Effects of single cage housing on stress, cognitive and seizure parameters in the rat and mouse pilocarpine models of epilepsy

Keeping social contact in experimental epilepsy

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Conflict of Interest

The authors declare no conflicts of interest.

ABSTRACT

Many experimental approaches require housing rodents in individual cages, including in epilepsy research. However, rats and mice are social animals; and individual housing constitutes a stressful situation. The goal of the present study was to determine the effects of individual housing as compared to conditions maintaining social contact on stress markers and epilepsy. Control male mice socially housed during pretest and then transferred to individual cages for six weeks displayed anhedonia, increased anxiety and biological markers of stress as compared to pretest values or mice kept socially housed during six weeks. Pilocarpine-treated mice housed together showed increased levels of anhedonia, anxiety and stress markers as well as decreased cognitive performance as compared to the control group. The differences were more significant in pilocarpine-treated mice housed individually. Anxiety correlated linearly with cognitive performance and stress markers independently of the experimental conditions. In the male rat pilocarpine model, seizures were sixteen times more frequent in singly housed animals as compared to animals kept in pairs. Daily interactions with an experimenter in otherwise singly housed animals was sufficient to produce results identical to those found in animals kept in pairs. We propose that social isolation produces a severe phenotype in terms of stress and seizure frequency as compared to animals maintaining social contact (at least in these two models), a factor that needs to be taken into account for data interpretation, in particular for preclinical studies.

Significance Statement

Many experimental approaches require housing rodents in individual cages, a stressful condition for social animals, even in an enriched environment context. Using the pilocarpine model of epilepsy in rats and mice, we report that singly housing animals develop a more severe phenotype in terms of stress and epilepsy as compared to animals maintaining social contact.

We propose that social isolation adds a degree of complexity for the interpretation of data, which may be particularly relevant for preclinical studies.

KEYWORDS: Epilepsy, Stress, Social isolation, Single housing

Introduction

Social isolation (single housing) and barren environment are stressful to rodents; for a recent review cf. (Arakawa, 2018; Mumtaz et al., 2018). Social isolation of rodents after weaning is often used as an experimental manipulation to model early life stress in humans. It produces anxiety/depressive-like behavior and cognitive deficits and favors the emergence of pathological traits such as addictive behavior (Bianchi et al., 2006; Brenes et al., 2008; Ros-Simo and Valverde, 2012). These behavioral alterations are associated with biological modifications including oxidative stress, the production of stress hormones and inflammatory cytokines (Moller et al., 2013; Krugel et al., 2014; Shao et al., 2015; Butler et al., 2016). Social isolation performed in adult animals also results in behavioral and endocrinological alterations (Berry et al., 2012; Krugel et al., 2014; Ieraci et al., 2016; Filipovic et al., 2017). However, a general understanding of the consequences of social isolation is difficult to extract from these studies because they use different types of isolation, period and age of isolation, behavioral and molecular protocols, leading to conflicting reports (Arakawa, 2018). Since all studies report that social isolation does produce biological alterations, many regulation agencies recommend social housing and environmental enrichment to improve the welfare of rats and mice in animal facilities (Lidster et al., 2016). However, single housing of rodents may be imposed by the experimental protocol to obtain accurate measurements of biological parameters in individual animals, such as in the fields of toxicology, drug addiction, and neurological disorders. Experimental epilepsy is a typical example. In order to precisely assess seizures with continuous video-EEG recordings, instrumented animals are singly housed to prevent the severing of EEG wires and aggressive behavior from others and to clearly identify individuals

85 on the video. Several studies have shown that environmental enrichment has positive effects on
86 seizure frequency or severity in experimental models (Morelli et al., 2014; Kotloski and Sutula,
87 2015; Dezsi et al., 2016; Vrinda et al., 2017), but the effect of social isolation starting from the
88 beginning of the experimental procedure in the presence of environmental enrichment has not
89 been assessed.

90 There exist many types of experimental models of epilepsy in different rodent species
91 and strains (Levesque et al., 2016; Loscher, 2017; Becker, 2018). The effects of social isolation
92 should be studied for each model, as results may be model-, strain- and species-specific. For
93 example, Wistar and Sprague Dawley rats display different phenotypes in terms of depression-
94 like profile and cognitive deficits in the kainic acid and pilocarpine models (Inostroza et al.,
95 2011; Inostroza et al., 2012). Here we focus on the widely used pilocarpine model of
96 experimental epilepsy in adult male Swiss mice and Wistar rats (Levesque et al., 2016).
97 Numerous studies show a dysregulation of the HPA axis in patients and in experimental
98 epilepsy, which is model-dependent (Maguire and Salpekar, 2013; Wulsin et al., 2016; Mumtaz
99 et al., 2018). Since stress also results in the activation of the HPA axis, we reasoned that social
100 isolation in experimental models of epilepsy might produce a strong HPA axis response via the
101 contributions of both isolation- and epilepsy-induced stress (Gunn and Baram, 2017). Social
102 isolation during the neonatal or juvenile periods increases seizure susceptibility and produces a
103 behavioral phenotype (Lai et al., 2006; Amiri et al., 2017), but the effects of social isolation in
104 adult experimental models of epilepsy have not been investigated. The issue is important as
105 most mechanistic, pharmacological and interventional studies use recordings of singly housed
106 animals. We hypothesized that the combination of both isolation x epilepsy factors might
107 exacerbate the epilepsy phenotype as compared to animals maintaining social interactions.
108 Clinical studies support this hypothesis. Stressful life events are associated with an increased
109 risk of seizure occurrence in patients with epilepsy (Baldin et al., 2017; Kotwas et al., 2017).

110 In some patients, the fear to have a seizure and social isolation due to stigmatization may
111 increase the allostatic load, with a direct impact on seizure frequency (Kotwas et al., 2017). We
112 thus assessed the consequences of animal housing on stress response and epilepsy severity,
113 acting on a single experimental variable: social interaction (environmental enrichment was
114 provided). We also propose an alternate solution to maintain social contact in singly housed
115 animals, to decrease their stress level. We hope to foster discussions on how to interpret results
116 (including negative preclinical studies) obtained in different housing conditions.
117
118

119 **METHODS**

120 **Animals**

121 We used male Wistar rats (200 to 250g; Charles Rivers Laboratories, Les Oncins, France),
122 aged nine weeks (at their arrival in the laboratory). Swiss male mice (8 weeks old) were
123 obtained from the animal husbandry of the Faculty of Sciences, Cadi Ayyad University,
124 Marrakech (Morocco). The animals were kept under controlled environmental conditions (23±1
125 °C; night-day cycle (12 h-12 h)) with *ad libitum* access to food and water. Zeitgeber (ZT) 0 was
126 at 7:30 am (the time when the light was switched on in the animal facility). All procedures were
127 conducted per approved institutional protocols, and with the provisions for animal care and use
128 prescribed in the scientific procedures on living animals, European Council Directive:
129 EU2010/63 and by the Council Committee of Research Laboratories of the Faculty of Sciences,
130 Cadi Ayyad University, Marrakech. All efforts were made to minimize any animal suffering.
131

132 **Experimental design**

133 **In mice**

134 The experimental protocol is shown in Figure 1A. Swiss male mice were housed in groups
135 of 3 per cage. In order to validate stress in the same mice throughout the experimental protocol,

136 one mouse from each litter was randomly picked, ear-marked and returned to the same cage
137 with littermates. They were left together (group-housed) during one week without experimental
138 intervention to allow adaptation to the housing environment. After that, the ear-marked mouse
139 of each litter was subjected to different behavioral tests in order to obtain their pretest levels in
140 the social condition. Sucrose preference was assessed every day for one week. At the end of the
141 week, we evaluated anxiety levels (Day-3: elevated plus maze test – EPM) and memory
142 functions (Day -2-1: novel object recognition test -NOR). In this experimental protocol, we
143 consider two independent variables: housing (social and isolation) and treatment (control and
144 epilepsy), thus defining four groups of animals. After establishing pretest levels, cages were
145 randomly assigned to two groups to receive pilocarpine injection to trigger status epilepticus
146 (Pilo group– experimental procedure described hereafter) or saline (Non-pilo group). Both
147 groups were further divided into two groups: social condition (SC, n =18, 3/cage) and isolated
148 condition (IC, n = 6, 1/cage). For the isolated condition, ear-marked mice were taken away from
149 their home-cages and singly housed in cages identical to their home-cages. For the social
150 condition, the ear-marked mice remained in their home-cages with littermates. We refer to the
151 time following group assignment as the posttest period. Four weeks following the beginning of
152 the posttest period, mice already evaluated during the pretest period were again evaluated for
153 anhedonia (Day 28-35), EPM (Day 36) and NOR (37-38). Behavioral tasks (EPM and NOR)
154 were recorded and analyzed using Ethovision®XTNoldus 8.5 video tracking program (Noldus,
155 Netherlands) connected to a video camera (JVC). At the end of each behavioral session,
156 apparatuses were cleaned with a 75% ethanol solution to remove any of odor or trace. The
157 behavioral tests were performed between 8:00 and 12:00 a.m. during the light cycle to avoid
158 the circadian-related fluctuation in the performance of the mice. Before the beginning of
159 behavioral tests, the animals were transferred to the testing room in their home cages and left
160 there to habituate for 60 min.

At the end of the experimental protocol, we measured ACTH, CORT and BDNF levels as in rats (described below).

In rats

The experimental protocol is shown in Figure 7A. Animals were received in sets of 4 from the same litter from the vendor. Two main groups of animals were used: animals with spontaneous seizures following pilocarpine-induced status epilepticus (pilo group – experimental procedure described hereafter) and control animals (Non-pilo group). Pilo and Non-pilo animals were further divided into three groups:

- Isolated group: rats were singly housed and were not handled during the experimental period except for cage cleaning and body weight measurement once a week.
- Handled group: rats were singly housed and were handled daily until the end of the experiment (see below).
- Paired group: rats were kept in buddy pairs with social interaction upon arrival in the animal facility.

Handling was performed twice daily (ZT2 and ZT9) throughout the experimental period by the same experimenter. Briefly, each handling session consisted of stroking animals for 1 minute each in their cage. Then, each rat was gently handled by experimenter's hands (without wearing gloves), while being softly stroked from the head to the tail for 2 minutes. Finally, the rats were placed back in their home cage and fed by the experimenter for 2 minutes. Isolated and paired groups were left undisturbed, except for weekly cage cleaning and body weighing.

Animals from the Non-pilo group were randomized to the three housing groups when received. Animals from the Pilo group were randomized after pilocarpine-induced status epilepticus. Since we do not know the stress history of the animals when we receive them, randomization decreases this confounding factor.

186

187 **Sample size**

188 A preliminary analysis made on isolated (n=4) and handled (n=4) pilo groups for seizure
189 frequency allowed us to perform a power analysis. We determined that n=3 per group was
190 enough. The experimental procedure in the pilo groups was conducted twice. The first and
191 second group of simultaneously recorded animals being composed of (n=4 isolated, n=4
192 handled, n=6 paired) and (n=3 isolated, n=4 handled, n=6 paired), respectively. In the paired
193 groups, only one animal was instrumented. Seizure analysis was thus performed in 6 animals.
194 Results were similar in the two sets, and data were pooled together.

195 For the mice experiments, the number of subjects was defined based on the study design.
196 Preliminary analysis was conducted with n=3 for the social condition and n=3 for the isolated
197 condition where the results were non-significant. In this case, a second study was done with a
198 sample size of 3 per group.

199

200 **Status epilepticus induction and electrode implantation**

201 In rats, status epilepticus (SE) was induced by a single i.p injection of pilocarpine (Pilo)
202 320 mg/kg, one week after receiving the animals from the vendor. In mice, we used repeated
203 100 mg/kg i.p. injections every 20 min until SE onset 17 days after receiving the animals (one
204 week for habituation and ten days for pretest of behavioral tasks). To reduce peripheral effects,
205 animals were pre-treated with Methyl-scopolamine (1mg/kg) 30 min prior to Pilo injection. SE
206 was stopped by diazepam (10 mg/kg i.p., twice within a 15 min interval) after 60 min and 90
207 min of SE; respectively. At the end of these injections, mice and rats were hydrated with saline
208 (2ml i.p. twice within two h) and fed with a porridge made of soaked pellets, until they resumed
209 normal feeding behavior. All drugs were obtained from Sigma.

210 In rats, four weeks following SE, the telemetry implant was surgically inserted intraperitoneally
211 under anesthesia (ketamine [1 mg/ kg]/xylasine [0.5 mg/kg] i.p) and connected to screws on the
212 surface of the brain by two electrodes; one above the cortex (4.0 mm anteroposterior, 2.0 mm
213 mediolaterally, compared with bregma), the second, above the cerebellum as reference. The
214 EEG signal was transmitted by radio to a data acquisition and processing system (DSI). In the
215 paired group, both animals developed epilepsy, but only one rat was equipped with the
216 telemetry system as two animals cannot be recorded simultaneously with EEG transmitters in
217 the same cage, while the other was monitored with video only. Animals were left to recover
218 during one week before switching on the transmitter.
219
220 **Monitoring of spontaneous recurrent seizures**
221 Continuous (24/7) EEG recordings started in the 6th week after SE, a period sufficient to reach
222 stability in seizure frequency (Williams et al., 2009), and were stopped at week 10. We verified
223 that animals displayed stable seizure activity, quantifying seizure frequency during each
224 successive week. Spontaneous recurrent seizures were detected and quantified using both visual
225 inspections of the EEG and a semi-automatic way using Clampfit 10.2. All detected seizures
226 were verified and reconfirmed using "NeuroScore" software. We used the following spike
227 detection parameters: 300 µV amplitude threshold with a rejection value of 4 mV, 1 ms
228 minimum duration, 300 ms maximum duration, 1 ms minimum spike interval, 1 s maximum
229 spike interval, 2 s minimum spike train duration, 10 minimum spike number. All detected
230 events were then visually inspected. These conservative parameters led to the detection of false
231 positives, which were removed manually. In five animals, the entire EEG recording was
232 visually inspected to confirm that no seizures escaped the semi-automatic detection (no false
233 negative). Video recordings were performed during the light phase in the animal facility with
234 the DSI system. They were used to assess seizure severity according to Racine's scale after

235 their electrophysiological detection. Rats kept in pairs never had spontaneous seizures
236 simultaneously, which allowed a correct assessment of seizure severity of the EEG monitored
237 animal. Finally, keeping animals in pairs did not prevent/alter continuous EEG recordings in
238 the equipped animals (no loss of signal).
239
240 **Behavioral and biological parameters**
241 Body weights of mice and rats were measured weekly at ZT 2.
242
243 **Sucrose consumption test**
244 In mice, the baseline for sucrose consumption was assessed every day for one week, seven
245 days after their arrival in the laboratory. The sucrose preference was calculated at the 5th week
246 following the separation and treatment. In rats, sucrose consumption was assessed at week four
247 and week eight following animal reception. Sucrose and water intakes were measured daily at
248 ZT 2.
249 Briefly, animals were given a free choice between two bottles, one with 1% sucrose
250 solution and another with tap water. The location of the bottles was alternated every day to
251 prevent possible effects of side preference in drinking behavior. The consumption of water and
252 sucrose solution was estimated by weighting the bottles. For the paired, the volume of sweet
253 water consumed by rat was taken as the total consumed volume divided by 2. For the social
254 groups, the volume of sweet water consumed by mouse was taken as the total consumed volume
255 divided by the total number of animals at each time. Sweet water consumption corresponds to
256 sucrose preference, which is calculated as a percentage of the volume of sucrose intake over
257 the total volume of fluid intake using the following equation:
258 Sucrose preference= V (sucrose solution)/ (V (sucrose solution) +V (water)) X100%.
259

260 **Elevated plus maze test**

261 The elevated plus maze has been described as a simple method for assessing anxiety-like
262 behavior in rodents (Pellow et al., 1985; Lapiz-Bluhm et al., 2008). The elevated plus maze
263 consisted of four arms (two open without walls and two enclosed arms by 15 cm high walls) 50
264 cm long and 5 cm wide, which were joined at a square central area (5 × 5 cm) to form a plus
265 sign. The maze floor and the side/end walls of the enclosed arms were made of clear Plexiglas.
266 The room illumination of the elevated plus maze apparatus was under an approximate
267 brightness of 200 lux. Briefly, the test consisted of placing the mouse gently in the central arena
268 of the elevated plus maze, facing the junction of an open and closed arm. The mouse was
269 allowed to freely explore the maze for 5 min while the duration and frequency of entries into
270 open arms were recorded. Anxiety index (Rao and Sadananda, 2016) was calculated as: 1 -
271 ((open-arm time/total time) + (open-arm entries/total entries))/2.

