Effects of single cage housing in the rat and mouse pilocarpine models of epilepsy

Keeping social contact in experimental epilepsy

Manouze H[1,2], Ghestem A[2], Poillerat V[3], Bennis M[1], Ba-M'hamed S[1], Benoliel JJ[3,4], Becker C[3*], Bernard C[2*]

[1]Lab of Pharmacology, Neurobiology & Behavior (URAC-37), Cadi Ayyad University, Marrakech, Morocco

[2]Aix Marseille Univ, Inserm, INS, Institut de Neurosciences des Systèmes, Marseille, France

[3]Université Paris Descartes, Sorbonne Paris Cité, Faculté de Médecine, F-75006, Paris, France

[4]AP-HP, Hôpital de la Pitié-Salpêtrière, Service de Biochimie Endocrinienne et Oncologique, F-75013, Paris, France

CBer, CBec, AG and JJB Designed Research; HM, AG, VP and MB Performed Research and Analyzed data; CBer, CBec, SBM and JJB Wrote the paper

Corresponding author: Christophe.bernard@univ-amu.fr

* Equally contributing last author

2 Figures, 1 Table

150 words in the abstract

106 words in the Significance Statement

399 words in the Introduction

494 words in the Discussion

Acknowledgments

This work was supported by the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement no602102 (EPITARGET), and Fondation pour la Recherche sur le Cerveau. HM was supported by NEUREN Project (PIRSES-GA-2012-318997).

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 housed individually for 6 weeks displayed anhedonia, anxiety and biological markers of stress as compared to mice housed 3 per cage. Pilocarpine-treated mice housed together showed increased levels anhedonia, anxiety and stress markers as compared to the control group. The differences were larger in pilocarpine treated mice housed individually. In the male rat pilocarpine model, we also found increased stress markers in isolated animals as compared to rats kept in pairs. Seizures were 16 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. This may be particularly relevant 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. This may be particularly relevant for preclinical studies.

KEY WORDS: Epilepsy, Stress, Social isolation, Single housing

Introduction

Social isolation (single housing) and barren environment are stressful to rodents; for a recent review cf. (Mumtaz et al., 2018). In fact, social isolation of rodents after weaning is often used as an experimental manipulation to model early life stress in humans. Social isolation for 8 weeks after weaning produces anxiety/depressive-like behaviour and cognitive deficits and favours the emergence of pathological traits such as addictive behaviour (Filipovic et al., 2017). These behavioural alterations are associated with biological modifications including oxidative stress, the production of stress hormones and inflammatory cytokines (Krugel et al., 2014; Shao et al., 2015; Butler et al., 2016; Filipovic et al., 2017). Therefore, many regulation agencies recommend social housing and environmental enrichment to improve 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 behaviour from others, to clearly identify individuals on the video, etc. Several studies have shown that environment enrichment has positive effects on seizure frequency or severity in experimental models (Morelli et al., 2014; Kotloski and Sutula, 2015; Dezsi et al., 2016; Vrinda et al., 2017), but the effect of social isolation starting from the beginning of the experimental procedure has not been assessed.

There exist many types of experimental models of epilepsy in different rodent species and strains (Levesque et al., 2016; Loscher, 2017; Becker, 2018). The effects of social isolation should be studied for each model, as results may be model-, strain- and species-

specific. For example, Wistar and Sprague Dawley rats display different phenotypes in terms of depression-like profile and cognitive deficits in the kainic acid and pilocarpine models (Inostroza et al., 2011; Inostroza et al., 2012). Here we focus on the widely used pilocarpine model of experimental epilepsy in adult male Swiss mice and Wistar rats (Levesque et al., 2016). Numerous studies show a dysregulation of the HPA axis in patients and in experimental epilepsy, which is model-dependent (Maguire and Salpekar, 2013; Wulsin et al., 2016; Mumtaz et al., 2018). Since stress also results in the activation of the HPA axis, we reasoned that social isolation in experimental models of epilepsy may produce a strong HPA axis response via the contributions of both -isolation and epilepsy-induced stress (Gunn and Baram, 2017). We also hypothesized that the combination of both factors may exacerbate the epilepsy phenotype as compared to animals maintaining social interactions. Clinical studies support this hypothesis. Stressful life events are associated with increased risk of seizure occurrence in patients with epilepsy (Baldin et al., 2017; Kotwas et al., 2017). In some patients, the fear to have a seizure and social isolation due to stigmatization may increase the allostatic load, with a direct impact on seizure frequency (Kotwas et al., 2017). We thus assessed the consequences of animal housing on stress response and epilepsy severity, acting on a single experimental variable: social interaction (environmental enrichment was provided). We also propose an alternate solution to maintain social contact in singly housed animals, to decrease their stress level. We hope to foster discussions on how to interpret results (including negative preclinical studies) obtained in different housing conditions.

METHODS

Animals

We used male Wistar rats (200 to 250g; Charles Rivers Laboratories, Les Oncins, France), aged of 9 weeks (at their arrival in laboratory). Swiss male mice (8 weeks old) were obtained

from the animal husbandry of the Faculty of Sciences, Cadi Ayyad University, Marrakech (Morocco). The animals were kept under controlled environmental conditions (23±1 °C; night-day cycle (12 h-12 h)) with *ad libitum* access to food and water. Zeitgeber (ZT) 0 was at 7:30 am (time when the light was switched on in the animal facility). All procedures were conducted in accordance with approved institutional protocols, and with the provisions for animal care and use prescribed in the scientific procedures on living animals, European Council Directive: EU2010/63and by the Council Committee of Research Laboratories of the Faculty of Sciences, Cadi Ayyad University, Marrakech. All efforts were made to minimize any animal suffering.

Experimental design

In mice

The experimental protocol is shown in Figure 1A. Swiss male mice were housed in groups of 3 per cage. In order to validate the stress state of same mouse throughout experimental protocol, one mouse from each litter was randomly picked, ear-marked and returned to its own cage. They were left together during one week without experimental intervention to allow adaptation to a novel housing environment. After one week, we performed different behavioral tests for each ear-marked mouse in order to obtain reference levels. Sucrose preference was assessed every day during 1 week. At the end of the week, we evaluated anxiety levels (Day-3: elevated plus maze – EPM) and performance in the novel object recognition (NOR) test (Day -2-1). Animals were then randomly assigned to two groups to receive a pilocarpine injection to trigger status epilepticus (pilo group– experimental procedure described hereafter) or saline (non-pilo group). Both groups were further divided into two groups: group-housing (social condition – SC, n =18, 3/cage) and one individual-housing condition (isolated condition – IC, n = 6, 1/cage). Individual housing used ear marked

mice. After 4 weeks exposure to different housing conditions, ear-marked mice from social and isolated conditions were tested for anhedonia (Day 46-53), EPM (Day 54) and NOR (55-56). Behavioral tasks (EPM and NOR) were recorded and analyzed using Ethovision®XTNoldus 8.5 video tracking program (Noldus, Netherlands) connected to a video camera (JVC). At the end of each behavioral session apparatuses were cleaned with a 75% ethanol solution to remove any of odor or trace. The behavioral tests were performed between 8:00 and 12:00 a.m. during the light cycle to avoid the circadian-related fluctuation in the performance of the mice. Before the beginning of behavioral tests, the animals were transferred to the testing room in their home cages and left 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 2A. 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 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 have no knowledge of stress history of the animals when we receive them, randomization decreases this confounding factor.

