Vulnerability to stress consequences induced by repeated social defeat in rats: Contribution of the angiotensin II type 1 receptor in cardiovascular alterations associated to low brain derived neurotrophic factor

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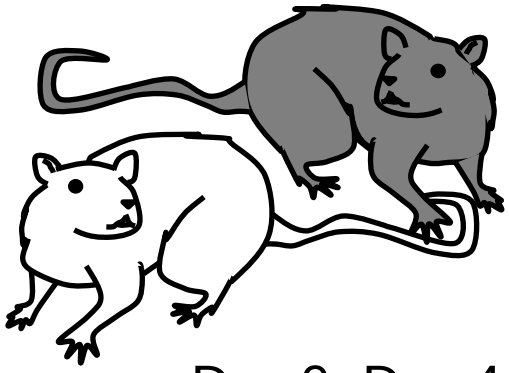
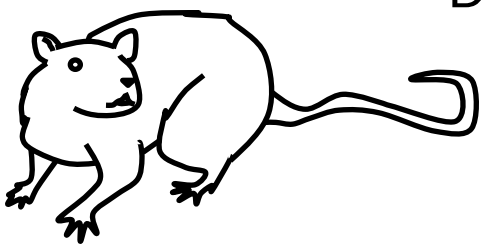
Abstract: After social stress, rats become vulnerable to depression, and this state is characterized by persistent low blood levels of brain-derived neurotrophic factor (BDNF). The aim of this study was to determine whether low BDNF levels are associated with long term autonomic changes. Defeated animals were subjected to four daily episodes of social defeats. Twenty five days later, defeated rats with low BDNF levels (Dlow) still displayed elevated sympathetic tone (as indicated by an elevated low frequency to high frequency ratio (LF/HF) in heart rate) and elevated blood pressure, as well as reduced baroreflex sensitivity (BRS). In contrast, those with higher BDNF levels (Dhigh) similar to controls, did not. Dlow animals persistent cardiovascular changes were abolished by acute inhibition of the dorsomedial nucleus of the hypothalamus (DMH). These cardiovascular changes were also prevented by chronic sub-cutaneous osmotic infusion of losartan, an angiotensin II type 1 receptor (AT1) receptor antagonist, started immediately after social defeat. In conclusion, the results show that greater vulnerability to stress consequences following a traumatic event is associated with an elevated LF/HF ratio, a persistent high blood pressure and a low BRS, all due to an AT1 receptor activation.

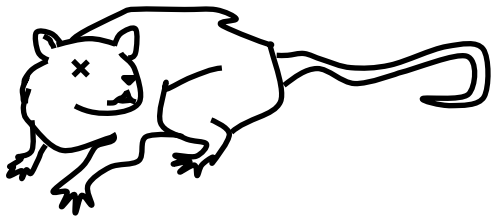
Graphical Abstract



Day 0- Day 4 Social defeat

Day 30





Non Vulnerability to cardiovascular alteration

Vulnerability to cardiovascular alteration

Non persistent activation of the DMH

Persistent activation of the DMH

Non Persistent Autonomic alterations

Persistent Autonomic alterations

Vulnerability to stress consequences induced by repeated social defeat in rats: contribution of the angiotensin II type 1 receptor in cardiovascular alterations associated to low brain derived neurotrophic factor.

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Highlights

- Previous work has shown that vulnerability to stress consequences after social defeat is linked to reduced levels of serum Brain-Derived Neurotrophic Factor (BDNF) 25 days after the last confrontation.
- We show here that these vulnerable rats also display persistent elevated sympathetic tone and blood pressure, and reduced baroreflex sensitivity (BRS).
- These persistent cardiovascular changes are mediated by the dorsomedial nucleus of the hypothalamus (DMH) as shown by acute local inhibition with muscimol.
- Angiotensin receptor 1 (AT_1) receptors are also involved in the persistent autonomic modifications because chronic sub-cutaneous infusion of the AT_1 antagonist losartan prevented them.
- The results show that persistent cardiovascular responses to a psychosocial stress associated to low serum BDNF levels can lead to vulnerability to stress consequences and that the DMH and AT_1 receptors contribute to these cardiovascular changes.

Abstract

After social stress, rats become vulnerable to depression, and this state is characterized by persistent low blood levels of brain-derived neurotrophic factor (BDNF). The aim of this study was to determine whether low BDNF levels are associated with long term autonomic changes. Defeated animals were subjected to four daily episodes of social defeats. Twenty five days later, defeated rats with low BDNF levels (Dlow) still displayed elevated sympathetic tone (as indicated by an elevated low frequency to high frequency ratio (LF/HF) in heart rate) and elevated blood pressure, as well as reduced baroreflex sensitivity (BRS). In contrast, those with higher BDNF levels (Dhigh) similar to controls, did not. Dlow animals persistent cardiovascular changes were abolished by acute inhibition of the dorsomedial nucleus of the hypothalamus (DMH). These cardiovascular changes were also prevented by chronic sub-cutaneous osmotic infusion of losartan, an angiotensin II type 1 receptor (AT_1) receptor antagonist, started immediately after social defeat. In conclusion, the results show that greater vulnerability to stress consequences following a traumatic event is associated with an elevated LF/HF ratio, a persistent high blood pressure and a low BRS, all due to an AT_1 receptor activation.

Keywords: social defeat, angiotensin, sympathetic, brain-derived neurotrophic factor, stress

1. Introduction

Decades of clinical, epidemiological and experimental research in humans and animals have strengthened the association between psychosocial stress, cardiovascular morbidity (Verrier & Lown, 1984; Rozanski et al., 1999; Sgoifo et al., 2009), and exaggerated sympathetic activation (Wittstein et al., 2005); however, the central origin of this dysfunction and the mechanisms involved remain unknown. The association between psychosocial stress and sympathetic activation can be studied with translational and naturalistic stress models that mimic the social competition originating from adverse home and work environments (Rozanski, 2005). A good example is anticipation of social defeat in the rat, one of the most powerful animal model to induce arrhythmias (Sgoifo et al., 1999).

A recent study (Claverie et al., 2016) using a social defeat procedure of 4 consecutive days of confrontation, has shown that animals sensitized to stress and vulnerable to depression are characterized by persistent low blood levels of brain-derived neurotrophic factor (BDNF) up to 1 month after the last confrontation. Previous work from our group analysing cardiovascular changes after social defeat demonstrated higher values of heart rate (HR), mean blood pressure (MBP), low frequency upon high frequency ratio (LF/HF) in heart rate variability (HRV), as well as a decreased HR response to pharmacological and spontaneous variations in blood pressure (vagal baroreflex sensitivity, BRS), 6 days after the last day of 4 consecutive days of confrontation, ie at Day 10 (Sévoz-Couche et al., 2013). This increase in LF/HF, associated with an increase in blood pressure (BP), indicates an increase in sympathetic tone (Pagani et al., 1986), while the reduced BRS is a sign of reduced parasympathetic tone. These changes were found to be due to activation of the dorsomedial nucleus of the hypothalamus (DMH) (Sévoz-Couche et al., 2013), a region involved in defence responses, including vasodilation in skeletal muscles and vasoconstriction in the viscera (Wang et al., 2010; Carrive P, 2011). The first aim of this study was therefore to determine if animals with persistent low BDNF blood levels at D30, considered as vulnerable, have persistent HRV and BRS changes, and if this is the case, if they are due to a long-term DMH activation.

