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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

18 Central neuropeptide Y (NPY) signaling participates in the regulation of cardiac autonomic outflow,
19 particularly via activation of NPY-Y1 receptors (Y1Rs). However, the specific brain areas and
20 neural pathways involved have not been completely identified yet. Here, we evaluate the role of
21 **hippocampal** Y1Rs in the modulation of the autonomic control of cardiac function using a
22 conditional knockout mouse model. Radiotelemetric transmitters were implanted in 4-month-old
23 male mice exhibiting reduced forebrain expression (rfb) of the Y1R ($Npy1r^{rfb}$, n=10) and their
24 corresponding controls ($Npy1r^{2lox}$, n=8). ECG signals were recorded (i) during resting conditions,
25 (ii) under selective pharmacological manipulation of cardiac vagal activity, and (iii) during acute and
26 chronic psychosocial stress challenges, and analyzed via time- and frequency-domain analysis of
27 heart rate variability. **$Npy1r^{rfb}$ mice showed a lower $Npy1r$ mRNA density in the dentate gyrus and
28 in the CA1 region of the hippocampus.** Under resting undisturbed conditions, $Npy1r^{rfb}$ mice
29 exhibited (i) a higher heart rate, (ii) a reduced overall heart rate variability, and (iii) lower values of
30 the indices of vagal modulation compared to $Npy1r^{2lox}$ counterparts. **Following pharmacological
31 vagal inhibition, heart rate was higher in control but not in $Npy1r^{rfb}$ mice compared to their
32 respective baseline values, suggesting that tonic vagal influences on heart rate were reduced in
33 $Npy1r^{rfb}$ mice. The magnitude of the heart rate response to an acute psychosocial stressor was
34 smaller in $Npy1r^{rfb}$ mice compared to $Npy1r^{2lox}$ counterparts (likely due to a concurrent lower vagal
35 withdrawal), even though absolute heart rate values did not differ between the two groups. These
36 findings suggest that reduced hippocampal Y1R expression leads to a decrease in resting vagal
37 modulation and heart rate variability, which, in turn, may determine a reduced cardiac autonomic
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
45 are mediated by multiple G-protein coupled receptor subtypes, which are characterized by different
46 tissue localization and pharmacological properties [6-8]. In particular, accumulating evidence
47 suggests that central Y1 receptors (Y1Rs) play a critical role in the inhibition of the sympathetic
48 outflow to peripheral tissues. For example, it has been shown in rats that **i.c.v. (lateral ventricle)**
49 administration of the Y1R agonist [Leu³¹,Pro³⁴]NPY reduces basal noradrenaline release from the
50 paraventricular nucleus of the hypothalamus (PVN) [9], the major site of autonomic regulation via
51 projections to the rostral ventro-lateral medulla and the spinal cord. Moreover, NPY, acting on
52 Y1Rs, decreases noradrenaline overflow from both the hypothalamus and the medulla in vitro [10].
53 A recent study has elegantly demonstrated that NPY released from the arcuate nucleus (ARC)
54 suppresses tyrosine hydroxylase (TH) expression in the PVN via activation of Y1Rs [11]. This
55 effect was associated with a reduction in TH expression in the locus coeruleus and other regions
56 (such as the A1/C1 cell groups) in the brain stem [11], which would ultimately lead to a reduced
57 sympathetic outflow to peripheral tissues [11]. Supporting this view, nanoinjection of NPY into the
58 PVN decreases heart rate (HR), sympathetic nerve activity and baroreflex control of HR in
59 anesthetized rats via activation of Y1Rs and Y5Rs [12]. In mice, Tovote and colleagues observed
60 that **bilateral i.c.v. injections** of NPY dose-dependently induced bradycardia and also blunted the
61 tachycardic response to a fear conditioning challenge [13], the latter via Y1R-mediated inhibition of
62 stress-induced sympathetic activation [13]. Similarly, global knockout of the Y1 receptor gene in
63 mice led to a larger HR activation during social defeat stress [14]. These findings support the view
64 that NPY-Y1R signaling may play an important role also in constraining stress-induced sympathetic
65 activation [15, 16]. While the relationship between NPY-Y1R signaling in the ARC/PVN axis and
66 sympathetic nervous system has been documented, there is little known about the neural
67 pathways and mechanisms that control it. Indeed, in contrast with the above reported observations
68 that are suggestive of a central sympathetic inhibitory role of Y1Rs, global knockout of the NPY-

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

73 Recently, a conditional knockout mouse model in which the inactivation of the Y1 receptor gene is
74 restricted to excitatory neurons in **the hippocampus**, in particular when mice were reared by foster
75 mothers exhibiting high levels of maternal care, has been developed [17]. Therefore, this animal
76 model may be useful for understanding the contribution of **hippocampal** Y1Rs for central autonomic
77 cardiovascular control. In order to reach this goal, autonomic modulation of cardiac function was
78 assessed in the above mentioned conditional knockout mouse model during (i) baseline conditions,
79 (ii) selective pharmacological autonomic manipulations, and (iii) acute and chronic psychosocial
80 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

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

116

117 2.2. Transmitter implantation and radiotelemetry system

118 ECG (sampling frequency 2 KHz), body temperature (T, $^{\circ}C$) and locomotor activity (LOC, counts
119 per minute, cpm) signals were recorded by a radiotelemetry system. It consisted of transmitters
120 (TA10ETA-F20, Data Sciences Int., St.Paul, MN, USA) and platform receivers (RPC-1, Data
121 Science Int., St.Paul, MN, USA), which were connected to a computer containing Art-Gold 1.10
122 data acquisition system (Data Science Int., St.Paul, MN, USA). Transmitter implantation was
123 performed under isoflurane anesthesia (2% in 100% oxygen), as previously described [18]. The

124 transmitter's body was implanted intraperitoneally and two electrodes (wire loops) were fixed to the
125 dorsal surface of the xyphoid process and in the anterior mediastinum close to the right atrium,
126 respectively. This surgical procedure guarantees high quality ECG recordings, even during
127 sustained physical activity. After surgery, mice were individually housed, injected with Gentamicin
128 sulphate (Aagent, Fatro, 1ml/kg, S.C.) and allowed two weeks of recovery before the beginning of
129 the experimental recordings.