Sample size

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

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

Status epilepticus induction and electrode implantation

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

In rats, four weeks following SE, the telemetry implant was surgically inserted intraperitoneally under anesthesia (ketamine [1 mg/ kg]/xylasine [0.5 mg/kg] i.p) and connected to screws on the surface of the brain by two electrodes; one above the cortex (4.0 mm anteroposterior, 2.0 mm mediolaterally, compared with bregma), the second, above the cerebellum as reference. The EEG signal was transmitted by radio to a data acquisition and processing system (DSI). In the paired group, both animals developed epilepsy but only one rat was equipped with the telemetry system as two animals cannot be recorded simultaneously with EEG transmitters in the same cage, while the other was monitored with video only. Animals were left to recover during one week before switching on the transmitter.

Monitoring of spontaneous recurrent seizures

Continuous (24/7) video-EEG recordings started in the 6th week after SE, a period sufficient to reach stability in seizure frequency (Williams et al., 2009), and were stopped at week 10. We verified that animals displayed stable seizure activity, quantifying seizure frequency during each successive week. Spontaneous recurrent seizures were detected and quantified

using both visual inspections of the EEG and a semi-automatic way usingClampfit 10.2. All detected seizures were verified and reconfirmed using "NeuroScore" software. Video recordings were used to assess seizure severity according to Racine's scale during the light phase in the animal facility. Rats kept in pairs never had spontaneous seizures simultaneously, which allowed a correct assessment of seizure severity of the EEG monitored animal. Finally, keeping animals in pairs did not prevent/alter continuous EEG recordings in the equipped animals (no loss of signal).

Behavioral and biological parameters

Body weights of mice and rats were measured weekly at ZT 2.

Sucrose consumption test

In mice, the baseline for sucrose consumption was assessed every day for 1 week, 7 days after their arrival in laboratory. The sucrose preference was calculated at the 5th week following the separation and treatment. In rats, sucrose consumption was assessed at week 4 and week 8 following animal reception. Sucrose and water intakes were measured daily at ZT 2.

Briefly, animals were given a free choice between two bottles, one with 1% sucrose solution and another with tap water. The location of the bottles was alternated every day to prevent possible effects of side preference in drinking behaviour. The consumption of water and sucrose solution was estimated by weighting the bottles. For the paired, the volume of sweet water consumed by rat was taken as the total consumed volume divided by 2. For the social groups, the volume of sweet water consumed by mouse was taken as the total consumed volume divided by the total number of animals at each time. Sweet water

consumption corresponds to sucrose preference, which is calculated as a percentage of the volume of sucrose intake over the total volume of fluid intake using the following equation:

Sucrose preference= V (sucrose solution)/ (V (sucrose solution) +V (water)) X100%.

Elevated plus maze test

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

Novel object recognition test

The novel object recognition test is widely used to evaluate object recognition memory in rodents (Ennaceur and Aggleton, 1994; Reger et al., 2009; Gaskin et al., 2010). The apparatus consisted of an open field (50× 50 × 50 cm high) made of Plexiglas with the inside painted matt black. The objects to be discriminated were available as three plastic objects. 24h before testing, mice were first habituated in the open field arena in the absence of any object for 10min. During the training trial, two identical objects (approximately 10 cm) were placed in the back corner of the box. The mouse was then positioned at the midpoint of the wall

opposite to the objects and the total time spent exploring the two objects was recorded for 10 min. During the test trial (60 min after training trial), one object used during training was replaced with a novel object and both of them were placed in the middle of the back wall. The animal was then allowed to explore freely for 5 min and the time spent exploring of each object was recorded. The discrimination between the novel and familiar objects was calculated by the discrimination index [(time spent exploring the novel object - time spent exploring the familial object) / total time x 100)].

ACTH, corticosterone and BDNF levels

Isolated, handled and paired rats in the non-pilo group were killed by decapitation after light anesthesia with isoflurane at the beginning of the 8th week following the separation at ZT4 and at the 6thweeks after the separation and treatment at ZT4 for pilo and non-pilo mice. Animals were decapitated in a quiet separate room, one by one, with the bench cleaned between animals. Trunk blood was collected in less than 5 sec after decapitation in dry tube and EDTA tube in order to obtain serum and plasma samples, respectively. The plasma was prepared by a 15 min centrifugation at 1600g, 4°C. The ACTH concentration was determined according the manufacturer's instructions (Clinisciences, France). For corticosterone and BDNF levels, blood was centrifuged at 3500g for 10 min at 4°C and the serum was stored at 80°C until used. Corticosterone and BDNF concentrations were determined according to the manufacturer's instructions (Coger, Promega, France).

Statistical analysis

Statistical analysis was performed using Sigma Plot 11.0 software. All data is presented as mean ± SEM. The effect on body weight and anhedonia (rat) was measured using the ANOVA with repeated measures followed by Bonferroni *post-hoc* analysis. The biological

and seizures parameters were tested according to the one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test. The effects of group and pilo treatment on anxiety index, discrimination index, ACTH, BDNF and corticosterone were measured using the two-way ANOVA followed by Bonferroni *post-hoc* analysis. The effects on anhedonia was measured using the non-parametric *Mann–Whitney U*. The significance threshold was set at $p<0.05$.

RESULTS

We first tested the consequences of social isolation on behavioral parameters and stress markers in adult pilo and non-pilo mice (**Figure 1A**).

Repeated measures ANOVA showed a significant effect of time ($F_{(5,100)}$=128.08, p<0.0001[a]) on body weight evolution; while the experimental group ($F_{(3,20)}$ =2.23, p=0.11[a]) and the interaction of group and age ($F_{(15,100)}$ =1.18, p=0.29[a]) had no effect (**Figure 1B**). At the 4th and 5th week post-social isolation, *post-hoc* analysis showed that both pilo-SC and pilo-IC groups gained significantly less weight than the non pilo-SC and the non pilo-IC group. There was no significant difference between all groups until the 3rdweek. Thus, the pilo group gained less weight than the non-pilo group, but housing conditions had no effect in both groups.