272

273 **Novel object recognition test**

274 The novel object recognition test is widely used to evaluate object recognition memory in
275 rodents (Ennaceur and Aggleton, 1994; Reger et al., 2009; Gaskin et al., 2010). The apparatus
276 consisted of an open field (50× 50 × 50 cm high) made of Plexiglas with the inside painted matt
277 black. The objects to be discriminated were available as three plastic objects. 24h before testing,
278 mice were first habituated in the open field arena in the absence of any object for 10 min. During
279 the training trial, two identical objects (approximately 10 cm) were placed in the back corner
280 of the box. The mouse was then positioned at the midpoint of the wall opposite to the objects
281 and the total time spent exploring the two objects was recorded for 10 min. During the test trial
282 (60 min after training trial), one object used during training was replaced with a novel object
283 and both of them were placed in the middle of the back wall. The animal was then allowed to
284 explore freely for 5 min and the time spent exploring each object was recorded. The

285 discrimination between the novel and familiar objects was calculated by the discrimination
286 index [(time spent exploring the novel object - time spent exploring the familial object) / total
287 time x 100)].
288
289 **ACTH, corticosterone and BDNF levels**
290 Isolated, handled and paired rats in the Non-pilo group were killed by decapitation after light
291 anesthesia with isoflurane at the beginning of the 8th week following the separation at ZT4 and
292 the 6th weeks after the separation and treatment at ZT4 for Pilo and Non-pilo mice. Animals
293 were decapitated in a quiet separate room, one by one, with the bench cleaned between animals.
294 Trunk blood was collected in less than 5 sec after decapitation in a dry tube and an EDTA tube
295 in order to obtain serum and plasma samples, respectively. The plasma was prepared by a 15
296 min centrifugation at 1600 g, 4°C. The ACTH concentration was determined according to the
297 manufacturer's instructions (Clinisciences, France). For corticosterone and BDNF levels, blood
298 was centrifuged at 3500 g for 10 min at 4°C, and the serum was stored at 80°C until used.
299 Corticosterone and BDNF concentrations were determined according to the manufacturer's
300 instructions (Coger, Promega, France).
301
302 **Statistical analysis**
303 The effect on body weight and anhedonia was measured using the ANOVA with repeated
304 measures followed by Bonferroni *post-hoc* analysis using Sigma Plot 11.0 software.
305 For estimation based on confidence intervals, we directly introduced the raw data in
306 https://www.estimationstats.com/ and downloaded the results and graphs. The mean difference
307 for two comparisons is shown with Cumming estimation plot. The raw data is plotted on the
308 upper axes. For each group, summary measurements (mean ± standard deviation) are shown as
309 gapped lines. Each mean difference is plotted on the lower axes as a bootstrap sampling

distribution. Five thousand bootstrap samples were taken; the confidence interval was bias-corrected and accelerated. Mean differences are depicted as dots; 95% confidence intervals are indicated by the ends of the vertical error bars. In order to measure the effect size, we used unbiased *Cohen's d* (also known as standardized mean difference). We also provide *P* value(s) the likelihood(s) of observing the effect size(s), *if the null hypothesis of zero difference is true*, using the test mentioned in the text.

Data sharing

All raw data is stored on our NAS server and available upon request.

320
321 **RESULTS**

322 **Effect of social isolation on weight gain and anhedonia in control and pilo mice**

323 Repeated measures ANOVA showed a significant effect of time ($F_{(5,100)}$=128.08,
324 p<0.0001[a]) on body weight evolution; while the experimental group ($F_{(3,20)}$ =2.23, p=0.11[a]) and
325 the interaction of group and age ($F_{(15,100)}$ =1.18, p=0.29[a]) had no significant effect (**Figure 1B**).
326 *Post-hoc* analysis showed that the Pilo-IC group gained significantly less weight than the Non-
327 pilo-SC group (p<0.05) at weeks four and five. There was no significant difference between all
328 groups in body weight before social isolation until the 3rd week of isolation.

329 We used the sucrose preference test to assess anhedonia (lack of interest in rewarding
330 stimuli). The typical behavior of animals is a bias toward the sweetened drink. Lack of
331 preference for the sweetened drink indicates anhedonia, which signs stress-related disorders
332 (Gold, 2015). The ANOVA repeated measures showed an effect of group on sweet water
333 consumption during the pretest period ($F_{(3,20)}$=8.33, p<0.001[b]) and the posttest period
334 ($F_{(3,20)}$=295.85, p<0.0001[c]) with no time effect in both periods (pretest: $F_{(6,120)}$=0.20, p=0.97[b]
335 and posttest: $F_{(6,120)}$=0.13, p=0.99[c]) (**Figure 1C**). During the pretest period, the *post-hoc*
336 analysis did not show a significant difference in sucrose preference between the different
337 groups.

338 During the posttest period, the *post-hoc* analysis revealed that sweet water consumption
339 was significantly lower by 15% and 20% in the Pilo-SC and Pilo-IC groups than in the Non-
340 pilo-SC and Non-pilo-IC groups (p<0.001). Both Non-pilo-IC and Pilo-IC groups had a
341 significant decrease in sucrose preference as compared to Non-pilo-SC (p<0.05, p<0.01 and
342 p<0.001) and Pilo-SC (p<0.05 and p<0.01) groups, respectively (**Figure 1C**). Therefore, social
343 isolation induces anhedonia in the control (Non-pilo) group. The Pilo group socially housed
344 also displayed anhedonia as compared to the control group. The anhedonia phenotype was
345 further increased in the socially isolated Pilo group.

346 In the following, we continue to use statistical inference analysis (null hypothesis
347 significance testing) with *P* values, but we also include estimation based on confidence intervals
348 (CI), which provides better insight into the structure and interpretation of the results. An
349 estimation based on confidence intervals would have been too cumbersome for the weight and
350 anhedonia data.
351
352 **Social isolation increases anxiety levels in control and pilo mice**
353 We then tested animals for anxiety-like behavior. All groups were tested before
354 experimental manipulation (pretest) and again afterward (posttest). As expected, anxiety levels
355 at pretest were very similar across groups, with each group mean within 3% of the others
356 (**Figure 2A**, compare Pre scores across groups). Thus, the random assignment did not induce a
357 bias. There was notable diversity, though, in pretest scores, with some animals scoring
358 relatively low (0.3) and others relatively high (0.45). These differences, however, were not
359 clearly predictive of posttest scores (r = 0.04 95% CI[-0.37,0 .43], p = 0.87).
360 For animals maintaining social housing during the posttest period, there was no average
361 change in anxiety index scores (Mean difference score (M_{diff}) = 0.00 95% CI[-0.08, 0.09], d =
362 0.0 95% CI[-1.2, 1.3], p = 0.91 for Wilcoxon test, Column 1 of **Figure 2A-B**). Note that the
363 confidence interval is long, so moderate effects in either direction cannot be ruled out (two
364 animals showed increased anxiety levels and four decreased levels). Pilo treatment for socially
365 housed mice produced a large increase in anxiety scores (M_{diff} = 0.19 95% CI[0.12, 0.24], d =
366 3.2 95% CI[1.6, 5.5], p = 0.03 for Wilcoxon test, Column 2 of **Figure 2A-B**).
367 We then compared the magnitudes of the changes induced by housing and treatment
368 (**Figure 2C**, left). We found a substantial effect of Pilo treatment: $M_{\text{diff/diff}}$ = 0.19 95% CI[0.05,
369 0.30], d = 1.6 95% CI[0.4, 4.3], p = 0.07 for Mann-Whitney test. Note that there is considerable
370 uncertainty about the magnitude of the Pilo effect under this condition, with the confidence

371 interval stretching down towards negligible effects. This uncertainty is due to high variation in
372 change scores in the standard social housing mice; the overlap in the change scores of SC-alone
373 vs. SC+Pilo pushing up the *p*-value just above the traditional significance level.

374 We then investigated the effects of social isolation and epilepsy. Consistent with the
375 literature on social deprivation, isolated housing produced a marked increase in anxiety index
376 scores, with all animals increasing from pretest to posttest (M_{diff} = 0.11 95% CI[0.06, 0.19], *d*
377 = 1.8 95% CI[1.1, 2.8], *p* = 0.03 for Wilcoxon test, Column 3 of **Figure 2A-B**). Pilo treatment
378 for isolated mice produced an even larger increase in anxiety scores (M_{diff} = 0.33 95% CI[0.27,
379 0.40], *d* = 5.0 95% CI[4.0, 6.0], *p* = 0.03 for Wilcoxon test, Column 4 of **Figure 2A-B**).
380 Comparing these changes (**Figure 2C**, right) shows that Pilo treatment produces a large change
381 above and beyond the effect of isolated housing: $M_{\text{diff/diff}}$ = 0.21 95% CI[0.13, 0.28], *d* = 3.0
382 95% CI[1.5, 4.8], *p* = 0.005 for Mann-Whitney test. The confidence interval suggests that all
383 compatible effect sizes are large.

384 The estimated effects of Pilo treatment were similar for both isolated ($M_{\text{diff/diff}}$ = 0.21,
385 95% CI[0.13, 0.28]) and socially housed animals ($M_{\text{diff/diff}}$ = 0.19, 95% CI[0.05, 0.30]). Thus,
386 the data do not provide strong evidence that housing condition modifies the effect of Pilo
387 treatment, and a formal test for an interaction was not significant ($F(1,20) = 0.11$, $p = 0.74$ for
388 a Treatment x Housing interaction in a non-parametric ANOVA performed on change scores).
389 This means that "epilepsy" increases anxiety levels independently from the housing conditions
390 and that the social isolation-induced increase in anxiety level adds to the "epilepsy"-induced
391 one, resulting in highest anxiety levels in the Pilo-IC group as compared to all other groups.
392

393 **Effects of housing and epilepsy in the Novel Object Recognition test**

394 Next, we assessed the effect of social isolation on memory performance using the NOR
395 test. As for anxiety levels, cognitive performance was similar at pretest across groups, with

396 each group mean within 6% of the others (**Figure 3A**, compare Pre scores across groups). There
397 was notable diversity, though, in pretest scores, with some animals scoring relatively low (50%)
398 and others relatively high (90%). These differences, however, were not clearly predictive of
399 posttest scores (r = -0.297 95% CI[-0.626, 0.121], p = 0.16).

400 For animals maintaining social housing during the posttest period, there was no average
401 change in dicrimination index scores (Mean difference score (M_{diff}) = -1.29% 95% CI[-16.4,
402 13.5], d = -0.08 95% CI[-1.71, 1.05], p = 0.92 for Wilcoxon test, Column 1 of **Figure 3A-B**).
403 Note that the confidence interval is long, so moderate effects in either direction cannot be ruled
404 out (two animals showed similar performance, two increased and two decreased). Pilo treatment
405 for socially housed mice produced a large decrease in performance in the NOR test (M_{diff} = -
406 49% 95% CI[-59, -38], d = -4.69 95% CI[-6.65, -3.03], p = 0.03 for Wilcoxon test, Column 2
407 of **Figure 3A-B**).

408 We then compared the magnitudes of the changes induced by housing and treatment
409 (**Figure 3C**, left). We found a large effect of Pilo treatment: $M_{\text{diff/diff}}$ = -47% 95% CI[-65, -27],
410 d = -2.6 95% CI[-4.4, -1.3], p = 0.008 for Mann-Whitney test.