130

131 2.3. General experimental outline

132 The timeline of the experimental protocol is depicted in Figure 1. Specific experimental procedures
133 and data analysis are described in the following sections. After recovery from surgery, mice were
134 weighed and left undisturbed in their home cages for 6 days (days 1-6). On days 7 and 9, they
135 underwent pharmacological autonomic challenges. From day 12 to day 25, mice were housed in
136 the cage of a dominant mouse in order to mimic a chronic psychosocial stress condition. At the end
137 of the experimental protocol, mice were sacrificed by decapitation. Brains were rapidly removed
138 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

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

147

148 2.5. Pharmacological autonomic challenge

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

151 design. Continuous ECG recordings were performed prior to (for 30 min, baseline condition) and
152 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
156 paradigm [18, 19], which is based on the classical resident-intruder test [20]. Six-month-old CD1
157 mice were housed with a female partner for 1 week and then individually housed and trained to
158 aggressively defend their territory from same sex mice intruders that were younger and lighter.
159 These dominant animals served as residents. Each experimental mouse was introduced as an
160 intruder in the home cage of a resident male; once there, it was attacked and subordinated by the
161 resident mouse (social defeat). After 10 min, the two animals were separated by a perforated
162 polystyrene-metal partition that divided the cage into two equal-area sections. Thus, the intruder
163 mouse was protected from direct physical contact but it was in constant olfactory, auditory and
164 visual contact with the resident (psychosocial challenge). The partition was removed five times
165 during the experimental protocol (Fig. 1) at an unpredictable time between 9:00 and 13:00 h and
166 repositioned after 2 min to prevent injuries. After each interaction, the experimental mice were
167 closely inspected for any improper injury and were excluded from further procedures in case they
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

174 HRV analysis was carried out on ECG recordings using ChartPro 5.0 software (ADInstrument,
175 Sydney, Australia), based on the recommendations for HRV analysis in mice [21]. Initially, each
176 ECG recording was split in 2-min temporal segments (0-2 min; 2-4 min; etc.). Ectopic beats and
177 recording artifacts were then removed following visual inspection of unprocessed ECG signals.
178 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
180 (RMSSD, ms) was quantified; this index reflects short-term, high-frequency variations of RR
181 interval, which are mainly due to cardiac parasympathetic activity [22]. In the frequency-domain,
182 the power spectrum was obtained with a fast Fourier transform-based method (Welch's
183 periodogram: 256 points, 50% overlap, and Hamming window). The following parameters were
184 evaluated: i) the total power of the spectrum (ms^2), which reflects all the cyclic components
185 responsible for variability [23], and ii) the power (ms^2) of the high frequency band (HF, 1.5-5.0 Hz),
186 which reflects the activity of the parasympathetic nervous system and is related to respiratory sinus
187 arrhythmia [24].

188

189 2.8. In Situ Hybridization for Npy1r mRNA

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

200 .

201 2.9. Statistical analysis

202 All statistical analyses were performed using the software package SPSS (version 22). Two-way
203 ANOVA for repeated measures with "group" as between-subject factor (2 levels: Npy1r^{rfb} and
204 Npy1r^{2lox}) was applied for ECG and telemetric data obtained from: i) baseline around-the-clock
205 recordings, with "time" as within-subject factor (6 levels: day 1, 2, 3, 4, 5 and 6); ii) pharmacological
206 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 $p < 0.05$.

212

213 3. Results

214

215 3.1. Body weight

216 Prior to surgery, body weight was 25.0 ± 0.9 g for Npy1r^{fb} mice and 26.0 ± 1.2 g for Npy1r^{2lox} mice
217 (n.s.). No significant differences between the two groups were found in body weight at the
218 beginning of the experimental protocol (day 1) (Npy1r^{fb}: 26.3 ± 0.3 g vs. Npy1r^{2lox} 27.2 ± 0.8 g).

219

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

221 In Npy1r^{fb} mice, Npy1r mRNA expression was significantly lower in hippocampal CA1 pyramidal
222 cell layers ($t = -3.1$, $p < 0.05$) and in dentate gyrus granule cell layers ($t = -3.4$, $p < 0.05$) compared with
223 their control littermates, whereas no difference was observed in CA3 pyramidal cell layers between
224 the two groups (Fig. 2).

225

226 3.3. Daily rhythms of HR, HRV, T and LOC during baseline conditions

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

241

242 3.4. Cardiac autonomic response to the pharmacological autonomic manipulation

243 Cardiac autonomic responses to vehicle or methylscopolamine injection are depicted in **Figure 4**.

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

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

262

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

264 **Cardiac autonomic and LOC responses to the first episode of social defeat are depicted in Figure**
265 **5. Two-way ANOVA yielded a significant effect of time for HR (F=18.9, p<0.01) and LOC (F=4.4,**
266 **p<0.05) values. In baseline conditions, HR was significantly higher in Npy1r^{rfb} than in Npy1r^{2lox}**
267 **mice (t=2.2, p<0.05) (Fig. 5A). No differences in absolute values of HR were observed between the**
268 **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
270 significantly smaller in $Npy1r^{fb}$ than in $Npy1r^{2lox}$ mice ($t=-4.6$, $p<0.01$) (Fig. 5B). Consistently, in
271 baseline conditions $Npy1r^{fb}$ mice had significantly lower HF spectral power values compared with
272 $Npy1r^{2lox}$ mice ($t=2.5$, $p<0.05$) (Fig. 5C). During the 10-min fight period, HF absolute values were
273 similar between the two groups (Fig. 5C), with the magnitude of the stress-induced reduction in HF
274 values being significantly smaller in $Npy1r^{fb}$ than in $Npy1r^{2lox}$ mice ($t=2.5$, $p<0.05$) (Fig. 5D). No
275 differences in LOC values were observed between $Npy1r^{fb}$ and $Npy1r^{2lox}$ mice in baseline
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

279 HR, HRV, T and LOC values during the light and dark phases of the chronic psychosocial stress
280 period are summarized in Table1. Two-way ANOVA revealed that chronic exposure to
281 psychosocial stress led to (i) higher HR values ($F_{light}=20.0$, $p<0.01$; $F_{dark}=19.8$, $p<0.01$), and (ii)
282 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
283 compared to baseline conditions (Table 1). However, there were no differences between the two
284 groups in HR, HRV parameters, T and LOC during both stress rhythm 1 and stress rhythm 2
285 periods (Table 1).