We used the sucrose preference test to assess anhedonia (lack of interest in rewarding stimuli). The typical behaviour of animals is a bias toward the sweetened drink. Lack of preference for the sweetened drink indicates anhedonia, which signs stress-related disorders (Gold, 2015). At baseline (before changing housing conditions), animals from different groups (different cages) did not show differences in sucrose preference (**Figure 1C**). Four weeks later, our results demonstrated that mice injected by pilo consumed less of sweet water than mice injected with vehicle. In addition, mice in isolated condition had sweet water consumption lower than those in social condition (**Figure 1C**). From day 1 until day 7, the non-parametric *Mann–Whitney U* test, applied on sweet water consumption, revealed that the pilo-SC (day 1: U=0.00; Z=-10.89; p<0.01; day 7: U=0.00; Z=-9.35; p<0.01 [B-1]) and pilo-IC (day1: U=0.00; Z=-10.23; p<0.01; day 7: U=0.00; Z=-10.09; p<0.01 [B-1]) groups consumed significantly less of sweet water than the non pilo-SC and non pilo-IC groups. Additionally, the *Mann–Whitney U* showed that both non pilo-IC (day1: U=2.00, Z= -4.87, p<0.05; day 7: U=1.50; Z= -4.72; p<0.01 [B-1]) and pilo-IC (day 1: U=1.00; Z=-4.42; p<0.01; day 7: U=5.00;

Z= -5.00; $p<0.05$ [B-1]) groups had a significant decrease in sucrose preference as compared to non pilo-SC and pilo-SC groups, respectively. Moreover, the Mann–Whitney U test did not show any effect of day on sweet consumption ($p>0.05$). Therefore, social isolation induces anhedonia in the control (non pilo) group. The pilo group socially housed also displayed anhedonia as compared to the control group, but the anhedonia phenotype was increased in the socially isolated pilo group.

We then tested animals for anxiety-like behavior. All groups tested before separation had similar anxiety levels (**Figure 1D**). Two-way ANOVA demonstrated significant difference in anxiety index recorded among the factors group ($F_{(3,40)}=12.93$, $p<0.0001$[b]) and time ($F_{(1,40)}=64.32$, $p<0.0001$[b]) as well as the interaction of group × time ($F_{(3,100)}=11.84$, $p<0.0001$[b]). *Post-hoc* comparisons confirmed that the pilo-treated groups showed a significant increase in anxiety ($p<0.01$ and $p<0.001$) with respect to the non pilo groups (**Figure 1D**). In the isolated condition, the anxiety index was significantly larger in both pilo and non pilo mice as compared to mice in social housing ($p<0.05$). These results show that social isolation readily increases anxiety levels in the control group. The pilo group maintaining social contact displayed increased anxiety as compared to the control group (epilepsy effect), but anxiety was further exacerbated in the pilo isolated group (combining the effects of epilepsy and social isolation).

Next, we assessed the effect of social isolation on memory performance using the NOR test. All groups performed similarly at baseline before separation and treatment (**Figure 1E**). The two-way ANOVA test showed significant difference in the discrimination index among the factors group ($F_{(3,40)}=6.95$, $p=0.0007$[c]) and time ($F_{(1,40)}=43.30$, $p<0.0001$[c])as well as the interaction of group × time ($F_{(3,100)}=8.89$, $p<0.0001$[c]) (**Figure1E**).The discrimination index for the pilo-treated group was notably lower than that of the non pilo group ($p<0.0001$). However, there was no significant effect of housing conditions in NOR performance in either

pilo or non pilo groups. Hence, the cognitive deficits in the NOR test are only due to the epilepsy condition.

Finally, we looked at the effect of social isolation on biological markers. For ACTH and corticosterone, two stress-related hormones, two-way ANOVA analysis showed a significant effect of treatment ($F_{(1,20)}$=74.62, p<0.0001; $F_{(1,20)}$=117.3, p<0.0001[d,e]) and social isolation ($F_{(1,20)}$=20.55, p=0.0002[d,e]; $F_{(1,20)}$=18.56, p=0.0003[d,e]) (**Figure 1F**). Our results showed a significant increase of ACTH and corticosterone levels in pilo as compared to non pilo mice (p<0.001). Moreover, this increase was observed in isolated condition as compared to social housing (p<0.05).

Strong stressful situations can trigger a state of vulnerability to depression in some individuals, which can be identified with low serum BDNF levels (Blugeot et al., 2011; Becker et al., 2015). We thus assessed serum BDNF in the different groups. Although serum BDNF level was not affected in the pilo group as compared to the non pilo group when animals were socially housed ($F_{(1,20)}$=0.47, p=0.5011[f]); social isolation had a significant effect ($F_{(1,20)}$=16.96, p=0.0005[f]) in both pilo and non pilo groups (**Figure 1F**). *Post-hoc* comparison confirmed that isolated mice showed a significant decrease in BDNF (p<0.05) when compared to socially housed mice. There was no significant difference between pilo and non pilo groups.

Together, the results obtained in control (non pilo) mice demonstrate that social isolation has a strong effect on many behavioral and biological stress indicators. They recapitulate the main features of social isolation performed after weaning to model early life stress (Mumtaz et al., 2018). In animals maintained in groups, the epilepsy had a direct effect on stress indicators. These effects were exacerbated in singly housed pilo mice. We conclude that social isolation produces a stronger stress phenotype (with the tests used here) in the pilo mouse experimental model of epilepsy. The lack of EEG recording devices for mice

prevented us to test the effect of social isolation on epilepsy severity. This issue was investigated in the pilocarpine rat model.

Because of their larger size, it was not technically possible to maintain rats in small colonies while performing wireless 24/7 recordings. For the social group, animals were kept as pairs (only one was equipped with wireless transmitter, but both received pilocarpine injection). We added a third group (handled) of singly housed animals but which could interact with the experimenter everyday (see methods). The rationale for this group is that some EEG studies are performed with wired systems, which precludes group housing, as animals tend to severe the wires. We first analysed general stress markers in control (non pilo) animals to determine whether social isolation had similar effects to those described in mice. The ANOVA repeated measures showed a significant effect of social condition ($F_{(2,17)}$=41.58, p<0.0001[g]) and time ($F_{(6,102)}$ =266.83, p<0.0001[g]) on the gain of body weight (**Figure 2B**). At the 4th week, *post-hoc* analysis showed that the isolated group gained significantly less weight than the handled and the paired group. There was no significant difference between all groups from the 1st to the 3th week.

We used the sucrose preference test to assess anhedonia. ANOVA repeated measures showed a main effect of social isolation on sweet water consumption (**Figure 2C**) already during the third week ($F_{(2,12)}$=21.66, p<0.0001[h]) up to the last week ($F_{(2,12)}$=97.85, p<0.0001[i]) 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, p>0.05 ; respectively [h,i]). During the third and the last week, *post-hoc* analysis revealed that sweet water consumption was significantly lower in the isolated than in the handled (p<0.01, p<0.001) and paired groups (p<0.05, p<0.01 and p<0.001). Thus, just two weeks of social isolation is sufficient to produce a state of anhedonia.