In a previous study in the rat, Porter et al. showed that repeated heat/restraint in the first 2 weeks post weaning reduce the BRS and increase blood pressure and that this

effect can be inhibited by a chronic infusion of losartan, a specific AT_1 receptor antagonist (Porter et al., 2004). This suggests that AT_1 receptors could play a pivotal role in emotional stress-induced hypertension and reduced vagal tone. Therefore, the second aim of this study was to explore the possibility that sub-cutaneous chronic infusion of losartan could reduce these persistent autonomic alterations in the vulnerable defeated animals.

2. Materials and methods

2.1 General procedures

Experiments were carried out in Sprague Dawley male rats (n= 68), Centre d'Elevage R. Janvier, Le Genest-Saint-Isle, France) weighing 290–310 g (6-8 weeks old). Animals were housed in individual cages (length x width x height: 45 x 25 x 17 cm) for 1 week before the beginning of the experiments. Wildtype Groningen (WTG) male rats (400–500 g) served as resident rats in confrontation encounters (Sgoifo et al., 1998). These rats were originally bred at the University of Groningen (The Netherlands) under conventionally clean conditions. The same WTG rats were used for all successive series of experiments. All animals were kept under controlled environmental conditions (i.e. 22 ± 1°C, 60% relative humidity, 12 h light–dark cycles and ad libitum food and water). Procedures involving animals and their care were all performed in accordance with the institutional guidelines, which follow national and European laws and policies (2010/63/EU). Ethical approval was obtained for this project (#2493).

2.1.1 Social defeat paradigm. Social defeat consisted of four daily conditioning sessions that involved the same pairs of residents and intruders (Becker et al., 2001; Claverie et al., 2016; Blugeot et al., 2017). The 45 min sessions were divided into two consecutive periods. During the first period (30 min), intruders were placed individually in a protective cage within the resident animal's home cage. The protective cage allowed unrestricted visual, auditory and olfactory contact with the resident but prevented close physical contact. During the second period (15 min), the protective cage was removed, either with the resident remaining present, which allowed for physical confrontation with the intruder (3–4 confrontations of ~10 s, during each of which the intruding (defeated, D) animal was always dominated by the resident rat) or with the resident removed, which gave the intruder access to the

entire resident home cage (non defeated control intruders, ND). The control intruders were therefore never physically attacked and defeated by the resident and did show any sign of stress and/or anxiety. Animals that were wounded as a result of the SD procedure were excluded from the experiment (2% of stressed animals).

2.1.2 Body weight. Body weights in D and ND rats were recorded daily at 9:00 a.m., starting 7 days before the social defeat and continuing until the end of the protocol.

2.1.3 Elevated plus maze (EPM) test. An EPM test (described in details elsewhere, Rivat et al., 2010) was used to evaluate anxiety-related behaviour in animals at Day 9. The time spent in the various arms of the maze, and the number of entries into the open and closed arms of the maze, were recorded using custom-made software.

2.1.4 Cardiovascular and ECG recordings. At Day 20 or Day 30 (Fig. 1), as a follow-up of the studies on Day 10 (Sevoz-Couche et al., 2013), the rats were anaesthetized with pentobarbital sodium (Ceva Santé Animale, Libourne, France; 60 mg/kg i.p.) (Sévoz-Couche et al., 1998) and placed in a stereotaxic frame with their head fixed in the flat skull position. Mean and pulsatile arterial pressure (MAP and PAP, respectively) was monitored via a femoral artery catheter. ECG was recorded using stainless steel pins placed subcutaneously into the forepaws and hind paws. These signals were amplified and filtered (Universal Amplifier; Gould, Courtabœuf, France). The ECG signal was then relayed to a 1401 interface (1401 Plus; CED, Cambridge, UK), which was connected to a computer running Spike 2 (version 6.08) software (CED). Waveform data were imported off-line into Spike CED (version 6.0). The RR interval signal was derived from the ECG.-Heart rate (HR) and HRV were measured from 90-s segments 5 min after saline or pharmacological blockade of the DMH in Study 1, and at baseline in Study II.

2.1.5 HRV analysis*.* Power spectra were calculated using fast Fourier transformation (size 256, Hanning window (Padley et al., 2005)), giving a final frequency resolution of 0.04 Hz. They were performed on the time interval between two consecutive beats (RR interval) derived from the ECG. Low frequency (LF) and high frequency (HF) powers were calculated within the 0.2–0.7 and 0.7–2.5 Hz frequency ranges, respectively (Sevoz-Couche et al., 2013).

2.1.6 Spontaneous baroreflex activation. We used the sequence method to calculate spontaneous HR BRS (spontaneous BRS, Sevoz-Couche et al., 2013). Spontaneous BRS was calculated as the mean slope of R—R interval sequences for all sequences detected during 90 s segments of data, 5 min after saline or pharmacological blockade of the DMH in Study 1, and at baseline in Study II.

2.1.7 Pharmacological blockade of the DMH in anaesthetized animals. Microinjections (0.1 µl) of saline and muscimol (5 mM) into the DMH were performed at P = 3 mm, L = 0.5 mm and V = 8 mm from the bregma (Netzer et al., 2011) in the same rats. Saline was injected first followed 10 min later by muscimol.

***2.1.8 Chronic pharmacological blockade of angiotensin II receptors*.** In another series of experiments, pumps (ALZET and 2ML2) supplying losartan (5 mg/kg per day) (Foucart et al., 1996) were implanted subcutaneously (Sévoz-Couche et al., 2013) in the backs of ND and D rats in the morning after completion of the social defeat (Day 5). The infusion of losartan continued from Day 5 to Day 19. Control experiments were made without losartan.

2.1.9 BDNF analysis .Blood samples (200 µl) were collected from the tail vein at different time points (Day −3, Day 10, and Day 30) in awake rats. After centrifugation, the serum was separated and stored at −20°C. Serum BDNF concentrations were determined using a 1:25 dilution in blood and a 1:10 dilution in the RVLM using a commercial assay (Promega Corporation, Madison, WI, USA) in 96-well plates (Corning Costar® EIA plate, New York, NY, USA) in accordance with the manufacturer's instructions. The BDNF concentration was expressed in ng/mL in blood and pg/mg in tissue.

2.1.9 Histology. DMH microinjection sites were identified from the tip of the micropipette track in 50 µm sections of brain tissue that were fixed in 10% formalin solution and cryoprotected in 20% sucrose solution for 5 days. Brain sections were stained with thionin to identify structures. The most ventral point of each injection track was determined. Only rats with correctly positioned injection sites were used for data analysis.

