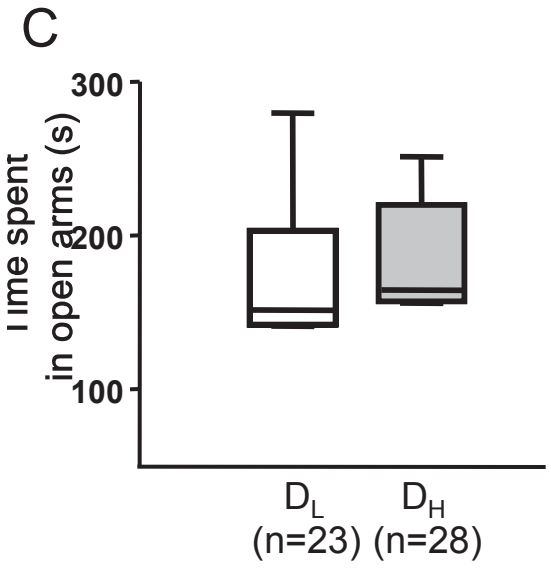


Figure 7





Defeat	$F(1,49)=0.20$, $p=0.88$
Time	$F(35,1715)=644.96$, $p<0.001$
Defeat X Time	$F(35,1715)=0.35$, $p=0.99$



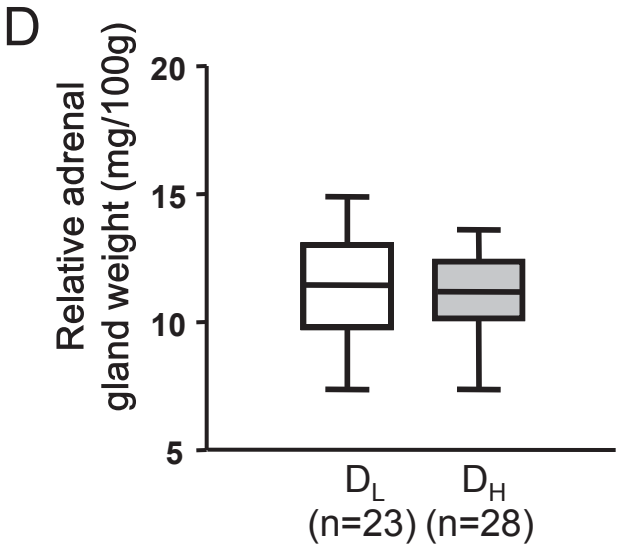
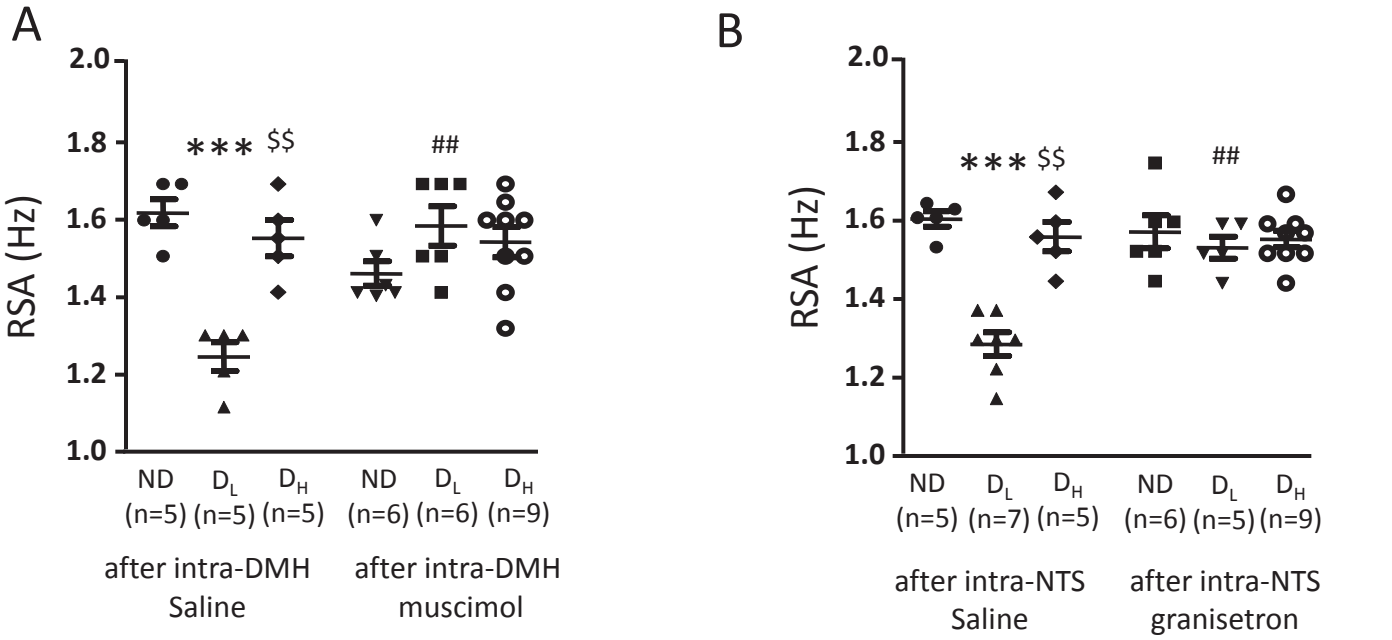