We then tested stress-related ACTH and corticosterone hormones. One-way ANOVA revealed a significant effect of social isolation on hormone levels (ACTH: $F_{(2,17)}$=5.05,

$p<0.02^{j}$; corticosterone: $F_{(2,17)}=8.42$, $p<0.01^{j}$; **Figure 2D**). The *post-hoc* analysis showed that ACTH and corticosterone levels were significantly increased in the isolated group as compared to the handled (t=2.69, p<0.05 and t=3.57, p<0.01; respectively) and paired (t=2.93, p<0.05; t=3.70, p<0.01; respectively) groups.

Finally, we assessed serum BDNF levels. One-way ANOVA revealed a significant effect of social isolation on BDNF levels ($F_{(2,17)}= 6.18$, $p<0.01^{l}$). BDNF levels were significantly lower in the isolated group as compared to the handled (t=2.38, p<0.05) and paired (t=3.47, p<0.01) groups (**Figure 1D**). There was no significant difference between the handled and paired groups in all tested biological parameters (ACTH: t=0.18; corticosterone: t=0.42 and BDNF: t=1.02, p>0.05).

We conclude that social isolation in the strains of control rats and mice used here produces aphenotype associated with stress. We then compared how different housing conditions influence the epilepsy phenotype.

One-way ANOVA analysis showed a significant difference between groups for seizure parameters (frequency, duration and severity) ($F_{(2,26)}=10.73$, $p<0.001$; $F_{(2,26)}=30.90$, $p<0.0001$; $F_{(2,26)}=4.07$, $p<0.05$; respectively ^{m,n,o}) (**Figure 3**). Figure 3-1 shows seizure frequency for each week for each experimental group, and for each individual animal. *Post-hoc* analysis showed that the seizure frequency and duration were considerably increased in the isolated group as compared to the handled and paired groups (handled: t=4.29 and t=7.82, p<0.001; paired: t= 3.45 and t=4.07, p<0.01; respectively). The handled group significantly demonstrated lower Racine scores (seizure severity) as compared to the isolated group (t=2.72, p<0.05); whereas, there was no significant difference between the paired and isolated groups (t=1.94, p>0.05). We only assessed the severity of the seizures during the light phase in the animal facility. Finally, there was no significant difference between the handled and paired groups for seizure frequency and seizure severity. Thus, lack of social interaction

dramatically increased the seizure phenotype, with seizures being 16 times more frequent in isolated animals as compared to the those socially housed or those having daily interaction with experimenters.

DISCUSSION

Our results show that single housing exacerbates the phenotype in Sprague Dawley rats and Swiss mice in the pilocarpine experimental model of epilepsy. It is important to note that daily social interaction with the experimenter was sufficient to prevent the development of a severe epilepsy phenotype in rats although they were singly housed. It was as efficient as keeping animals in pairs. Although we could not quantify it, we noted that isolated pilo animals were very aggressive and displayed escaping behaviour when cages were changed every week. Their cages were close to one another, suggesting that visual contact was not sufficient to prevent the effect of social isolation. Abnormal reaction to handling is a direct consequence of single housing (Hatch et al., 1965). In contrast, rats and mice socially housed (or in daily contact with an experimenter) remained calm when handled. In the laboratory, we are also performing electrophysiological recordings with high density silicon probes in experimental rat models of epilepsy. The equipment is protected with a copper mesh hat, which precludes social housing. We are using large cages, divided in two, separated by a grid allowing physical interaction. Each compartment contains one animal with epilepsy. Animals can make nose contact and interact with their whiskers through the bars. Animals do not show any sign of anxiety, and wire connectors are easily inserted, as in control animals (no struggle). This type of housing allows wired recordings in rats and mice with epilepsy, whilst maintaining a certain level of social interaction.

Our data on control animals confirms the large body of literature reporting that social isolation constitutes a major stressful situation that can lead to a depression-like profile (Mumtaz et al., 2018). Among the numerous markers that can be assessed, we focused on general ones such ACTH, corticosterone and anhedonia. When rats and mice are singly housed, ACTH and corticosterone levels are increased (Veenema et al., 2005; Djordjevic et al., 2012). In pilocarpine experimental models of epilepsy, ACTH and corticosterone levels are also increased (Mazarati et al., 2009; Inostroza et al., 2012; Ngoupaye et al., 2013). A direct comparison of ACTH and corticosterone values between studies is difficult as they depend upon the time of collection and the way blood is collected (*in vivo* or *post mortem* as done here). Despite the differences in experimental procedures, all results are qualitatively similar (increase due social isolation or epilepsy). To the best of our knowledge, we here provide the first evaluation of the contribution of both factors: social isolation and epilepsy. We demonstrate that epilepsy in socially housed mice produces an activation of the HPA axis and anhedonia, responses which are amplified in isolated mice with epilepsy. We found the same exacerbation in the rat experimental model between isolated and paired rats. A strong activation of the HPA axis in isolated animals with epilepsy may contribute to the expression of co-morbidities such as anxiety, cognitive deficits and depression-like behaviour.

In rats with epilepsy, we found that spontaneous seizures were 16 times more frequent in isolated animals as compared to animals kept in pairs or singly housed animals but with daily interactions with experimenter. During the 3 weeks of continuous recordings (starting 6 weeks after pilocarpine-induced status epilepticus), we did not find an increase in seizure frequency (Figure 3-1), suggesting that a global steady state activity had been reached (Williams et al., 2009). Interictal activity and seizures follow a multidien rhythm in the pilocarpine experimental rat model of epilepsy (Baud et al., 2019) as in humans (Baud et al., 2018). The fact that seizures occur at specific phases of this rhythm is not a confounding

factor as its frequency is between 5 and 7 days in this rat model (Baud et al., 2019), which means that we captured at least 3 cycles. In the present study, isolated animals had a high seizure frequency (≈16/day). It is difficult to compare this value with the existing literature as housing conditions are rarely mentioned. In addition, the beginning (how long after status epilepticus) and duration of 24/7 EEG recordings vary from one study to another. In the rat pilocarpine model, typical values for singly housed animals in Sprague Dawley rats are: 3/day during the early phase of epilepsy (Behr et al., 2017), 8/day (Paolone et al., 2019), 1-13/day (Tai et al., 2017), 10/day (Bankstahl et al., 2012); similar to values reported in Wistar rats: ≈4.5/day (Bajorat et al., 2011; Bajorat et al., 2018). Our study, although on the high end, remains in the same order of the magnitude as previously published work (we limited our analysis of the literature to the ongoing decade). We evaluated seizure severity only during the light phase, which constitutes a limitation of the study. We cannot rule out that seizures occurring during the dark phase may be less severe (including subclinical electrographic seizures).