2.1.10 Statistical analysis.Study I. A K-means clustering method based on serum BDNF values obtained before social defeat (Day -3) and at the end of the procedure

(Day 30) was first used to subdivide the Defeated animals into those with low (Dlow) and higher (Dhigh) BDNF level subgroups at D30, as described by Claverie et al (2016). The K-means clustering algorithm groups animals into a given number of subgroups (in our case vulnerable vs nonvulnerable animals), in a way that minimizes intragroup variability and maximizes intergroup variability (Claverie et al., 2016). The three groups (ND, Dlow and Dhigh) were then compared using ANOVAs. Chronological changes in serum BDNF levels (Day -3, Day 10, Day 20 and Day 30) and body weight (daily from Day -3 to Day 30) were assessed with repeated measure two-way ANOVA where the repeated factor was time and the other factor was defeat (ND, Dlow, Dhigh). The effects of a vehicle versus drug treatment (muscimol) in the DMH on cardiovascular parameters at Day 30 was assessed with a two-way repeated measures ANOVA where the repeated factor was treatment (muscimol or vehicle) and the other factor was defeat (ND, Dlow, Dhigh). Finally, differences in EPM between the three groups were analysed using a one-way ANOVA.

Study II. Chronological changes in serum BDNF levels (Day -3, Day 10 and Day 20) and body weight (daily from Day -3 to Day 20) were assessed with repeated measure two-way ANOVA where the repeated factor was time and the other factor was defeat (ND and D). The changes in cardiovascular parameters in ND and D rats at D20 with and without chronic infusion of losartan were compared with two-way ANOVA where the factors were treatment (with or without losartan) and the other factor was defeat (ND and D).

Post hoc analysis with multiple comparisons using the Bonferroni correction was applied after ANOVA when necessary, and results were considered significant if $P < 0.05$. Analyses were performed using Prism 7.03 (GraphPad Software).

2.2 Experimental overview (Fig. 1)

2.2.1 Study I: Behavioural and cardiovascular changes evoked by social defeat up to Day 30, and the contribution of the DMH. Social defeat lasted from Day 1 to Day 4. Study I was ended at Day 30, 26 days after the last session of social defeat. Serum BDNF concentration was analysed from blood samples taken in ND (n= 13) and D (n= 30) animals at Day -3 before social defeat, and at Day 10, Day 20 and Day 30 to determine subgroups using the K-means clustering method. The animals were tested on Day 9 in the EPM to assess their level of anxiety. On Day 30 they were

anaesthetized and prepared for acute recording of ECG and blood pressure, and to analyse MBP, HR, HRV and BRS. They were then microinjected with muscimol or saline in the DMH to assess the role of DMH neurons in long-term cardiovascular alterations.

2.2.2 Study II: Role of AT_1 receptors in the cardiovascular changes evoked by social defeat up to Day 20. Study II ended 16 days after the last session of social defeat (Day 20) and was designed to determine the effect of a chronic infusion of losartan on the long-term cardiovascular alterations induced by defeat. Serum BDNF concentration was analysed from blood samples taken in ND and D animals at Day -3 before social defeat, and at Day 10 and Day 20. The animals were anesthetised on Day 20 and prepared for recording of ECG and blood pressure. A total of 10 animals (ND, n= 4 and D, n= 6) received subcutaneously infusions with losartan. Another 15 animals (ND, n= 7 and D, n= 8) treated in exactly the same conditions except that they did not receive any infusion, served as control.

3. Results

3.1 Study I: Physiological, behavioural and cardiovascular changes evoked by social defeat up to Day 30, and the contribution of the DMH.

3.1.1 Serum BDNF changes: identification of subgroups of Defeated animals

Using the K-means clustering method based on serum BDNF levels obtained before the social defeat (Day -3) and at the end of the procedure (Day 30), we identified two groups of Defeated animals: Defeated vulnerable (Dlow, n= 13) which had reduced levels of BDNF, and Defeated non-vulnerable (Dhigh, n= 17) which had levels of BDNF comparable to Non-Defeated ND (n= 13) rats (Fig. 2A).

The time course of serum BDNF levels from Day -3 to Day 30 was then analysed (Fig. 2B). An interaction between time and defeat groups was found [$F(6,120]= 5.74$, $P<0.001$]. Before social defeat, BDNF levels were similar in ND rats and in the two subgroups of Defeated rats, Dlow and Dhigh (Day -3, $P = 0.95$ and P=0.86, respectively, Fig. 2B). At Day 10 and Day 20, BDNF levels were lower in both Dlow and Dhigh rats compared to ND animals because of the social defeat. However at Day 30, Dhigh rats had recovered, while Dlow rats had not; their BDNF levels were lower than in ND and Dhigh rats.

All further analyses in Study I were based on the division of the D group into Dlow and Dhigh rats.

3.1.2 Cardiovascular changes at Day 30: changes in, HR MAP and HRV in ND, Dlow and Dhigh rats and contribution of the DMH.

3.1.2.1 HR and MAP: An interaction between treatment (saline vs muscimol) and groups (ND vs Dlow and Dhigh) was found for MAP [F(2,40]= 19.06, P<0.0001] but not HR (Fig 3A) at Day 30 in anesthetized animals. MAP was higher in Dlow than in Dhigh or ND rats after saline but not after muscimol, while HR was not significantly different between Dlow and ND or Dhigh rats (*P* = 0.21 each), after saline or muscimol.

3.1.2.2 HRV: An interaction between treatment and defeat groups was found for HF [F(2,40)= 7.36, P=0.0018] and LF/HF [F(2,40)=21.27, P<0.0001]. HF was lower and LF or LF/HF were higher in Dlow than in Dhigh or ND animals after saline but not after muscimol (Fig 3B).

3.1.2.3 BRS: An interaction between treatment and groups was found for BRS [F(2,40)= 15.01, P<0.0001] (Fig 3C). Spontaneous BRS was lower in Dlow than in ND and Dhigh animals after saline but not after muscimol.

A representative site of DMH microinjection is shown in Fig 3D.

3.1.3 Behavioural and other physiological changes.

To determine if there was a difference in the stress impact between Dlow and Dhigh rats, additional behavioural and physiological variables were investigated, including a behavioural test of anxiety in the elevated plus maze and body weight.

3.1.3.1 EPM*:* At Day 9, both Dlow and Dhigh rats spent more time in the closed arms and less time in the open arms of the elevated plus maze than ND rats did (Supplemental Fig. 1).

3.1.3.2 Body weight. Changes in body weight before, during and after the social defeat are shown in Supplemental Fig. 2. There was a significant interaction between time and defeat groups [F(72,1440]= 4.44, P<0.0001]. ND, Dlow and Dhigh rats had the same weight in the days preceding the social defeat; however, as expected from

previous studies (Sévoz-Couche et al., 2013), defeated rats stopped putting on weight from the first session of social defeat, while ND rats continued their normal growth. By the end of the social defeat, all defeated rats were lighter than ND rats (Dlow vs Dhigh vs ND (mean ± S.E.M.): 402 ± 2 vs 402 ± 4 vs 432 ± 4 g at Day 5), but they gradually recovered in the following weeks. There was no difference between Dlow and Dhigh rats at any time (P = 0.84).

3.2 Study II: HRV and cardiovascular changes induced by social defeat up to Day 20 in rats with or without osmotic infusion of the AT_1 receptor antagonist losartan.

