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# Reduced NPY Y1 receptor hippocampal expression and signs of decreased vagal modulation of heart rate in mice

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2	Reduced NPY Y1 receptor hippocampal expression and signs of decreased vagal
3	modulation of heart rate in mice
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#### 17 Abstract

Central neuropeptide Y (NPY) signaling participates in the regulation of cardiac autonomic outflow, 18 19 particularly via activation of NPY-Y1 receptors (Y1Rs). However, the specific brain areas and neural pathways involved have not been completely identified yet. Here, we evaluate the role of 20 hippocampal Y1Rs in the modulation of the autonomic control of cardiac function using a 21 22 conditional knockout mouse model. Radiotelemetric transmitters were implanted in 4-month-old male mice exhibiting reduced forebrain expression (rfb) of the Y1R (Npy1r<sup>rfb</sup>, n=10) and their 23 corresponding controls (Npy1r<sup>2lox</sup>, n=8). ECG signals were recorded (i) during resting conditions, 24 (ii) under selective pharmacological manipulation of cardiac vagal activity, and (iii) during acute and 25 26 chronic psychosocial stress challenges, and analyzed via time- and frequency-domain analysis of heart rate variability. Npy1r<sup>fb</sup> mice showed a lower Npy1r mRNA density in the dentate gyrus and 27 in the CA1 region of the hippocampus. Under resting undisturbed conditions, Npy1r<sup>fb</sup> mice 28 exhibited (i) a higher heart rate, (ii) a reduced overall heart rate variability, and (iii) lower values of 29 the indices of vagal modulation compared to Npy1r<sup>2lox</sup> counterparts. Following pharmacological 30 vagal inhibition, heart rate was higher in control but not in Npy1r<sup>fb</sup> mice compared to their 31 respective baseline values, suggesting that tonic vagal influences on heart rate were reduced in 32 Npy1r<sup>fb</sup> mice. The magnitude of the heart rate response to an acute psychosocial stressor was 33 smaller in Npy1r<sup>fb</sup> mice compared to Npy1r<sup>2lox</sup> counterparts (likely due to a concurrent lower vagal 34 withdrawal), even though absolute heart rate values did not differ between the two groups. These 35 findings suggest that reduced hippocampal Y1R expression leads to a decrease in resting vagal 36 modulation and heart rate variability, which, in turn, may determine a reduced cardiac autonomic 37 responsiveness to acute stress challenges. 38

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40 Keywords: NPY; Stress; Heart; Autonomic; knockout; Parasympathetic.

#### 41 1. Introduction

42 Neuropeptide Y (NPY) is widely distributed in the central nervous system [1], with particularly high 43 concentrations in the hypothalamus [2] and limbic system [3, 4]. NPY is involved in the regulation 44 of a variety of complex functions that range from food intake to emotional state [5]. These effects are mediated by multiple G-protein coupled receptor subtypes, which are characterized by different 45 tissue localization and pharmacological properties [6-8]. In particular, accumulating evidence 46 47 suggests that central Y1 receptors (Y1Rs) play a critical role in the inhibition of the sympathetic outflow to peripheral tissues. For example, it has been shown in rats that i.c.v. (lateral ventricle) 48 administration of the Y1R agonist [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY reduces basal noradrenaline release from the 49 paraventricular nucleus of the hypothalamus (PVN) [9], the major site of autonomic regulation via 50 projections to the rostral ventro-lateral medulla and the spinal cord. Moreover, NPY, acting on 51 Y1Rs, decreases noradrenaline overflow from both the hypothalamus and the medulla in vitro [10]. 52 A recent study has elegantly demonstrated that NPY released from the arcuate nucleus (ARC) 53 54 suppresses tyrosine hydroxylase (TH) expression in the PVN via activation of Y1Rs [11]. This effect was associated with a reduction in TH expression in the locus coeruleus and other regions 55 (such as the A1/C1 cell groups) in the brain stem [11], which would ultimately lead to a reduced 56 sympathetic outflow to peripheral tissues [11]. Supporting this view, nanoinjection of NPY into the 57 PVN decreases heart rate (HR), sympathetic nerve activity and baroreflex control of HR in 58 59 anesthetized rats via activation of Y1Rs and Y5Rs [12]. In mice, Tovote and colleagues observed that bilateral i.c.v. injections of NPY dose-dependently induced bradycardia and also blunted the 60 tachycardic response to a fear conditioning challenge [13], the latter via Y1R-mediated inhibition of 61 62 stress-induced sympathetic activation [13]. Similarly, global knockout of the Y1 receptor gene in mice led to a larger HR activation during social defeat stress [14]. These findings support the view 63 that NPY-Y1R signaling may play an important role also in constraining stress-induced sympathetic 64 activation [15, 16]. While the relationship between NPY-Y1R signaling in the ARC/PVN axis and 65 sympathetic nervous system has been documented, there is little known about the neural 66 pathways and mechanisms that control it. Indeed, in contrast with the above reported observations 67 68 that are suggestive of a central sympathetic inhibitory role of Y1Rs, global knockout of the NPY-

Y1R gene in mice led to resting bradycardia, which was likely due to a reduced basal sympathetic tone and increased parasympathetic activity [14]. This apparent contradiction might be ascribed to compensatory effects that are due to gene inactivation in early development and/or might underlie different roles played by Y1Rs at different levels in the central autonomic network.

Recently, a conditional knockout mouse model in which the inactivation of the Y1 receptor gene is restricted to excitatory neurons in the hippocampus, in particular when mice were reared by foster mothers exhibiting high levels of maternal care, has been developed [17]. Therefore, this animal model may be useful for understanding the contribution of hippocampal Y1Rs for central autonomic cardiovascular control. In order to reach this goal, autonomic modulation of cardiac function was assessed in the above mentioned conditional knockout mouse model during (i) baseline conditions. (ii) selective pharmacological autonomic manipulations, and (iii) acute and chronic psychosocial stress challenges, via time- and frequency-domain analysis of HR variability (HRV). 

#### 96 2. Methods

97 2.1. Animals, housing conditions and ethics statements

Experiments were performed on 4-month-old male conditional knockout mice (Npv1r<sup>rfb</sup>, n=10) and 98 their control littermates (Npy1r<sup>2lox</sup>, n=8) that were matched for body weight. Npy1r<sup>rfb</sup> and Npy1r<sup>2lox</sup> 99 mice (background strain: C57BL/6J) were generated following the breeding scheme described by 100 Bertocchi et al. (see [17] for details). Briefly, Npy1r<sup>2lox</sup>/Tg<sup>αCamKII-tTA/LC1</sup> mice (named herein Npy1r<sup>fb</sup>) 101 were generated using doxycycline dependent control of the Cre-LoxP system. Using this approach, 102 Bertocchi et al. [17] achieved the deletion of Npy1r in the hippocampal CA1 and CA3 pyramidal 103 and in the dentate gyrus granule cell layers. Since the phenotype of Npy1r<sup>fb</sup> mutants becomes 104 105 evident only in males reared by foster mothers showing high level of maternal cares versus adopted pups, immediately after birth the Npy1r<sup>rfb</sup> male mice used in our study were fostered to 106 Dox-naive CD1 dams, which display high levels of maternal cares (unpublished data). Littermates 107 comprising Npy1r<sup>2lox</sup>/Tg<sup> $\alpha$ CamKII-tTA</sup>, Npy1<sup>r2lox</sup>/Tg<sup>LC1</sup>, and Npy1r<sup>2lox</sup> genotypes were used as controls 108 (named herein Npy1r<sup>2lox</sup> controls), as in Bertocchi et al. [17]. After weaning, mice were kept in same 109 sex sibling groups in 40x20x20 cages in rooms with humidity- (50 ± 10%) and temperature- (22 ± 2 110 °C) controlled conditions, with food and water available ad libitum. Experiments were performed 111 during the light phase of the 12-h light/dark cycle (lights on at 7 a.m.). All experimental procedures 112 and protocols were approved by the Veterinarian Animal Care and Use Committee of Parma 113 University and conducted in accordance with the European Community Council Directives of 22 114 September 2010 (2010/63/UE). 115

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117 2.2. Transmitter implantation and radiotelemetry system

ECG (sampling frequency 2 KHz), body temperature (T, °C) and locomotor activity (LOC, counts per minute, cpm) signals were recorded by a radiotelemetry system. It consisted of transmitters (TA10ETA-F20, Data Sciences Int., St.Paul, MN, USA) and platform receivers (RPC-1, Data Science Int., St.Paul, MN, USA), which were connected to a computer containing Art-Gold 1.10 data acquisition system (Data Science Int., St.Paul, MN, USA). Transmitter implantation was performed under isoflurane anesthesia (2% in 100% oxygen), as previously described [18]. The transmitter's body was implanted intraperitoneally and two electrodes (wire loops) were fixed to the dorsal surface of the xyphoid process and in the anterior mediastinum close to the right atrium, respectively. This surgical procedure guarantees high quality ECG recordings, even during sustained physical activity. After surgery, mice were individually housed, injected with Gentamicin sulphate (Aagent, Fatro, 1ml/kg, S.C.) and allowed two weeks of recovery before the beginning of the experimental recordings.