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

The field of epilepsy research makes use of many different experimental models, strains and species. Results are clearly model-, strain- and species-specific, which may explain some conflicting results. We propose that another factor to consider is housing conditions. Some patients with epilepsy may experience social isolation, which correlates

with more severe forms of epilepsy (Kotwas et al., 2017). Results obtained in experimental animal models kept in isolation may be highly relevant to such patient situation, or any other situation associated with high levels of stress. Results obtained in socially housed animals may be relevant to other subsets of patients, with a less severe phenotype. Our results may have important consequences in terms of data interpretation, in particular when testing anti-epileptogenesis or anti-seizure strategies. When animals are kept in isolation, two intermingled factors need to be taken into account: social isolation-induced stress and epilepsy. Different interpretations can thus be provided to the existing body of literature in experimental epilepsy. Some studies report a large decrease in seizure frequency when animals are treated with a given compound. It is possible that the compound may have targeted the isolation-dependent stress component of the phenotype and not epilepsy itself. A reduction in seizure frequency by a factor of 16 would be equivalent to the difference we report between paired and isolated animals. For example, inhibition of kelch like ECH associated protein 1 (Keap1) reduces seizure frequency by a factor 20 in isolated kainic acid-treated Sprague-Dawley animals (Shekh-Ahmad et al., 2018). Keap1 controls nuclear factor erythroid 2-related factor 2 (Nrf2), a key regulator of antioxidant defence mechanisms. The authors suggest that these results support “the hypothesis that reactive oxygen species generation is a key event in the development of epilepsy” (Shekh-Ahmad et al., 2018). Since social isolation produces reactive oxygen species (Filipovic et al., 2017), it is possible that activating antioxidant defence mechanisms may have targeted the isolation-induced component of the seizure phenotype. Indeed, treating animals with a strong antioxidant had no effect on seizure frequency in the kainic acid rat model when animals interacted daily with experimenters, whilst the treatment was very efficient when animals are characterized by high levels of reactive oxygen species before status epilepticus (in press). These considerations provide another possible interpretation of the Keap1 data (Shekh-Ahmad et al., 2018).

However, their conclusion that antioxidant treatment is strongly disease modifying in the context of social isolation is not only valid but also highly clinically relevant to socially isolated patients. Finally, many (often unpublished) preclinical studies performed in isolated animals did not evidence any significant effect on seizure frequency. Since the social isolation stress component could act as a confounding factor producing a strong phenotype, there may be false negative results; i.e. the tested drugs may be very efficient in experimental animals maintaining social interaction.

Although we cannot claim that maintaining social interaction between rodents in laboratory conditions corresponds to “normality” (as in the wild), single housing does produce strong biological alterations that render data interpretation more complex, in both physiological and pathological conditions. We recommend that material and methods should systematically report housing conditions, and that all efforts should be made to maintain social interaction. Finally, these results were obtained in one experimental model (pilocarpine), in two species, one strain for each species and only in males. Similar studies should be performed in females, other strains and models such kainic acid, kindling, tetanus toxin etc. Our results and conclusions should not be seen as a criticism of previous works. Rather varying housing conditions provide a unique opportunity to study, with the same experimental model, different situations found in patients with high and low stress levels.

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Figure Legends

Figure 1: Effect of social isolation conditions on behavioral and biological parameters in mice injected or not with pilocarpine. (A) Experimental protocol in non-pilo and pilo mice. **(B)** The average body weight over 6 weeks, (**C**) the sweet water consumption measured during the pre and post-social isolation. **(D)** Anxiety-like behavior and memory function **(E)** are shown for isolated (IC) and social mice (SC) of the pilo and non-pilo group. (**F**) The levels of ACTH concentration, serum corticosterone and serum BDNF are shown for isolated (n= 6) and social group (n=6) of the non-pilo and pilo mice group. Data are mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p< 0.001$ in comparison with the vehicle group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ in comparison with social condition group. $^{\S\S}p<0.01$ and $^{\S\S\S}p<0.001$ compared to the baseline.

Figure 2: Effect of social isolation conditions on behavioral and biological parameters in rats. (A) Experimental protocol in non-pilo and pilo rat groups. **(B)** The average body weight over seven weeks, (**C**) the sweet water consumption measured during the first and last week, and **(D)** the levels of ACTH concentration, serum corticosterone and serum BDNF levels are shown for isolated (n= 5), interaction (handled, n= 5) and paired (n= 10 [5 couples]) rats of the non-pilo group. The isolated group displayed strong behavioral and biological alterations as compared to the two other groups. Data are mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ in comparison with the isolated group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ in comparison with the handled group.

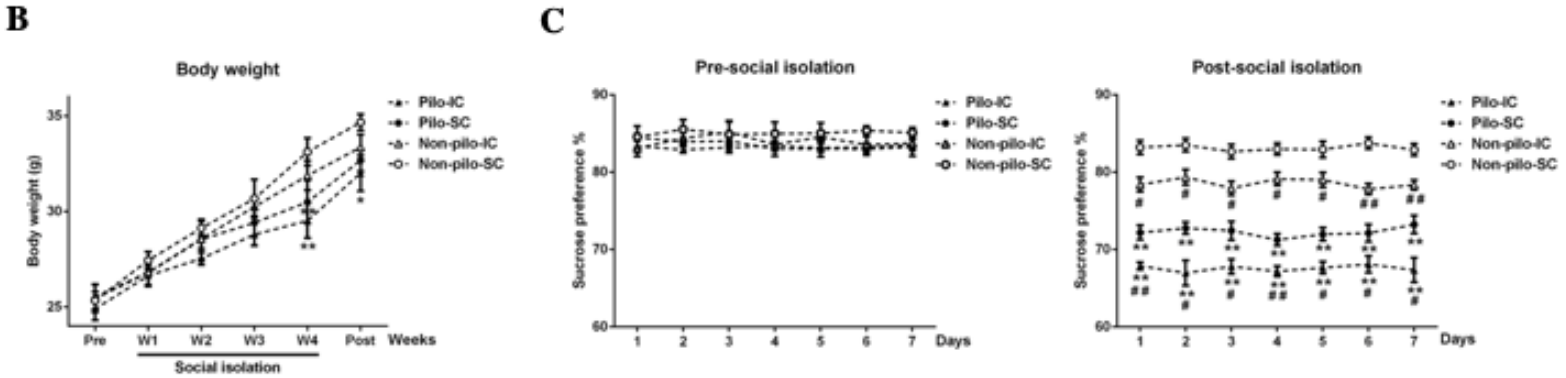
Figure 3: **Effect of social isolation conditions on spontaneous seizures in rats**. The average seizure frequency (number of seizures per hour), duration and severity are shown for isolated, interaction (handled) and paired rats (n=11, n=12 and n=6) of the pilo group. Isolated rats