The time course of serum BDNF levels from Day -3 to Day 20 was analysed in ND and D animals, without or with losartan infusion starting at the end of social defeat (Fig. 4A). In both cases, an interaction between time and defeat groups was found between time and defeat groups ([F(2,26)= 5.54, P = 0.012] and [F(2,16)= 7.30, P=0.0056], respectively). Initially, i.e. before social defeat, BDNF levels were similar in ND and D rats (Day -3, P = 0.88 and P = 0.94, respectively). As expected, at Day 10 and Day 20, BDNF levels were lower in D rats compared to ND rats because of the social defeat. This reduction was the same with or without losartan infusion. In addition, clear cardiovascular changes were observed at D20 in the D rats compared to the ND rats (higher MAP and LH/HF, and lower BRS). However, and in contrast to the serum BDNF levels, these cardiovascular changes were completely abolished by the losartan infusion (Fig. 4B). Statistical analysis confirmed a significant interaction between the two conditions for MAP ([F(1,21)= 4.46, P = 0.04]), LF/HF ([F(1,21)= 5.14, P = 0.03]) and BRS ([F(1,21)= 8.93, P = 0.006]).

4. Discussion

The main finding of the study is that our model of social defeat can induce vulnerability to stress consequences, with low BDNF levels associated to a persistent increase in the LF/HF ratio and blood pressure. At Day 30, this corresponds to approximately 50% of the defeated animals. Importantly, increased activity in the DMH and chronic activation of AT_1 receptors contribute to this effect.

4.1 Psychophysiological parameters in Defeated Dlow and Dhigh animals

Apart from its well-characterised actions as a trophic factor, accumulating evidence suggests that BDNF exhibits “non-trophic” neuroprotective actions; for example, serum and hippocampal BDNF levels are reduced in mood disorders (Kreinin et al., 2015). In the short term, low BDNF levels appear to be compensatory, protecting against deleterious chronic stress-related events, rather than maladaptive (Ortiz et al., 2014). Regardless, low serum BDNF is now considered to be a predictive biomarker of vulnerability to depression. Four weeks (Day 30) after a priming stressful event (social defeat procedure), 42% of animals displayed persistent decreased serum and hippocampus BDNF concentrations, reduced hippocampal volume and neurogenesis, CA3 dendritic retraction and decrease in spine density, as well as amygdala neuron hypertrophy, constituting latent vulnerability traits to depression (Blugeot et al., 2011). In this group, called vulnerable, a subsequent mild stress evoked a rise of serum corticosterone levels and a “depressive” phenotype, in contrast to nonvulnerable animals. Subsequent studies using a K-means clustering based on blood levels of BDNF found that animals with higher episodes of epileptic seizures had persistent low levels of BDNF, and epilepsia sensitization was therefore considered to be associated to vulnerability to depression (Claverie et al., 2016). We confirm, by using the same methodology, that the social defeat procedure splits the animal population into two groups: those that recover to pre-stress BDNF levels by Day 30 (Dhigh, which may be considered to be non-vulnerable to depression as found in Claverie’s study) and those that do not (Dlow, which are possibly vulnerable to depression).

The impact of chronic stress was not different between Dlow and Dhigh rats. There was no difference in the expected weight loss between these groups during the social defeat or in the following recovery. In addition, an EPM test applied at Day 9 showed that both Dlow and Dhigh rats spent less time in the open arms, showing that both had developed an anxiety-like state. Therefore, the vulnerability to depression is not associated to a higher sensitization to anxiety.

4.2 Cardiovascular alterations induced by social defeat and contribution of the DMH

Stress and depression are associated to cardiovascular alterations. In particular, depressive symptoms are associated to lower HRV (Walter et al., 2019), increased LF/HF ratio (Kikuchi et al., 2009) and reduced BRS (Davydov et al., 2007). In our

study, animals with reduced BDNF have also similar persistent cardiovascular alterations. Dlow animals were sensitized by the stressful defeat because they had persistent exaggerated sympathetic activity at Day 30. First, we observed that the LF/HF ratio and MAP (but not HR) were higher than in ND and Dhigh animals in control conditions (i.e. after saline into the DMH). Of note, the HF band (a marker of parasympathetic activity) was decreased in Dlow rats, and that was confirmed with a reduced spontaneous BRS. These data show that the Dlow subgroup is less resilient to stress considering sympathetic and parasympathetic activity, and the indicators of this low resilience in terms of autonomic balance may be higher LF/HF ratio and MAP, and a lower HF and BRS. Therefore, vulnerability to stress induced by social defeat is associated with not only lower BDNF levels but also with cardiovascular alterations.

DMH Muscimol treatment in Dlow animals reversed the increase in the LF/HF ratio and MAP, as well as the decrease in HF and BRS, thus showing that the DMH is also responsible for this persistent increase in sympathetic activity and decrease in parasympathetic tone. It suggests that the DMH remains active in the vulnerable subpopulation of D rats to keep the sympathovagal balance tilted towards sympathetic dominance.

4.3 Contribution of AT_1 receptors in social defeat-induced cardiovascular alterations.

Several studies converge to the pivotal role of Angiotensin II in animal models of hypertension (Campagnaro et al., 2012; Li et al., 2015; Soares et al., 2017). In particular, central AT_1 receptors may play an important role in mediating the hypertensive response to acute emotional stress (Mayorov & Head, 2003). Moreover, chronic infusion of losartan was found to prevent the increase in blood pressure and decrease in BRS induced by stress (Porter et al., 2004). These results suggest that AT_1 receptors could be involved in persistent defeat-induced cardiovascular alterations (in particular increase in MAP and LF/HF ratio) in Dlow and Dhigh animals.

As it was not possible to determine the time of recovery from Study I, we could not accurately discriminate D rats based on BDNF levels at Day 20. Therefore in Study II that ended at Day 20 (with or without losartan), defeated animals were not separated

in two subgroups. Under these conditions, BDNF was similarly lower at Day 10 and Day 20 in D than in ND animals, whether they had received losartan or not. This shows that the reduction in BDNF after stress is not mediated by activation of AT_1 receptors. However, the change in MAP LF/HF ratio and BRS observed in D animals was completely abolished by losartan. At this point it is not possible to determine if those rats that would have been Dhigh had already recovered in terms of cardiovascular stress effects, but we can be reasonably confident that those rats that would have been Dlow did recover. So it appears that losartan reduced cardiovascular alterations at least in Dlow at Day 20.

The site of action (central or peripheral) of losartan can't be specifically determined, since the osmotic diffusion was done subcutaneously. Losartan could have acted peripherally. Recently, a study by Chiu et al showed that a reduction of blood pressure elevation by oral gavage of losartan in spontaneously hypertensive rats occurred through the suppression of LARG expression and MYPT-1 phosphorylation in vascular smooth muscle cells (Chiu et al., 2019). Also, AT_1 receptor antagonists are known to counteract the remodelling of small arteries in hypertension (Schiffrin, 2002).