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#### 131 2.3. General experimental outline

The timeline of the experimental protocol is depicted in Figure 1. Specific experimental procedures and data analysis are described in the following sections. After recovery from surgery, mice were weighed and left undisturbed in their home cages for 6 days (days 1-6). On days 7 and 9, they underwent pharmacological autonomic challenges. From day 12 to day 25, mice were housed in the cage of a dominant mouse in order to mimic a chronic psychosocial stress condition. At the end of the experimental protocol, mice were sacrificed by decapitation. Brains were rapidly removed and immediately frozen in liquid nitrogen, then stored at -80° C until analysis.

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#### 140 2.4. Daily rhythms of HR, HRV, T and LOC

ECG, T and LOC signals were sampled around-the-clock for 2 min every 30 min in baseline conditions (Basal Rhythms, days 1-6) and during chronic psychosocial stress (Stress Rhythms 1, days 16-19; Stress Rhythms 2, days 22-25) (Fig. 1). Heart rate (HR, beats per minute, bpm), HRV parameters (see ECG analysis section for details), T and LOC were calculated as mean values of the 12-h light and 12-h dark phases of the daily cycle for each recording day. Subsequently, they were further averaged as mean values of the light and dark phase for each recording period.

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148 2.5. Pharmacological autonomic challenge

On day 7 and 9 (Fig. 1), mice were intraperitoneally injected with either i) vehicle (0.9% NaCl, 1 ml/kg) or ii) methylscopolamine (muscarinic receptor antagonist, 0.1 mg/kg), with a rotational

design. Continuous ECG recordings were performed prior to (for 30 min, baseline condition) and
 following (for 60 min) each injection, with the mice in their home cages.

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154 2.6. Chronic psychosocial stress

155 In the present study we applied a modified version of the standard chronic psychosocial stress paradigm [18, 19], which is based on the classical resident-intruder test [20]. Six-month-old CD1 156 mice were housed with a female partner for 1 week and then individually housed and trained to 157 aggressively defend their territory from same sex mice intruders that were younger and lighter. 158 159 These dominant animals served as residents. Each experimental mouse was introduced as an intruder in the home cage of a resident male; once there, it was attacked and subordinated by the 160 resident mouse (social defeat). After 10 min, the two animals were separated by a perforated 161 polystyrene-metal partition that divided the cage into two equal-area sections. Thus, the intruder 162 mouse was protected from direct physical contact but it was in constant olfactory, auditory and 163 visual contact with the resident (psychosocial challenge). The partition was removed five times 164 165 during the experimental protocol (Fig. 1) at an unpredictable time between 9:00 and 13:00 h and repositioned after 2 min to prevent injuries. After each interaction, the experimental mice were 166 closely inspected for any improper injury and were excluded from further procedures in case they 167 168 were wounded. During the first episode of agonistic interaction, continuous ECG recordings were 169 performed for 30 min in baseline conditions (with the mice in their home cage), during the 10-min 170 agonistic interaction, and for 60 min after the intruders were again separated from the residents by 171 the partition.

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# 173 2.7. Heart rate variability (HRV) analysis

HRV analysis was carried out on ECG recordings using ChartPro 5.0 software (ADInstrument,
Sydney, Australia), based on the recommendations for HRV analysis in mice [21]. Initially, each
ECG recording was split in 2-min temporal segments (0-2 min; 2-4 min; etc.). Ectopic beats and
recording artifacts were then removed following visual inspection of unprocessed ECG signals.
Subsequently, HR and time- and frequency-domain parameters of HRV were quantified for each 2-

179 min time period. In the time domain, the root mean square of successive R-R interval differences (RMSSD, ms) was quantified; this index reflects short-term, high-frequency variations of RR 180 interval, which are mainly due to cardiac parasympathetic activity [22]. In the frequency-domain, 181 the power spectrum was obtained with a fast Fourier transform-based method (Welch's 182 periodogram: 256 points, 50% overlap, and Hamming window). The following parameters were 183 evaluated: i) the total power of the spectrum (ms<sup>2</sup>), which reflects all the cyclic components 184 responsible for variability [23], and ii) the power (ms<sup>2</sup>) of the high frequency band (HF, 1.5-5.0 Hz), 185 which reflects the activity of the parasympathetic nervous system and is related to respiratory sinus 186 arrhythmia [24]. 187

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189 2.8. In Situ Hybridization for Npy1r mRNA

In situ hybridization was performed on 14-µm-thick coronal brain sections, according to the 190 protocol reported by Wisden and Morris [25], in Npy1r<sup>rfb</sup> (n=4) and Npy1r<sup>2lox</sup> (n=4) mice. Four 191 different 40-/45-mer oligonucleotide S35-labeled probes were simultaneously used to increase the 192 193 signal and the reaction was carried out as previously described [17]. The area of interest of clearly distinguishable nuclei was defined following the boundaries of the labeled regions [dentate gyrus 194 granule cell layer (DG)], whereas three to four spots were used for each slice in poorly contrasted 195 regions [CA1 pyramidal cell layer (CA1) and CA3 pyramidal cell layer (CA3)]. Optical densities (OD 196 unit) were measured and averaged after a rodboard calibration. Background was measured by 197 averaging three to five spots in the surrounding blank region of the autoradiogram, then subtracted 198 from the corresponding nucleus value. 199

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201 2.9. Statistical analysis

All statistical analyses were performed using the software package SPSS (version 22). Two-way ANOVA for repeated measures with "group" as between-subject factor (2 levels: Npy1r<sup>fb</sup> and Npy1r<sup>2lox</sup>) was applied for ECG and telemetric data obtained from: i) baseline around-the-clock recordings, with "time" as within-subject factor (6 levels: day 1, 2, 3, 4, 5 and 6); ii) pharmacological challenges, with "time" as within-subject factor (4 levels: baseline; post injection 1, 2, 3); iii) first

episode of social defeat, with "time" as within-subject factor (8 levels: baseline, fight, sensory contact 1, 2, 3, 4, 5 and 6); iv) around-the-clock recordings during chronic social stress, with "time" as within-subject factor (3 levels: baseline, stress 1 and stress 2). Follow-up analyses were conducted using Student's "t" tests, with a Bonferroni correction for multiple comparisons for each outcome variable separately. Statistical significance was set at p<0.05.

213 3. Results

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215 3.1. Body weight

Prior to surgery, body weight was  $25.0\pm0.9$  g for Npy1r<sup>rfb</sup> mice and  $26.0\pm1.2$  g for Npy1r<sup>2lox</sup> mice (n.s.). No significant differences between the two groups were found in body weight at the beginning of the experimental protocol (day 1) (Npy1r<sup>rfb</sup>:  $26.3\pm0.3$  g vs. Npy1r<sup>2lox</sup> 27.2\pm0.8 g).

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220 3.2. Expression of Npy1r mRNA in the hippocampus of control and conditional mutants

In Npy1r<sup>rb</sup> mice, Npy1r mRNA expression was significantly lower in hippocampal CA1 pyramidal

cell layers (t=-3.1, p<0.05) and in dentate gyrus granule cell layers (t=-3.4, p<0.05) compared with

their control littermates, whereas no difference was observed in CA3 pyramidal cell layers between

the two groups (Fig. 2).

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# 3.3. Daily rhythms of HR, HRV, T and LOC during baseline conditions

The daily rhythms of HR, HRV indices, T and LOC in resting conditions are depicted in Figure 3 227 and summarized in Table 1. Two-way ANOVA yielded a main effect of "group" for HR (F=20.1, 228 p<0.01), total power (F=7.0, p<0.05), RMSSD (F=5.3, p<0.05), and HF (F=4.6, p<0.05) values. 229 Follow-up analyses revealed that Npv1r<sup>rfb</sup> mice had significantly higher HR values than Npv1r<sup>2lox</sup> 230 mice during both the light (t=2.9, p<0.05) and dark (t=3.9, p<0.01) phases of the daily cycle (Fig. 231 3A and Table 1). Npv1r<sup>rb</sup> mice exhibited significantly lower values of total power than Npv1r<sup>2lox</sup> 232 mice during both the light (t=2.6, p<0.05) and the dark (t=3.0, p<0.01) phases of the light-dark 233 cycle (Fig. 3B and Table 1). RMSSD values also resulted significantly lower in Npy1r<sup>rb</sup> mice than 234 Npv1r<sup>2lox</sup> counterparts in both phases (light: t=2.4, p<0.05; dark: t=2.5, p<0.05) (Fig. 3C and Table 235 1). Also spectral power values in the HF band were lower in Npy1r<sup>rfb</sup> compared to Npy1r<sup>2lox</sup> mice 236 (light: t=2.4, p<0.05; dark: t=1.9, p=0.07) (Fig. 3D and Table 1). In addition, Npy1r<sup>fb</sup> mice had 237 significantly higher T values than Npy1r<sup>2lox</sup> mice during the light phase (t=2.0, p=0.05) (Fig. 3E and 238 Table 1). Lastly, no significant differences between the two groups were observed for LOC values 239 (Fig. 3F and Table 1). 240

242 **3.4.** Cardiac autonomic response to the pharmacological autonomic manipulation