Figure 8

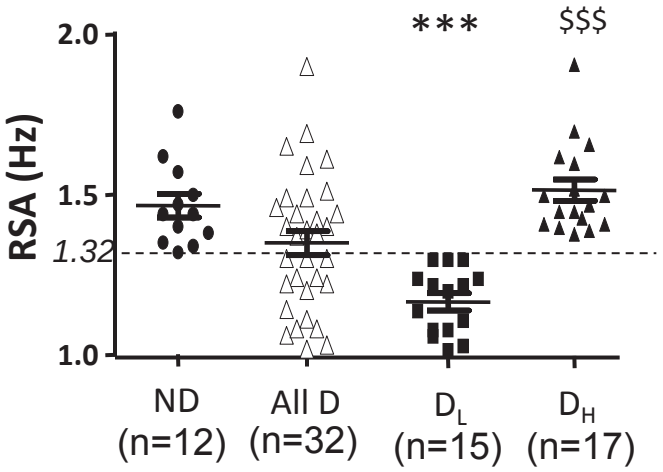


Defeat (ND or D_H or D_L)	$F(2,30)=16.5$, $p<0.001$
Treatment (muscimol or saline)	$F(2,30)=2.1$, $p=0.15$
Defeat X Treatment	$F(2,30)=4.3$, $p=0.02$

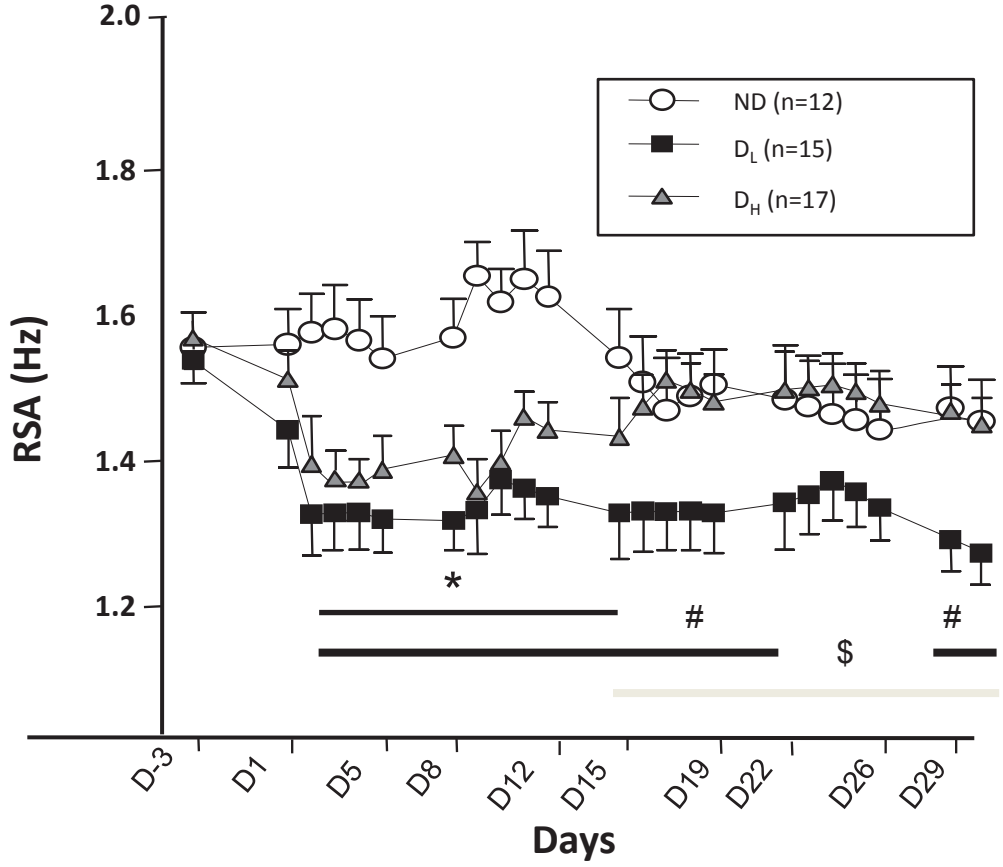
Defeat (ND or D_H or D_L)	$F(2,31)=10.4$, $p=<0.001$
Treatment (granisetron or saline)	$F(2,31)=4.1$, $p=0.050$
Defeat X Treatment	$F(2,31)=6.0$, $p=0.006$

Figure 9

A



B



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