Losartan could also have acted centrally. One possibility is that losartan acted on AT_1 receptors located in the DMH itself. Indeed, an interesting study performed by Shekhar et al showed that injections of losartan into the DMH of "panic-prone" rats blocked the anxiety-like and physiological components of DMH lactate-induced panic-like responses. However, this local Angiotensin receptors- mediated effect may be specific to the DMH lactate response because it was not observed with panic-like responses induced by direct activation of the DMH by NMDA (Shekhar et al., 2006). It is more likely that Angiotensin acted in regions that receive projections from the DMH and are involved in cardiovascular regulation, i.e. the rostral ventrolateral medulla (RVLM) and the nucleus tractus solitarius (NTS) (Thompson et al., 1996). The RVLM is the main premotor sympathoexcitatory vasopressor centre (Brown & Guyenet, 1984). It has a high density of AT_1 receptors (Allen et al., 1998) and activation of these receptors increases blood pressure through ROS production (de Oliveira-Sales et al., 2010). Upregulation of RVLM AT_1 receptors causes hypertension in rats subjected to electric foot-shocks (Du et al., 2013), suggesting that these receptors may be responsible for stress-induced hypertension in D

animals. If correct, there may be a link between RVLM AT_1 receptors and the DMH. Direct projections from the DMH to the RVLM exist (Fontes et al., 2001; Horiuchi et al., 2004), but the angiotensin link could also be indirect. In support of an involvement of the NTS in persisting cardiovascular alterations in social defeat, a significant decrease in systolic blood pressure and ROS production in the NTS was found in male SHRs after a 2 weeks treatment with losartan (Cheng et al., 2010). In addition, losartan reduced not only hypertension and LF/HF ratio but also BRS, and we showed previously that the NTS was involved in short-term BRS suppression in social defeat. In line with this result, in another study using adenoviral vector experiments, Paton et al. showed that Angiotensin II activates eNOS and ROS in the NTS to depress the baroreflex (Paton et al., 2001).

Therefore, this study clearly establishes a role for AT_1 receptors in persisting cardiovascular alterations after social defeat, and shows that losartan can be an effective treatment against these deleterious modifications. A hypothetical summary diagram showing a likely link between DMH, RVLM and/or NTS in persistent cardiovascular modifications linked to angiotensin after social defeat is presented Fig. 5. However, more experiments are needed to determine the exact location of AT_1 receptors, as well as the mechanism involved (either AT_1 receptor increase or hyperactivation, and a possible link with oxidative stress).

5 Limitations to the study

Different studies by our laboratory have shown that low serum BDNF levels are associated with vulnerability to depression (Blugeot et al., 2011; Bouvier et al., 2017). Another work used solely a k-means discrimination based on BDNF levels to directly identify animals as potentially vulnerable to depression (Claverie et al., 2016). Using the same procedure, we could separate defeated animals in two subgroups based on their serum BDNF levels (Dlow and Dhigh animals). We clearly evidenced that another consequence of social defeat is long-lasting cardiovascular alterations, similar to those found in depression. Based on the studies mentioned above, it is possible that the Dlow animals of this study were also vulnerable to depression. However, only the use of parameters such as anhedonia or behavioural tests would allow to confirm this possibility.

6 Novelty and significance

Patients susceptible of developing persistent autonomic dysfunction (and possibly depression) after stressful encounters could be identified using sympathetic (higher LF/HF ratio and elevated blood pressure) and vagal (reduced baroreflex sensitivity) indicators in combination with measurement of serum levels of BDNF (low levels). Furthermore, such patients (e.g. after ischemic stroke) (Sörös & Hachinski, 2012) could be systemically treated with losartan to restore their autonomic balance.

7 Sources of Funding

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8 Acknowledgments

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Figures legends

Fig. 1. Experimental overview of the different groups of experiments

In Study I, serum BDNF levels were measured at Day -3, Day 10, Day 20 and Day 30 in the same animals to determinate vulnerability to depression. EPM was performed at Day 9, and HRV parameters, HR and MAP were obtained under anaesthesia at Day 30, 5 min after intra-DMH microinjections (saline [veh] or muscimol [musc]). In Study II, HRV parameters, HR and MAP were obtained at Day 20 in rats without or with osmotic infusion of losartan from Day 5 to Day 19.

Fig. 2. Classifications of defeated animals in Dlow or Dhigh subgroups

(A) Defeated animals were classified as Dlow or Dhigh subgroups according to the K-means clustering method based on baseline (Day -3) and recovery (Day 30) serum BDNF levels.

(B) Time course of serum BDNF levels in ND (n= 13), Dlow (n= 13) and Dhigh (n= 17) rats, before (Day −3) and after (Day 10, Day 20 and Day 30) the social defeat procedure. BDNF values were similar between all groups at Day −3, but decreased in Dlow and Dhigh rats at Day 10 before recovering in Dhigh rats only at Day 30. Values are the mean ± S.E.M. * $P < 0.05$ and ** $P < 0.01$, $^{\#}P < 0.05$.

Fig. 3. Effect of DMH inhibition on HRV parameters in ND, Dlow and Dhigh anaesthetized rats

At Day 30, heart rate (HR, A) was not different, mean arterial pressure (MAP, A) and the LF/HF ratio (B) were higher, and the baroreflex sensitivity (BRS, C) was lower in Dlow (n=13) compared to ND (n=13) and Dhigh (n=17) rats after saline but not after muscimol into the DMH. Values are the mean ± S.E.M. *P < 0.05 and ***P < 0.001, $^{\#\#}P$ <0.01 and $^{\#\#\#}$P<0.001. (D) Representative injection site (arrow) in the DMH (approximately −2.9 mm from the bregma). *3V*, third ventricle; *DMH,* dorsomedial nucleus of the hypothalamus; *f*, fornix; *mt*, mammillothalamic tract; *PeF*, perifornical area; *VMH*, ventromedial nucleus of the hypothalamus.

Fig. 4. **BDNF level and cardiovascular changes induced by social defeat with or without losartan osmotic treatment.**

(A) Time course of serum BDNF levels in ND and D rats without losartan (los-, control, n=7 and n=8, respectively) or with losartan (los+, experimental, n=4 and n=6, respectively), before (Day −3) and after (Day 10 and Day 20) the social defeat procedure in Study II. As in Study I, BDNF values were similar between ND and D groups at Day −3, but decreased D rats at Day 10 and Day 20, independently of the losartan treatment. Values are the mean ± S.E.M. * P < 0.05.

(B) At D20 control defeated rats (D) had a higher MAP and LF/HF ratio and a lower BRS than ND rats (los-). This difference was abolished by chronic losartan infusion for 2 weeks following social defeat (los+). Values are the mean ± S.E.M. * P < 0.05, ** P < 0.01 and *** P < 0.001.

Fig. 5. Hypothetical functional interaction between the DMH, the RVLM and the NTS underlying persistent cardiovascular changes associated with the social defeat.

This summary diagram shows that the social defeat activates the DMH to produce different long-term autonomic modifications: a decrease in the parasympathetic drive leading to a reduced BRS and an increase in the sympathetic drive to produce a persistent hypertension and modification in frequential HRV (increase in the LF/HF ratio). Osmotic infusion of losartan, a specific angiotensin II type 1 receptor antagonist, prevented these alterations, suggesting that angiotensin II may be

responsible for these stress effects. Possible mechanisms involve the RVLM and the NTS, two regions targeted by the DMH. Angiotensin II may act at on AT_1 RVLM receptors located on presympathetic neurones to increase the sympathetic drive. AT_1 receptors can also be activated into the NTS to increase ROS production and activate the sympathetic drive on one hand, and inhibit baroreceptor neurons that project on the nucleus ambiguous (NAmb) on the other hand, resulting in both hypertension and reduced BRS.