Cardiac autonomic responses to vehicle or methylscopolamine injection are depicted in Figure 4. 243 Before vehicle injection, HR was significantly higher in Npy1r<sup>rfb</sup> than in Npy1r<sup>2lox</sup> mice (t=2.6, 244 p<0.05) (Fig. 4A). In the same period, HRV analysis revealed that Npy1r<sup>fb</sup> mice had significantly 245 lower values of RMSSD (t=2.1, p=0.05) and HF spectral power (t=2.2, p<0.05) compared to 246 Npy1r<sup>2lox</sup> mice (Fig. 4C, E). The injection of vehicle provoked an increase in HR and a reduction in 247 RMSSD and HF values in both groups, with no group differences in the mean values of these 248 parameters (Fig. 4A, C, E). All parameters returned to baseline values within 40 min. During the 249 last 20-min recording period (min 40-60), mean HR was significantly higher in Npy1r<sup>rfb</sup> compared to 250 Npy1r<sup>2lox</sup> mice (t=2.2, p<0.05) (Fig. 4A). In the same period, RMSSD and HF mean values were 251 lower in Npy1r<sup>rfb</sup> than in Npy1r<sup>2lox</sup> mice (RMSSD: t=2.0, p=0.06; HF: t=2.6, p<0.05) (Fig. 4C, E). 252

Before methylscopolamine injection, HR was significantly higher in Npy1r<sup>fb</sup> compared to Npy1r<sup>2lox</sup> 253 mice (t=2.3, p<0.05). Blockade of muscarinic receptors with methylscopolamine provoked a 254 significant reduction of RMSSD (Npy1r<sup>fb</sup>: t=8.5, p<0.01; Npy1r<sup>2lox</sup>: t=6.5, p<0.01) and HF (Npy1r<sup>fb</sup>: 255 t=5.4, p<0.01; Npy1r<sup>2lox</sup>: t=4.1, p<0.01) values in both groups that persisted through the 60-min 256 recording period (Fig. 4D, F). There were no differences in the peak HR response to 257 methylscopolamine injection between the two groups (Fig. 4B). However, contrary to what has 258 been observed during vehicle (control) condition, during the last 20-min recording period (min 40-259 60) mean HR was (i) similar between the two groups, and (ii) significantly higher in Npy1r<sup>2lox</sup> 260 compared to the respective baseline value (t=2.2, p<0.05). 261

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263 **3.5**. HR, HRV, and LOC response to the first episode of social defeat

Cardiac autonomic and LOC responses to the first episode of social defeat are depicted in Figure 5. Two-way ANOVA yielded a significant effect of time for HR (F=18.9, p<0.01) and LOC (F=4.4, p<0.05) values. In baseline conditions, HR was significantly higher in Npy1r<sup>rfb</sup> than in Npy1r<sup>2lox</sup> mice (t=2.2, p<0.05) (Fig. 5A). No differences in absolute values of HR were observed between the two groups during the 10-min fight period and the following 60-min sensory contact phase (Fig. 269 5A). However, the magnitude of the HR response during the 10-min fight period (delta values) was significantly smaller in Npy1r<sup>rfb</sup> than in Npy1r<sup>2lox</sup> mice (t=-4.6, p<0.01) (Fig. 5B). Consistently, in 270 baseline conditions Npv1r<sup>rb</sup> mice had significantly lower HF spectral power values compared with 271 Npy1r<sup>2lox</sup> mice (t=2.5, p<0.05) (Fig. 5C). During the 10-min fight period, HF absolute values were 272 similar between the two groups (Fig. 5C), with the magnitude of the stress-induced reduction in HF 273 values being significantly smaller in Npy1r<sup>rfb</sup> than in Npy1r<sup>2lox</sup> mice (t=2.5, p<0.05) (Fig. 5D). No 274 differences in LOC values were observed between Npy1r<sup>rfb</sup> and Npy1r<sup>2lox</sup> mice in baseline 275 276 conditions and in response to the social challenge (Fig. 5E, F).

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# 278 **3.6**. Daily rhythms of HR, HRV, T and LOC during chronic psychosocial stress

HR, HRV, T and LOC values during the light and dark phases of the chronic psychosocial stress period are summarized in Table1. Two-way ANOVA revealed that chronic exposure to psychosocial stress led to (i) higher HR values ( $F_{light}$ =20.0, p<0.01;  $F_{dark}$ =19.8, p<0.01), and (ii) lower RMSSD ( $F_{light}$ =4.9, p<0.05), total power ( $F_{dark}$ =7.2, p<0.05) and HF ( $F_{light}$ =6.7, p<0.05) values compared to baseline conditions (Table 1). However, there were no differences between the two groups in HR, HRV parameters, T and LOC during both stress rhythm 1 and stress rhythm 2 periods (Table 1).

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4. Discussion

This study demonstrates that reduced expression of hippocampal Y1Rs in mice is associated with an increase in heart rate and a decrease in vagal neural modulation at rest.

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In the present study, HRV analysis was carried out in order to characterize cardiac autonomic 297 neural modulation of Npy1r<sup>fb</sup> mice under different experimental conditions. During both the light 298 and the dark phases of the daily cycle, Npy1r<sup>fb</sup> mice exhibited a lower cardiac vagal modulation, 299 300 as indexed by RMSSD and HF, which was likely responsible for the resting tachycardia and reduced overall HRV (total power) observed in these mice. Indeed, such changes could not be 301 302 ascribed to a different level of somatomotor activity between the two groups. Rather, an important 303 confirmation of the involvement of autonomic mechanisms in determining resting tachycardia in Npy1r<sup>rb</sup> mice comes from the pharmacological blockade of muscarinic receptors with 304 methylscopolamine. Under this condition of potent and sustained pharmacological inhibition of 305 306 cardiac vagal modulation, and after recovery from the stress of injection (see vehicle injection 307 condition for a comparison), the group difference in baseline heart rate was abolished. This was due to the fact that heart rate under pharmacological vagal inhibition was higher in control but not 308 in Npy1r<sup>fb</sup> mice compared to their respective baseline values. Consequently, it may be reasonable 309 to hypothesize that tonic vagal influences on heart rate were reduced in Npv1r<sup>fb</sup> mice, leading to 310 resting tachycardia. Interestingly, we found that baseline body temperature values were also 311 somewhat higher in Npy1r<sup>fb</sup> mice. In rodents, brown adipose tissue (BAT) is the main site of 312 nonshivering thermogenesis, and it is under tight control by the sympathetic nervous system [26]. 313 Therefore, signs of hyperthermia in Npy1r<sup>fb</sup> mice might suggest an increased sympathetic drive to 314 315 BAT.

In this study, Npy1r<sup>rb</sup> mice Y1R expression is significantly reduced in the dentate gyrus and the CA1 region of the hippocampus, where Y1Rs have been associated with glutamate-positive and NPY-positive neurons [27]. The ventral CA1 hippocampal field is thought to be linked by multisynaptic connections to the sympathetic-related regions of the hypothalamus, suggesting its possible involvement in a higher-brain autonomic circuit [28]. In addition, the hippocampus

321 connects to nucleus tractus solitarius-projecting regions of the medial prefrontal cortex (mPFC), such as the infralimbic cortex [29], indicating that hippocampal actions on autonomic function may 322 also be routed through the mPFC. Importantly, electrical and chemical stimulation of the 323 hippocampal formation in the anesthetized and the awake rat decreases heart rate, blood pressure 324 325 and respiratory rate [30, 31]. Interestingly, pharmacological manipulation of vagal activity with methylatropine revealed that the cardiovascular, but not the respiratory, responses were mediated 326 partially by vagal influences and partially by sympathetic influences [30]. Taken together, these 327 preliminary observations indicate that hippocampal Y1Rs may participate in the control of 328 autonomic outflow to peripheral tissues under baseline, undisturbed conditions. In a previous 329 study, Npy1r<sup>rfb</sup> mice have been characterized as having reduced body weight, less adipose tissue, 330 and lower serum leptin levels [17]. Here, mice did not differ for body weight, either prior or after 331 surgery recovery, as they were matched to avoid possible bias. Therefore, we might cautiously 332 assume that body weight is not a major determinant of the autonomic phenotype described in 333 Npy1r<sup>fb</sup> mice. On the other hand, the hippocampus is an important component of the neuronal 334 circuitry controlling anxiety-related behaviors and Npy1r<sup>rb</sup> mice have been previously shown to 335 display higher levels of anxiety in the elevated plus maze and open field tests [17]. These findings 336 are consistent with a previous observation that overexpression of virally-transduced NPY in the 337 mouse hippocampus produces anxiolytic-like effects [32]. Moreover, in humans NPY 338 haploinsufficiency is correlated with trait anxiety [33]. Given that anticipation of future aversive 339 340 events is a key aspect of anxiety disorders [34] and that anxiety is thought to be related to greater anticipatory reactivity in the brain [35], it is tempting to speculate that reduced vagal tone and HRV 341 in Npv1r<sup>fb</sup> mice might reflect heightened anxiety-related behavior. Following this line of reasoning, 342 reduced expression of Y1Rs on neurons lying in hippocampal areas or their related circuits might 343 344 account for the imbalance in the autonomic control of resting heart rate that characterizes high levels of anxiety in humans [36] and animal models [37]. 345

