On the difficulty to interpret results when animals are singly housed: experimental epilepsy as a prototypical example

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ABSTRACT

Many experimental approaches require housing rodents in individual cages, a stressful condition for social animals, which may act as a confounding factor for data interpretation. Comparing three groups of Wistar rats - singly housed, singly housed with daily social interaction, and animals kept in pairs - we found that socially isolated animals displayed classical markers of behavioral stress and anhedonia. We then investigated how social isolation may impact the development of epilepsy, a prototypical experimental condition requiring single housing for seizure monitoring. We found that seizures were 25 times more frequent in singly housed animals without social interaction as compared to the other two groups. Isolated animals also showed increased oxidative stress levels. Data obtained from singly housed animals without social interaction is difficult to interpret because results are obtained in a context of behavioral and oxidative stress. Preclinical studies may be strongly affected by such confounding factor.

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

Introduction

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 behavior and cognitive deficits, also the emergence of pathological traits such as addictive behavior. These behavioral alterations are associated with biological modifications such as oxidative stress, the production of stress hormones and inflammatory cytokines (Krugel et al., 2014, Shao et al., 2015, Butler et al., 2016). Single housing of animals is also done to obtain accurate measurements of biological parameters in individual animals, in particular in preclinical studies, such as in the fields of toxicology, drug addiction, and neurological disorders. Although social isolation is done for practical purposes and not for modeling early life stress, it is possible that biological alterations resulting from single housing may act as confounding factors in such studies; an issue that remains to be investigated. For example, how does an underlying state of oxidative stress, inflammation etc. due to social isolation impact the development of a neurological disorder or the efficacy of its therapeutic treatments? In other words, what are the consequences of a perturbed homeostasis resulting from single housing on the interpretation of results?

Epilepsy research provides an ideal condition to address this issue. Continuous video-EEG recordings in singly housed animals are usually performed to monitor seizure activity. Single housing may increase stress levels thus interfering with epilepsy itself and its co-morbidities (depression and cognitive deficits). Clinical studies clearly show that 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). Hence,

social isolation may act as a confounding factor and change the phenotype of epilepsy in experimental models. We thus assessed the consequences of animal housing on epilepsy severity and specific biological markers, acting on a single experimental variable: social interaction.

METHODS

Animals

Sixty three adult male Wistar rats (200 to 250g; Charles Rivers Laboratories, Les Oncins, France.), aged of 9 weeks (at their arrival in laboratory) were used in this study. 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).

Handling procedure and experimental groups

The experimental protocol is shown in **Figure 1A**. Animals were received in groups of 4 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 pairs with social interaction.

Handling was performed twice daily (ZT 2 and ZT 9) 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 assigned to the three housing groups when received. Animals from the pilo group were assigned after pilocarpine-induced status epilepticus. We used different timings for the non-pilo and pilo groups in order to compare the biological parameters in both groups at the same time (**Figure 1A**).

Status epilepticus induction and electrode implantation

Status epilepticus (SE) was induced by an i.p injection of pilocarpine (pilo) (320mg/kg) one week after receiving the animals from the vendor, a standard experimental procedure. 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 of SE. At the end of these injections, the rats were hydrated with saline (2ml i.p. twice within 2 h) and fed with baby food until they resumed normal feeding behavior. All drugs were obtained from Sigma.

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 hippocampus (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 9 weeks. We verified that animals displayed stable seizure activity, quantifying seizure frequency during each successive week. Seizure frequency was similar for each week of the recording period (not shown). Spontaneous recurrent seizures were detected and quantified using both visual inspections of the EEG and a semi-automatic way using Clampfit 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 (1972). 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 in the non-pilo group

Body weight

Bodyweights were measured weekly at ZT 2.

Sucrose consumption test

Experiments were performed as described by (Becker et al., 2008) in non-pilo animals two weeks and six weeks following animal reception. Sucrose and water intakes were measured daily at ZT 2. Briefly, rats 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 behavior. The consumption of water and sucrose solution was estimated by weighting the bottles. For the paired group, the sweet water consumption was considered as the mean of volume consumed by both rats. 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%.

ACTH, corticosterone and BDNF levels

Isolated, handled and paired rats in the non-pilo group were killed by decapitation at the beginning of the eighth week. Trunk blood was collected 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).