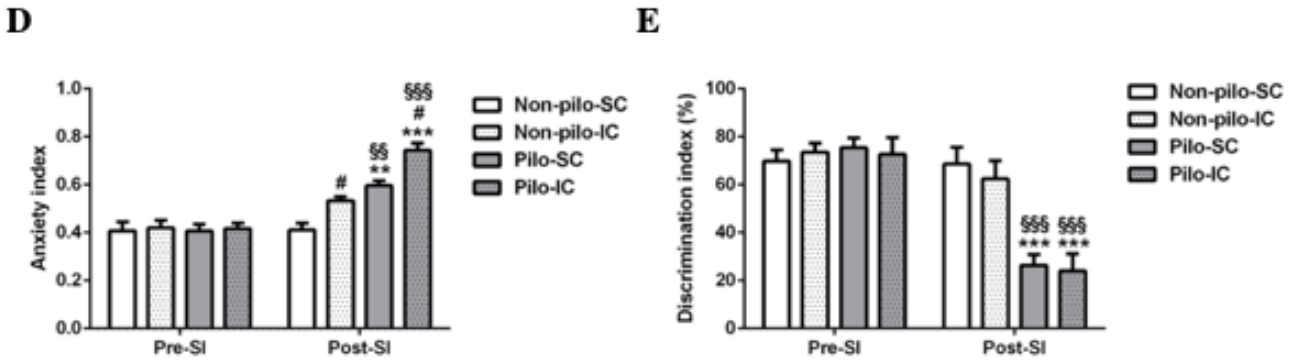
displayed a very severe epileptic phenotype as compared to the other groups. Values are expressed as mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001 in comparison with the isolated group.

Figure 3-1: Distribution of seizures per week and for each animal. Top left panel. The average seizure frequency (seizures per hour) over the three weeks of EEG recording is shown for isolated (n=11), handled (n=12) and paired rats (n=6). No significant difference was shown between weeks in seizure frequency for all experimental groups. Other panels show the average seizure frequency per week for each animal. Two animals (out of 11) in the isolated group had a seizure frequency similar to that found in paired or handled animals (0.03/h).

Data are mean ± SEM. ANOVA two way followed by Bonferroni *post-hoc*, *p<0.05 and **p<0.01 refers to handled or paired groups vs. isolated group comparison.

A
Anhedonia test
EPM test
NOR test
Anhedonia test
EPM test
NOR test
-10
-4
-3
-2
-1
0
7
14
21
28
35
36
37
38
39
Days
Pilo or NaCl injection + Separation
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
Pre-social isolation
Post-social isolation
Sucrose preference %
Days


D
Anxiety index
Pre-SI
Post-SI
E
Discrimination index (%)
Pre-SI
Post-SI
Non-pilo-SC
Non-pilo-IC
Pilo-SC
Pilo-IC

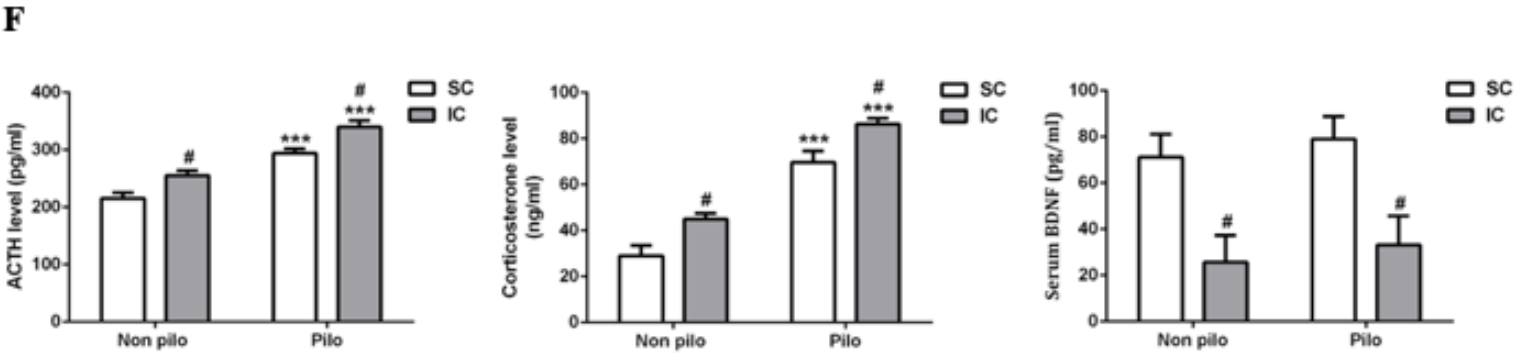

F
ACTH level (pg/ml)
Corticosterone level (ng/ml)
Serum BDNF (pg/ml)
Non pilo
Pilo
SC
IC