In this study, absolute values of HR were similar between mutant and control mice during an acute psychosocial challenge (social defeat). Elevated HR during social defeat was mediated by a decreased vagal tone compared to baseline levels in both groups. However, given that the

concurrent stress-induced increase in HR and decrease in vagal tone were smaller in Npy1r<sup>fb</sup> 349 mice, it could be hypothesized that a failure to further decrease cardiac vagal activity contributes to 350 the smaller stress-induced increase in HR in Npv1r<sup>fb</sup> mice. Two other factors may account for the 351 reduced HR response to the social challenge in these mutant mice: (i) a somewhat lower level of 352 353 somatomotor activity, (ii) the fact that HR may have reached its physiological maximum, thus masking the reported differences in baseline HR. Stressful stimuli are processed in multiple limbic 354 355 forebrain structures, including the amygdala, hippocampus and prefrontal cortex [38]. Limbic stress effector pathways converge on crucial subcortical relay sites, providing for downstream processing 356 357 of limbic information. In particular, numerous studies link the hippocampus with inhibition of the HPA axis [38, 39]. For example, hippocampal stimulation decreases glucocorticoid secretion in rats 358 and humans [40, 41], whereas hippocampal damage increases stress-induced and in some cases, 359 basal glucocorticoid secretion [39]. Not surprisingly, Npy1r<sup>rb</sup> mice show enhanced hypothalamic 360 CRH immunoreactivity and higher serum corticosterone levels [17]. It has been hypothesized that 361 the selective inactivation of Y1Rs in principal excitatory neurons of hippocampus might stimulate 362 363 HPA axis via the glutamatergic output [17]. Although these limbic circuits are also thought to participate in autonomic integration, their precise role(s) in stress-induced responses is not yet 364 defined. The results of this study provide preliminary evidence that hippocampal Y1R expression 365 might also be involved in the regulation of cardiac autonomic responses to acute stress challenges. 366 On the other hand, no evident differences were observed in HR and HRV parameters in response 367 to the chronic psychosocial challenge employed in this study. 368

In conclusion, the results of this study provide preliminary evidence that conditional reduction of 369 370 hippocampal Y1Rs leads to signs of decreased cardiac vagal tone and HRV in mice at rest. On the other hand, the role of hippocampal Y1Rs in the modulation of cardiac autonomic stress response 371 372 requires deeper investigation. We acknowledge that the robustness of these findings is certainly 373 limited by several factors which need to be addressed in future experiments. For example, Y1R deficiency tied to certain hippocampal (e.g. glutamatergic) neurons might be useful for revealing 374 the precise neurobiological pathways underlying the autonomic phenotype of Npy1r<sup>rb</sup> mice. 375 Moreover, Y1R expression should be investigated in other brain areas that may play a role in the 376

cardiovascular and behavioral effects of hippocampal Y1 receptor deficiency, such as the infralimbic area of the mPFC. Finally, the potential contribution of other phenotypic features of Npy1r<sup>rfb</sup> mice (such as, reduced body weight, increased anxiety, and increased NPY and CRH content [17]) to the autonomic changes described in this study needs to be taken into account. Nevertheless, this conditional knockout mouse model might be useful for gaining a deeper understanding of the role of Y1Rs in emotional-processing areas of the brain for autonomic nervous system control.

385 Figure captions

386 Figure 1. Timeline of the experimental procedures.

387

Figure 2. Expression of Npy1r mRNA in hippocampal CA1 and CA3 pyramidal cell layers and in the dentate gyrus (DG) granule cell layers in Npy1r<sup>rfb</sup> mice (n=4) and Npy1r<sup>2lox</sup> (n=4) mice. Relative optical densities (OD) are expressed as means  $\pm$  SEM. \* indicates a significant difference between Npy1r<sup>rfb</sup> and Npy1r<sup>2lox</sup> mice (p<0.05).

392

Figure 3. Time course of changes in heart rate, heart rate variability parameters, body temperature and locomotor activity during the light (L) and the dark (D) phases of baseline daily rhythm recordings, in Npy1r<sup>rfb</sup> (n = 10) and Npy1r<sup>2lox</sup> (n = 8) mice. Values are reported as means  $\pm$  SEM of data obtained by averaging multiple 2 min segments acquired every 30 min for the 12-h light and the 12-h dark phase of the daily cycle. RMSSD = root mean square of successive R-R interval differences; HF = high frequency; LF = low frequency. Statistical results are reported in the results' section.

400

Figure 4. Time course of changes in heart rate (panels A and B), RMSSD (panels C and D) and high frequency (HF) power (panels E and F) in baseline conditions (bas) and following the injection of vehicle (left panels) or methylscopolamine (right panels) in Npy1r<sup>fb</sup> (n = 10) and Npy1r<sup>2lox</sup> (n = 8) mice. Values are expressed as means  $\pm$  SEM. \* indicates a significant difference between Npy1r<sup>fb</sup> and Npy1r<sup>2lox</sup> mice (p<0.05); <sup>#</sup> indicates a significant difference vs. the respective baseline value (p<0.05).

407

Figure 5. Left panels show the time course of changes in heart rate (A), high frequency (HF) power (C), and locomotor activity (E) values in baseline conditions (bas) and during the first episode of social defeat, in Npy1r<sup>fb</sup> (n= 10) and Npy1r<sup>2lox</sup> (n= 8) mice. Right panels show delta values of heart rate (B), HF power (D), and locomotor activity (F) which were calculated as the difference between

- 412 mean "fight" and mean baseline values. Absolute and delta values are expressed as means ±
- 413 SEM. \* indicates a significant difference between Npy1r<sup>rfb</sup> and Npy1r<sup>2lox</sup> mice (p<0.05).

# 415 Tables

416 Table 1. Daily rhythms of HR, HRV parameters, body temperature and locomotor activity in 417 baseline condition and during chronic psychosocial stress.

418

		BASELINE RHYTHMS		STRESS RHYTHMS 1		STRESS RHYTHMS 2	
		Light	Dark	Light	Dark	Light	Dark
HR	Npy1r <sup>rfb</sup>	504±7*	567±7*	552±14 <sup>#</sup>	597±15	515±11	582±13
(bpm)	Npy1r <sup>2lox</sup>	466±12	532±5	$533 \pm 14^{\#}$	$590 \pm 10^{\#}$	497±11	571±7 <sup>#</sup>
TOTAL power	Npy1r <sup>rfb</sup>	78.7±8.3*	45.2±3.2*	39.3±6.4 <sup>#</sup>	29.0±3.8 <sup>#</sup>	91.9±15.8	41.4±5.6
(ms²)	Npy1r <sup>2lox</sup>	119.7±14.5	61.4±4.4	51.9±14.1 <sup>#</sup>	$28.7 \pm 5.6^{\#}$	87.6±20.6	45.0±7.9
RMSSD	Npy1r <sup>rfb</sup>	4.6±0.4*	3.2±0.3*	2.7±0.3 <sup>#</sup>	$2.3\pm0.2^{\#}$	4.6±0.6	3.2±0.4
(ms)	Npy1r <sup>2lox</sup>	6.4±0.7	4.2±0.3	$3.4\pm0.8^{\#}$	$2.8 \pm 0.4^{\#}$	$4.3\pm0.7^{\#}$	3.7±0.7
HF power	Npy1r <sup>rfb</sup>	7.4±1.4*	3.8±0.8	2.6±0.5 <sup>#</sup>	2.1±0.4	7.1±1.7	3.6±0.9
(ms²)	Npy1r <sup>2lox</sup>	13.4±2.2	5.9±0.8	5.1±2.5 <sup>#</sup>	$3.2\pm0.9^{\#}$	$6.0 \pm 1.5^{\#}$	5.1±1.7
Т	Npy1r <sup>rfb</sup>	36.2±0.2*	37.3±0.2	36.3±0.2	37.2±0.2	36.1±0.2	37.2±0.2
(°C)	Npy1r <sup>2lox</sup>	35.8±0.1	37.0±0.1	35.9±0.1	37.0±0.2	35.8±0.1	37.1±0.1
LOC	Npy1r <sup>rfb</sup>	4.0±0.4	8.8±1.1	4.4±0.8	11.5±2.3	4.4±0.6	9.4±1.5
(cpm)	Npy1r <sup>2lox</sup>	4.6±0.5	11.4±1.5	5.2±0.7	12.2±1.7	4.9±0.7	12.4±1.5

419

420 Values are reported as mean values ± SEM of data obtained by averaging multiple 2 min segments acquired every 30 min over a 6-day period for baseline rhythms and over a 4-day period 421 for stress rhythms 1 and stress rhythms 2, in Npy1r<sup>rfb</sup> (n = 10) and Npy1r<sup>2lox</sup> (n = 8) mice. HR= heart 422 423 rate; HRV= heart rate variability; RMSSD = root mean square of successive R-R interval differences; HF = high frequency; LF = low frequency; T = body temperature; LOC = locomotor 424 activity. \* indicates a significant difference between Npy1r<sup>fb</sup> and Npy1r<sup>2lox</sup> mice; <sup>#</sup> indicates a 425 significant difference between stress rhythm value and the respective baseline rhythm value. 426 (p<0.05). 427