Supplemental Figures

Supplemental Fig. 1. Assessment of anxiety profile in ND, Dlow and Dhigh rats in the elevated plus maze test at Day 9 (Study I)

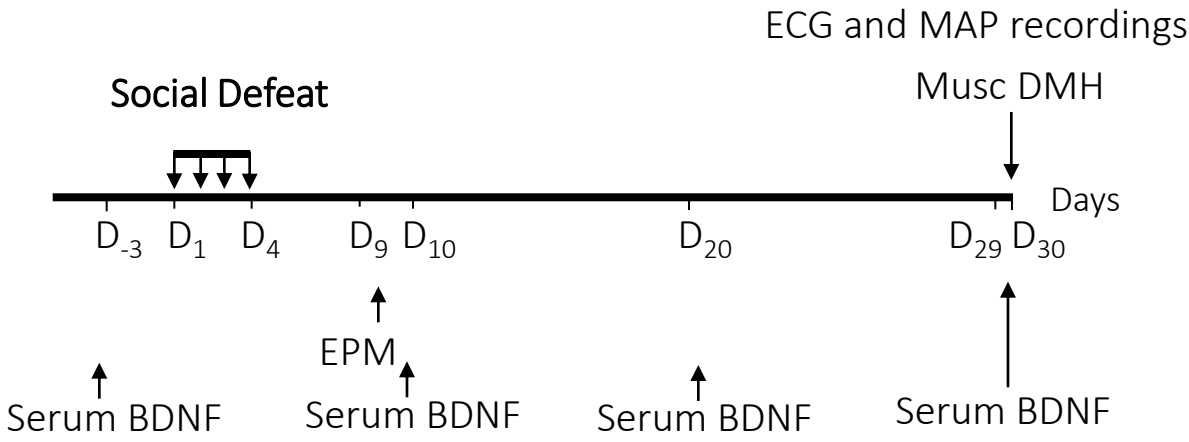
At D9, both Dlow and Dhigh rats spent more time in the closed arms and less time in the open compared to ND animals. Box and whisker graphs indicate the minimum and maximum values and the median. **P* < 0.05 and ***P* < 0.01.

Supplemental Fig. 2. Long-term effects (Day -3 to Day 30) of social defeat on body weight in ND, Dlow and Dhigh rats (Study I)

There was an interaction between group and time effect. Body weights of Dlow and Dhigh animals were lower than those of ND rats during the first days of the conditioning sessions, until at least D13. Each point represents the mean ± S.E.M. of all ND, Dlow or Dhigh rats. **P* < 0.05

Figure 1

Study I



Study II

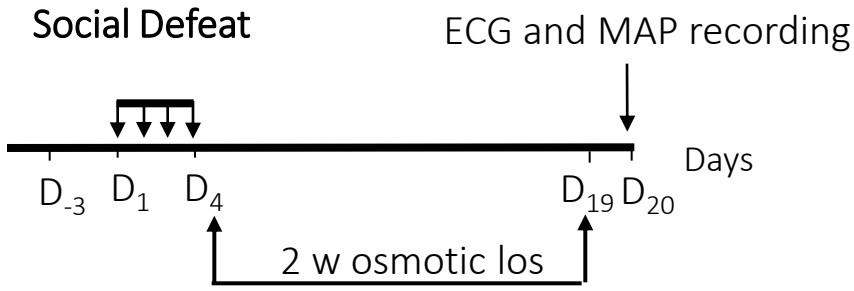


Figure 2

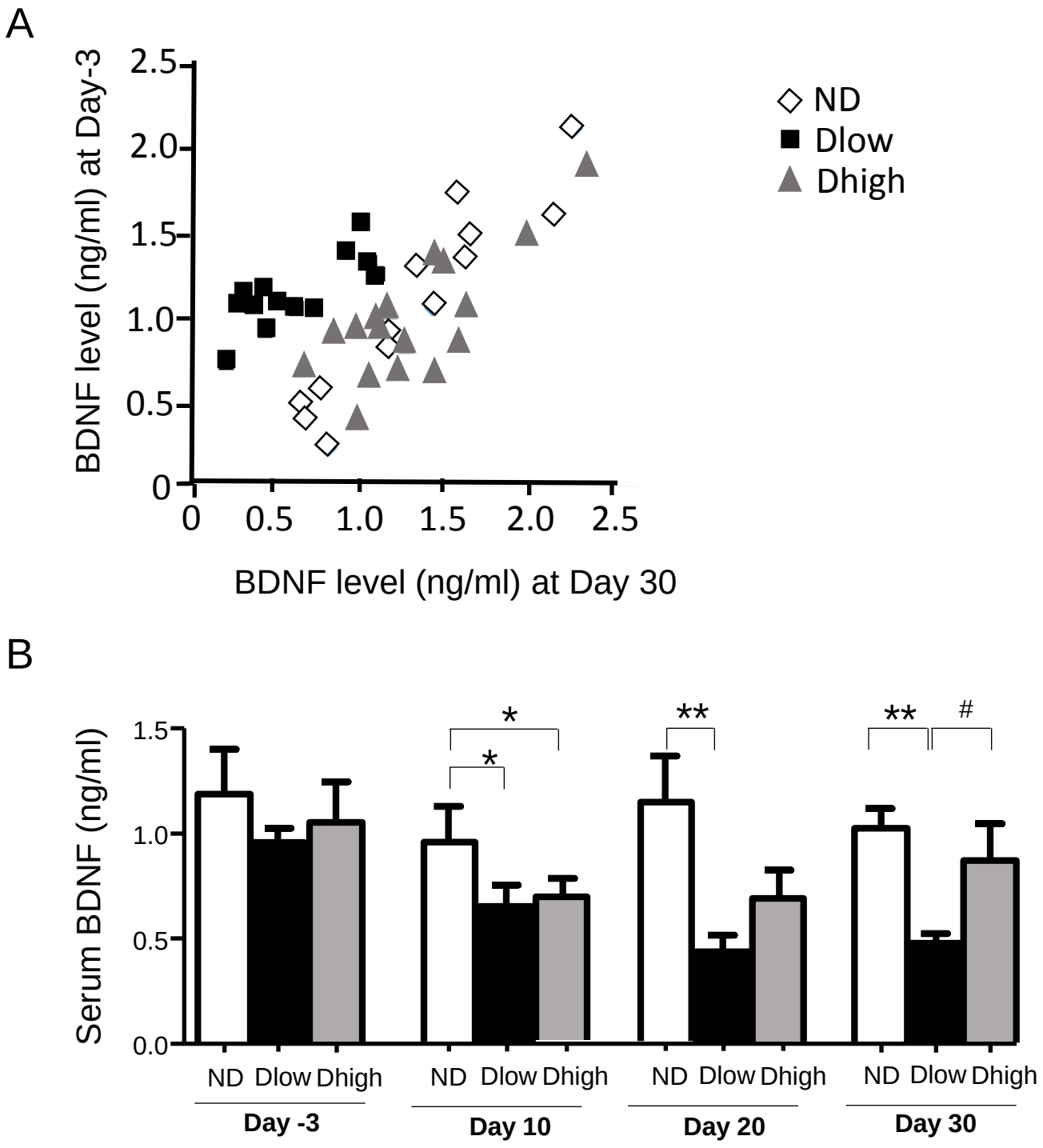

A
BDNF level (ng/ml) at Day-3
2.5
2.0
1.5
1.0
0.5
0
0
0.5
1.0
1.5
2.0
2.5
BDNF level (ng/ml) at Day 30
ND
Dlow
Dhigh
B
Serum BDNF (ng/ml)
1.5
1.0
0.5
0.0
*
*
**
**
#
ND Dlow Dhigh
ND Dlow Dhigh
ND Dlow Dhigh
ND Dlow Dhigh
Day -3
Day 10
Day 20
Day 30