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293 4. Discussion

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

296

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

316 **In this study, Npy1r^{fb} mice Y1R expression is significantly reduced in the dentate gyrus and the**
317 **CA1 region of the hippocampus**, where Y1Rs have been associated with glutamate-positive and
318 NPY-positive neurons [27]. The ventral CA1 hippocampal field is thought to be linked by
319 multisynaptic connections to the sympathetic-related regions of the hypothalamus, suggesting its
320 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),
322 such as the infralimbic cortex [29], indicating that hippocampal actions on autonomic function may
323 also be routed through the mPFC. Importantly, electrical and chemical stimulation of the
324 hippocampal formation in the anesthetized and the awake rat decreases heart rate, blood pressure
325 and respiratory rate [30, 31]. Interestingly, pharmacological manipulation of vagal activity with
326 methylatropine revealed that the cardiovascular, but not the respiratory, responses were mediated
327 partially by vagal influences and partially by sympathetic influences [30]. Taken together, **these**
328 **preliminary observations indicate that hippocampal Y1Rs may participate in the control of**
329 **autonomic outflow to peripheral tissues under baseline, undisturbed conditions. In a previous**
330 **study, Npy1r^{flb} mice have been characterized as having reduced body weight, less adipose tissue,**
331 **and lower serum leptin levels [17]. Here, mice did not differ for body weight, either prior or after**
332 **surgery recovery, as they were matched to avoid possible bias. Therefore, we might cautiously**
333 **assume that body weight is not a major determinant of the autonomic phenotype described in**
334 **Npy1r^{flb} mice. On the other hand,** the hippocampus is an important component of the neuronal
335 circuitry controlling anxiety-related behaviors and Npy1r^{flb} mice have been previously shown to
336 display higher levels of anxiety in the elevated plus maze and open field tests [17]. These findings
337 are consistent with a previous observation that overexpression of virally-transduced NPY in the
338 mouse hippocampus produces anxiolytic-like effects [32]. Moreover, in humans NPY
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
341 anticipatory reactivity in the brain [35], it is tempting to speculate **that reduced vagal tone and HRV**
342 **in Npy1r^{flb} mice might reflect heightened anxiety-related behavior. Following this line of reasoning,**
343 **reduced expression of Y1Rs on neurons lying in hippocampal areas or their related circuits might**
344 **account for the imbalance in the autonomic control of resting heart rate that characterizes high**
345 **levels of anxiety in humans [36] and animal models [37].**
346 **In this study, absolute values of HR were similar between mutant and control mice during an acute**
347 **psychosocial challenge (social defeat). Elevated HR during social defeat was mediated by a**
348 **decreased vagal tone compared to baseline levels in both groups. However, given that the**

349 concurrent stress-induced increase in HR and decrease in vagal tone were smaller in Npy1r^{fb}
350 mice, it could be hypothesized that a failure to further decrease cardiac vagal activity contributes to
351 the smaller stress-induced increase in HR in Npy1r^{fb} mice. Two other factors may account for the
352 reduced HR response to the social challenge in these mutant mice: (i) a somewhat lower level of
353 somatomotor activity, (ii) the fact that HR may have reached its physiological maximum, thus
354 masking the reported differences in baseline HR. Stressful stimuli are processed in multiple limbic
355 forebrain structures, including the amygdala, hippocampus and prefrontal cortex [38]. Limbic stress
356 effector pathways converge on crucial subcortical relay sites, providing for downstream processing
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
359 and humans [40, 41], whereas hippocampal damage increases stress-induced and in some cases,
360 basal glucocorticoid secretion [39]. Not surprisingly, Npy1r^{fb} mice show enhanced hypothalamic
361 CRH immunoreactivity and higher serum corticosterone levels [17]. It has been hypothesized that
362 the selective inactivation of Y1Rs in principal excitatory neurons of hippocampus might stimulate
363 HPA axis via the glutamatergic output [17]. Although these limbic circuits are also thought to
364 participate in autonomic integration, their precise role(s) in stress-induced responses is not yet
365 defined. The results of this study provide preliminary evidence that hippocampal Y1R expression
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.

369 In conclusion, the results of this study provide preliminary evidence that conditional reduction of
370 hippocampal Y1Rs leads to signs of decreased cardiac vagal tone and HRV in mice at rest. On the
371 other hand, the role of hippocampal Y1Rs in the modulation of cardiac autonomic stress response
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
374 deficiency tied to certain hippocampal (e.g. glutamatergic) neurons might be useful for revealing
375 the precise neurobiological pathways underlying the autonomic phenotype of Npy1r^{fb} mice.
376 Moreover, Y1R expression should be investigated in other brain areas that may play a role in the

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

384

385 Figure captions

386 Figure 1. Timeline of the experimental procedures.

387

388 **Figure 2. Expression of Npy1r mRNA in hippocampal CA1 and CA3 pyramidal cell layers and in**
389 **the dentate gyrus (DG) granule cell layers in Npy1r^{rfb} mice (n=4) and Npy1r^{2lox} (n=4) mice. Relative**
390 **optical densities (OD) are expressed as means ± SEM. * indicates a significant difference between**
391 **Npy1r^{rfb} and Npy1r^{2lox} mice (p<0.05).**

392

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

400

401 Figure 4. Time course of changes in heart rate (panels A and B), RMSSD (panels C and D) and
402 high frequency (HF) power (panels E and F) in baseline conditions (bas) and following the injection
403 of vehicle (left panels) or methylscopolamine (right panels) in Npy1r^{rfb} (n = 10) and Npy1r^{2lox} (n = 8)
404 mice. Values are expressed as means ± SEM. * indicates a significant difference between Npy1r^{rfb}
405 and Npy1r^{2lox} mice (p<0.05); # indicates a significant difference vs. the respective baseline value
406 (p<0.05).

407

408 **Figure 5. Left panels show the time course of changes in heart rate (A), high frequency (HF) power**
409 **(C), and locomotor activity (E) values in baseline conditions (bas) and during the first episode of**
410 **social defeat, in Npy1r^{rfb} (n= 10) and Npy1r^{2lox} (n= 8) mice. Right panels show delta values of heart**
411 **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 \pm
413 SEM. * indicates a significant difference between Npy1r^{flb} and Npy1r^{2lox} mice (p<0.05).
414

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 (bpm)	Npy1r ^{ffb}	504±7*	567±7*	552±14 [#]	597±15	515±11	582±13
	Npy1r ^{2lox}	466±12	532±5	533±14 [#]	590±10 [#]	497±11	571±7 [#]
TOTAL power (ms ²)	Npy1r ^{ffb}	78.7±8.3*	45.2±3.2*	39.3±6.4 [#]	29.0±3.8 [#]	91.9±15.8	41.4±5.6
	Npy1r ^{2lox}	119.7±14.5	61.4±4.4	51.9±14.1 [#]	28.7±5.6 [#]	87.6±20.6	45.0±7.9
RMSSD (ms)	Npy1r ^{ffb}	4.6±0.4*	3.2±0.3*	2.7±0.3 [#]	2.3±0.2 [#]	4.6±0.6	3.2±0.4
	Npy1r ^{2lox}	6.4±0.7	4.2±0.3	3.4±0.8 [#]	2.8±0.4 [#]	4.3±0.7 [#]	3.7±0.7
HF power (ms ²)	Npy1r ^{ffb}	7.4±1.4*	3.8±0.8	2.6±0.5 [#]	2.1±0.4	7.1±1.7	3.6±0.9
	Npy1r ^{2lox}	13.4±2.2	5.9±0.8	5.1±2.5 [#]	3.2±0.9 [#]	6.0±1.5 [#]	5.1±1.7
T (°C)	Npy1r ^{ffb}	36.2±0.2*	37.3±0.2	36.3±0.2	37.2±0.2	36.1±0.2	37.2±0.2
	Npy1r ^{2lox}	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 (cpm)	Npy1r ^{ffb}	4.0±0.4	8.8±1.1	4.4±0.8	11.5±2.3	4.4±0.6	9.4±1.5
	Npy1r ^{2lox}	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
 421 segments acquired every 30 min over a 6-day period for baseline rhythms and over a 4-day period
 422 for stress rhythms 1 and stress rhythms 2, in Npy1r^{ffb} (n = 10) and Npy1r^{2lox} (n = 8) mice. HR= heart
 423 rate; HRV= heart rate variability; RMSSD = root mean square of successive R-R interval
 424 differences; HF = high frequency; LF = low frequency; T = body temperature; LOC = locomotor
 425 activity. * indicates a significant difference between Npy1r^{ffb} and Npy1r^{2lox} mice; # indicates a
 426 significant difference between stress rhythm value and the respective baseline rhythm value.
 427 (p<0.05).