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2	Reduced NPY Y1 receptor hippocampal expression and signs of decreased vagal
3	modulation of heart rate in mice
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#### 17 Abstract

Central neuropeptide Y (NPY) signaling participates in the regulation of cardiac autonomic outflow, 18 19 particularly via activation of NPY-Y1 receptors (Y1Rs). However, the specific brain areas and neural pathways involved have not been completely identified yet. Here, we evaluate the role of 20 hippocampal Y1Rs in the modulation of the autonomic control of cardiac function using a 21 22 conditional knockout mouse model. Radiotelemetric transmitters were implanted in 4-month-old male mice exhibiting reduced forebrain expression (rfb) of the Y1R (Npy1r<sup>rfb</sup>, n=10) and their 23 corresponding controls (Npy1r<sup>2lox</sup>, n=8). ECG signals were recorded (i) during resting conditions, 24 (ii) under selective pharmacological manipulation of cardiac vagal activity, and (iii) during acute and 25 26 chronic psychosocial stress challenges, and analyzed via time- and frequency-domain analysis of heart rate variability. Npy1r<sup>fb</sup> mice showed a lower Npy1r mRNA density in the dentate gyrus and 27 in the CA1 region of the hippocampus. Under resting undisturbed conditions, Npy1r<sup>fb</sup> mice 28 exhibited (i) a higher heart rate, (ii) a reduced overall heart rate variability, and (iii) lower values of 29 the indices of vagal modulation compared to Npy1r<sup>2lox</sup> counterparts. Following pharmacological 30 vagal inhibition, heart rate was higher in control but not in Npy1r<sup>fb</sup> mice compared to their 31 respective baseline values, suggesting that tonic vagal influences on heart rate were reduced in 32 Npy1r<sup>fb</sup> mice. The magnitude of the heart rate response to an acute psychosocial stressor was 33 smaller in Npy1r<sup>rfb</sup> mice compared to Npy1r<sup>2lox</sup> counterparts (likely due to a concurrent lower vagal 34 withdrawal), even though absolute heart rate values did not differ between the two groups. These 35 findings suggest that reduced hippocampal Y1R expression leads to a decrease in resting vagal 36 modulation and heart rate variability, which, in turn, may determine a reduced cardiac autonomic 37 38 responsiveness to acute stress challenges.

39

40 Keywords: NPY; Stress; Heart; Autonomic; knockout; Parasympathetic.

#### 41 1. Introduction

42 Neuropeptide Y (NPY) is widely distributed in the central nervous system [1], with particularly high 43 concentrations in the hypothalamus [2] and limbic system [3, 4]. NPY is involved in the regulation 44 of a variety of complex functions that range from food intake to emotional state [5]. These effects are mediated by multiple G-protein coupled receptor subtypes, which are characterized by different 45 tissue localization and pharmacological properties [6-8]. In particular, accumulating evidence 46 47 suggests that central Y1 receptors (Y1Rs) play a critical role in the inhibition of the sympathetic outflow to peripheral tissues. For example, it has been shown in rats that i.c.v. (lateral ventricle) 48 administration of the Y1R agonist [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY reduces basal noradrenaline release from the 49 paraventricular nucleus of the hypothalamus (PVN) [9], the major site of autonomic regulation via 50 projections to the rostral ventro-lateral medulla and the spinal cord. Moreover, NPY, acting on 51 Y1Rs, decreases noradrenaline overflow from both the hypothalamus and the medulla in vitro [10]. 52 A recent study has elegantly demonstrated that NPY released from the arcuate nucleus (ARC) 53 54 suppresses tyrosine hydroxylase (TH) expression in the PVN via activation of Y1Rs [11]. This effect was associated with a reduction in TH expression in the locus coeruleus and other regions 55 (such as the A1/C1 cell groups) in the brain stem [11], which would ultimately lead to a reduced 56 sympathetic outflow to peripheral tissues [11]. Supporting this view, nanoinjection of NPY into the 57 PVN decreases heart rate (HR), sympathetic nerve activity and baroreflex control of HR in 58 59 anesthetized rats via activation of Y1Rs and Y5Rs [12]. In mice, Tovote and colleagues observed that bilateral i.c.v. injections of NPY dose-dependently induced bradycardia and also blunted the 60 tachycardic response to a fear conditioning challenge [13], the latter via Y1R-mediated inhibition of 61 62 stress-induced sympathetic activation [13]. Similarly, global knockout of the Y1 receptor gene in mice led to a larger HR activation during social defeat stress [14]. These findings support the view 63 that NPY-Y1R signaling may play an important role also in constraining stress-induced sympathetic 64 activation [15, 16]. While the relationship between NPY-Y1R signaling in the ARC/PVN axis and 65 sympathetic nervous system has been documented, there is little known about the neural 66 pathways and mechanisms that control it. Indeed, in contrast with the above reported observations 67 68 that are suggestive of a central sympathetic inhibitory role of Y1Rs, global knockout of the NPY-

Y1R gene in mice led to resting bradycardia, which was likely due to a reduced basal sympathetic tone and increased parasympathetic activity [14]. This apparent contradiction might be ascribed to compensatory effects that are due to gene inactivation in early development and/or might underlie different roles played by Y1Rs at different levels in the central autonomic network.

Recently, a conditional knockout mouse model in which the inactivation of the Y1 receptor gene is restricted to excitatory neurons in the hippocampus, in particular when mice were reared by foster mothers exhibiting high levels of maternal care, has been developed [17]. Therefore, this animal model may be useful for understanding the contribution of hippocampal Y1Rs for central autonomic cardiovascular control. In order to reach this goal, autonomic modulation of cardiac function was assessed in the above mentioned conditional knockout mouse model during (i) baseline conditions. (ii) selective pharmacological autonomic manipulations, and (iii) acute and chronic psychosocial stress challenges, via time- and frequency-domain analysis of HR variability (HRV). 

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#### 96 2. Methods

97 2.1. Animals, housing conditions and ethics statements

Experiments were performed on 4-month-old male conditional knockout mice (Npy1r<sup>fb</sup>, n=10) and 98 their control littermates (Npy1r<sup>2lox</sup>, n=8) that were matched for body weight. Npy1r<sup>fb</sup> and Npy1r<sup>2lox</sup> 99 mice (background strain: C57BL/6J) were generated following the breeding scheme described by 100 Bertocchi et al. (see [17] for details). Briefly, Npy1r<sup>2lox</sup>/Tg<sup>αCamKII-tTA/LC1</sup> mice (named herein Npy1r<sup>rfb</sup>) 101 were generated using doxycycline dependent control of the Cre-LoxP system. Using this approach, 102 Bertocchi et al. [17] achieved the deletion of Npy1r in the hippocampal CA1 and CA3 pyramidal 103 and in the dentate gyrus granule cell layers. Since the phenotype of Npy1r<sup>fb</sup> mutants becomes 104 105 evident only in males reared by foster mothers showing high level of maternal cares versus adopted pups, immediately after birth the Npy1r<sup>rfb</sup> male mice used in our study were fostered to 106 Dox-naive CD1 dams, which display high levels of maternal cares (unpublished data). Littermates 107 comprising Npy1r<sup>2lox</sup>/Tg<sup>αCamKII-tTA</sup>, Npy1<sup>r2lox</sup>/Tg<sup>LC1</sup>, and Npy1r<sup>2lox</sup> genotypes were used as controls 108 (named herein Npy1r<sup>2lox</sup> controls), as in Bertocchi et al. [17]. After weaning, mice were kept in same 109 sex sibling groups in 40x20x20 cages in rooms with humidity- (50 ± 10%) and temperature- (22 ± 2 110 °C) controlled conditions, with food and water available ad libitum. Experiments were performed 111 during the light phase of the 12-h light/dark cycle (lights on at 7 a.m.). All experimental procedures 112 and protocols were approved by the Veterinarian Animal Care and Use Committee of Parma 113 University and conducted in accordance with the European Community Council Directives of 22 114 September 2010 (2010/63/UE). 115

116

117 2.2. Transmitter implantation and radiotelemetry system

ECG (sampling frequency 2 KHz), body temperature (T, °C) and locomotor activity (LOC, counts per minute, cpm) signals were recorded by a radiotelemetry system. It consisted of transmitters (TA10ETA-F20, Data Sciences Int., St.Paul, MN, USA) and platform receivers (RPC-1, Data Science Int., St.Paul, MN, USA), which were connected to a computer containing Art-Gold 1.10 data acquisition system (Data Science Int., St.Paul, MN, USA). Transmitter implantation was performed under isoflurane anesthesia (2% in 100% oxygen), as previously described [18]. The transmitter's body was implanted intraperitoneally and two electrodes (wire loops) were fixed to the dorsal surface of the xyphoid process and in the anterior mediastinum close to the right atrium, respectively. This surgical procedure guarantees high quality ECG recordings, even during sustained physical activity. After surgery, mice were individually housed, injected with Gentamicin sulphate (Aagent, Fatro, 1ml/kg, S.C.) and allowed two weeks of recovery before the beginning of the experimental recordings.