411 We then investigated the effects of social isolation and epilepsy. Isolated housing
412 produced a modest decrease in performance (M_{diff} = -11% 95% CI[-23, 9], d = -0.8 95% CI[-
413 2.9, 0.8], p = 0.3 for Wilcoxon test, Column 3 of **Figure 3A-B**). Note that there is considerable
414 uncertainty about the magnitude of the isolation effect, with the confidence interval stretching
415 from positive no negative values. This uncertainty is due to one animal, which performance
416 dramatically increased once isolated, pushing up the p value above the traditional significance
417 level. If we do not take this animal in consideration, the decrease in performance was large
418 (M_{diff} = 21.1% 95.0% CI[-29.4, -12.3], d = -2.75 95.0% CI[-6.11, -2.02], p = 0.043). Replication
419 studies are now required to determine whether most control animals perform less once isolated
420 whilst others show an increase of performance (e.g. the animal suffered/was dominated when

421 socially housed). However, we can reasonably propose that social isolation decreased
422 performance in the NOR test is most control mice. Pilo treatment for isolated mice produced a
423 very large decrease in performance (M_{diff} = -49% 95% CI[-68, -32], d = -2.8 95% CI[-3.7, -2.0],
424 p = 0.02 for Wilcoxon test, Column 4 of **Figure 3A-B**). Comparing these changes (**Figure 3C**,
425 right) shows that Pilo treatment produces a robust change above and beyond the effect of
426 isolated housing: $M_{diff/diff}$ = -38% 95% CI[-71, -12], d = -1.3 95% CI[-2.4, -0.2], p = 0.044 for
427 Mann-Whitney test. The confidence interval suggests that all compatible effect sizes are large.
428 The estimated effects of Pilo treatment were similar for both isolated ($M_{diff/diff}$ = -38%,
429 95% CI[-74, -1]) and socially housed animals ($M_{diff/diff}$ = -48%, 95% CI[-71, -24]). The large
430 confidence interval for isolated animals is due to the Non-pilo animal showing increased
431 performance after isolation. Thus, the data do not provide strong evidence that housing
432 condition modifies the effect of Pilo treatment, and a formal test for an interaction was not
433 significant ($F(1,20) = 0.28$, $p = 0.6$ for a Treatment x Housing interaction in a non-parametric
434 ANOVA performed on change scores). This means that "epilepsy" decreases cognitive
435 performance in the NOR test independently from the housing conditions, but that the social
436 isolation-induced decrease in performance adds to the "epilepsy"-induced one, resulting in poor
437 performance in the Pilo-IC group as compared to all other groups.
438 We conclude that housing conditions affect cognitive performance in the NOR test in
439 most individuals (although not for the group) and that epilepsy is associated with similar
440 substantial cognitive deficits in the NOR test irrespective of housing conditions.
441
442 **Anxiety and novel object recognition performance co-vary independently of housing**
443 **and treatment conditions**

444 Anxiety correlates with poor performance in a variety of cognitive tasks (Moran, 2016).
445 Given the wide distribution of data points for the two behavioral tests used here, we tested for
446 a correlation between the two variables. We found a strong linear relationship between
447 anxiety levels and the discrimination index, $r = -0.858$ 95% CI[-0.918, -0.758], $p < 0.0001$,
448 (**Figure 4A**). In pretest, all animals were distributed around the regression line, spanning
449 various levels of anxiety/discrimination values, reflecting the individual differences between
450 mice in pretest condition. In posttest conditions, mice that remained socially housed were
451 distributed within the pretest values. Social isolation in the control (Non-pilo) group did not
452 change the anxiety/discrimination relationship but shifted the points towards higher
453 anxiety/lower performance. Pilo treatment in socially housed mice resulted in a further shift
454 while combining both Pilo treatment and isolation produced the maximum shift. Thus, the
455 relationship between anxiety/discrimination values remained constant independently of the
456 experimental conditions, i.e. there is a strong and stable rule of covariance between anxiety
457 and discrimination levels. The experimental conditions appear only to change the position
458 along the regression line.
459
460 **Social isolation and epilepsy increase corticosterone and ACTH levels**
461 We then assessed biological markers obtained at the end of the experimental protocol
462 (**Figure 1A**). Using treatment as reference (**Figure 5A**, right panel), the unpaired mean
463 difference between Pilo-SC and Non-pilo-SC corticosterone levels was 40.7 ng/mL [95.0%CI
464 27.2, 51.5] and the unpaired mean difference between Pilo-IC and Non-pilo-IC was 41.3 ng/mL
465 [95.0%CI 34.6, 47.2], corresponding to an increase of corticosterone by 55% and 91%,
466 respectively. In both conditions, the two-sided P value of the Mann-Whitney test was 0.00507.
467 The unbiased Cohen's d was 3.228 and 6.204 for the SC and IC groups, respectively. The
468 difference of differences (**Figure 6A**) was 0.595 ng/mL [95.0%CI -15.202, 16.392], indicating

469 no significant interaction between treatment and housing. Using social housing as reference
470 (**Figure 5A**, left panel), the unpaired mean difference between Non-pilo-SC and Non-pilo-IC
471 corticosterone levels was -16.0 ng/mL [95.0%CI -23.5, -4.43] and the unpaired mean difference
472 between Pilo-SC and Pilo-IC corticosterone levels was -16.6 ng/mL [95.0%CI -26.4, -6.68],
473 corresponding to an increase in corticosterone level in the isolated group by 59% and 95%,
474 respectively. In both conditions, the two-sided *P* value of the Mann-Whitney test was 0.0306.
475 The unbiased Cohen's *d* was 1.656 and 1.591 for the Non-pilo and Pilo groups, respectively.
476 The effect of isolation had similar effects in both control and pilo groups (1.6 standard
477 deviation). We conclude that both epilepsy and social isolation produce a significant increase
478 in corticosterone levels, the most substantial effect occurring in the Pilo-IC group.

479 For ACTH, using Non-pilo treatment as reference (**Figure 5B**, right panel), the unpaired
480 mean difference between Pilo-SC and Non-pilo-SC ACTH levels was 78.9 pg/mL [95.0%CI
481 48.9, 96.3] and the unpaired mean difference between Pilo-IC and Non-pilo-IC was 84.8 pg/mL
482 [95.0%CI 63.0, 1.14e+02], corresponding to an increase in ACTH level in the Pilo group by
483 37% and 33%, respectively. The two-sided *P* values of the Mann-Whitney test were 0.00824
484 and 0.00507, respectively. The unbiased Cohen's *d* was 3.336 and 3.189 for the SC and IC
485 groups, respectively, suggesting that epilepsy had strong but similar effects (3 standard
486 deviation) in both socially housed and isolated mice. The difference of differences (**Figure 6B**)
487 was 5.914 pg/mL [95.0%CI -33.628, 45.457], indicating no significant interaction between
488 treatment and housing. Using social housing as reference (**Figure 5B**, left panel), the unpaired
489 mean difference between Non-pilo-SC and Non-pilo-IC ACTH levels was -40.0 pg/mL
490 [95.0%CI -60.8, -12.4] and the unpaired mean difference between Pilo-SC and Pilo-IC ACTH
491 levels was -45.9 pg/mL [95.0%CI -77.7, -27.0], corresponding to an increase in ACTH level in
492 the isolated group by 21% and 17%, respectively. The two-sided *P* values of the Mann-Whitney
493 test were 0.0202 and 0.00507, respectively. The unbiased Cohen's *d* was 1.596 and 1.817 for

494 the Non-pilo and Pilo groups, respectively. The effect of isolation had similar effects in both
495 control and Pilo groups (1.6 standard deviation). We conclude that both epilepsy and social
496 isolation produce a significant increase in ACTH levels, the largest effect occurring in the Pilo-
497 IC group.

498 Since ACTH and corticosterone levels are stress markers, not surprisingly, we found a
499 strong linear relationship between anxiety levels and corticosterone $r = 0.936$ 95% CI[0.856,
500 0.972], $p < 0.0001$ (**Figure 4B**) as well as for ACTH $r = 0.978$ 95% CI[0.948, 0.99], $p < 0.0001$
501 (**Figure 4C**) levels. We note again the strong co-variance of anxiety level with
502 corticosterone/ACTH levels. The distribution of points along the regression line reflects the
503 variability of individuals across housing/treatment conditions. The housing/treatment
504 conditions do not impact upon the co-variance rule itself. As for the discrimination index
505 (**Figure 4A**), the conditions merely shift the distribution of points toward higher values as a
506 function of the severity of the effect, the Pilo isolated group displaying the greater anxiety-
507 corticosterone-ACTH levels, and the control Non-pilo social the lowest levels.

508

509 **Social isolation decreases serum BDNF levels in control and pilo mice**

510 Strong stressful situations can trigger a state of vulnerability to depression in some
511 individuals, which can be identified with low serum BDNF levels (Blugeot et al., 2011; Becker
512 et al., 2015). We thus assessed serum BDNF in the different groups. Using Non-pilo treatment
513 as reference (**Figure 5C**, right panel), the unpaired mean difference between Pilo-SC and Non-
514 pilo-SC BDNF levels was 7.76 pg/mL [95.0%CI -17.5, 33.5] and the unpaired mean difference
515 between Pilo-IC and Non-pilo-IC was 7.45 pg/mL [95.0%CI -23.5, 37.3]. The two-
516 sided P values of the Mann-Whitney test were 0.378. The unbiased Cohen's d was 0.293 and
517 0.232 for the SC and IC groups, respectively. These results suggest that epilepsy does not affect
518 significantly serum BDNF levels in mice in both housing conditions. Using social housing as

519 reference (**Figure 5C**, left panel), the unpaired mean difference between Non-pilo-SC and Non-
520 pilo-IC BDNF levels was 45.5 pg/mL [95.0%CI 13.0, 69.9] and the unpaired mean difference
521 between Pilo-SC and Pilo-IC was 45.9 pg/mL [95.0%CI 13.4, 71.3], corresponding to a
522 decrease in the isolated group by 57% and 51%, respectively. The two-sided *P* values of
523 the Mann-Whitney test were 0.0306 for Non-pilo and Pilo groups. The unbiased Cohen's *d* was
524 -1.577 and -1.527 for the Non-pilo and Pilo groups, respectively. The effect of isolation had
525 similar effects in both control and Pilo groups (1.5 standard deviation). The difference of
526 differences (**Figure 6C**) was -0.312 pg/mL [95.0%CI -46.608, 45.983], indicating no
527 significant interaction between treatment and housing. Thus, housing conditions has strong
528 effects on serum BDNF levels in both Pilo and Non-pilo groups.

529 In contrast with the condition-independent co-variance rules found previously for
530 corticosterone and ACTH, individual BDNF data show a more complex relationship. In both
531 control (Non-pilo) and epilepsy (Pilo) conditions, we found a strong linear relationship between
532 anxiety levels and serum BDNF levels, the housing condition just shifting the distribution of
533 points, $r = -0.923$ 95% CI[-0.948, -0.7439], $p < 0.0001$ and $r = -0.882$ 95% CI[-0.967, -0.625],
534 $p < 0.0001$ for Non-pilo and Pilo groups, respectively (**Figure 6D**). However, the epilepsy
535 condition clearly shifts the relationship to the right of the graph, i.e. as compared to the control
536 condition, a similar level of serum BDNF level is associated with a higher level of anxiety in
537 Pilo mice. Said differently, the epilepsy condition changes the setpoint (the *y* intercept of the
538 linear regression function) of the relationship between anxiety and serum BDNF levels, without
539 an apparent change of the slope (the co-variance rule).

540

541 Together, the results obtained in control (Non-pilo) mice demonstrate that social isolation
542 has a strong effect on many behavioral and biological stress indicators. They recapitulate the
543 main features of social isolation performed after weaning to model early life stress (Mumtaz et

544 al., 2018). In animals maintained in groups, epilepsy had a direct effect on stress indicators.
545 These effects were exacerbated in singly housed Pilo mice. We conclude that social isolation
546 produces a stronger phenotype (for the tests used here) in the Pilo mouse experimental model
547 of epilepsy. The mouse study was designed to evaluate two independent variables (housing and
548 epilepsy) on several parameters relevant to stress and cognitive performance. A limitation of
549 this study is that we did not evaluate spontaneous seizures with video/EEG. For this, we would
550 have had to double the number of animals to evaluate the two independent variables. Indeed,
551 the surgery, the implantation of an invasive device, and the treatment (anesthesia, anti-
552 inflammatory) may by themselves change the animal's biology and thus change the
553 relationships between anxiety and cognitive performance, etc. requiring a study design
554 including animals equipped with EEG transmitters and not-equipped animals. Future studies
555 should address this issue. However, we directly investigated the effect of social conditions on
556 seizures in the pilocarpine rat model.
557
558 **Effect of social isolation in control rats**
559 Since we could not perform a full two-condition study like in mice, we first wanted to
560 determine whether the general effects of isolation in control animals were similar in the two
561 species, simplifying the experimental protocol in control rats (Figure 7A). We compared three
562 groups of rats: singly housed animals (Isolated), animals kept in pairs (Paired) and isolated
563 animals interacting with the experimentor every day (Handled). The rationale for designing
564 these three groups is the constraints imposed by the experimental part done in the pilocarpine
565 model of epilpsy (see below). Because of their larger size, it was not technically possible to
566 maintain rats in small colonies while performing wireless 24/7 recordings. For the social group,
567 animals were kept in pairs. The justification of the Handled group is that some EEG studies are

568 performed with wired systems, which precludes group housing, as animals tend to sever the
569 wires.

570 We first tested the effect of isolation in control rats. The ANOVA repeated measures
571 showed a significant effect of social condition ($F_{(2,17)}$=41.58, p<0.0001[d]) and time ($F_{(6,102)}$
572 =266.83, p<0.0001[d]) on the gain of body weight (**Figure 7B**). At the 4th week, *post-hoc* analysis
573 showed that the Isolated group gained significantly less weight (by 7%) than the Handled and
574 the Paired group. There was no significant difference between all groups from the 1st to the 3rd
575 week.

576 We used the sucrose preference test to assess anhedonia. ANOVA repeated measures
577 showed a main effect of social isolation on sweet water consumption (**Figure 7C**) already
578 during the third week ($F_{(2,12)}$=21.66, p<0.0001[e]) up to the last week ($F_{(2,12)}$=97.85, p<0.0001[f])
579 with no time effect in both weeks (first week: $F_{(6,72)}$ =0.11, p>0.05 and last week: $F_{(6,72)}$ = 1.09,
580 p>0.05 ; respectively [e,f]). During the third and the last week, *the post-hoc* analysis revealed that
581 sweet water consumption was significantly lower (by 5-10%) in the Isolated than in the Handled
582 (p<0.01, p<0.001) and Paired groups (p<0.05, p<0.01 and p<0.001). Thus, just two weeks of
583 social isolation is sufficient to produce a state of anhedonia in control rats.