428

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432

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534

535

17 **Abstract**

18 Central neuropeptide Y (NPY) signaling participates in the regulation of cardiac autonomic outflow,
19 particularly via activation of NPY-Y1 receptors (Y1Rs). However, the specific brain areas and
20 neural pathways involved have not been completely identified yet. Here, we evaluate the role of
21 hippocampal Y1Rs in the modulation of the autonomic control of cardiac function using a
22 conditional knockout mouse model. Radiotelemetric transmitters were implanted in 4-month-old
23 male mice exhibiting reduced forebrain expression (rfb) of the Y1R ($Npy1r^{rfb}$, n=10) and their
24 corresponding controls ($Npy1r^{2lox}$, n=8). ECG signals were recorded (i) during resting conditions,
25 (ii) under selective pharmacological manipulation of cardiac vagal activity, and (iii) during acute and
26 chronic psychosocial stress challenges, and analyzed via time- and frequency-domain analysis of
27 heart rate variability. $Npy1r^{rfb}$ mice showed a lower $Npy1r$ mRNA density in the dentate gyrus and
28 in the CA1 region of the hippocampus. Under resting undisturbed conditions, $Npy1r^{rfb}$ mice
29 exhibited (i) a higher heart rate, (ii) a reduced overall heart rate variability, and (iii) lower values of
30 the indices of vagal modulation compared to $Npy1r^{2lox}$ counterparts. Following pharmacological
31 vagal inhibition, heart rate was higher in control but not in $Npy1r^{rfb}$ mice compared to their
32 respective baseline values, suggesting that tonic vagal influences on heart rate were reduced in
33 $Npy1r^{rfb}$ mice. The magnitude of the heart rate response to an acute psychosocial stressor was
34 smaller in $Npy1r^{rfb}$ mice compared to $Npy1r^{2lox}$ counterparts (likely due to a concurrent lower vagal
35 withdrawal), even though absolute heart rate values did not differ between the two groups. These
36 findings suggest that reduced hippocampal Y1R expression leads to a decrease in resting vagal
37 modulation and heart rate variability, which, in turn, may determine a reduced cardiac autonomic
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
45 are mediated by multiple G-protein coupled receptor subtypes, which are characterized by different
46 tissue localization and pharmacological properties [6-8]. In particular, accumulating evidence
47 suggests that central Y1 receptors (Y1Rs) play a critical role in the inhibition of the sympathetic
48 outflow to peripheral tissues. For example, it has been shown in rats that i.c.v. (lateral ventricle)
49 administration of the Y1R agonist [Leu³¹,Pro³⁴]NPY reduces basal noradrenaline release from the
50 paraventricular nucleus of the hypothalamus (PVN) [9], the major site of autonomic regulation via
51 projections to the rostral ventro-lateral medulla and the spinal cord. Moreover, NPY, acting on
52 Y1Rs, decreases noradrenaline overflow from both the hypothalamus and the medulla in vitro [10].
53 A recent study has elegantly demonstrated that NPY released from the arcuate nucleus (ARC)
54 suppresses tyrosine hydroxylase (TH) expression in the PVN via activation of Y1Rs [11]. This
55 effect was associated with a reduction in TH expression in the locus coeruleus and other regions
56 (such as the A1/C1 cell groups) in the brain stem [11], which would ultimately lead to a reduced
57 sympathetic outflow to peripheral tissues [11]. Supporting this view, nanoinjection of NPY into the
58 PVN decreases heart rate (HR), sympathetic nerve activity and baroreflex control of HR in
59 anesthetized rats via activation of Y1Rs and Y5Rs [12]. In mice, Tovote and colleagues observed
60 that bilateral i.c.v. injections of NPY dose-dependently induced bradycardia and also blunted the
61 tachycardic response to a fear conditioning challenge [13], the latter via Y1R-mediated inhibition of
62 stress-induced sympathetic activation [13]. Similarly, global knockout of the Y1 receptor gene in
63 mice led to a larger HR activation during social defeat stress [14]. These findings support the view
64 that NPY-Y1R signaling may play an important role also in constraining stress-induced sympathetic
65 activation [15, 16]. While the relationship between NPY-Y1R signaling in the ARC/PVN axis and
66 sympathetic nervous system has been documented, there is little known about the neural
67 pathways and mechanisms that control it. Indeed, in contrast with the above reported observations
68 that are suggestive of a central sympathetic inhibitory role of Y1Rs, global knockout of the NPY-

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

73 Recently, a conditional knockout mouse model in which the inactivation of the Y1 receptor gene is
74 restricted to excitatory neurons in the hippocampus, in particular when mice were reared by foster
75 mothers exhibiting high levels of maternal care, has been developed [17]. Therefore, this animal
76 model may be useful for understanding the contribution of hippocampal Y1Rs for central autonomic
77 cardiovascular control. In order to reach this goal, autonomic modulation of cardiac function was
78 assessed in the above mentioned conditional knockout mouse model during (i) baseline conditions,
79 (ii) selective pharmacological autonomic manipulations, and (iii) acute and chronic psychosocial
80 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

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

116

117 2.2. Transmitter implantation and radiotelemetry system

118 ECG (sampling frequency 2 KHz), body temperature (T , $^{\circ}C$) and locomotor activity (LOC, counts
119 per minute, cpm) signals were recorded by a radiotelemetry system. It consisted of transmitters
120 (TA10ETA-F20, Data Sciences Int., St.Paul, MN, USA) and platform receivers (RPC-1, Data
121 Science Int., St.Paul, MN, USA), which were connected to a computer containing Art-Gold 1.10
122 data acquisition system (Data Science Int., St.Paul, MN, USA). Transmitter implantation was
123 performed under isoflurane anesthesia (2% in 100% oxygen), as previously described [18]. The

124 transmitter's body was implanted intraperitoneally and two electrodes (wire loops) were fixed to the
125 dorsal surface of the xyphoid process and in the anterior mediastinum close to the right atrium,
126 respectively. This surgical procedure guarantees high quality ECG recordings, even during
127 sustained physical activity. After surgery, mice were individually housed, injected with Gentamicin
128 sulphate (Aagent, Fatro, 1ml/kg, S.C.) and allowed two weeks of recovery before the beginning of
129 the experimental recordings.