130

131 2.3. General experimental outline

The timeline of the experimental protocol is depicted in Figure 1. Specific experimental procedures and data analysis are described in the following sections. After recovery from surgery, mice were weighed and left undisturbed in their home cages for 6 days (days 1-6). On days 7 and 9, they underwent pharmacological autonomic challenges. From day 12 to day 25, mice were housed in the cage of a dominant mouse in order to mimic a chronic psychosocial stress condition. At the end of the experimental protocol, mice were sacrificed by decapitation. Brains were rapidly removed and immediately frozen in liquid nitrogen, then stored at -80° C until analysis.

139

#### 140 2.4. Daily rhythms of HR, HRV, T and LOC

ECG, T and LOC signals were sampled around-the-clock for 2 min every 30 min in baseline conditions (Basal Rhythms, days 1-6) and during chronic psychosocial stress (Stress Rhythms 1, days 16-19; Stress Rhythms 2, days 22-25) (Fig. 1). Heart rate (HR, beats per minute, bpm), HRV parameters (see ECG analysis section for details), T and LOC were calculated as mean values of the 12-h light and 12-h dark phases of the daily cycle for each recording day. Subsequently, they were further averaged as mean values of the light and dark phase for each recording period.

147

148 2.5. Pharmacological autonomic challenge

On day 7 and 9 (Fig. 1), mice were intraperitoneally injected with either i) vehicle (0.9% NaCl, 1 ml/kg) or ii) methylscopolamine (muscarinic receptor antagonist, 0.1 mg/kg), with a rotational

design. Continuous ECG recordings were performed prior to (for 30 min, baseline condition) and
 following (for 60 min) each injection, with the mice in their home cages.

153

154 2.6. Chronic psychosocial stress

155 In the present study we applied a modified version of the standard chronic psychosocial stress paradigm [18, 19], which is based on the classical resident-intruder test [20]. Six-month-old CD1 156 mice were housed with a female partner for 1 week and then individually housed and trained to 157 aggressively defend their territory from same sex mice intruders that were younger and lighter. 158 159 These dominant animals served as residents. Each experimental mouse was introduced as an intruder in the home cage of a resident male; once there, it was attacked and subordinated by the 160 resident mouse (social defeat). After 10 min, the two animals were separated by a perforated 161 polystyrene-metal partition that divided the cage into two equal-area sections. Thus, the intruder 162 mouse was protected from direct physical contact but it was in constant olfactory, auditory and 163 visual contact with the resident (psychosocial challenge). The partition was removed five times 164 165 during the experimental protocol (Fig. 1) at an unpredictable time between 9:00 and 13:00 h and repositioned after 2 min to prevent injuries. After each interaction, the experimental mice were 166 closely inspected for any improper injury and were excluded from further procedures in case they 167 168 were wounded. During the first episode of agonistic interaction, continuous ECG recordings were 169 performed for 30 min in baseline conditions (with the mice in their home cage), during the 10 min 170 agonistic interaction, and for 60 min after the intruders were again separated from the residents by 171 the partition.

172

# 173 2.7. Heart rate variability (HRV) analysis

HRV analysis was carried out on ECG recordings using ChartPro 5.0 software (ADInstrument,
Sydney, Australia), based on the recommendations for HRV analysis in mice [21]. Initially, each
ECG recording was split in 2-min temporal segments (0-2 min; 2-4 min; etc.). Ectopic beats and
recording artifacts were then removed following visual inspection of unprocessed ECG signals.
Subsequently, HR and time- and frequency-domain parameters of HRV were quantified for each 2-

179 min time period. In the time domain, the root mean square of successive R-R interval differences (RMSSD, ms) was quantified; this index reflects short-term, high-frequency variations of RR 180 interval, which are mainly due to cardiac parasympathetic activity [22]. In the frequency-domain, 181 the power spectrum was obtained with a fast Fourier transform-based method (Welch's 182 periodogram: 256 points, 50% overlap, and Hamming window). The following parameters were 183 evaluated: i) the total power of the spectrum (ms<sup>2</sup>), which reflects all the cyclic components 184 responsible for variability [23], and ii) the power (ms<sup>2</sup>) of the high frequency band (HF, 1.5-5.0 Hz), 185 which reflects the activity of the parasympathetic nervous system and is related to respiratory sinus 186 arrhythmia [24]. 187

188

189 2.8. In Situ Hybridization for Npy1r mRNA

In situ hybridization was performed on 14-µm-thick coronal brain sections, according to the 190 protocol reported by Wisden and Morris [25], in Npy1r<sup>rfb</sup> (n=4) and Npy1r<sup>2lox</sup> (n=4) mice. Four 191 different 40-/45-mer oligonucleotide S35-labeled probes were simultaneously used to increase the 192 193 signal and the reaction was carried out as previously described [17]. The area of interest of clearly distinguishable nuclei was defined following the boundaries of the labeled regions [dentate gyrus 194 granule cell layer (DG)], whereas three to four spots were used for each slice in poorly contrasted 195 regions [CA1 pyramidal cell layer (CA1) and CA3 pyramidal cell layer (CA3)]. Optical densities (OD 196 unit) were measured and averaged after a rodboard calibration. Background was measured by 197 averaging three to five spots in the surrounding blank region of the autoradiogram, then subtracted 198 from the corresponding nucleus value. 199

200

201 2.9. Statistical analysis

All statistical analyses were performed using the software package SPSS (version 22). Two-way ANOVA for repeated measures with "group" as between-subject factor (2 levels: Npy1r<sup>fb</sup> and Npy1r<sup>2lox</sup>) was applied for ECG and telemetric data obtained from: i) baseline around-the-clock recordings, with "time" as within-subject factor (6 levels: day 1, 2, 3, 4, 5 and 6); ii) pharmacological challenges, with "time" as within-subject factor (4 levels: baseline; post injection 1, 2, 3); iii) first

207 episode of social defeat, with "time" as within-subject factor (8 levels: baseline, fight, sensory 208 contact 1, 2, 3, 4, 5 and 6); iv) around-the-clock recordings during chronic social stress, with "time" 209 as within-subject factor (3 levels: baseline, stress 1 and stress 2). Follow-up analyses were 210 conducted using Student's "t" tests, with a Bonferroni correction for multiple comparisons for each 211 outcome variable separately. Statistical significance was set at p<0.05.

213 3. Results

# 214

3.1. Body weight

Prior to surgery, body weight was  $25.0\pm0.9$  g for Npy1r<sup>fb</sup> mice and  $26.0\pm1.2$  g for Npy1r<sup>2lox</sup> mice (n.s.). No significant differences between the two groups were found in body weight at the beginning of the experimental protocol (day 1) (Npy1r<sup>fb</sup>:  $26.3\pm0.3$  g vs. Npy1r<sup>2lox</sup> 27.2\pm0.8 g).

219

3.2. Expression of Npy1r mRNA in the hippocampus of control and conditional mutants In Npy1r<sup>rfb</sup> mice, Npy1r mRNA expression was significantly lower in hippocampal CA1 pyramidal cell layers (t=-3.1, p<0.05) and in dentate gyrus granule cell layers (t=-3.4, p<0.05) compared with their control littermates, whereas no difference was observed in CA3 pyramidal cell layers between the two groups (Fig. 2).

225

#### 3.3. Daily rhythms of HR, HRV, T and LOC during baseline conditions

The daily rhythms of HR, HRV indices, T and LOC in resting conditions are depicted in Figure 3 227 and summarized in Table 1. Two-way ANOVA yielded a main effect of "group" for HR (F=20.1, 228 p<0.01), total power (F=7.0, p<0.05), RMSSD (F=5.3, p<0.05), and HF (F=4.6, p<0.05) values. 229 Follow-up analyses revealed that Npy1r<sup>fb</sup> mice had significantly higher HR values than Npy1r<sup>2lox</sup> 230 mice during both the light (t=2.9, p<0.05) and dark (t=3.9, p<0.01) phases of the daily cycle (Fig. 231 3A and Table 1). Npv1r<sup>rb</sup> mice exhibited significantly lower values of total power than Npv1r<sup>2lox</sup> 232 mice during both the light (t=2.6, p<0.05) and the dark (t=3.0, p<0.01) phases of the light-dark 233 cycle (Fig. 3B and Table 1). RMSSD values also resulted significantly lower in Npy1r<sup>fb</sup> mice than 234 Npv1r<sup>2lox</sup> counterparts in both phases (light: t=2.4, p<0.05; dark: t=2.5, p<0.05) (Fig. 3C and Table 235 1). Also spectral power values in the HF band were lower in Npy1r<sup>fb</sup> compared to Npy1r<sup>2lox</sup> mice 236 (light: t=2.4, p<0.05; dark: t=1.9, p=0.07) (Fig. 3D and Table 1). In addition, Npy1r<sup>fb</sup> mice had 237 significantly higher T values than Npy1r<sup>2lox</sup> mice during the light phase (t=2.0, p=0.05) (Fig. 3E and 238 Table 1). Lastly, no significant differences between the two groups were observed for LOC values 239 (Fig. 3F and Table 1). 240