584 We then tested stress-related corticosterone and ACTH hormones in control rats. For
585 corticosterone, using the isolated condition as reference (**Figure 8A**), the unpaired mean
586 difference between Isolated and Handled corticosterone levels was -8.17 ng/mL [95.0%CI -
587 12.2, -4.2] and the unpaired mean difference between Isolated and Paired was -7.04 ng/mL
588 [95.0%CI -11.1, -3.47], corresponding to a 60% and 48% increase in Isolated animals,
589 respectively. The two-sided P values of the Mann-Whitney test were 0.0216 and 0.0167,
590 respectively. The unbiased Cohen's *d* was -2.095 and -1.926 for the Handled and Paired groups,
591 respectively. Social isolation thus had a strong effect on corticosterone levels in control rats.
592 The two distributions for the Handled and Paired groups were similar (unbiased Cohen's *d* was

593 0.367). For ACTH, using the isolated condition as reference (**Figure 8B**), the unpaired mean
594 difference between Isolated and Handled corticosterone levels was -1.14e+02 pg/mL [95.0%CI
595 -2.22e+02, -63.7] and the unpaired mean difference between Isolated and Paired was -1.07e+02
596 pg/mL [95.0%CI -1.48e+02, -50.5], corresponding to a 52% and 47% increase in Isolated
597 animals, respectively. The two-sided P values of the Mann-Whitney test were 0.0122 and
598 0.0235, respectively. The unbiased Cohen's *d* was -1.579 and -1.685 for the Handled and Paired
599 groups, respectively. Social isolation thus had also a strong effect on ACTH levels. The two
600 distributions for the Handled and Paired groups were similar (unbiased Cohen's *d* was 0.086).
601 Finally, we assessed serum BDNF levels. Using the isolated condition as reference
602 (**Figure 8C**), the unpaired mean difference between Isolated and Handled BDNF levels was
603 0.32 pg/mL [95.0%CI 0.151, 0.524] and the unpaired mean difference between Isolated and
604 Paired was 0.458 pg/mL [95.0%CI 0.198, 0.697], corresponding to a 16% and 22% decrease in
605 Isolated animals, respectively. The two-sided P values of the Mann-Whitney test were 0.0216
606 and 0.012, respectively. The unbiased Cohen's *d* was 1.749 and 1.464 for the Handled and
607 Paired groups, respectively. Social isolation thus had also a strong effect decreasing BDNF
608 levels in rats, as in control mice (Figure 5). The two distributions for the handled and paired
609 groups were similar (unbiased Cohen's *d* was 0.457).
610 We conclude that, as in mice, social isolation in control rats produces a phenotype
611 associated with stress. Interestingly, daily handling of otherwise isolated animals prevented the
612 occurrence of the phenotypic markers of stress. They did not defer from paired animals for the
613 measures considered here. We then compared how different housing conditions influence the
614 epilepsy phenotype (the experimental protocol is shown in **Figure 7A**).
615
616 **Social isolation worsens epilepsy severity in Pilo rats**

617 Typical seizures included low voltage fast activity, a tonic phase and a tonic-clonic phase,
618 which were associated with the typical behavioral expression (using video recordings
619 performed during the light phase). Most seizures terminated with a logarithmic slowing down
620 of the activity (based on visual inspection), characteristic of a homoclinic bifurcation (El
621 Houssaini et al., 2015; Saggio et al., 2017). Since the recording device provides an AC signal,
622 we could not detect the eventual presence of DC shift at seizure onset, which would have
623 indicated seizures of the Epileptor class (saddle node/homoclinic), the most common form of
624 seizure dynamics found across species, including in humans (Jirsa et al., 2014). Interictal spikes
625 could precede seizure onset, but this was not found systematically, including within the same
626 animal. We did not find noticeable differences in the EEG between seizures occurring in the
627 three different groups (Isolated, Handled, Paired). The pilocarpine experimental model of
628 epilepsy is characterized by the presence of different types of bursts of spikes, which dynamics
629 are very different from seizures (Chauviere et al., 2012). These bursts of spikes were not
630 considered as seizures.

631 Since all Pilo rats were EEG recorded 24/7, we first verified the stability of seizure
632 activity per week. **Figure 9** shows that the average seizure frequency per week was remarkably
633 stable for each recorded animal in each group, i.e. seizure frequency did not tend to increase or
634 decrease over time. We thus pooled the seizures recorded in each animal over the three weeks
635 to assess the effects of social isolation.

636 The unpaired mean difference in seizure frequency between Isolated and Handled animals
637 (**Figure 10A**) was -0.665 seizure/h [95.0%CI -1.04, -0.372] and the unpaired mean difference
638 between Isolated and Paired was -0.65 seizure/h [95.0%CI -1.04, -0.356]. The two-sided P
639 value of the Mann-Whitney test were 0.0004 and 0.0104, respectively. The unbiased Cohen's
640 *d* was -1.547 and -1.26 for the Handled and Paired groups with respect to Isolated, respectively.

641 The distributions between Handled and Paired groups were similar (unbiased Cohen's *d* was
642 0.623).

643 The unpaired mean difference in seizure duration between Isolated and Handled (**Figure**
644 **10B**) was -36.8 s [95.0%CI -48.3, -27.8]. The unpaired mean difference between Isolated and
645 Paired was -23.5 s [95.0%CI -35.6, -16.0]. The two-sided P value of the Mann-Whitney test
646 were 5.5e-05 and 0.00149, respectively. The unbiased Cohen's *d* was -2.857 and -1.72 for the
647 Handled and Paired groups with respect to Isolated, respectively. The distributions between
648 Handled and Paired groups were different (unbiased Cohen's *d* was 1.658). Although their
649 confidence intervals overlapped (no significant difference in seizure duration), the distribution
650 was narrower in the Paired group overlapping with the distribution of more prolonged duration
651 seizures in the Handled group (**Figure 10B**).

652 Seizure severity was assessed with the video during the light phase only. The unpaired
653 mean difference between Isolated and Handled groups (**Figure 10C**) was -0.421 [95.0%CI -
654 0.648, -0.194]. The unpaired mean difference between Isolated and Paired was -0.359 [95.0%CI
655 -0.772, -0.0377]. The two-sided P value of the Mann-Whitney test were 0.0121 and 0.26,
656 respectively. The unbiased Cohen's *d* was -1.343 and -1.177 for the Handled and Paired groups
657 with respect to Isolated, respectively. The distributions between Handled and Paired groups
658 were not different (unbiased Cohen's *d* was 0.135).

659 We conclude that social isolation dramatically worsened the seizure phenotype, with
660 seizures being 16 times more frequent in isolated animals as compared to those socially housed
661 or those having daily interaction with experimenters. Interestingly, daily interactions in
662 otherwise singly housed animals had a similar effect on epilepsy severity as compared to
663 animals maintaining social interaction.

664

665

666 **DISCUSSION**

667 Our results show that single housing exacerbates the phenotype in male Wistar rats and
668 Swiss mice in the pilocarpine experimental model of epilepsy. It is important to note that daily
669 social interaction with the experimenter was sufficient to prevent the development of a severe
670 epilepsy phenotype in rats although they were singly housed. It was as efficient as keeping
671 animals in pairs. Although we could not quantify it, we noted that isolated Pilo animals were
672 very aggressive and displayed escaping behavior when cages were changed every week. Their
673 cages were close to one another, suggesting that visual contact was not sufficient to prevent the
674 effect of social isolation. Abnormal reaction to handling is a direct consequence of single
675 housing (Hatch et al., 1965).

676 In contrast, rats and mice socially housed (or in daily contact with an experimenter)
677 remained calm when handled. In the laboratory, we are also performing electrophysiological
678 recordings with high-density silicon probes in experimental rat models of epilepsy. The
679 equipment is protected with a copper mesh hat, which precludes social housing. We are using
680 large cages, divided in two, separated by a grid allowing physical interaction. Each
681 compartment contains one animal with epilepsy. Animals can make nose contact and interact
682 with their whiskers through the bars. Animals do not show any sign of anxiety, and wire
683 connectors are easily inserted, as in control animals (no struggle). This type of housing allows
684 wired recordings in rats and mice with epilepsy, while maintaining a certain level of social
685 interaction.

686 Our data on control animals confirms the vast body of literature reporting that social
687 isolation constitutes a major stressful situation that can lead to a depression-like profile
688 (Mumtaz et al., 2018). Among the numerous markers that can be assessed, we focused on
689 general ones such as ACTH, corticosterone, and anhedonia. When rats and mice are singly
690 housed, ACTH and corticosterone levels are increased (Veenema et al., 2005; Djordjevic et al.,

691 2012). Visual inspection of the raw data seems to indicate a bimodal distribution of anxiety
692 levels in pretest conditions in control mice (Figure 2A). It will be interesting to determine
693 whether these animals correspond to animals susceptible and resilient to depression (Han and
694 Nestler, 2017). When exposed to an intense stress (test), control rats also split into two
695 populations, vulnerable and non-vulnerable to depression (Blugeot et al., 2011; Becker et al.,
696 2015; Claverie et al., 2016; Bouvier et al., 2017; Becker et al., 2019). Thus, in pretest
697 conditions, rats and mice are not homogeneous, as they are going to react differently when
698 challenged by external factors such as stress or epileptogenesis (Bernard, 2016; Medel-Matus
699 et al., 2017).

700 In pilocarpine experimental models of epilepsy, ACTH and corticosterone levels are
701 increased (Mazarati et al., 2009; Inostroza et al., 2012; Ngoupaye et al., 2013). A direct
702 comparison of ACTH and corticosterone values between studies is difficult as they depend
703 upon the time of collection and the way blood is collected (*in vivo* or *post mortem* as done here).
704 Despite the differences in experimental procedures, all results are qualitatively similar (increase
705 due to social isolation or epilepsy). To the best of our knowledge, we here provide the first
706 evaluation of the contribution of both factors: social isolation and epilepsy. We demonstrate
707 that epilepsy in socially housed mice produces an activation of the HPA axis and anhedonia,
708 responses which are amplified in isolated mice with epilepsy. A strong activation of the HPA
709 axis in isolated animals with epilepsy may contribute to the expression of co-morbidities such
710 as anxiety, cognitive deficits, and depression-like behavior.

711 The results obtained in mice are also worth discussing in a general manner. Animals
712 were randomly selected at baseline for their future group assignment (treatment/housing). The
713 first striking observation is how distributed are the values of anxiety and discrimination index
714 of individual mice. The "control" population is far from homogeneous. As discussed above,
715 this heterogeneity can be used to generate diverse groups of mice after an insult, e.g. mice

716 susceptible and resilient to depression thus mimicking human conditions (Akil et al., 2018).
717 Such heterogeneity is also found in rats, enabling to explain why some animals develop a severe
718 form of epilepsy and/or co-morbidities and not others (Blugeot et al., 2011; Becker et al., 2015;
719 Becker et al., 2019). Many factors can account for such heterogeneity in anxiety/cognitive
720 performance at baseline in mice, including different genetic backgrounds and life experience
721 (e.g. handling by the dam, dominant/dominated relations in the cage). Since we tested animals
722 always at the same time of the day, circadian variations may not play a role. Multidien cycles
723 (slower cycles with days-weeks-months periods) have been demonstrated in human (Baud et
724 al., 2018) and rats (Baud et al., 2019) with epilepsy. Whether control animals display
725 endogenous multidien cycles remains to be determined. If they do, anxiety/cognitive
726 performance may vary as a function of these cycles, and since such slow cycles are not
727 synchronized in animals recorded simultaneously (Baud et al., 2019), the anxiety/cognitive
728 performance points will be distributed according to which position in the slow cycle a given
729 animal is when it is tested.

730 Another striking observation we made is the linear relationship found between anxiety
731 and ACTH/corticosterone/discrimination levels. The relationship was still present after
732 isolation and Pilo treatment. This means that these variables are tied by a strong co-variance
733 rule. It will be interesting to test other variables (e.g. stability of place cells, fear conditioning,
734 working memory) with anxiety levels. Given the damage produced by pilocarpine-induced
735 status epilepticus in numerous brain regions, we could have expected a deviation from the co-
736 variance rule. Instead, animals just "slide down" the regression line towards stronger
737 phenotypic expression. Said differently, epilepsy did not "reprogram" the relationship between
738 anxiety and ACTH/corticosterone/discrimination levels. However, epilepsy did reprogram the
739 anxiety/BDNF relationship. BDNF is involved in numerous functions and phenotypic traits, in
740 particular, stress (Murinova et al., 2017) and epilepsy (Iughetti et al., 2018). Although there

741 exists a linear relationship between anxiety and serum BNDF, the relationship is shifted towards
742 larger anxiety value in Pilo mice, while the slope remains mostly unaffected. This suggests that
743 epilepsy changes the general rule linking anxiety and serum BDNF levels. Together, our results
744 further support the concept that rodents, like humans, should be considered as individuals. Even
745 if as a group (on average) an effect can be evidenced, there are significant discrepancies
746 between animals, including animals not responding as the group average. Again, many factors
747 can account for such behavior, but we argue that discounting them may make us miss an
748 important biological phenomenon that the observation revealed. It is therefore not surprising
749 that following status epilepticus animals display different seizure severities.

750 In rats with epilepsy, we found that spontaneous seizures were, in average, 16 times
751 more frequent in isolated animals as compared to animals kept in pairs or singly housed animals
752 but with daily interactions with experimenter. Looking at individuals in the Isolated group, we
753 noted a substantial dispersion of seizure frequency. Some Isolated animals had a low seizure
754 frequency, in the range of the Handled/Paired groups (perhaps signing a resilience to stress-
755 induced isolation or resilience to pilocarpine-induced status epilepticus). Even though, at
756 baseline, animals may display a high variability (cf. the dispersion of ACTH, corticosterone
757 and BDNF values in control rats), which may explain different fates as a function of housing
758 conditions, the consequences of isolation are quite dramatic. During the three weeks of
759 continuous recordings (starting six weeks after pilocarpine-induced status epilepticus), we did
760 not find an increase in seizure frequency (Figure 9), suggesting that a global steady state activity
761 had been reached (Williams et al., 2009). Interictal activity and seizures follow a multidien
762 rhythm in the pilocarpine experimental rat model of epilepsy (Baud et al., 2019) as in humans
763 (Baud et al., 2018). The fact that seizures occur at specific phases of this rhythm is not a
764 confounding factor as the frequency of the multidien rhythm is between five and seven days in
765 this rat model (Baud et al., 2019), which means that we captured at least three cycles.

766 In the present study, isolated animals had a high seizure frequency (≈16/day). It is
767 difficult to compare this value with the existing literature as housing conditions are rarely
768 mentioned. In addition, the beginning (how long after status epilepticus) and duration of 24/7
769 EEG recordings vary from one study to another. In the rat pilocarpine model, typical values for
770 singly housed animals in Sprague Dawley rats are: 3/day during the early phase of epilepsy
771 (Behr et al., 2017), 8/day (Paolone et al., 2019), 1-13/day (Tai et al., 2017), 10/day (Bankstahl
772 et al., 2012); similar to values reported in Wistar rats: ≈4.5/day (Bajorat et al., 2011; Bajorat et
773 al., 2018). Our study, although on the high end, remains in the same order of the magnitude as
774 previously published work (we limited our analysis of the literature to the ongoing decade). We
775 evaluated seizure severity only during the light phase, which constitutes a limitation of the
776 study. We cannot rule out that seizures occurring during the dark phase may be less severe
777 (including subclinical electrographic seizures). It is important to note that the International
778 League Against Epilepsy released suggestions on common data elements for physiological
779 (Gorter et al., 2018), behavioral (Mazarati et al., 2018) and EEG (Ono et al., 2018) studies
780 performed in experimental animal models of epilepsy. Although our study was planned before
781 the release of such information, adopting common data elements will help to compare different
782 studies in the future.