130

131 2.3. General experimental outline

132 The timeline of the experimental protocol is depicted in Figure 1. Specific experimental procedures
133 and data analysis are described in the following sections. After recovery from surgery, mice were
134 weighed and left undisturbed in their home cages for 6 days (days 1-6). On days 7 and 9, they
135 underwent pharmacological autonomic challenges. From day 12 to day 25, mice were housed in
136 the cage of a dominant mouse in order to mimic a chronic psychosocial stress condition. At the end
137 of the experimental protocol, mice were sacrificed by decapitation. Brains were rapidly removed
138 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

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

147

148 2.5. Pharmacological autonomic challenge

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

151 design. Continuous ECG recordings were performed prior to (for 30 min, baseline condition) and
152 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
156 paradigm [18, 19], which is based on the classical resident-intruder test [20]. Six-month-old CD1
157 mice were housed with a female partner for 1 week and then individually housed and trained to
158 aggressively defend their territory from same sex mice intruders that were younger and lighter.
159 These dominant animals served as residents. Each experimental mouse was introduced as an
160 intruder in the home cage of a resident male; once there, it was attacked and subordinated by the
161 resident mouse (social defeat). After 10 min, the two animals were separated by a perforated
162 polystyrene-metal partition that divided the cage into two equal-area sections. Thus, the intruder
163 mouse was protected from direct physical contact but it was in constant olfactory, auditory and
164 visual contact with the resident (psychosocial challenge). The partition was removed five times
165 during the experimental protocol (Fig. 1) at an unpredictable time between 9:00 and 13:00 h and
166 repositioned after 2 min to prevent injuries. After each interaction, the experimental mice were
167 closely inspected for any improper injury and were excluded from further procedures in case they
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

174 HRV analysis was carried out on ECG recordings using ChartPro 5.0 software (ADInstrument,
175 Sydney, Australia), based on the recommendations for HRV analysis in mice [21]. Initially, each
176 ECG recording was split in 2-min temporal segments (0-2 min; 2-4 min; etc.). Ectopic beats and
177 recording artifacts were then removed following visual inspection of unprocessed ECG signals.
178 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
180 (RMSSD, ms) was quantified; this index reflects short-term, high-frequency variations of RR
181 interval, which are mainly due to cardiac parasympathetic activity [22]. In the frequency-domain,
182 the power spectrum was obtained with a fast Fourier transform-based method (Welch's
183 periodogram: 256 points, 50% overlap, and Hamming window). The following parameters were
184 evaluated: i) the total power of the spectrum (ms^2), which reflects all the cyclic components
185 responsible for variability [23], and ii) the power (ms^2) of the high frequency band (HF, 1.5-5.0 Hz),
186 which reflects the activity of the parasympathetic nervous system and is related to respiratory sinus
187 arrhythmia [24].

188

189 2.8. In Situ Hybridization for Npy1r mRNA

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

200 .

201 2.9. Statistical analysis

202 All statistical analyses were performed using the software package SPSS (version 22). Two-way
203 ANOVA for repeated measures with "group" as between-subject factor (2 levels: Npy1r^{rfb} and
204 Npy1r^{2lox}) was applied for ECG and telemetric data obtained from: i) baseline around-the-clock
205 recordings, with "time" as within-subject factor (6 levels: day 1, 2, 3, 4, 5 and 6); ii) pharmacological
206 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 $p < 0.05$.

212

213 3. Results

214

215 3.1. Body weight

216 Prior to surgery, body weight was 25.0 ± 0.9 g for Npy1r^{rfb} mice and 26.0 ± 1.2 g for Npy1r^{2lox} mice
217 (n.s.). No significant differences between the two groups were found in body weight at the
218 beginning of the experimental protocol (day 1) (Npy1r^{rfb}: 26.3 ± 0.3 g vs. Npy1r^{2lox} 27.2 ± 0.8 g).

219

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

221 In Npy1r^{rfb} mice, Npy1r mRNA expression was significantly lower in hippocampal CA1 pyramidal
222 cell layers ($t = -3.1$, $p < 0.05$) and in dentate gyrus granule cell layers ($t = -3.4$, $p < 0.05$) compared with
223 their control littermates, whereas no difference was observed in CA3 pyramidal cell layers between
224 the two groups (Fig. 2).

225

226 3.3. Daily rhythms of HR, HRV, T and LOC during baseline conditions

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

241

242 3.4. Cardiac autonomic response to the pharmacological autonomic manipulation

243 Cardiac autonomic responses to vehicle or methylscopolamine injection are depicted in Figure 4.

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

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

262

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

264 Cardiac autonomic and LOC responses to the first episode of social defeat are depicted in Figure
265 5. Two-way ANOVA yielded a significant effect of time for HR ($F=18.9$, $p<0.01$) and LOC ($F=4.4$,
266 $p<0.05$) values. In baseline conditions, HR was significantly higher in Npy1r^{rfb} than in Npy1r^{2lox}
267 mice ($t=2.2$, $p<0.05$) (Fig. 5A). No differences in absolute values of HR were observed between the
268 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
270 significantly smaller in Npy1r^{fb} than in Npy1r^{2lox} mice ($t=-4.6$, $p<0.01$) (Fig. 5B). Consistently, in
271 baseline conditions Npy1r^{fb} mice had significantly lower HF spectral power values compared with
272 Npy1r^{2lox} mice ($t=2.5$, $p<0.05$) (Fig. 5C). During the 10-min fight period, HF absolute values were
273 similar between the two groups (Fig. 5C), with the magnitude of the stress-induced reduction in HF
274 values being significantly smaller in Npy1r^{fb} than in Npy1r^{2lox} mice ($t=2.5$, $p<0.05$) (Fig. 5D). No
275 differences in LOC values were observed between Npy1r^{fb} and Npy1r^{2lox} mice in baseline
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