3.4. Cardiac autonomic response to the pharmacological autonomic manipulation

Cardiac autonomic responses to vehicle or methylscopolamine injection are depicted in Figure 4. 243 Before vehicle injection, HR was significantly higher in Npy1r<sup>rfb</sup> than in Npy1r<sup>2lox</sup> mice (t=2.6, 244 p<0.05) (Fig. 4A). In the same period, HRV analysis revealed that Npy1r<sup>fb</sup> mice had significantly 245 lower values of RMSSD (t=2.1, p=0.05) and HF spectral power (t=2.2, p<0.05) compared to 246 Npy1r<sup>2lox</sup> mice (Fig. 4C, E). The injection of vehicle provoked an increase in HR and a reduction in 247 RMSSD and HF values in both groups, with no group differences in the mean values of these 248 parameters (Fig. 4A, C, E). All parameters returned to baseline values within 40 min. During the 249 last 20-min recording period (min 40-60), mean HR was significantly higher in Npy1r<sup>rfb</sup> compared to 250 Npy1r<sup>2lox</sup> mice (t=2.2, p<0.05) (Fig. 4A). In the same period, RMSSD and HF mean values were 251 lower in Npy1r<sup>rfb</sup> than in Npy1r<sup>2lox</sup> mice (RMSSD: t=2.0, p=0.06; HF: t=2.6, p<0.05) (Fig. 4C, E). 252

Before methylscopolamine injection, HR was significantly higher in Npy1r<sup>fb</sup> compared to Npy1r<sup>2lox</sup> 253 mice (t=2.3, p<0.05). Blockade of muscarinic receptors with methylscopolamine provoked a 254 significant reduction of RMSSD (Npy1r<sup>fb</sup>: t=8.5, p<0.01; Npy1r<sup>2lox</sup>: t=6.5, p<0.01) and HF (Npy1r<sup>fb</sup>: 255 t=5.4, p<0.01; Npy1r<sup>2lox</sup>: t=4.1, p<0.01) values in both groups that persisted through the 60-min 256 recording period (Fig. 4D, F). There were no differences in the peak HR response to 257 methylscopolamine injection between the two groups (Fig. 4B). However, contrary to what has 258 been observed during vehicle (control) condition, during the last 20-min recording period (min 40-259 60) mean HR was (i) similar between the two groups, and (ii) significantly higher in Npy1r<sup>2lox</sup> 260 compared to the respective baseline value (t=2.2, p<0.05). 261

262

# 3.5. HR, HRV, and LOC response to the first episode of social defeat

Cardiac autonomic and LOC responses to the first episode of social defeat are depicted in Figure 5. Two-way ANOVA yielded a significant effect of time for HR (F=18.9, p<0.01) and LOC (F=4.4, p<0.05) values. In baseline conditions, HR was significantly higher in Npy1r<sup>rfb</sup> than in Npy1r<sup>2lox</sup> mice (t=2.2, p<0.05) (Fig. 5A). No differences in absolute values of HR were observed between the two groups during the 10-min fight period and the following 60-min sensory contact phase (Fig.

269 5A). However, the magnitude of the HR response during the 10-min fight period (delta values) was significantly smaller in Npy1r<sup>rfb</sup> than in Npy1r<sup>2lox</sup> mice (t=-4.6, p<0.01) (Fig. 5B). Consistently, in 270 baseline conditions Npv1r<sup>rb</sup> mice had significantly lower HF spectral power values compared with 271 Npy1r<sup>2lox</sup> mice (t=2.5, p<0.05) (Fig. 5C). During the 10-min fight period, HF absolute values were 272 similar between the two groups (Fig. 5C), with the magnitude of the stress-induced reduction in HF 273 values being significantly smaller in Npy1r<sup>fb</sup> than in Npy1r<sup>2lox</sup> mice (t=2.5, p<0.05) (Fig. 5D). No 274 differences in LOC values were observed between Npy1r<sup>rfb</sup> and Npy1r<sup>2lox</sup> mice in baseline 275 276 conditions and in response to the social challenge (Fig. 5E, F).

277

# 278 3.6. Daily rhythms of HR, HRV, T and LOC during chronic psychosocial stress

HR, HRV, T and LOC values during the light and dark phases of the chronic psychosocial stress period are summarized in Table1. Two-way ANOVA revealed that chronic exposure to psychosocial stress led to (i) higher HR values ( $F_{light}$ =20.0, p<0.01;  $F_{dark}$ =19.8, p<0.01), and (ii) lower RMSSD ( $F_{light}$ =4.9, p<0.05), total power ( $F_{dark}$ =7.2, p<0.05) and HF ( $F_{light}$ =6.7, p<0.05) values compared to baseline conditions (Table 1). However, there were no differences between the two groups in HR, HRV parameters, T and LOC during both stress rhythm 1 and stress rhythm 2 periods (Table 1).

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4. Discussion

This study demonstrates that reduced expression of hippocampal Y1Rs in mice is associated with an increase in heart rate and a decrease in vagal neural modulation at rest.

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In the present study, HRV analysis was carried out in order to characterize cardiac autonomic 297 neural modulation of Npy1r<sup>fb</sup> mice under different experimental conditions. During both the light 298 and the dark phases of the daily cycle, Npy1r<sup>rb</sup> mice exhibited a lower cardiac vagal modulation, 299 300 as indexed by RMSSD and HF, which was likely responsible for the resting tachycardia and reduced overall HRV (total power) observed in these mice. Indeed, such changes could not be 301 302 ascribed to a different level of somatomotor activity between the two groups. Rather, an important 303 confirmation of the involvement of autonomic mechanisms in determining resting tachycardia in Npy1r<sup>fb</sup> mice comes from the pharmacological blockade of muscarinic receptors with 304 methylscopolamine. Under this condition of potent and sustained pharmacological inhibition of 305 306 cardiac vagal modulation, and after recovery from the stress of injection (see vehicle injection 307 condition for a comparison), the group difference in baseline heart rate was abolished. This was due to the fact that heart rate under pharmacological vagal inhibition was higher in control but not 308 in Npy1r<sup>fb</sup> mice compared to their respective baseline values. Consequently, it may be reasonable 309 to hypothesize that tonic vagal influences on heart rate were reduced in Npy1r<sup>fb</sup> mice, leading to 310 resting tachycardia. Interestingly, we found that baseline body temperature values were also 311 somewhat higher in Npy1r<sup>fb</sup> mice. In rodents, brown adipose tissue (BAT) is the main site of 312 nonshivering thermogenesis, and it is under tight control by the sympathetic nervous system [26]. 313 Therefore, signs of hyperthermia in Npy1r<sup>fb</sup> mice might suggest an increased sympathetic drive to 314 BAT. 315

In this study, Npy1r<sup>rfb</sup> mice Y1R expression is significantly reduced in the dentate gyrus and the CA1 region of the hippocampus, where Y1Rs have been associated with glutamate-positive and NPY-positive neurons [27]. The ventral CA1 hippocampal field is thought to be linked by multisynaptic connections to the sympathetic-related regions of the hypothalamus, suggesting its possible involvement in a higher-brain autonomic circuit [28]. In addition, the hippocampus

321 connects to nucleus tractus solitarius-projecting regions of the medial prefrontal cortex (mPFC), such as the infralimbic cortex [29], indicating that hippocampal actions on autonomic function may 322 also be routed through the mPFC. Importantly, electrical and chemical stimulation of the 323 hippocampal formation in the anesthetized and the awake rat decreases heart rate, blood pressure 324 325 and respiratory rate [30, 31]. Interestingly, pharmacological manipulation of vagal activity with methylatropine revealed that the cardiovascular, but not the respiratory, responses were mediated 326 partially by vagal influences and partially by sympathetic influences [30]. Taken together, these 327 preliminary observations indicate that hippocampal Y1Rs may participate in the control of 328 autonomic outflow to peripheral tissues under baseline, undisturbed conditions. In a previous 329 study, Npy1r<sup>rfb</sup> mice have been characterized as having reduced body weight, less adipose tissue, 330 and lower serum leptin levels [17]. Here, mice did not differ for body weight, either prior or after 331 surgery recovery, as they were matched to avoid possible bias. Therefore, we might cautiously 332 assume that body weight is not a major determinant of the autonomic phenotype described in 333 Npy1r<sup>fb</sup> mice. On the other hand, the hippocampus is an important component of the neuronal 334 circuitry controlling anxiety-related behaviors and Npy1r<sup>rb</sup> mice have been previously shown to 335 display higher levels of anxiety in the elevated plus maze and open field tests [17]. These findings 336 are consistent with a previous observation that overexpression of virally-transduced NPY in the 337 mouse hippocampus produces anxiolytic-like effects [32]. Moreover, in humans NPY 338 339 haploinsufficiency is correlated with trait anxiety [33]. Given that anticipation of future aversive 340 events is a key aspect of anxiety disorders [34] and that anxiety is thought to be related to greater anticipatory reactivity in the brain [35], it is tempting to speculate that reduced vagal tone and HRV 341 in Npv1r<sup>fb</sup> mice might reflect heightened anxiety-related behavior. Following this line of reasoning, 342 reduced expression of Y1Rs on neurons lying in hippocampal areas or their related circuits might 343 account for the imbalance in the autonomic control of resting heart rate that characterizes high 344 levels of anxiety in humans [36] and animal models [37]. 345