FIGURE 1

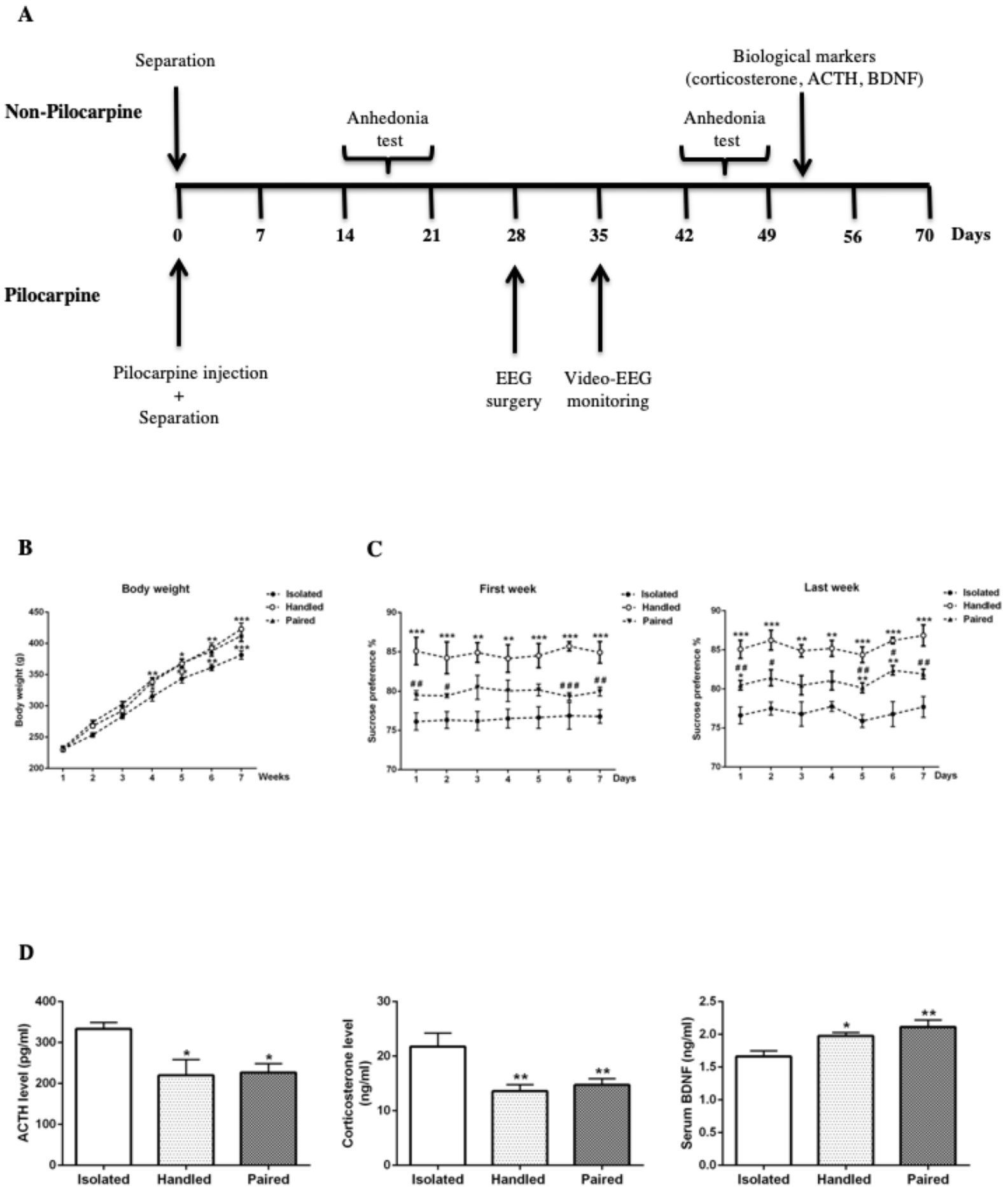
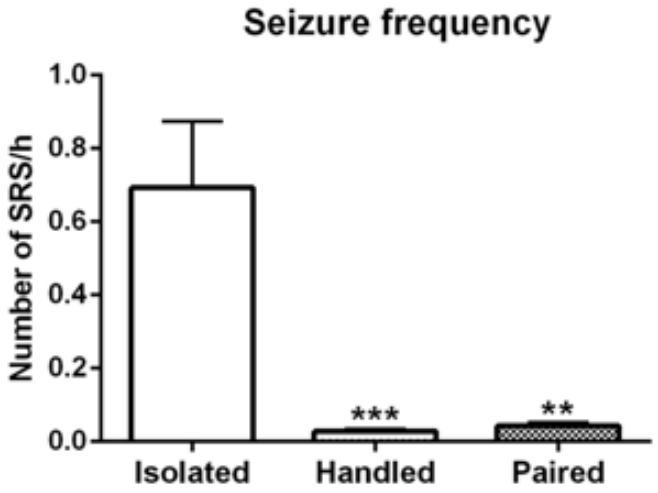
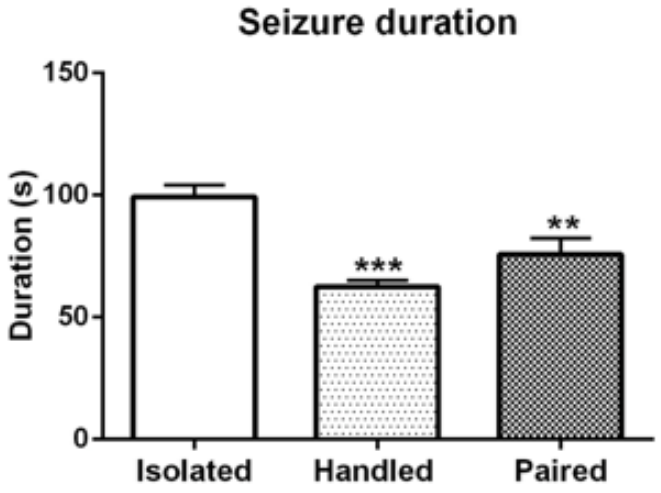

A
Separation
Non-Pilocarpine
Anhedonia test
Biological markers (corticosterone, ACTH, BDNF)
Anhedonia test
0
7
14
21
28
35
42
49
56
70
Days
Pilocarpine
Pilocarpine injection + Separation
EEG surgery
Video-EEG monitoring
B
Body weight
Isolated
Handled
Paired
Body weight (g)
Weeks
C
First week
Last week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

FIGURE 2


Seizure frequency
Number of SRS/h
1.0
0.8
0.6
0.4
0.2
0.0
***
**
Isolated
Handled
Paired


Seizure duration
Duration (s)
150
100
50
0
***
**
Isolated
Handled
Paired

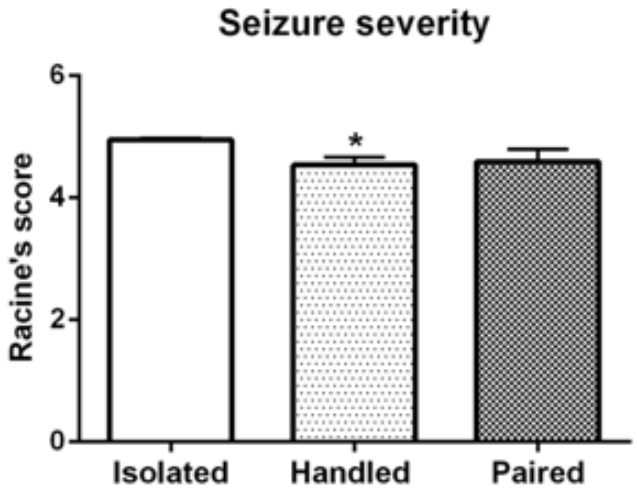

Seizure severity
Racine's score
6
4
2
0
*
Isolated
Handled
Paired

FIGURE 3

A- Statistical analysis of the effects of mice groups (grouped/isolated) and treatment (Veh and pilo) on behavioral, biological and biochemical parameters:

DATA	DATA STRUCTURE (Normality test)	TYPE OF TEST	POWER
Body weight (a)	yes	ANOVA with repeated measures Post hoc test :Bonferroni test	Group effect: F(3,20) = 2.23, P = 0.11 Time effect: F(5,100) = 128.08, P < 0.0001 Interaction: F(15,100) = 1.18, P = 0.29 (CI = 95%)
EPM (b)	yes	ANOVA with repeated measures Post hoc test :Bonferroni test	Group effect: F(3,40) = 12.93, P < 0.0001 Time effect: F(1,40) = 64.32, P < 0.0001 Interaction: F(3,40) = 11.84, P < 0.0001 (CI = 95%)
NOR (c)	yes	ANOVA with repeated measures Post hoc test :Bonferroni test	Group effect: F(3,40) = 6.95, P = 0.0007 Time effect: F(1,40) = 43.30, P < 0.0001 Interaction: F(3,40) = 8.89, P < 0.0001 (CI = 95%)
ACTH (d)	yes	Two way ANOVA Post hoc test : Bonferroni test	Group effect: F(1,20) = 20.55, P = 0.0002 Treatment effect: F(1,20) = 74.62, P < 0.0001 Interaction: F(1,20) = 0.09, P = 0.75 (CI = 95%)
Corticosterone (e)	yes	Two way ANOVA Post hoc test :	Group effect: F(1,20) = 18.56,

		Bonferroni test	P = 0.0003 Treatment effect: F(1,20) = 117.3, P < 0.0001 Interaction: F(1,20) = 0.006, P = 0.93 (CI = 95%)
BDNF (f)	yes	Two way ANOVA Post hoc test : Tukey test	Group effect: F(1,20) = 16.96, P = 0.0005 Treatment effect: F(1,20) = 0.4696, P = 0.5011 Interaction: F(1,20) = 0.0002, P = 0.9889 (CI = 95%)

B-Statistical analysis of the group (1) and day effects (2) on sucrose preference in mice using non-parametric *Mann–Whitney U* test after 4 weeks of isolation.