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

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293 4. Discussion

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

296

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

316 In this study, Npy1r^{fb} mice Y1R expression is significantly reduced in the dentate gyrus and the
317 CA1 region of the hippocampus, where Y1Rs have been associated with glutamate-positive and
318 NPY-positive neurons [27]. The ventral CA1 hippocampal field is thought to be linked by
319 multisynaptic connections to the sympathetic-related regions of the hypothalamus, suggesting its
320 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),
322 such as the infralimbic cortex [29], indicating that hippocampal actions on autonomic function may
323 also be routed through the mPFC. Importantly, electrical and chemical stimulation of the
324 hippocampal formation in the anesthetized and the awake rat decreases heart rate, blood pressure
325 and respiratory rate [30, 31]. Interestingly, pharmacological manipulation of vagal activity with
326 methylatropine revealed that the cardiovascular, but not the respiratory, responses were mediated
327 partially by vagal influences and partially by sympathetic influences [30]. Taken together, these
328 preliminary observations indicate that hippocampal Y1Rs may participate in the control of
329 autonomic outflow to peripheral tissues under baseline, undisturbed conditions. In a previous
330 study, *Npy1r^{flb}* mice have been characterized as having reduced body weight, less adipose tissue,
331 and lower serum leptin levels [17]. Here, mice did not differ for body weight, either prior or after
332 surgery recovery, as they were matched to avoid possible bias. Therefore, we might cautiously
333 assume that body weight is not a major determinant of the autonomic phenotype described in
334 *Npy1r^{flb}* mice. On the other hand, the hippocampus is an important component of the neuronal
335 circuitry controlling anxiety-related behaviors and *Npy1r^{flb}* mice have been previously shown to
336 display higher levels of anxiety in the elevated plus maze and open field tests [17]. These findings
337 are consistent with a previous observation that overexpression of virally-transduced NPY in the
338 mouse hippocampus produces anxiolytic-like effects [32]. Moreover, in humans NPY
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
341 anticipatory reactivity in the brain [35], it is tempting to speculate that reduced vagal tone and HRV
342 in *Npy1r^{flb}* mice might reflect heightened anxiety-related behavior. Following this line of reasoning,
343 reduced expression of Y1Rs on neurons lying in hippocampal areas or their related circuits might
344 account for the imbalance in the autonomic control of resting heart rate that characterizes high
345 levels of anxiety in humans [36] and animal models [37].

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

349 concurrent stress-induced increase in HR and decrease in vagal tone were smaller in Npy1r^{fb}
350 mice, it could be hypothesized that a failure to further decrease cardiac vagal activity contributes to
351 the smaller stress-induced increase in HR in Npy1r^{fb} mice. Two other factors may account for the
352 reduced HR response to the social challenge in these mutant mice: (i) a somewhat lower level of
353 somatomotor activity, (ii) the fact that HR may have reached its physiological maximum, thus
354 masking the reported differences in baseline HR. Stressful stimuli are processed in multiple limbic
355 forebrain structures, including the amygdala, hippocampus and prefrontal cortex [38]. Limbic stress
356 effector pathways converge on crucial subcortical relay sites, providing for downstream processing
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
359 and humans [40, 41], whereas hippocampal damage increases stress-induced and in some cases,
360 basal glucocorticoid secretion [39]. Not surprisingly, Npy1r^{fb} mice show enhanced hypothalamic
361 CRH immunoreactivity and higher serum corticosterone levels [17]. It has been hypothesized that
362 the selective inactivation of Y1Rs in principal excitatory neurons of hippocampus might stimulate
363 HPA axis via the glutamatergic output [17]. Although these limbic circuits are also thought to
364 participate in autonomic integration, their precise role(s) in stress-induced responses is not yet
365 defined. The results of this study provide preliminary evidence that hippocampal Y1R expression
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.

369 In conclusion, the results of this study provide preliminary evidence that conditional reduction of
370 hippocampal Y1Rs leads to signs of decreased cardiac vagal tone and HRV in mice at rest. On the
371 other hand, the role of hippocampal Y1Rs in the modulation of cardiac autonomic stress response
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
374 deficiency tied to certain hippocampal (e.g. glutamatergic) neurons might be useful for revealing
375 the precise neurobiological pathways underlying the autonomic phenotype of Npy1r^{fb} mice.
376 Moreover, Y1R expression should be investigated in other brain areas that may play a role in the

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

384

385 Figure captions

386 Figure 1. Timeline of the experimental procedures.

387

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

392

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

400

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

407

408 Figure 5. Left panels show the time course of changes in heart rate (A), high frequency (HF) power
409 (C), and locomotor activity (E) values in baseline conditions (bas) and during the first episode of
410 social defeat, in Npy1r^{rfb} (n= 10) and Npy1r^{2lox} (n= 8) mice. Right panels show delta values of heart
411 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 \pm
413 SEM. * indicates a significant difference between Npy1r^{flb} and Npy1r^{2lox} mice (p<0.05).
414

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 (bpm)	Npy1r ^{ffb}	504±7*	567±7*	552±14 [#]	597±15	515±11	582±13
	Npy1r ^{2lox}	466±12	532±5	533±14 [#]	590±10 [#]	497±11	571±7 [#]
TOTAL power (ms ²)	Npy1r ^{ffb}	78.7±8.3*	45.2±3.2*	39.3±6.4 [#]	29.0±3.8 [#]	91.9±15.8	41.4±5.6
	Npy1r ^{2lox}	119.7±14.5	61.4±4.4	51.9±14.1 [#]	28.7±5.6 [#]	87.6±20.6	45.0±7.9
RMSSD (ms)	Npy1r ^{ffb}	4.6±0.4*	3.2±0.3*	2.7±0.3 [#]	2.3±0.2 [#]	4.6±0.6	3.2±0.4
	Npy1r ^{2lox}	6.4±0.7	4.2±0.3	3.4±0.8 [#]	2.8±0.4 [#]	4.3±0.7 [#]	3.7±0.7
HF power (ms ²)	Npy1r ^{ffb}	7.4±1.4*	3.8±0.8	2.6±0.5 [#]	2.1±0.4	7.1±1.7	3.6±0.9
	Npy1r ^{2lox}	13.4±2.2	5.9±0.8	5.1±2.5 [#]	3.2±0.9 [#]	6.0±1.5 [#]	5.1±1.7
T (°C)	Npy1r ^{ffb}	36.2±0.2*	37.3±0.2	36.3±0.2	37.2±0.2	36.1±0.2	37.2±0.2
	Npy1r ^{2lox}	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 (cpm)	Npy1r ^{ffb}	4.0±0.4	8.8±1.1	4.4±0.8	11.5±2.3	4.4±0.6	9.4±1.5
	Npy1r ^{2lox}	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
 421 segments acquired every 30 min over a 6-day period for baseline rhythms and over a 4-day period
 422 for stress rhythms 1 and stress rhythms 2, in Npy1r^{ffb} (n = 10) and Npy1r^{2lox} (n = 8) mice. HR= heart
 423 rate; HRV= heart rate variability; RMSSD = root mean square of successive R-R interval
 424 differences; HF = high frequency; LF = low frequency; T = body temperature; LOC = locomotor
 425 activity. * indicates a significant difference between Npy1r^{ffb} and Npy1r^{2lox} mice; # indicates a
 426 significant difference between stress rhythm value and the respective baseline rhythm value.
 427 (p<0.05).