In this study, absolute values of HR were similar between mutant and control mice during an acute psychosocial challenge (social defeat). Elevated HR during social defeat was mediated by a decreased vagal tone compared to baseline levels in both groups. However, given that the

concurrent stress-induced increase in HR and decrease in vagal tone were smaller in Npy1r<sup>fb</sup> 349 mice, it could be hypothesized that a failure to further decrease cardiac vagal activity contributes to 350 the smaller stress-induced increase in HR in Npv1r<sup>fb</sup> mice. Two other factors may account for the 351 reduced HR response to the social challenge in these mutant mice: (i) a somewhat lower level of 352 353 somatomotor activity, (ii) the fact that HR may have reached its physiological maximum, thus masking the reported differences in baseline HR. Stressful stimuli are processed in multiple limbic 354 355 forebrain structures, including the amygdala, hippocampus and prefrontal cortex [38]. Limbic stress effector pathways converge on crucial subcortical relay sites, providing for downstream processing 356 357 of limbic information. In particular, numerous studies link the hippocampus with inhibition of the 358 HPA axis [38, 39]. For example, hippocampal stimulation decreases glucocorticoid secretion in rats and humans [40, 41], whereas hippocampal damage increases stress-induced and in some cases, 359 basal glucocorticoid secretion [39]. Not surprisingly, Npy1r<sup>rb</sup> mice show enhanced hypothalamic 360 CRH immunoreactivity and higher serum corticosterone levels [17]. It has been hypothesized that 361 the selective inactivation of Y1Rs in principal excitatory neurons of hippocampus might stimulate 362 363 HPA axis via the glutamatergic output [17]. Although these limbic circuits are also thought to participate in autonomic integration, their precise role(s) in stress-induced responses is not yet 364 defined. The results of this study provide preliminary evidence that hippocampal Y1R expression 365 366 might also be involved in the regulation of cardiac autonomic responses to acute stress challenges. 367 On the other hand, no evident differences were observed in HR and HRV parameters in response 368 to the chronic psychosocial challenge employed in this study.

In conclusion, the results of this study provide preliminary evidence that conditional reduction of 369 370 hippocampal Y1Rs leads to signs of decreased cardiac vagal tone and HRV in mice at rest. On the other hand, the role of hippocampal Y1Rs in the modulation of cardiac autonomic stress response 371 requires deeper investigation. We acknowledge that the robustness of these findings is certainly 372 373 limited by several factors which need to be addressed in future experiments. For example, Y1R deficiency tied to certain hippocampal (e.g. glutamatergic) neurons might be useful for revealing 374 the precise neurobiological pathways underlying the autonomic phenotype of Npy1r<sup>rb</sup> mice. 375 Moreover, Y1R expression should be investigated in other brain areas that may play a role in the 376

cardiovascular and behavioral effects of hippocampal Y1 receptor deficiency, such as the infralimbic area of the mPFC. Finally, the potential contribution of other phenotypic features of Npy1r<sup>rfb</sup> mice (such as, reduced body weight, increased anxiety, and increased NPY and CRH content [17]) to the autonomic changes described in this study needs to be taken into account. Nevertheless, this conditional knockout mouse model might be useful for gaining a deeper understanding of the role of Y1Rs in emotional-processing areas of the brain for autonomic nervous system control.

385 Figure captions

Figure 1. Timeline of the experimental procedures.

387

Figure 2. Expression of Npy1r mRNA in hippocampal CA1 and CA3 pyramidal cell layers and in the dentate gyrus (DG) granule cell layers in Npy1r<sup>rfb</sup> mice (n=4) and Npy1r<sup>2lox</sup> (n=4) mice. Relative optical densities (OD) are expressed as means  $\pm$  SEM. \* indicates a significant difference between Npy1r<sup>rfb</sup> and Npy1r<sup>2lox</sup> mice (p<0.05).

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Figure 3. Time course of changes in heart rate, heart rate variability parameters, body temperature and locomotor activity during the light (L) and the dark (D) phases of baseline daily rhythm recordings, in Npy1r<sup>rfb</sup> (n = 10) and Npy1r<sup>2lox</sup> (n = 8) mice. Values are reported as means  $\pm$  SEM of data obtained by averaging multiple 2 min segments acquired every 30 min for the 12-h light and the 12-h dark phase of the daily cycle. RMSSD = root mean square of successive R-R interval differences; HF = high frequency; LF = low frequency. Statistical results are reported in the results' section.

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Figure 4. Time course of changes in heart rate (panels A and B), RMSSD (panels C and D) and high frequency (HF) power (panels E and F) in baseline conditions (bas) and following the injection of vehicle (left panels) or methylscopolamine (right panels) in Npy1r<sup>fb</sup> (n = 10) and Npy1r<sup>2lox</sup> (n = 8) mice. Values are expressed as means  $\pm$  SEM. \* indicates a significant difference between Npy1r<sup>fb</sup> and Npy1r<sup>2lox</sup> mice (p<0.05); <sup>#</sup> indicates a significant difference vs. the respective baseline value (p<0.05).

407

Figure 5. Left panels show the time course of changes in heart rate (A), high frequency (HF) power (C), and locomotor activity (E) values in baseline conditions (bas) and during the first episode of social defeat, in Npy1r<sup>rfb</sup> (n= 10) and Npy1r<sup>2lox</sup> (n= 8) mice. Right panels show delta values of heart rate (B), HF power (D), and locomotor activity (F) which were calculated as the difference between

- 412 mean "fight" and mean baseline values. Absolute and delta values are expressed as means ±
- 413 SEM. \* indicates a significant difference between Npy1r<sup>rfb</sup> and Npy1r<sup>2lox</sup> mice (p<0.05).

# 415 Tables

416 Table 1. Daily rhythms of HR, HRV parameters, body temperature and locomotor activity in 417 baseline condition and during chronic psychosocial stress.

418

		BASELINE RHYTHMS		STRESS RHYTHMS 1		STRESS RHYTHMS 2	
		Light	Dark	Light	Dark	Light	Dark
HR	Npy1r <sup>rfb</sup>	504±7*	567±7*	552±14 <sup>#</sup>	597±15	515±11	582±13
(bpm)	Npy1r <sup>2lox</sup>	466±12	532±5	$533 \pm 14^{\#}$	$590 \pm 10^{\#}$	497±11	571±7 <sup>#</sup>
TOTAL power	Npy1r <sup>rfb</sup>	78.7±8.3*	45.2±3.2*	39.3±6.4 <sup>#</sup>	29.0±3.8 <sup>#</sup>	91.9±15.8	41.4±5.6
(ms²)	Npy1r <sup>2lox</sup>	119.7±14.5	61.4±4.4	51.9±14.1 <sup>#</sup>	28.7±5.6 <sup>#</sup>	87.6±20.6	45.0±7.9
RMSSD	Npy1r <sup>rfb</sup>	4.6±0.4*	3.2±0.3*	$2.7\pm0.3^{\#}$	$2.3\pm0.2^{\#}$	4.6±0.6	3.2±0.4
(ms)	Npy1r <sup>2lox</sup>	6.4±0.7	4.2±0.3	$3.4\pm0.8^{\#}$	$2.8 \pm 0.4^{\#}$	4.3±0.7 <sup>#</sup>	3.7±0.7
HF power	Npy1r <sup>rfb</sup>	7.4±1.4*	3.8±0.8	$2.6\pm0.5^{\#}$	2.1±0.4	7.1±1.7	3.6±0.9
(ms²)	Npy1r <sup>2lox</sup>	13.4±2.2	5.9±0.8	5.1±2.5 <sup>#</sup>	$3.2\pm0.9^{\#}$	6.0±1.5 <sup>#</sup>	5.1±1.7
Т	Npy1r <sup>rfb</sup>	36.2±0.2*	37.3±0.2	36.3±0.2	37.2±0.2	36.1±0.2	37.2±0.2
(°C)	Npy1r <sup>2lox</sup>	35.8±0.1	37.0±0.1	35.9±0.1	37.0±0.2	35.8±0.1	37.1±0.1
LOC	Npy1r <sup>rfb</sup>	4.0±0.4	8.8±1.1	4.4±0.8	11.5±2.3	4.4±0.6	9.4±1.5
(cpm)	Npy1r <sup>2lox</sup>	4.6±0.5	11.4±1.5	5.2±0.7	12.2±1.7	4.9±0.7	12.4±1.5

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420 Values are reported as mean values ± SEM of data obtained by averaging multiple 2 min segments acquired every 30 min over a 6-day period for baseline rhythms and over a 4-day period 421 for stress rhythms 1 and stress rhythms 2, in Npy1r<sup>fb</sup> (n = 10) and Npy1r<sup>2lox</sup> (n = 8) mice. HR= heart 422 423 rate; HRV= heart rate variability; RMSSD = root mean square of successive R-R interval differences; HF = high frequency; LF = low frequency; T = body temperature; LOC = locomotor 424 activity. \* indicates a significant difference between Npy1r<sup>fb</sup> and Npy1r<sup>2lox</sup> mice; <sup>#</sup> indicates a 425 significant difference between stress rhythm value and the respective baseline rhythm value. 426 (p<0.05). 427

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