Oxidative stress

Oxidative stress was evaluated in both pilo and non-pilo groups. The brains were immediately dissected on ice and the hippocampus removed, frozen in liquid nitrogen and stored at −80°C. In accordance with the manufacturer's instructions, the lipid peroxidation level was determined by measuring the level of thiobarbituric reactive species (TBARs) (Coger, France). This was expressed as nmol of MDA per mg of hippocampus (4 animals per group).

Statistical analysis

Statistical analysis was performed using SigmaPlot 11.0 software. All data is presented as mean ± SEM. The effect of social interaction on body weight and sucrose consumption was measured using the two-ways analysis of variance (ANOVA) with repeated measures followed by Holm-Sidak *post-hoc* analysis. The biological and seizures parameters were tested according to the one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple comparisons. A two-way ANOVA followed by *post-hoc* testing with the Student-Newman-Keuls multiple comparisons was used to measure variance of MDA level between groups. The significance threshold was set at $p<0.05$.

RESULTS

Since social isolation performed just after weaning produces important biological modifications, we first determined whether social isolation performed when adult animals (9 weeks old) were received from the vendor also produced biological alterations. We analyzed three groups of control animals: singly housed with no social interaction (isolated), singly housed with social interaction (handled), and animals kept

in pairs (paired). Two-ways ANOVA repeated measures showed a significant effect of social condition ($F_{(2,17)}$ =25.21, p<0.001) and time ($F_{(6,102)}$ =306.89, p<0.001) on the gain of body weight **(Figure 1B).** *Post hoc* analysis showed that the isolated group gained significantly less weight than the handled and the paired group at week 7 (t= 4.15, p<0.001 and t=3.77, p<0.01; respectively).

We used the sucrose preference test to assess anhedonia (lack of interest in rewarding stimuli). The typical behavior of animals is a bias toward the sweetened drink, whilst lack of preference for the sweetened drink indicates anhedonia. Anhedonia is present in stress-related disorders (Gold, 2015). Two-way ANOVA repeated measures showed a main effect of social isolation on sweet water consumption (**Figure 1C**) already during the third week ($F_{(2,17)}$=23.96, p<0.001) up to the last week ($F_{(2,17)}$=145.56, p<0.001) with no time effect in both weeks (first week: $F_{(6,102)}$ =0.11, p>0.05 and last week: $F_{(6,102)}$ = 1.01, p>0.05 ; respectively). 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<0.001) and paired groups (p<0.05, p<0.01 and p<0.001). There was no difference between the handled and paired groups. Thus, just two weeks of social isolation is sufficient to produce a state of anhedonia.

The state of anhedonia is related to stress-related ACTH and corticosterone hormones. One-way ANOVA revealed a strong effect of social isolation on hormone levels measured at the end of the 7th week (ACTH: $F_{(2,17)}$=5.05, p<0.05; corticosterone: $F_{(2,17)}$=8.08, p<0.01; **Figure1D**). *Post hoc* analysis showed that ACTH and corticosterone levels were significantly increased in the isolated group as compared to the handled (q=3.81, p<0.05 and q=5.14, p<0.01; respectively) and paired (q=4.14, p<0.01; q=5.06, p<0.01; respectively) groups. Together, these results demonstrate that animals experiencing social isolation display markers usually associated to stress.

We have recently demonstrated that vulnerability to depression is associated with decreased serum BDNF levels (Blugeot et al., 2011). In keeping with this, one-way ANOVA revealed a strong effect of social isolation on BDNF levels ($F_{(2,17)}$= 6.18, p<0.01). BDNF levels were significantly lower in the isolated group as compared to the handled (q=3.38, p<0.05) and paired (q=4.91, p<0.01) groups. There was no significant difference between the handled and paired groups in all tested biological parameters (ACTH: q=0.85; corticosterone: q=0.66 and BDNF: q=2.18, p>0.05). However, analysis of MDA levels did not reveal statistically significant increased oxidative stress in isolated animals (**Figure 2B**). Note that for the latter experiment, we did not collect brains from the paired group.

We conclude that lack of social interaction resulting from single housing has a strong impact on behavioral and physiological parameters. We then assessed how social isolation would impact experimental epilepsy.