428

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432

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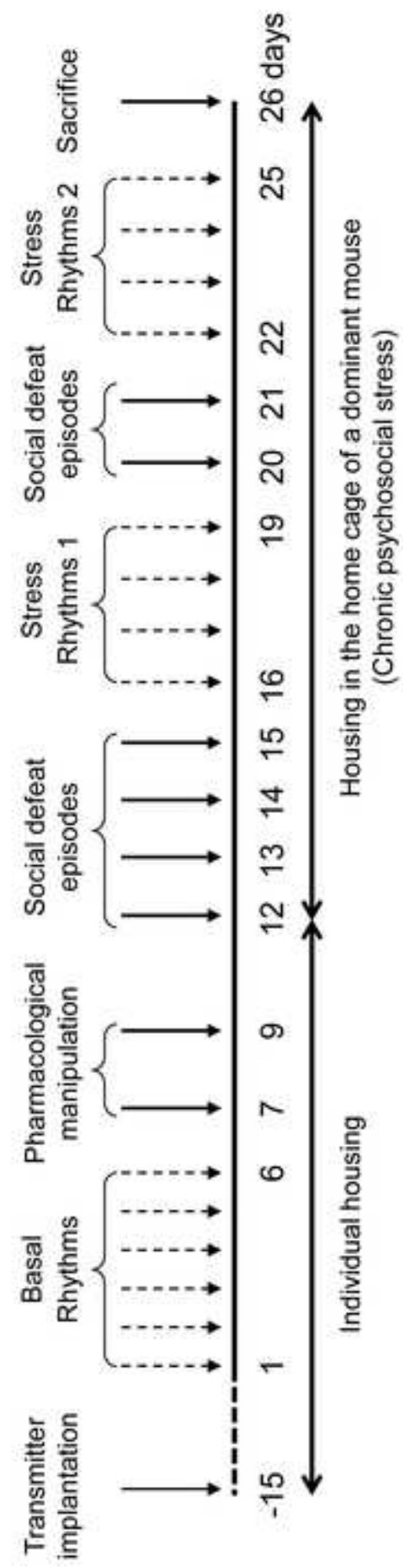


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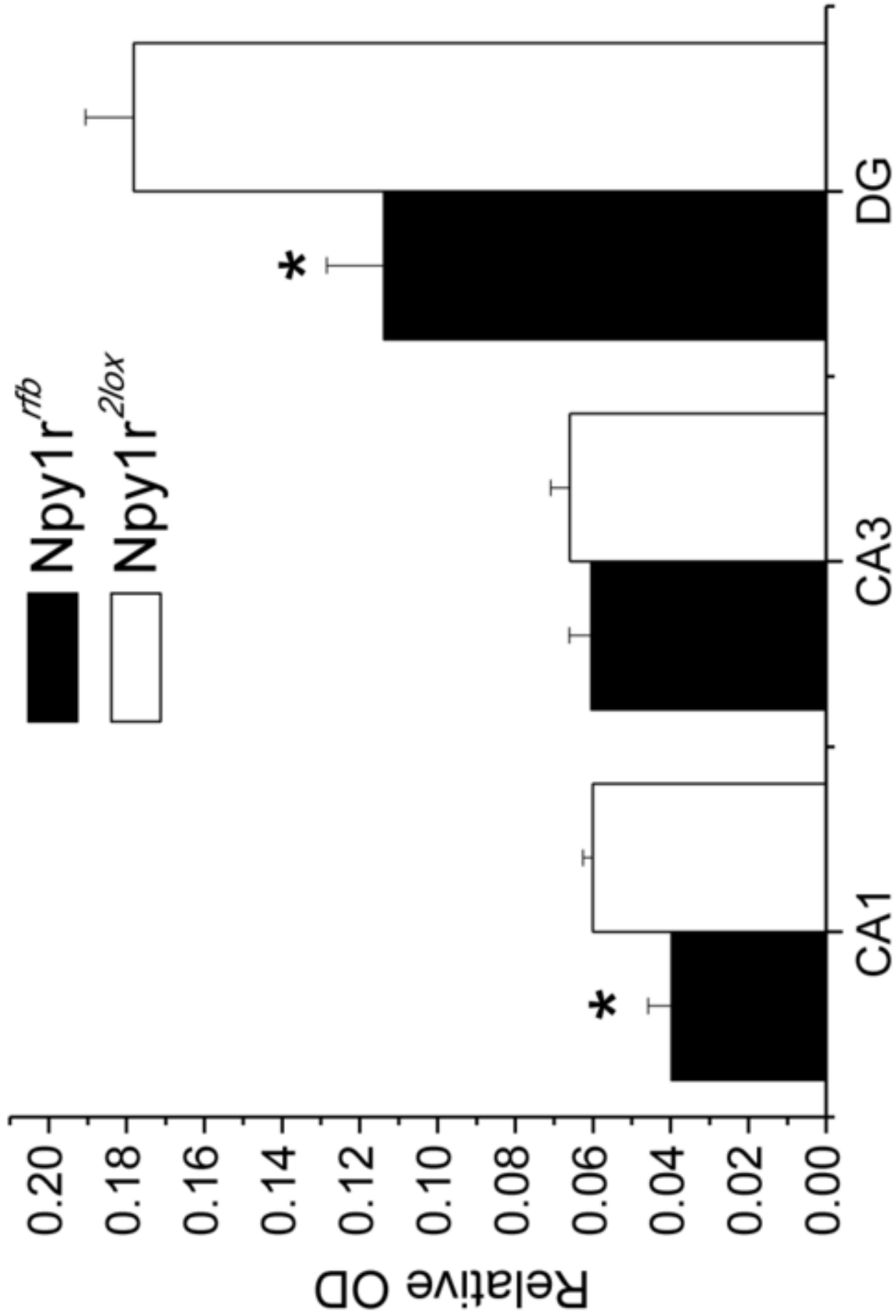