Figure 3

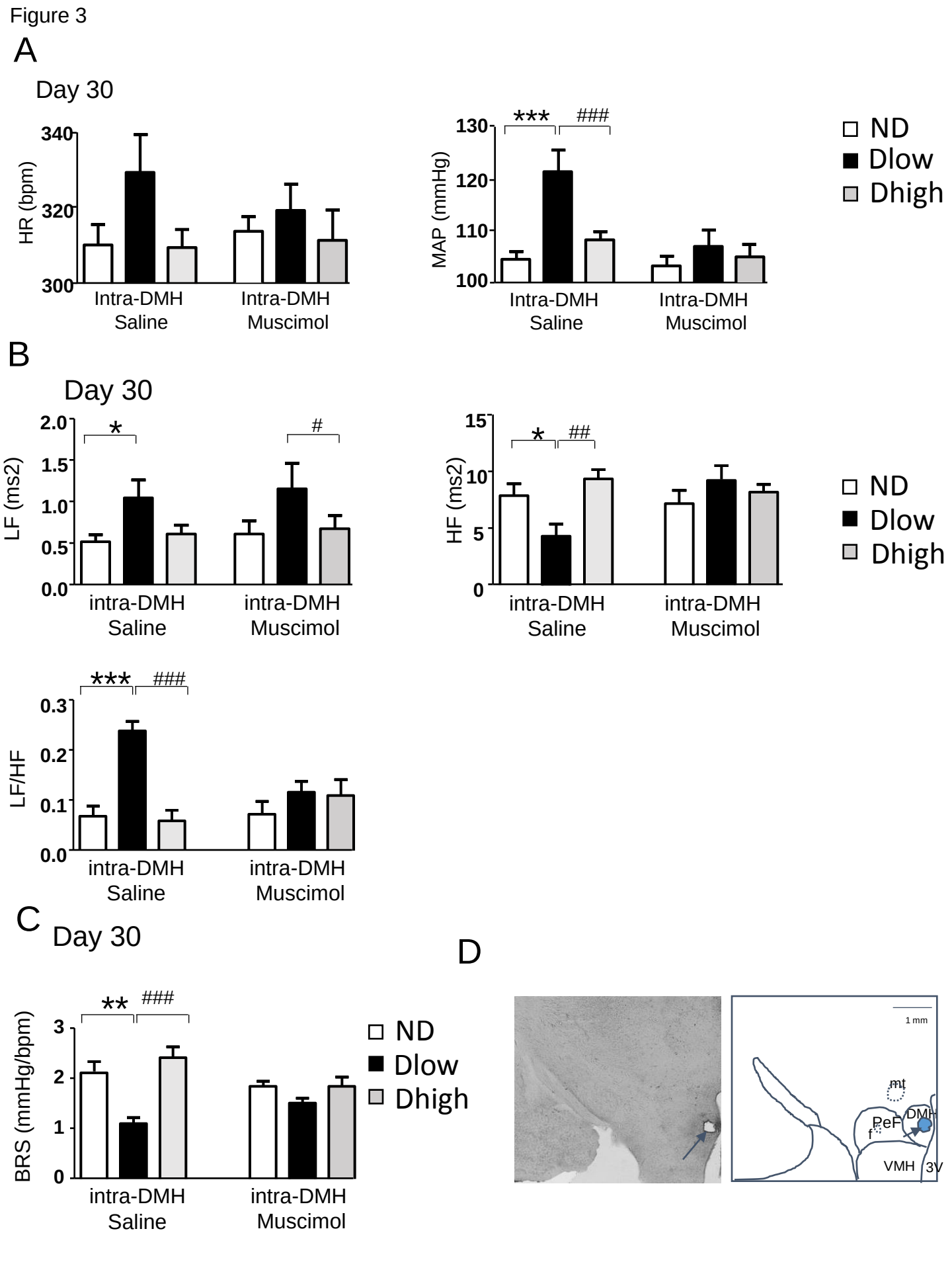

A
Day 30
HR (bpm)
340
320
300
Intra-DMH Saline
Intra-DMH Muscimol
MAP (mmHg)
130
120
110
100
***
###
ND
Dlow
Dhigh
B
Day 30
LF (ms2)
2.0
1.5
1.0
0.5
0.0
*
#
intra-DMH Saline
intra-DMH Muscimol
HF (ms2)
15
10
5
0
*
##
LF/HF
0.3
0.2
0.1
0.0
***
###
C
Day 30
BRS (mmHg/bpm)
3
2
1
0
**
###
D
1 mm
mt
DMH
ReF
f
VMH
3V