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; $F_{(2,26)}$=30.56, p<0.001; $F_{(2,26)}$=4.28, p<0.05; respectively) (**Figure 2A**). *Post-hoc* analysis showed that seizure frequency and duration were considerably increased in the isolated group as compared to the handled and paired groups (handled: q=6.05 and q=10.99, p<0.001; paired: q= 4.87 and q=5.77, p<0.01 and p<0.001; respectively). The handled group significantly demonstrated lower Racine scores (seizure severity) as compared to the isolated group (q=3.93, p<0.05); whereas, there was no significant difference between the handled and isolated groups (q=2.84, p>0.05). Finally, there was no significant difference between the handled and paired groups for all seizure parameters tested. Thus, lack of social interaction dramatically increased the seizure phenotype, in particular increasing seizure frequency by 2500%.

Finally, we assessed the level of oxidative stress, which correlates with seizure severity. Statistical analysis with two-way ANOVA, with treatment (pilo, non-pilo) as a within-subject variable and social isolation as a within-subject variable, showed a significant effect of treatment ($F_{(1,16)}$ =228.50) and social isolation ($F_{(2,16)}$ = 64. 54, $p<0.001$) on MDA levels (**Figure 2B**). Compared to the control group, *post-hoc* analysis revealed that MDA levels increased significantly in all groups treated with pilo ($q=21.39$, $p<0.001$). In addition, our results showed a significant increase of lipid peroxidation in pilo-isolated rats as compared to pilo-handled ($q=5.04$, $p<0.01$) and pilo-paired rats ($q=16.03$,$p<0.001$). Finally, the frequency of seizures and the level of MDA showed a significant and negative correlation (correlation coefficient 0.67, $p<0.05$, Spearman correlation) **(Figure 2C)**.

DISCUSSION

Our results show that single housing has a profound impact on seizure severity, generating a very severe phenotype (2500% increase in seizure frequency) as compared to the other two groups. It is important to note that daily social interaction with the experimenter was sufficient to prevent the development of a severe phenotype. It was as efficient as keeping animals in pairs. Although we could not quantify it scientifically, we noted that isolated pilo animals were very aggressive and displayed escaping behavior when cages were changed every week. In contrast, the other two groups remained calm when handled.

Since lack of social interaction produces a state of stress in non-pilo (control) animals, we propose that it constitutes a very favorable ground for the development of a severe epileptic phenotype. Status epilepticus would hit weakened neuronal networks

(with altered homeostasis). This is in keeping with the fact that animals vulnerable to depression (which display a high level of oxidative stress) also develop a more severe epilepsy than non vulnerable animals (Becker et al., 2015). Accordingly, we found that isolated animals displayed low levels of serum BDNF as those reported in animals vulnerable to depression (Blugeot et al., 2011, Bouvier et al., 2016). Social isolation may thus produce a general state of vulnerability to epilepsy, and to neurological disorders in general. Interestingly, environment enrichment, which can restore homeostasis, has positive effects on seizure frequency (Morelli et al., 2014, Yang et al., 2016).

Reactive oxygen species play a role in the development of seizures (Rauca et al., 2004, Militao et al., 2010). Our results are consistent with those reporting increased MDA levels in the same model of experimental epilepsy (Freitas, 2009, Tsai et al., 2010). Although causality cannot be established, we found a correlation between oxidative stress and epilepsy severity, with a strong effect in the isolated group. In this study, we have used the biological parameters commonly assessed in the context of altered homeostasis (stress hormones, oxidative stress), but it is likely that many other parameters are modified (inflammation, sleep patterns etc.).

Our results have important consequences for data interpretation (and perhaps for solving discrepancies in various fields). Results obtained in singly housed animals may be strongly influenced by the alterations occurring in neuronal circuits as a result of social isolation (already seen after two weeks). This is particularly true for the number of seizures (2500% increase) and their severity. Such “stress” confounding factor needs to be taken into account for a proper interpretation of observations obtained in singly housed animals. This may be particularly critical in the case of preclinical studies. A drug may be potent in experimental animals with social interaction, but its effects may remain undetected in singly housed animals (the stress factor would be too strong).