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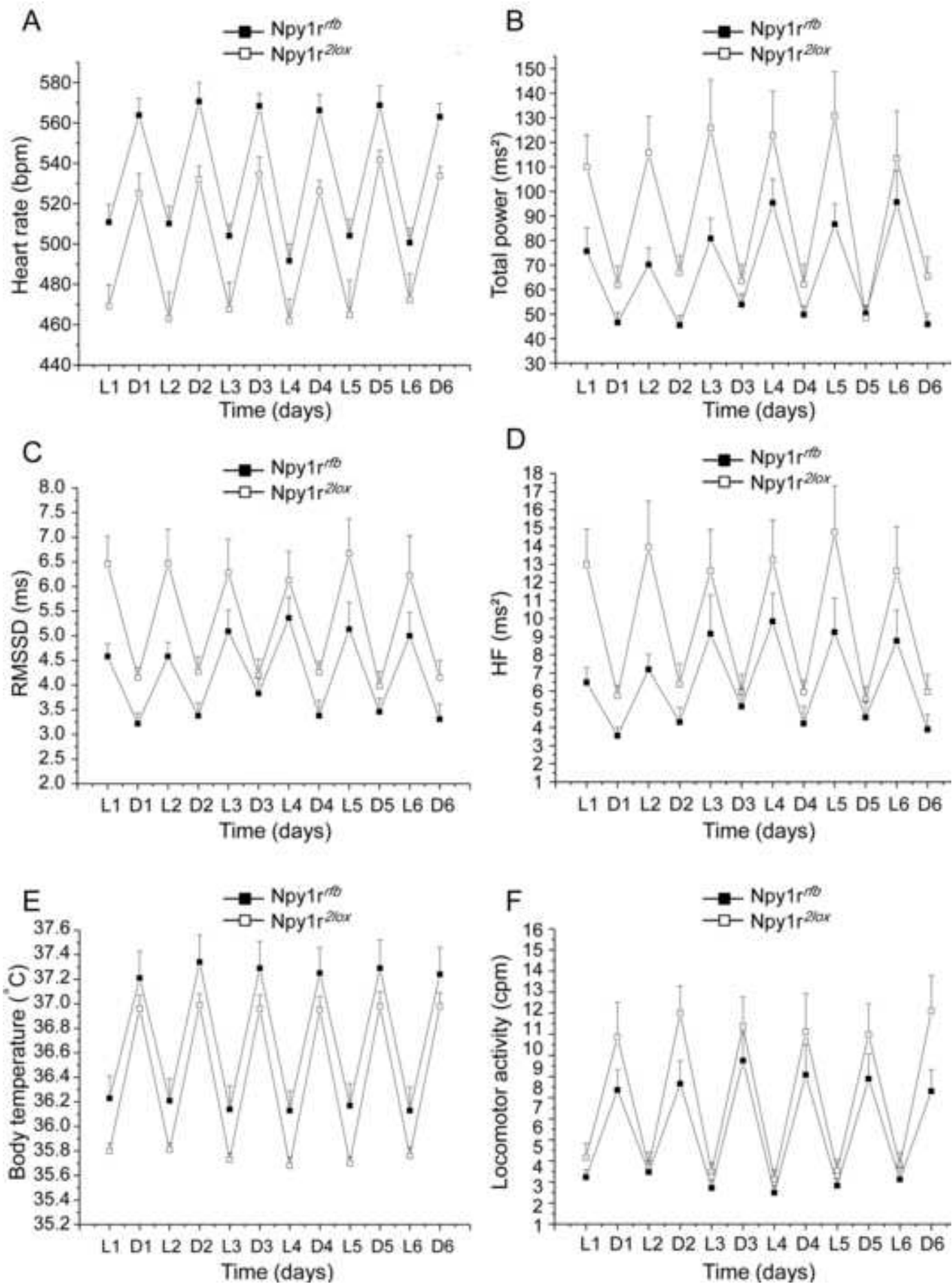


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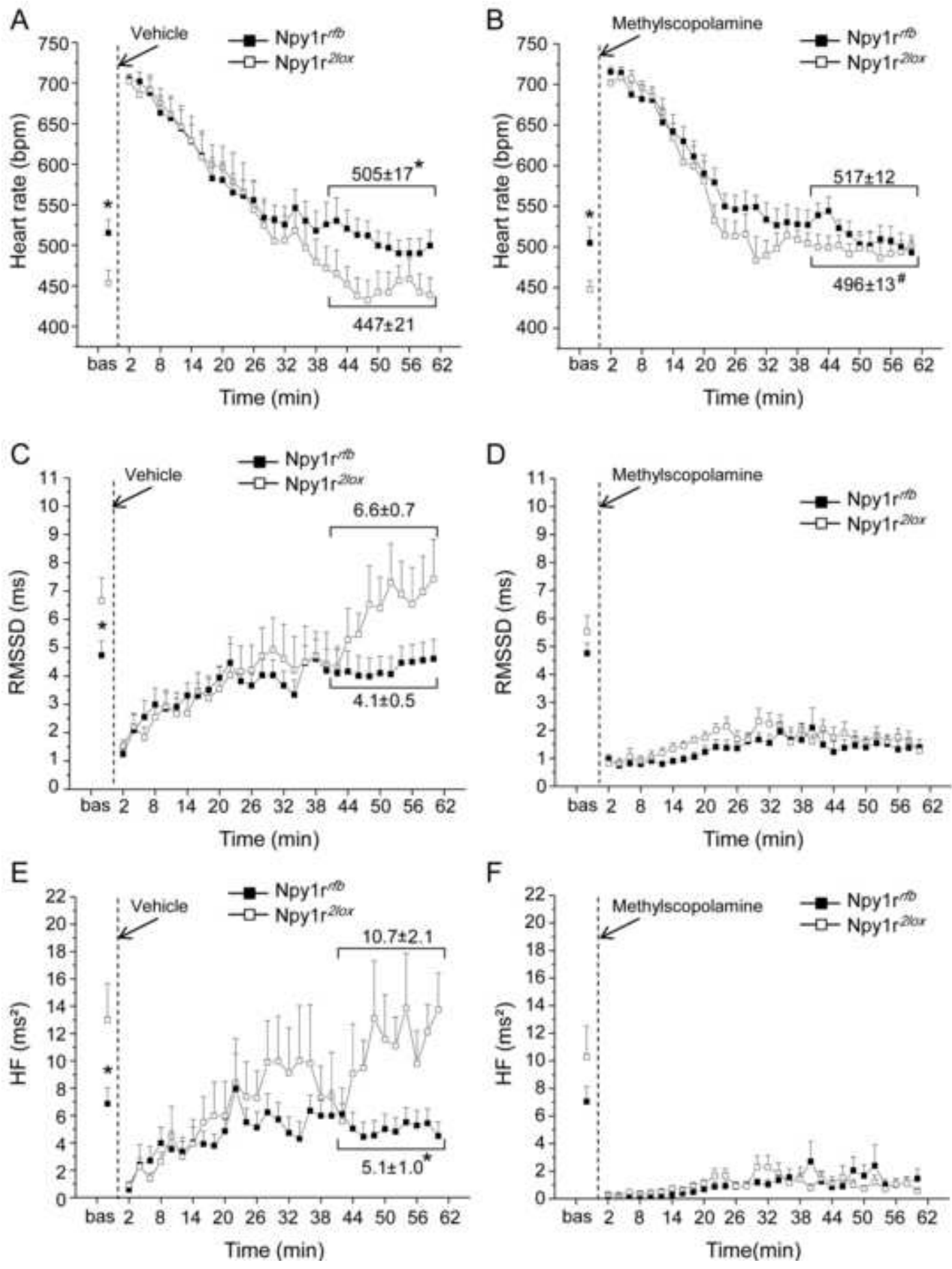


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