783 Finding the mechanisms underlying the difference in phenotypes between the different
784 housing conditions was beyond the scope of this study. We do not claim that the strong seizure
785 phenotype found in the Isolated group is solely due to the stronger activation of the HPA axis,
786 although it may contribute to it. We may tentatively propose that social isolation produces a
787 vulnerability phenotype, as suggested by the low levels of serum BDNF found in Isolated
788 animals (Becker et al., 2015). We suggest that epileptogenesis occurring in networks
789 "weakened" by social isolation-induced stress would result in a strong phenotype.

790 The field of epilepsy research makes use of many different experimental models, strains
791 and species. Results are model-, strain- and species-specific, which may explain some
792 conflicting results. We propose that another confounding factor to consider is housing
793 conditions. Some patients with epilepsy may experience social isolation, which correlates with
794 more severe forms of epilepsy (Kotwas et al., 2017). Results obtained in experimental animal
795 models kept in isolation may be highly relevant to such patient situation, or any other situation
796 associated with high levels of stress. Results obtained in socially housed animals may be
797 relevant to other subsets of patients, with a less severe phenotype. Our results have significant
798 consequences in terms of data interpretation, in particular when testing anti-epileptogenesis or
799 anti-seizure strategies. Our data provide strong evidence that the very large effect of Pilo under
800 IC is an effect of Pilo *and* rearing. This means that the phenotype measured in singly housed
801 animals is a blend of the treatment and the housing. It also means that any attempts to
802 modify/treat epilepsy in these conditions could be modifying/treating the epilepsy effect or the
803 housing effect, or both, because they are conflated. Some studies report a significant decrease
804 in seizure frequency when animals are treated with a given compound. It is possible that the
805 compound may have targeted the isolation-dependent stress component of the phenotype and
806 not epilepsy itself. This is analogous to the anxiety/cognitive performance linear relationship
807 we described. The two variables, housing and treatment, make the animals move up/down along
808 the regression line. If the treatment affects the stress-induced component, animals will move
809 back up to fewer seizures, but the biological component of epilepsy itself may not be affected
810 *per se*. A reduction in seizure frequency by a factor of 16 would be equivalent to the difference
811 we report between Paired and Isolated animals. For example, inhibition of kelch like ECH
812 associated protein 1 (Keap1) reduces seizure frequency by a factor 20 in isolated kainic acid-
813 treated Sprague-Dawley animals (Shekh-Ahmad et al., 2018). Keap1 controls nuclear factor
814 erythroid 2-related factor 2 (Nrf2), a key regulator of antioxidant defense mechanisms. The

815 authors suggest that these results support "the hypothesis that reactive oxygen species
816 generation is a key event in the development of epilepsy" (Shekh-Ahmad et al., 2018). Since
817 social isolation produces reactive oxygen species (Filipovic et al., 2017), it is possible that
818 activating antioxidant defense mechanisms may have targeted the isolation-induced component
819 of the seizure phenotype. Indeed, treating animals with a potent antioxidant did not affect
820 seizure frequency in the kainic acid rat model when animals interacted daily with
821 experimenters, while the treatment was very efficient when animals were characterized by high
822 levels of reactive oxygen species before status epilepticus (Becker et al., 2019). These
823 considerations provide another possible interpretation of the Keap1 results (Shekh-Ahmad et
824 al., 2018). However, the conclusion of Shekh-Ahmad and collaborators that antioxidant
825 treatment is strongly disease modifying in the context of social isolation is not only valid but
826 also highly clinically relevant to socially isolated patients. Finally, many (often unpublished)
827 preclinical studies performed in isolated animals did not evidence any significant effect on
828 seizure frequency. Since the social isolation stress component could act as a confounding factor
829 producing a strong phenotype, there may be many false negative results; i.e. the tested drugs
830 may have been very efficient in experimental animals maintaining social interaction.
831
832 Although we cannot claim that maintaining social interaction between rodents in
833 laboratory conditions corresponds to "normality" (as in the wild), single housing does produce
834 strong biological alterations that render data interpretation more complex, in both physiological
835 and pathological conditions. We recommend that material and methods should systematically
836 report housing conditions and that all efforts should be made to maintain social interaction.
837 Finally, these results were obtained in one experimental model (pilocarpine), in two species,
838 one strain for each species and only in males. Similar studies should be performed in females,
839 other strains and models such as kainic acid, kindling, tetanus toxin etc. Our results and

840 conclusions should not be seen as a criticism of previous works. Rather varying housing
841 conditions provide a unique opportunity to study, with the same experimental model, different
842 situations found in patients with high and low stress levels.

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1050

1051

1052 **Figure Legends**

1053

1054 **Figure 1: Effect of social isolation on body weight and anhedonia in control and epileptic**
1055 **mice. (A)** Experimental protocol. In pretest condition, up to day 0, mice were socially housed
1056 and evaluated for anhedonia, anxiety (EPM) and novel object recognition (NOR) test. Tested
1057 mice were then assigned randomly to different groups: Non-pilo social condition (SC), i.e.
1058 control like before D0, Non-pilo isolated condition (IC), i.e. the mice were transferred to an
1059 individual cage, Pilo SC, the mice received pilocarpine to induce status epilepticus, which
1060 triggers epileptogenesis, the process leading to the occurrence of spontaneous seizures, and Pilo
1061 IC. Four weeks following status epilepticus (posttest conditions), the same mice were evaluated
1062 for anhedonia, EPM and NOR tests. At the end of the experimental protocol, animals were
1063 killed to measure corticosterone, ACTH, and BDNF. **(B)** Evolution of averaged body weight
1064 over six weeks for the four groups. The Pilo IC groups gained less weight than the other groups.
1065 Data are mean ± SEM. $^{*}p<0.05$ in comparison with the control non-Pilo SC group. (**C**) Sweet
1066 water consumption was identical in all mice in pretest conditions (splitting them on their future
1067 group assignment). As compared to the control group (Non-pilo SC), the Non-pilo IC showed
1068 anhedonia, which was further increased in the Pilo-SC and exacerbated in the Pilo-IC group.
1069 Data are mean ± SEM. $^{***}p< 0.001$ in comparison with the non-Pilo SC group; $^{\#}p<0.05$,
1070 $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ in comparison with the corresponding social condition group.

1071

1072 **Figure 2: Effect of social isolation on anxiety levels in control and epileptic mice.** The
1073 paired mean difference for four comparisons between posttest (Non-pilo SC, Pilo SC, Non-pilo
1074 IC, and Pilo IC) and pretest (Pre) conditions are shown in the Cumming estimation plot. The
1075 raw data is plotted on the upper axes; each paired set of observations is connected by a line (**A**).
1076 On the lower axis (**B**), each paired mean difference is plotted as a bootstrap sampling
1077 distribution. Mean differences are depicted as black dots; 95% confidence intervals are

1078 indicated by the ends of the vertical error bars. Colored dots correspond to the posttest-pretest
1079 differences for each mouse. Although anxiety levels appear similar in average in pretest
1080 conditions, we note a wide dispersion of the raw data, i.e. individual animals can have very
1081 different levels of anxiety (compare pretest values in panel A). We also note the apparent
1082 existence of a bimodal distribution. **(B)** There was no change in anxiety level in the control
1083 group (Non-pilo mice maintained in social housing) although individuals displayed variability
1084 (first panel from the left). Social isolation in Non-pilo animals (third panel from the left)
1085 significantly increased anxiety levels as compared to pretest. The changes in anxiety levels were
1086 more extensive in the Pilo-SC group (second panel) and most extensive in the Pilo-IC group
1087 (fourth panel). **(C)** Each mean difference in changes in anxiety score is plotted as a bootstrap
1088 sampling distribution. Mean differences are depicted as dots; 95% confidence intervals are
1089 indicated by the ends of the vertical error bars. Epilepsy (in Pilo mice) produced the same
1090 increase in anxiety levels in SC and IC conditions (although the IC group starts from higher
1091 anxiety levels as compared to the SC group). This means that the effect of epilepsy in isolated
1092 mice on anxiety levels is a blend of the effect of epilepsy and isolation.
1093
1094 **Figure 3. Effect of social isolation on the novel object recognition test in control and**
1095 **epileptic mice.** The paired mean difference for four comparisons between posttest (Non-pilo
1096 SC, Pilo SC, Non-pilo IC, and Pilo IC) and pretest (Pre) conditions are shown in the Cumming
1097 estimation plot. The raw data is plotted on the upper axes; each paired set of observations is
1098 connected by a line (**A**). On the lower axis (**B**), each paired mean difference is plotted as a
1099 bootstrap sampling distribution. Mean differences are depicted as black dots; 95% confidence
1100 intervals are indicated by the ends of the vertical error bars. Colored dots correspond to the
1101 posttest-pretest differences for each mouse. As for anxiety levels, although the discrimination
1102 index appears similar in average in pretest conditions, we note a wide dispersion of the raw

1103 data, i.e. individual animals can have very different levels of performance in the NOR test
1104 (compare pretest values in panel A). **(B)** There was no change in performance in the control
1105 group (Non-pilo mice maintained in social housing) although individuals displayed variability
1106 (first panel from the left). Social isolation in Non-pilo animals (third panel from the left) did
1107 not change the performance at the group level (the confidence interval crosses the null line),
1108 but we note that five out of six of the isolated animals displayed a decrease in performance as
1109 compared to pretest. The changes in discrimination index were substantial in both Pilo-SC
1110 (second panel) and Pilo-IC (fourth panel) groups. **(C)** Each mean difference in changes in
1111 discrimination index score is plotted as a bootstrap sampling distribution. Mean differences are
1112 depicted as dots; 95% confidence intervals are indicated by the ends of the vertical error bars.
1113 Epilepsy (in Pilo mice) produced the same increase in anxiety levels in SC and IC conditions.
1114
1115 **Figure 4: Co-variance between anxiety levels and the discrimination index, corticosterone,**
1116 **ACTH and BDNF levels. (A)** The discrimination index is linearly correlated to anxiety levels.
1117 All mice are displayed. The dispersion of pairs of values for anxiety levels (Figure 2A) across
1118 0.25 unit and discrimination index (Figure 2B) across 50 units at baseline is clearly apparent
1119 (green dots). All mice are distributed close to the regression line (built from all data points).
1120 The Non-pilo group maintaining social housing (dark blue dots) remained in the cloud of
1121 baseline values. The Non-pilo isolated group (orange dots) showed a downward shift. Note the
1122 presence of the outlier with high cognitive performance and high anxiety level. Pilo animals
1123 displayed a further downward shift with higher anxiety levels and lower cognitive performance,
1124 with isolated animals showing the strongest phenotype. Corticosterone **(B)** and ACTH **(C)**
1125 levels are strongly linearly correlated to anxiety levels. As for the discrimination index, the
1126 phenotype increased in a group-dependent manner Non-pilo SC < Non-pilo IC < Pilo SC < Pilo

1127 IC. **(D)** The relationship between BDNF and anxiety levels was also linear but treatment-
1128 dependent. The regression line was shifted to the right (greater anxiety levels) in epileptic mice.
1129
1130 **Figure 5: Effect of social isolation on corticosterone, ACTH and BDNF levels in control**
1131 **and epileptic mice.** In panels A, B and C, the mean difference for two comparisons is shown
1132 in the Cumming estimation plot. All raw data is shown on the upper axes. For each group,
1133 summary measurements (mean ± standard deviation) are shown as gapped lines. Each mean
1134 difference is plotted on the lower axes as a bootstrap sampling distribution. Mean differences
1135 are depicted as black dots; 95% confidence intervals (CIs) are indicated by the ends of the
1136 vertical error bars. **(A)** Social isolation increased corticosterone levels by 15 units in both Non-
1137 pilo and Pilo groups (left panel). The effect of epilepsy was even stronger (40 units) in the SC
1138 and IC groups (right panel). **(B)** Social isolation increased ACTH levels by 40 units in both
1139 Non-pilo and Pilo groups (left panel). The effect of epilepsy was even stronger (80 units) in the
1140 SC and IC groups (right panel). **(C)** Social isolation decreased BDNF levels by 40 units in both
1141 Non-pilo and Pilo groups (left panel). The Epilepsy condition had no apparent effect on BDNF
1142 levels in both SC and IC groups.
1143
1144 **Figure 6: Housing x treatment interaction on corticosterone, ACTH and BDNF levels.**
1145 Means and 95% CIs are as in Figure 5. The (Pilo – Non-pilo) differences between the right two
1146 means and the left two means are marked by the purple lines ending in diamonds (indicating
1147 the direction of the change). The slanted dotted lines make a comparison of the two differences.
1148 The difference between the differences is marked by the triangle on the difference axis, with its
1149 CI. The analysis of the difference of differences did not reveal apparent housing x treatment
1150 interaction for corticosterone **(A)**, ACTH **(B)** and BDNF **(C)** levels.
1151

1152 **Figure 7: Effect of social isolation on body weight and anhedonia in control rats. (A)**
1153 Experimental protocol in Non-pilo and Pilo rat groups. In the control (Non-pilo) group (top part
1154 of the time axis) rats were assigned at Day 0 to Isolated, Handled and Paired groups. Anhedonia
1155 was assessed two weeks and six weeks later. During week 7, animals were killed for the analysis
1156 of corticosterone, ACTH and BDNF levels. In the Pilo group (bottom part of the time axis),
1157 animals were injected with pilocarpine at Day 0 and assigned to Isolated, Handled and Paired
1158 groups. Four weeks later, rats were equipped with wireless EEG transmitters, and recordings
1159 started one week later for three weeks. **(B)** Evolution of the average body weight over seven
1160 weeks in control (Non-pilo) rats for the three Isolated, Handled and Paired groups. The isolated
1161 group significantly gained less weight. (**C**) In control rats, Isolated animals already showed
1162 anhedonia during the first evaluation (two weeks after separation) as compared to Paired
1163 animals. Interestingly, Handled animals showed increased sweet water consumption as
1164 compared to the Paired group. The same result was found during the second evaluation period
1165 six weeks after group assignment. Data are mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$
1166 in comparison with the isolated group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ in comparison with
1167 the handled group.
1168
1169 **Figure 8: Effect of social isolation on corticosterone, ACTH and BDNF levels in control**
1170 **rats. (A)** Social isolation increased corticosterone levels by seven units in Isolated rats as
1171 compared to Handled and Paired animals. **(B)** Social isolation increased ACTH levels by 100
1172 units in Isolated rats as compared to Handled and Paired animals. **(C)** Social isolation decreased
1173 BDNF levels by 0.4 units in Isolated rats as compared to Handled and Paired animals. The
1174 effect of isolation was significant for the three biomarkers, the CIs being far from the null value.
1175 The overlap of the distributions between Handled and Paired animals suggests a lack of
1176 difference between the two conditions.