B-1	**Non pilo-IC vs Non pilo-SC**	**Pilo-IC vs Pilo-SC**	**Pilo-SC vs Non pilo-SC**	**Pilo-IC vs Non pilo-IC**
Day 1	U= 2.00; Z= -4.87; P= 0.0108 *	U= 1.00; Z=-4.42; P=0.0043**	U= 0.00 ; Z=-10.89; P= 0.0022**	U=0.00 ; Z=-10.23; p=0.0022**
Day 2	U= 4.00; Z= -3.96; P= 0.0238*	U= 2.50; Z= -5.56; P=0.0108*	U= 0.00 ; Z= -10.63 P= 0.0022**	U=0.00; Z=- 12.49 p= 0.0022**
Day 3	U= 3.00; Z=- 4.75 P=0.0152*	U= 4.5; Z= -4.98; P= 0.0303*	U= 0.00 ; Z= -10.17; P= 0.0022**	U= 0.00; Z= -10.20 p=0.002**
Day 4	U= 2.50; Z= -3.31 P= 0.0108*	U= 2.00 ; Z=-4.60; P=0.0087**	U= 0.00; Z=- 12.37; P= 0.002**	U= 0.00; Z=-11.77 p=0.002**
Day 5	U= 4.00; Z=-3.91 P=0.0238*	U= 2.00;Z=-4.44; P=0.0108 *	U=0.00; Z=-10.84; P= 0.0022**	U= 0.00; Z=-11.74; p= 0.0022**
Day 6	U= 0.00; Z=-5.84 P= 0.0022**	U= 5.00 ; Z=-4.06; P=0.0390*	U= 0.00 ; Z=-11.21 P= 0.0022**	U= 0.00; Z=-9.82; p= 0.0022**

Day 7	U= 1.50; Z= -4.72 P= 0.0065**	U=5.00;Z=-5.00; P=0.0390*	U= 0.00; Z=-9.35 P= 0.0022**	U= 0.00; Z=-10.09 p= 0.0022**

B-2	**Day 2 vs day 1**	**Day 3 vs day 1**	**Day 4 vs day 1**	**Day 5 vs day 1**	**Day 6 vs day 1**	**Day 7 vs day 1**
Non-pilo-SC	U= 14.50 ; Z=0.11 ; P=0.6147	U=16.50 : Z=-0.26 ; P=0.8528	U=15.00 ; Z=-0.27 ; P=0.6861	U=16.50 ; Z=-0.13 ; P=0.8355	U=15.00 ; Z=-0.27 ; P=0.6688	U=16.50 ; Z=-0.16 ; P=0.8550
Non-Pilo-IC	U=14.00 ; Z=1.18 ; P=0.5714	U=16.00 ; Z=-0.34 ; p=0.7900	U=13.00 ; Z= 0.90 ; P=0.4567	U=14.50 ; Z=0.72 ; P= 0.6234	U=16.50 ; Z=-0.40 ; P=0.8506	U=17.50 ; Z=0.09 ; P=0.9848
Pilo-SC	U= 14.50 ; Z= 0.53 P= 0.6212	U=16.50 ; Z= 0.37 ; P=0.8550	U=14.00 ; Z=-1.19 ; P= 0.5584	U=16.00 ; Z= -0.22 ; P= 0.7944	U=17.50 ; Z=0.00 ; P= 0.9784	U=13.00 ; Z= 0.84 : P= 0.4632
Pilo-IC	U=16.00 ; Z= -0.45 ; P= 0.7965	U=16.50 ; Z= 0.20 ; P= 0.8528	U=12.00 ; Z=-0.92 P= 0.3896	U=15.50 ; Z=-0.11 ; P=0.7403	U=15.00 ; Z= 0.33 ; P= 0.6732	U=15.00 ; Z=0.32 ; P=0.6753

C: Statistical analysis of the effects of rat groups (isolated/handled/paired) on behavioral, biological, biochemical and parameters

DATA	**DATA STRUCTURE (Normality test)**	**TYPE OF TEST**	**POWER**
Body weight (g)	yes	ANOVA with repeated measures Post hoc test :Bonferroni test	Group effect: F(2,17) = 41.58, P < 0.0001 Time effect: F(6,102) = 266.83, P < 0.0001 Interaction: F(12,102) = 2.10, P = 0.02 (CI = 95%)
Anhedonia First week (h)	yes	ANOVA with repeated measures Post hoc test :Bonferroni test	Group effect: F(2,12) = 21.66, P < 0.0001 Time effect: F(6,72) = 0.11, P = 0.99 Interaction: F(12,102) = 0.16, P = 0.99 (CI = 95%)
Anhedonia Last week (i)	yes	ANOVA with repeated measures Post hoc test :Bonferroni test	Group effect: F(2,12) = 97.85, P < 0.0001 Time effect: F(6,72) = 1.09,

			P = 0.37 Interaction: F(12,72) = 0.13, P = 0.99 (CI = 95%)
ACTH (j)	yes	One way ANOVA Post hoc test : Bonferroni test	F(2,17) = 5.05, P= 0.019 (CI = 95%)
Corticosterone (k)	yes	One way ANOVA Post hoc test : Bonferroni test	F(2,17) = 8.42, P = 0.0029 (CI = 95%)
BDNF (l)	yes	One way ANOVA Post hoc test : Bonferroni test	F(2,17) = 6.18, P = 0.0096 (CI = 95%)
Seizure frequency (m)	yes	One way ANOVA Post hoc test : Bonferroni test	F(2,26) = 10.73, P = 0.0004 (CI = 95%)
Seizure duration (n)	yes	One way ANOVA Post hoc test : Bonferroni test	F(2,26) = 30.90, P < 0.0001 (CI = 95%)
Seizure severity (o)	yes	One way ANOVA Post hoc test : Bonferroni test	F(2,26) = 4.07, P = 0.028 (CI = 95%)