Conversely, several experimental studies report promising anti-epileptogenic effects, with a large reduction (at least 10 times) of seizure frequency. Based on our findings, it is difficult to determine whether these results target the epilepsy-related and/or the stress-related factors. Similar conclusions can be reached regarding other neurological disorders or pathological conditions (e.g. drug addiction). However, studies performed with singly housed animals may be relevant in the context of social isolation. Indeed, patients with epilepsy are often stigmatized, and may experience social isolation.

Although we cannot claim that maintaining social interaction between rodents in laboratory conditions corresponds to "normality", singly 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 effort should be made to maintain social interaction.

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DISCLOSURE

The authors declare no conflicts of interest. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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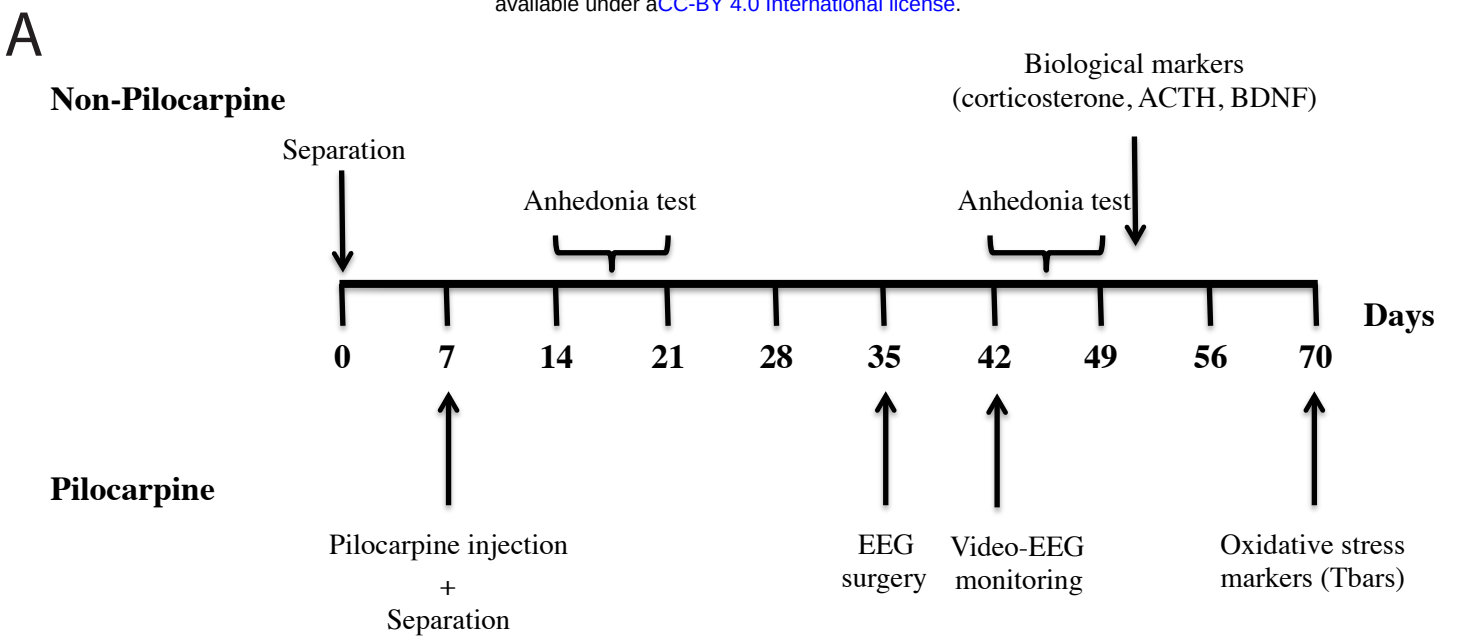
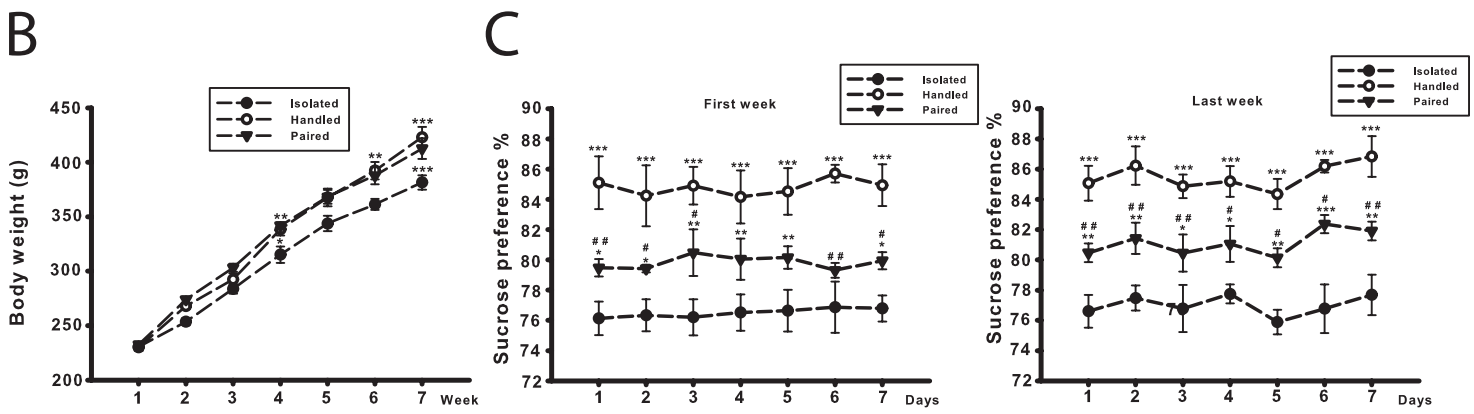
Figure Legends