1177

1178 **Figure 9: Distribution of seizures per week for each animal**. The average seizure frequency
1179 (seizures per hour) for each recording week is shown for **(A)** Isolated (n=11), **(B)** Handled
1180 (n=12) and **(C)** Paired rats (n=6). Note that the scale of seizure frequency in the Isolated group
1181 is one order of magnitude higher than the other two groups. Two animals (out of 11) in the
1182 Isolated group had a seizure frequency similar to that found in Paired or Handled animals
1183 (0.03/h). The three groups displayed stability (despite some individual variability) over the
1184 three weeks of recordings.

1185

1186 **Figure 10**: **Social isolation increases spontaneous seizures by a factor of 16 in rats**. **(A)** The
1187 seizure frequency (number of spontaneous recurrent seizures - SRS per hour), **(B)** duration and
1188 **(C)** severity are shown for Isolated, Handled and Paired rats (n=11, n=12, and n=6). Isolated
1189 rats displayed a very severe epileptic phenotype as compared to the other groups. Handled and
1190 Paired groups showed a similar phenotype.

1191

1192

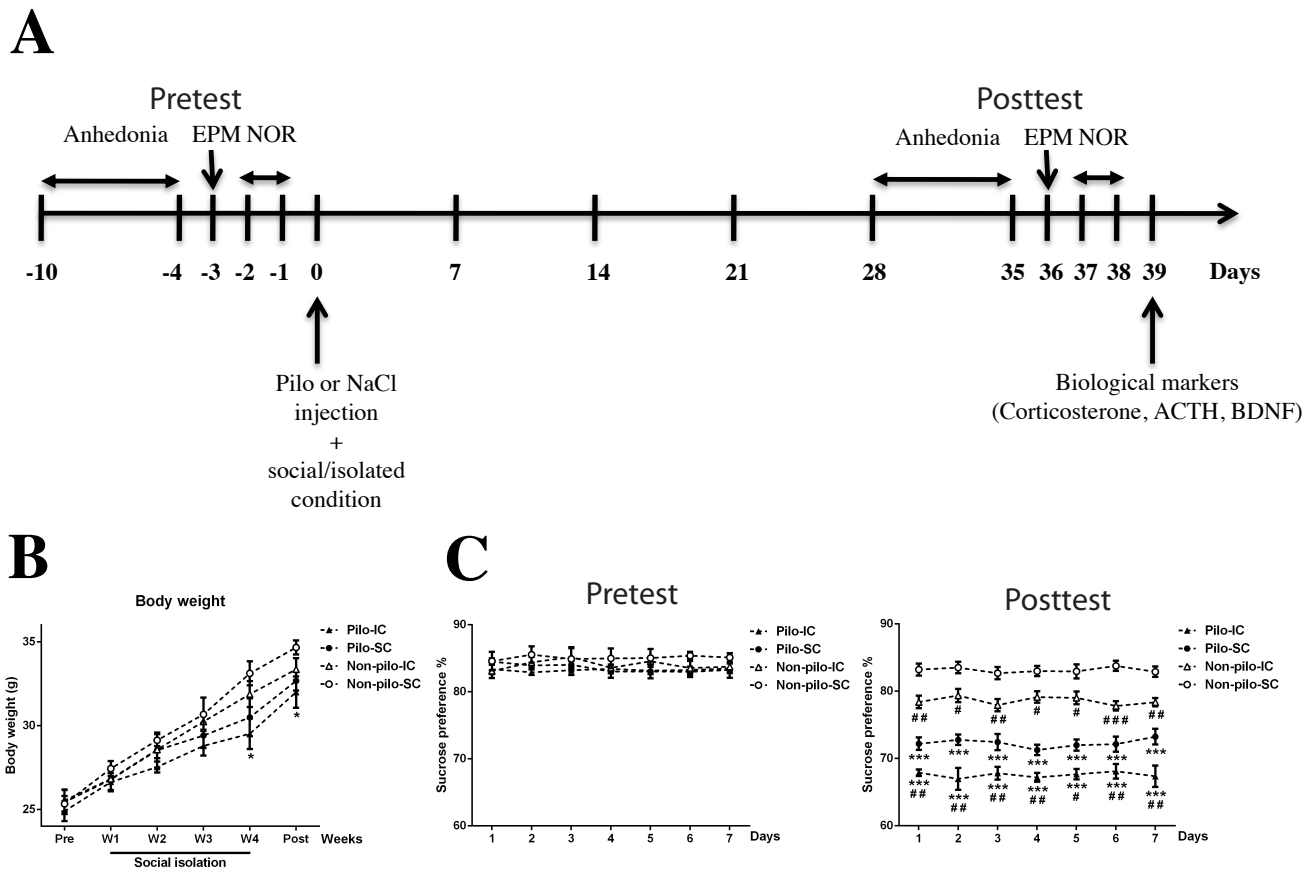
A
Pretest
Anhedonia
EPM NOR
Posttest
Anhedonia
EPM NOR
-10
-4
-3
-2
-1
0
7
14
21
28
35
36
37
38
39
Days
Pilo or NaCl
injection
+
social/isolated
condition
Biological markers
(Corticosterone, ACTH, BDNF)
B
Body weight
Body weight (g)
Pre
W1
W2
W3
W4
Post
Weeks
Social isolation
Pilo-IC
Pilo-SC
Non-pilo-IC
Non-pilo-SC
C
Pretest
Sucrose preference %
Days
Posttest
Sucrose preference %
Days

Figure 1

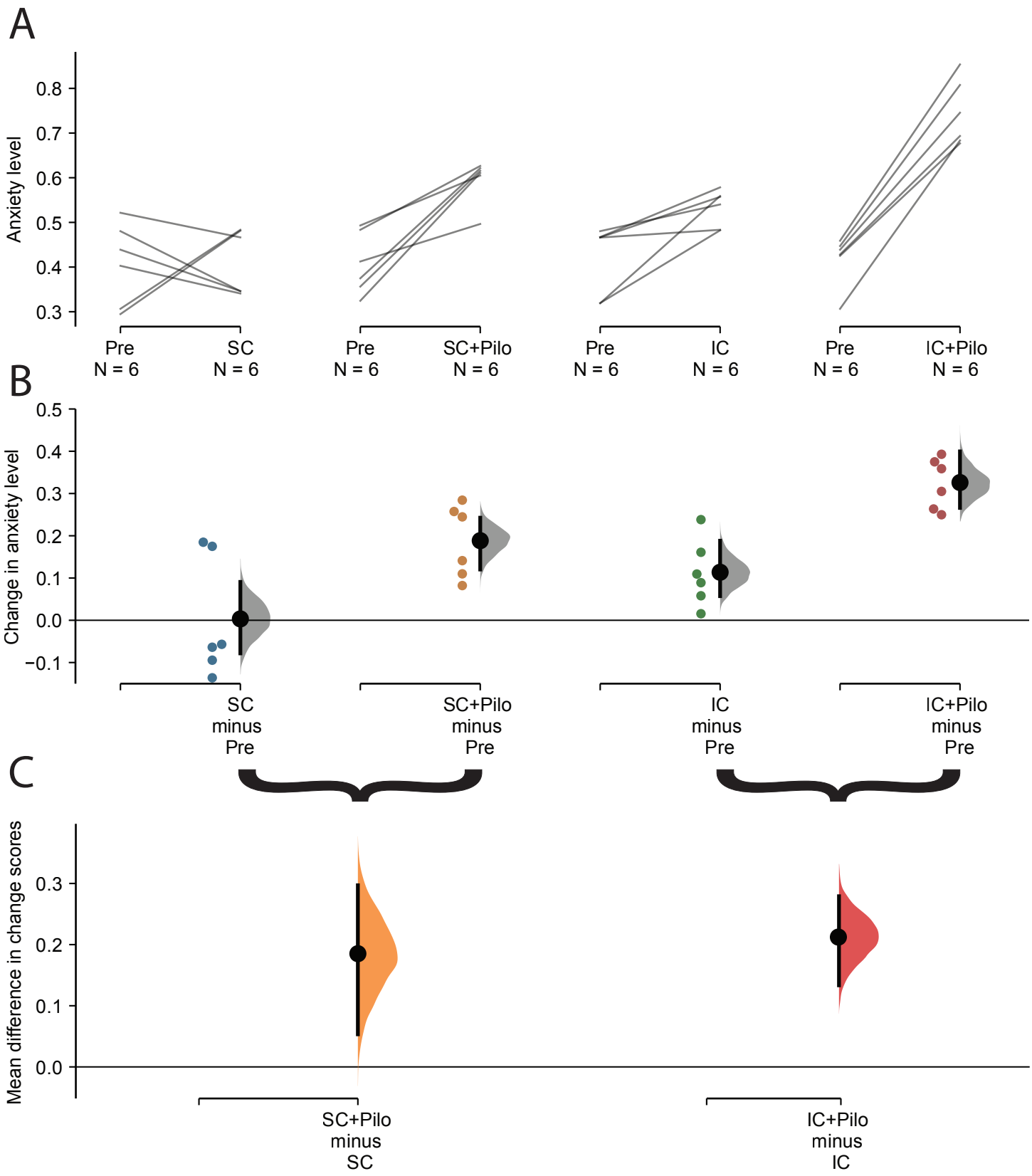
A
Anxiety level
0.8
0.7
0.6
0.5
0.4
0.3
Pre
N = 6
SC
N = 6
Pre
N = 6
SC+Pilo
N = 6
Pre
N = 6
IC
N = 6
Pre
N = 6
IC+Pilo
N = 6
B
Change in anxiety level
0.5
0.4
0.3
0.2
0.1
0.0
−0.1
SC
minus
Pre
SC+Pilo
minus
Pre
IC
minus
Pre
IC+Pilo
minus
Pre
C
Mean difference in change scores
0.3
0.2
0.1
0.0
SC+Pilo
minus
SC
IC+Pilo
minus
IC

Figure 2

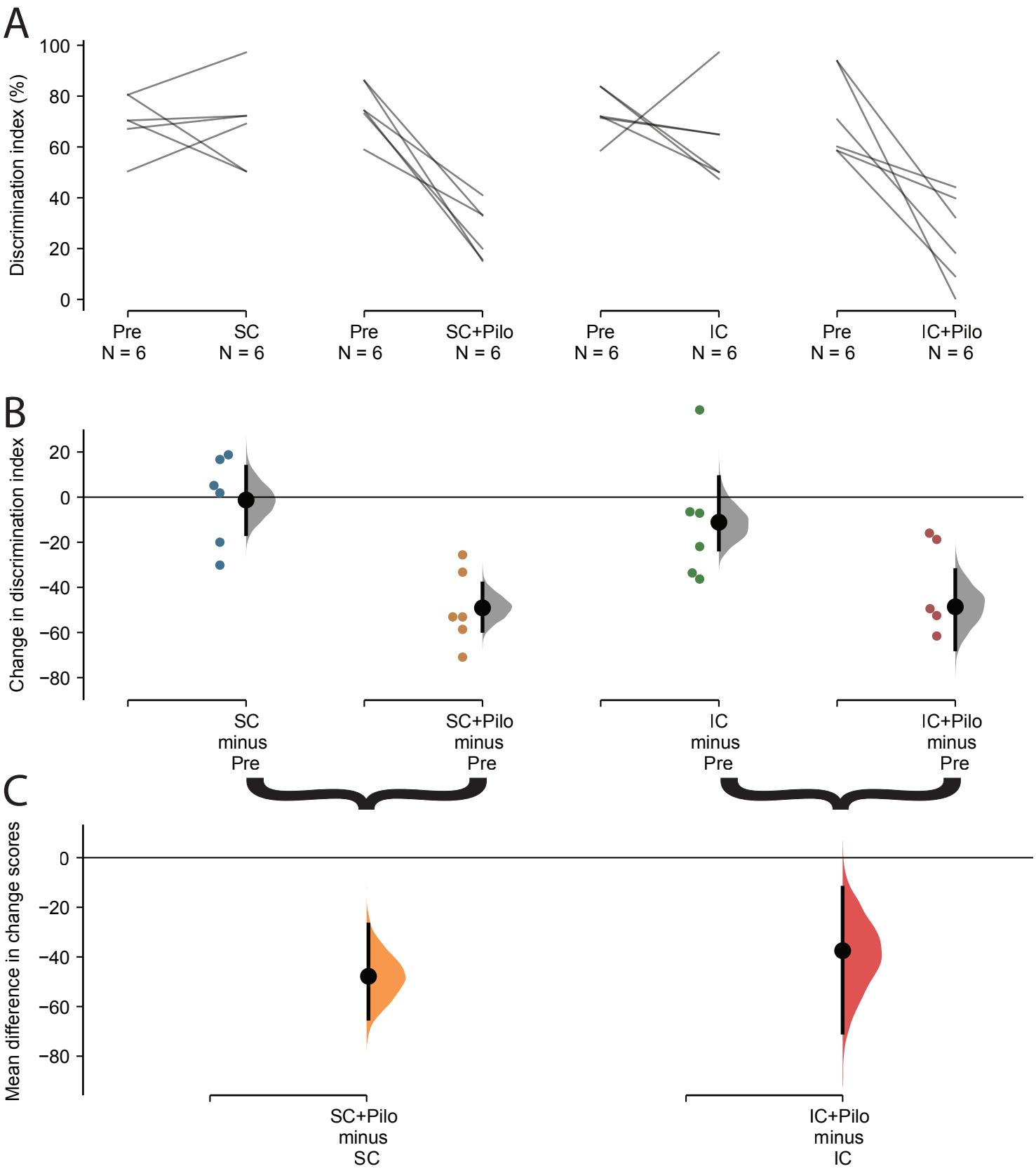
A
Discrimination index (%)
100
80
60
40
20
0
Pre
N = 6
SC
N = 6
Pre
N = 6
SC+Pilo
N = 6
Pre
N = 6
IC
N = 6
Pre
N = 6
IC+Pilo
N = 6
B
Change in discrimination index
20
0
−20
−40
−60
−80
SC
minus
Pre
SC+Pilo
minus
Pre
IC
minus
Pre
IC+Pilo
minus
Pre
C
Mean difference in change scores
0
−20
−40
−60
−80
SC+Pilo
minus
SC
IC+Pilo
minus
IC