Figure 4

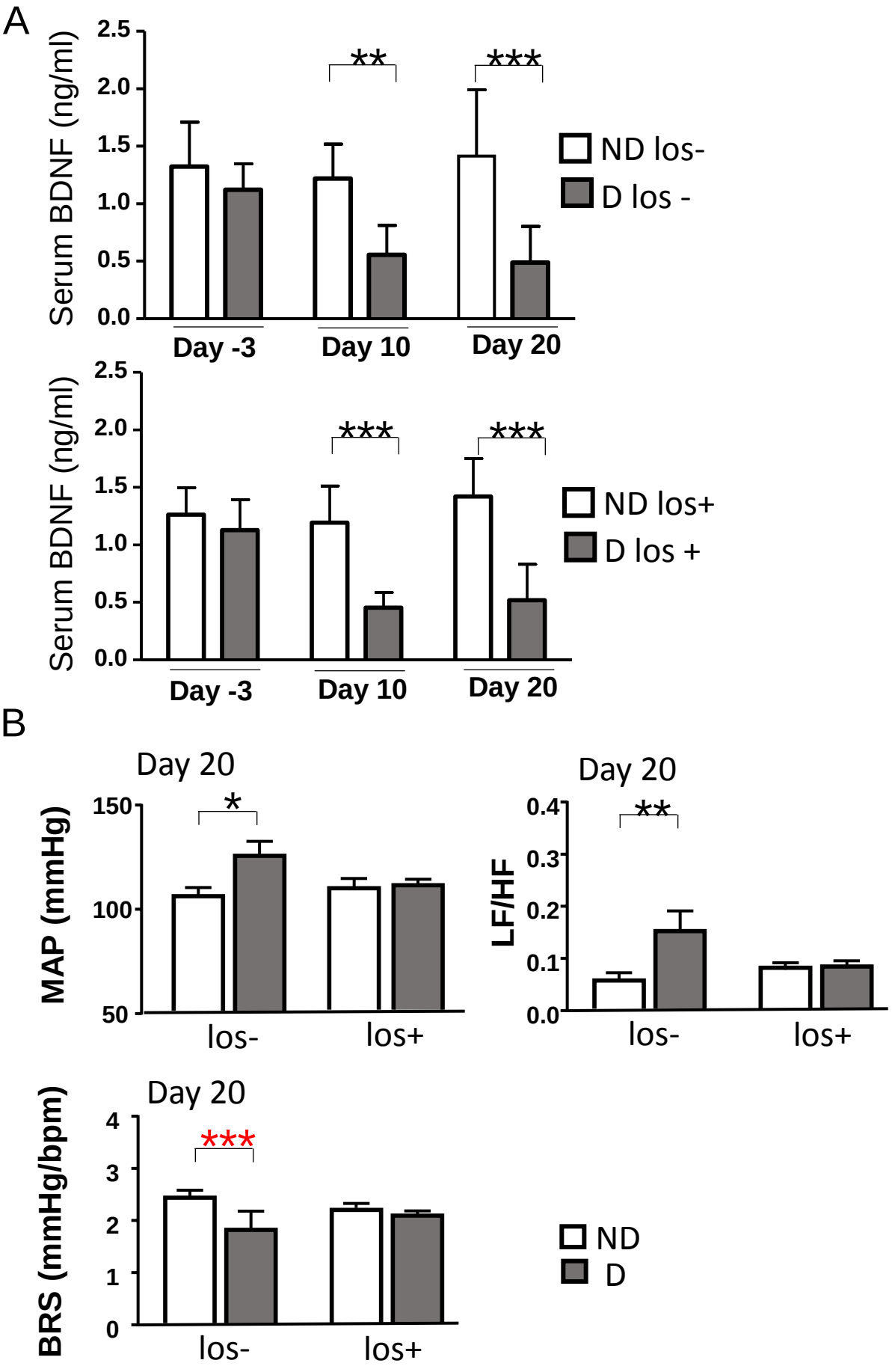
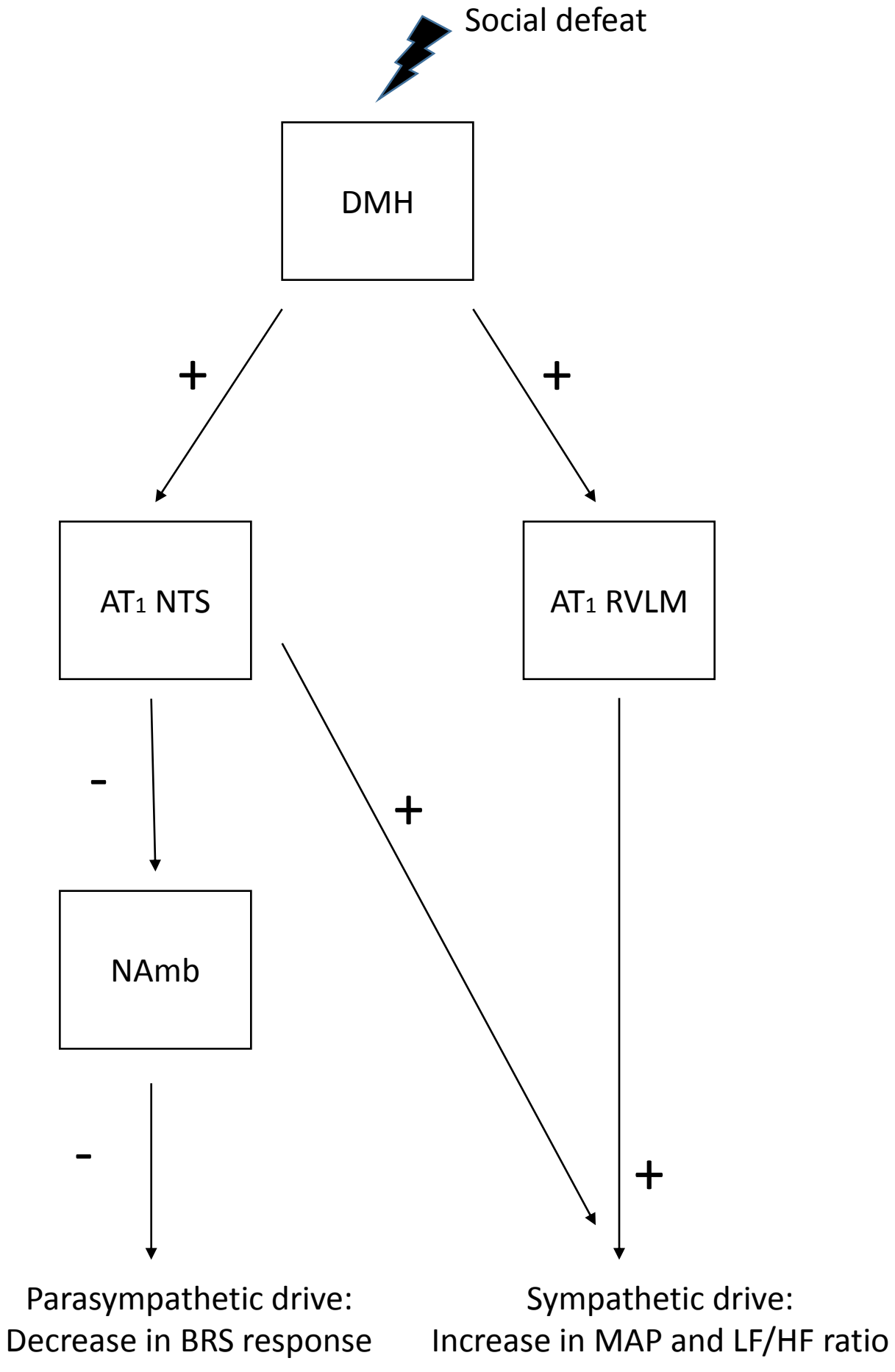

A
Serum BDNF (ng/ml)
2.5
2.0
1.5
1.0
0.5
0.0
**
***
ND los-
D los -
Day -3
Day 10
Day 20
Serum BDNF (ng/ml)
***
***
ND los+
D los +
Day -3
Day 10
Day 20
B
Day 20
MAP (mmHg)
150
100
50
*
los-
los+
Day 20
LF/HF
0.4
0.3
0.2
0.1
0.0
**
los-
los+
Day 20
BRS (mmHg/bpm)
4
3
2
1
0
***
los-
los+
ND
D

Figure 5


Social defeat
DMH
+
+
AT1 NTS
AT1 RVLM
-
+
NAmb
-
+
Parasympathetic drive:
Decrease in BRS response
Sympathetic drive:
Increase in MAP and LF/HF ratio

Supplementary material for online publication only

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***Response to Reviewers**

Answer to Editor In Chief

- Delete the running head.

  - Do not use a justified layout. Use the format: paragraph alignment -- left.

  - Delete the list of abbreviations.

  - Do not write 'et al' in italic.

  - Rename heading 2 into Materials and methods.

  - Please number all subheadings.

  - Number heading Discussion as 4.

  - Use Fig. (not Figure).

  - Use S.E.M. (not SEM).

We have modified the manuscript accordingly.

AUTHOR DECLARATION

We wish to confirm that there are no known conflicts of interest
associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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Signed by the corresponding author, on behalf of all authors

Caroline Sevoz-Couche