Figure 1: Effect of social isolation conditions on behavioral and biological parameters. (A) Experimental protocol in non-pilo and pilo 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 are shown for isolated (n= 5), interaction (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 interaction group.

Figure 2: **Effect of social isolation conditions on spontaneous seizures and on MDA level**. **(A)** The average seizure frequency (number of seizures per hour), duration and severity are shown for isolated, interaction and paired rats (n=11, n=12 and n=12, i.e. 6 pairs, respectively) of the pilo group. Isolated rats displayed a very severe epileptic phenotype as compared to the other groups. **(B)** The analysis of MDA levels in non-pilo rats (isolated and interaction) and pilo rats (isolated, interaction and paired rats) revealed high levels of oxidative stress in all pilo animals, and increased levels in isolated pilo rats as compared to the other two pilo groups. **(C)** Correlation between seizure frequency and MDA levels. Each data represents an individual animal, isolated (dark circles), interaction (white circles) and paired rats (dark triangles). Values are expressed as mean ± SEM. *** p< 0.001 in comparison with the non-pilo group and $^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001 in comparison with the isolated non-pilo group (n=4 per group).





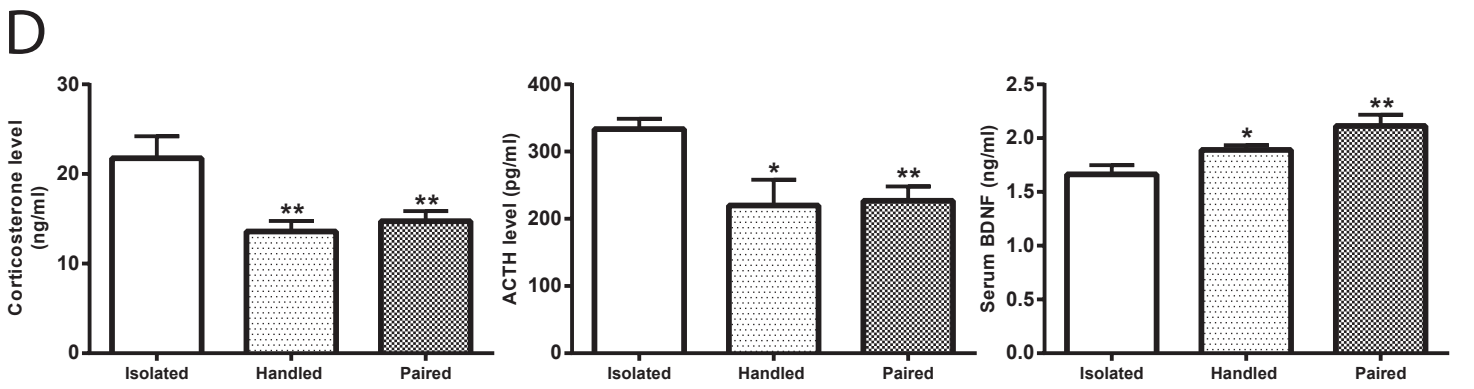


Figure 1

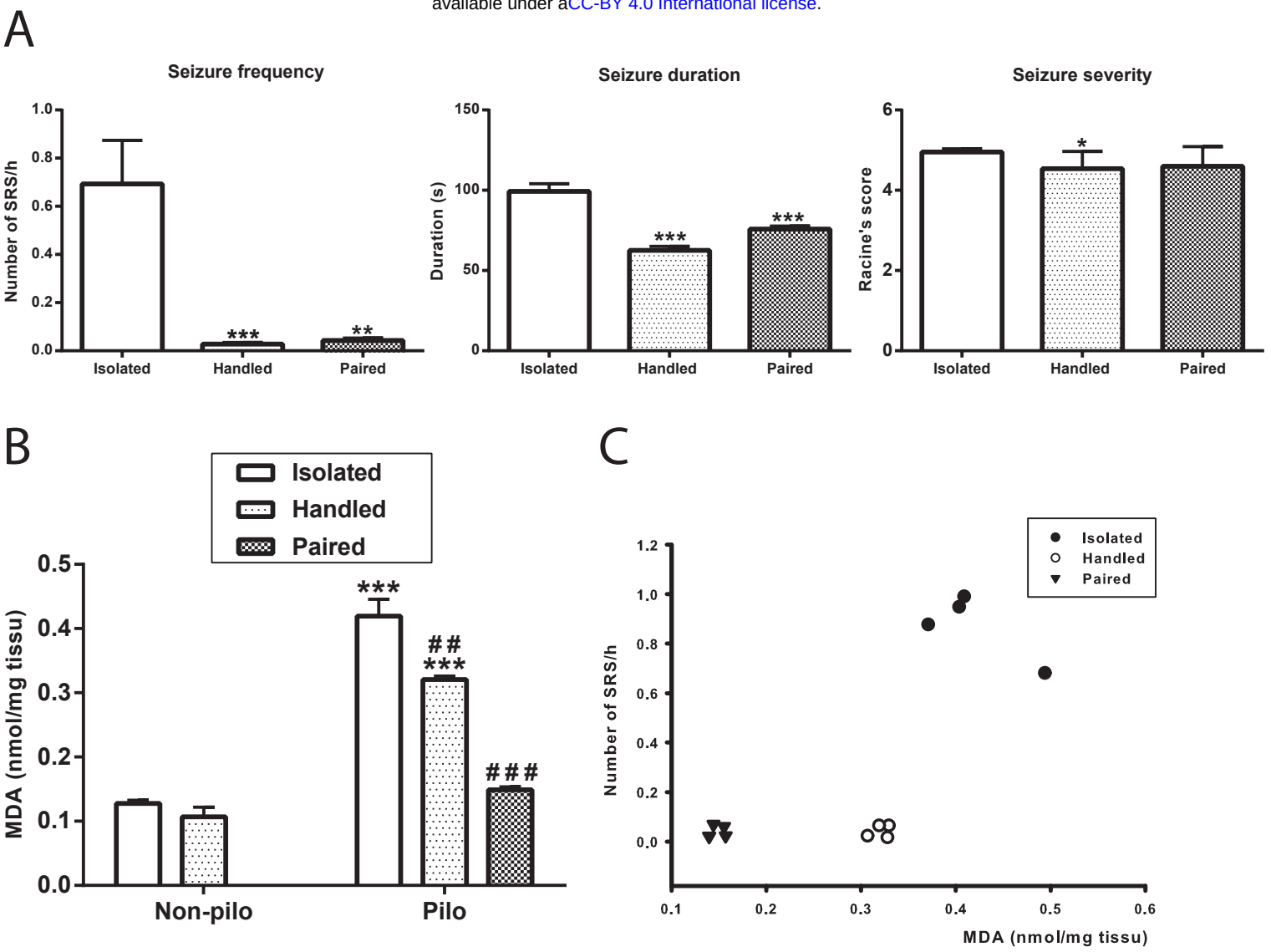


Figure 2