Figure 3

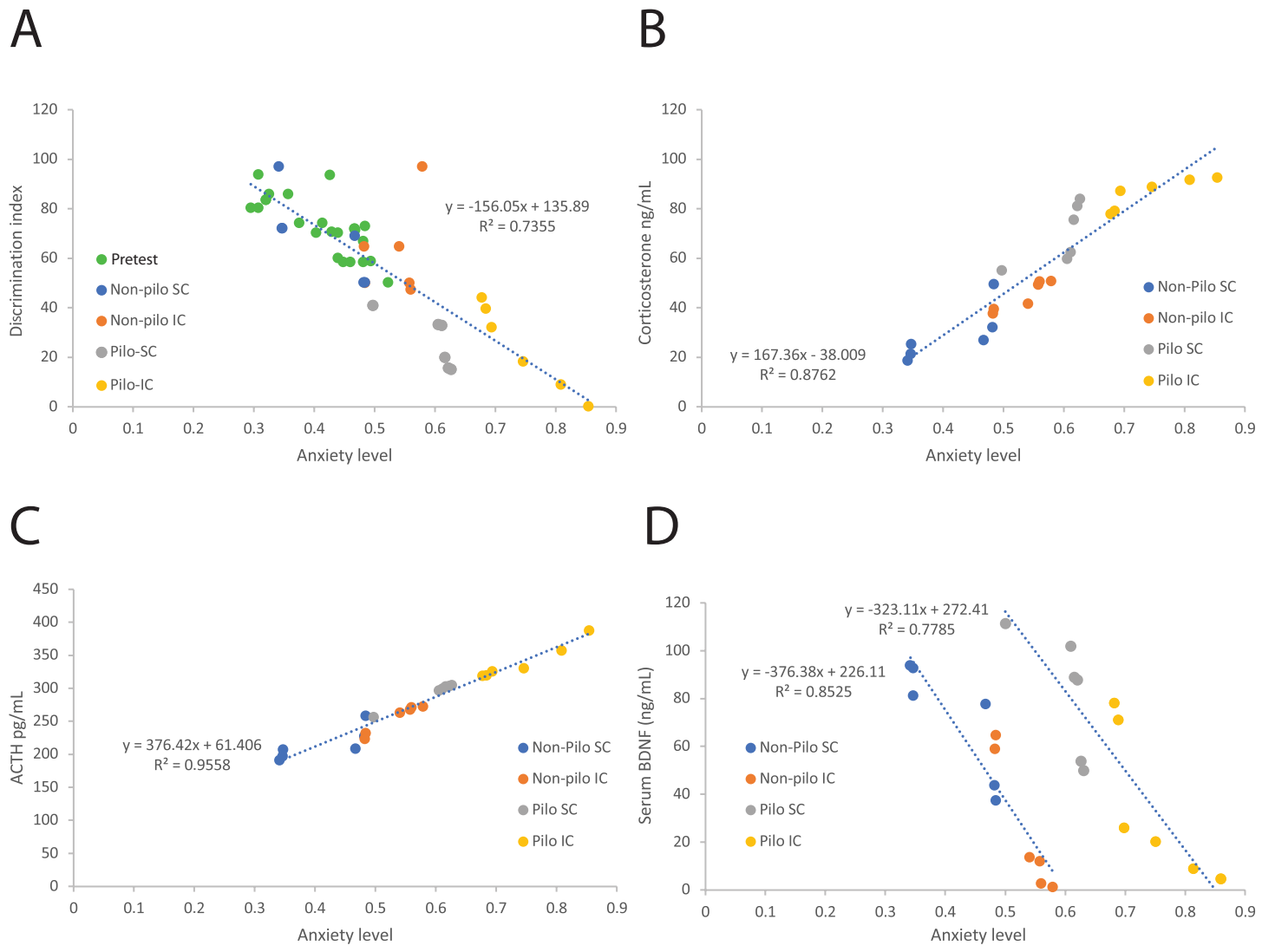
A
Discrimination index
Anxiety level
y = -156.05x + 135.89
R² = 0.7355
Pretest
Non-pilo SC
Non-pilo IC
Pilo-SC
Pilo-IC
B
Corticosterone ng/mL
Anxiety level
y = 167.36x - 38.009
R² = 0.8762
Non-Pilo SC
Non-pilo IC
Pilo SC
Pilo IC
C
ACTH pg/mL
Anxiety level
y = 376.42x + 61.406
R² = 0.9558
Non-Pilo SC
Non-pilo IC
Pilo SC
Pilo IC
D
Serum BDNF (ng/mL)
Anxiety level
y = -323.11x + 272.41
R² = 0.7785
y = -376.38x + 226.11
R² = 0.8525
Non-Pilo SC
Non-pilo IC
Pilo SC
Pilo IC

Figure 4

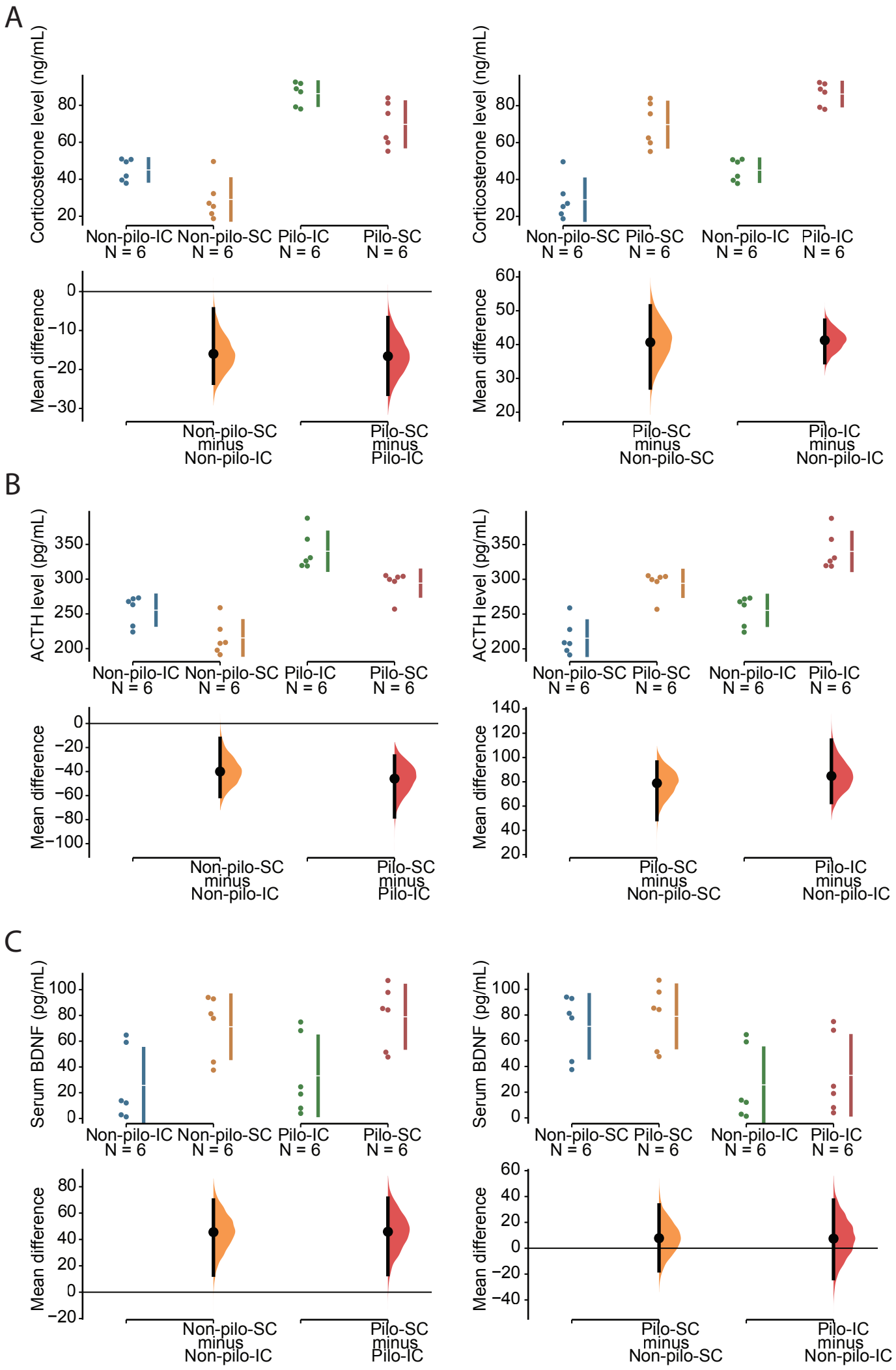
A
Corticosterone level (ng/mL)
Non-pilo-IC N = 6
Non-pilo-SC N = 6
Pilo-IC N = 6
Pilo-SC N = 6
Mean difference
Non-pilo-SC minus Non-pilo-IC
Pilo-SC minus Pilo-IC
Pilo-SC minus Non-pilo-SC
Pilo-IC minus Non-pilo-IC
B
ACTH level (pg/mL)
C
Serum BDNF (pg/mL)

Figure 5

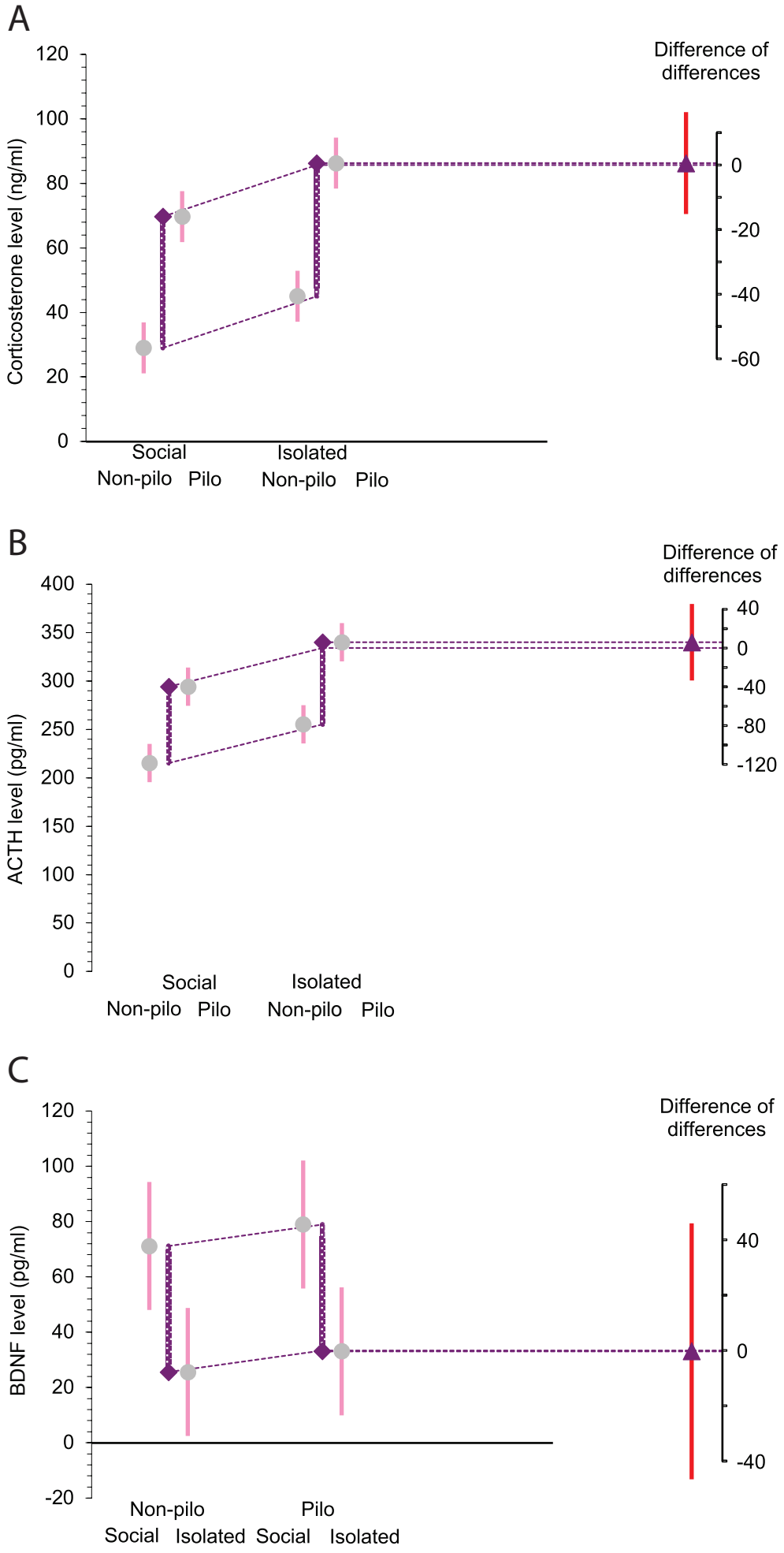
A
Corticosterone level (ng/ml)
120
100
80
60
40
20
0
Social
Non-pilo
Pilo
Isolated
Non-pilo
Pilo
Difference of differences
0
-20
-40
-60
B
ACTH level (pg/ml)
400
350
300
250
200
150
100
50
0
Social
Non-pilo
Pilo
Isolated
Non-pilo
Pilo
Difference of differences
40
0
-40
-80
-120
C
BDNF level (pg/ml)
120
100
80
60
40
20
0
-20
Non-pilo
Social
Isolated
Pilo
Social
Isolated
Difference of differences
40
0
-40

Figure 6

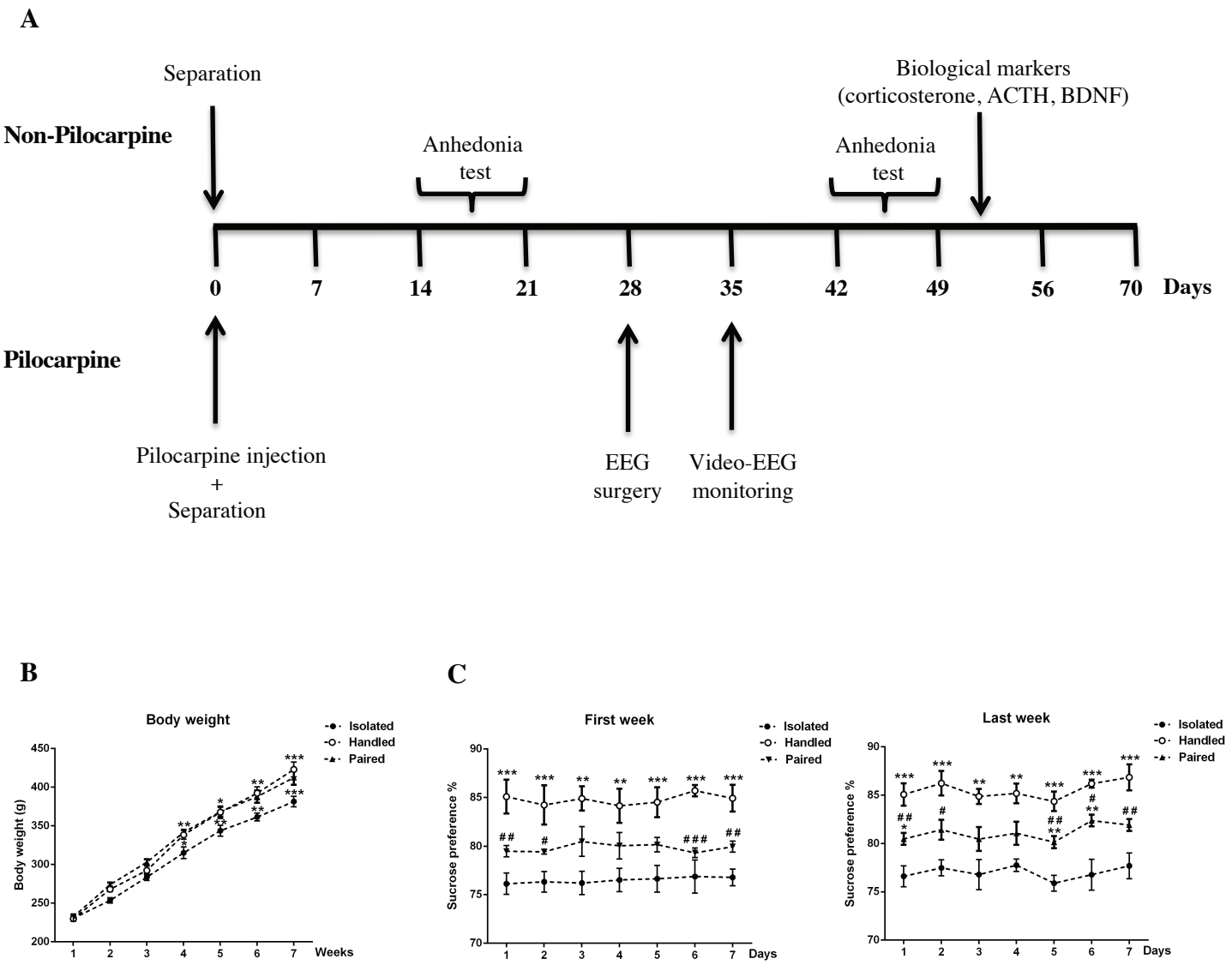
A
Separation
Non-Pilocarpine
Anhedonia test
Anhedonia test
Biological markers (corticosterone, ACTH, BDNF)
0
7
14
21
28
35
42
49
56
70
Days
Pilocarpine
Pilocarpine injection + Separation
EEG surgery
Video-EEG monitoring
B
Body weight
Isolated
Handled
Paired
Body weight (g)
Weeks
C
First week
Last week
Sucrose preference %
Days

Figure 7

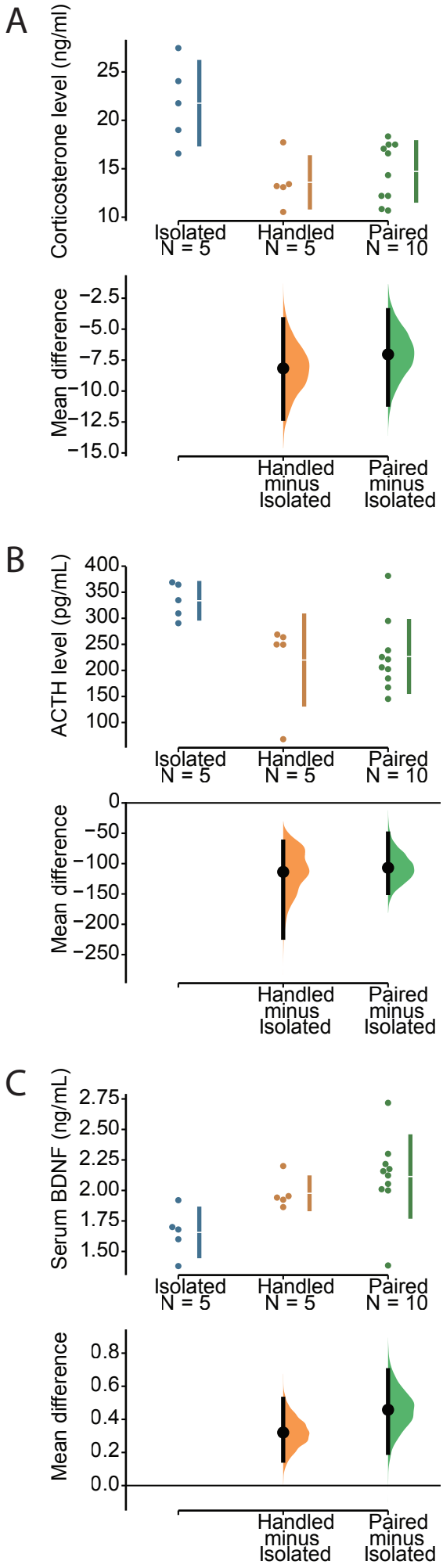
A
Corticosterone level (ng/ml)
25
20
15
10
Isolated
N = 5
Handled
N = 5
Paired
N = 10
Mean difference
−2.5
−5.0
−7.5
−10.0
−12.5
−15.0
Handled minus Isolated
Paired minus Isolated
B
ACTH level (pg/mL)
400
350
300
250
200
150
100
Isolated
N = 5
Handled
N = 5
Paired
N = 10
Mean difference
0
−50
−100
−150
−200
−250
Handled minus Isolated
Paired minus Isolated
C
Serum BDNF (ng/mL)
2.75
2.50
2.25
2.00
1.75
1.50
Isolated
N = 5
Handled
N = 5
Paired
N = 10
Mean difference
0.8
0.6
0.4
0.2
0.0
Handled minus Isolated
Paired minus Isolated

Figure 8

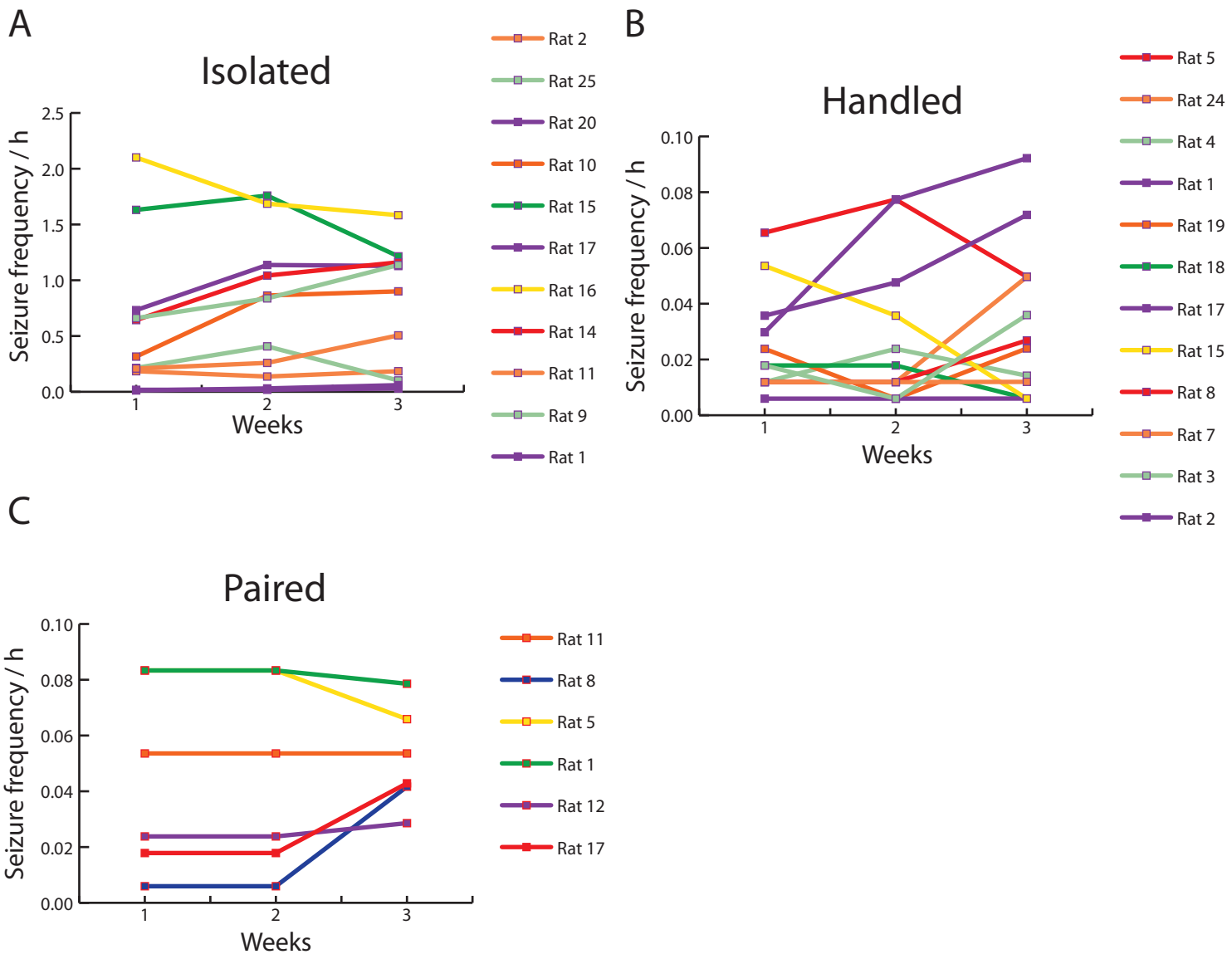
A
Isolated
Seizure frequency / h
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
Weeks
Rat 2
Rat 25
Rat 20
Rat 10
Rat 15
Rat 17
Rat 16
Rat 14
Rat 11
Rat 9
Rat 1
B
Handled
Seizure frequency / h
0.10
0.08
0.06
0.04
0.02
0.00
1
2
3
Weeks
Rat 5
Rat 24
Rat 4
Rat 1
Rat 19
Rat 18
Rat 17
Rat 15
Rat 8
Rat 7
Rat 3
Rat 2
C
Paired
Seizure frequency / h
0.10
0.08
0.06
0.04
0.02
0.00
1
2
3
Weeks
Rat 11
Rat 8
Rat 5
Rat 1
Rat 12
Rat 17

Figure 9

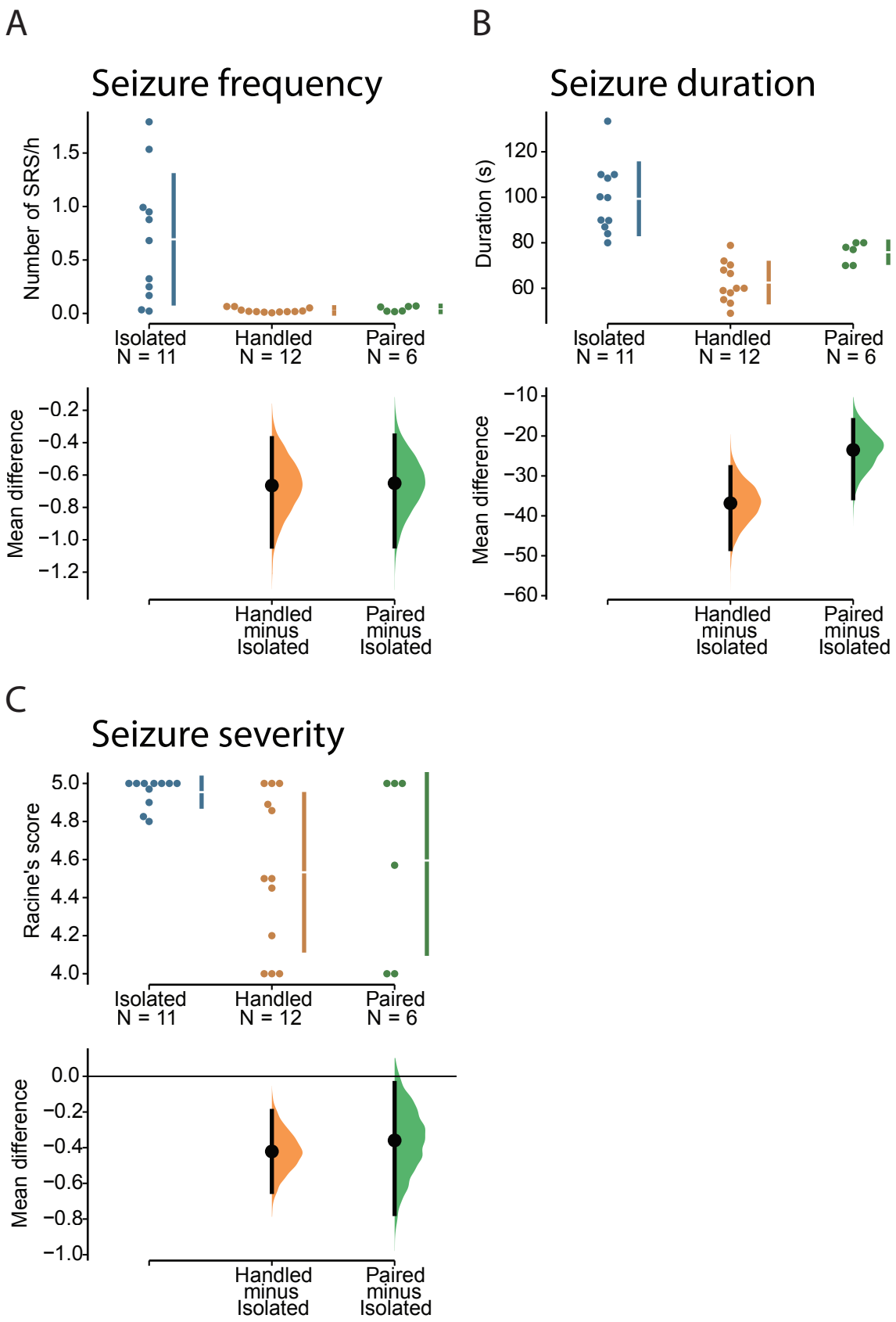
A
Seizure frequency
Number of SRS/h
1.5
1.0
0.5
0.0
Isolated
N = 11
Handled
N = 12
Paired
N = 6
Mean difference
−0.2
−0.4
−0.6
−0.8
−1.0
−1.2
Handled
minus
Isolated
Paired
minus
Isolated
B
Seizure duration
Duration (s)
120
100
80
60
Isolated
N = 11
Handled
N = 12
Paired
N = 6
Mean difference
−10
−20
−30
−40
−50
−60
Handled
minus
Isolated
Paired
minus
Isolated
C
Seizure severity
Racine's score
5.0
4.8
4.6
4.4
4.2
4.0
Isolated
N = 11
Handled
N = 12
Paired
N = 6
Mean difference
0.0
−0.2
−0.4
−0.6
−0.8
−1.0
Handled
minus
Isolated
Paired
minus
Isolated

Figure 10