The Slow Speed Analysis of Video-Monitoring is Essential for the Observation of Behavioral Manifestations in the Chronic Phase of Pilocarpine Model in Non-Se Female Rats

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Abstract

Only few studies have focus on animals that received Pilocarpine (Pilo) and did not develop behavioral status epilepticus (SE) and, whether they may become epileptic in the model's chronic phase. Authors observed mossy fiber sprouting in the hippocampus of Non-SE (NSE) rats (Scharfman et al., 2001), while others observed spontaneous and recurrent seizures (SRS) 6-8 months after animals received the drug (Navarro Mora et al., 2009). Neuronal excitability is influenced by female hormones, as well as, the occurrence of SE in castrated and non-castrated female rats. However, it is not known whether females that received Pilo and did not show SE, may have SRS. The aim of this work was to investigate whether castrated and non-castrated female rats that did not show behavioral SE after Pilo, will develop SRS in the following one-year. For that, animals received 360mg/kg of Pilo and were video-monitored for 12 months. SE females from castrated and non-castrated groups became epileptic since the first month after drug injection. Epileptic behaviors were identified watching recordings in the fast speed. Castrated and Non-castrated NSE animals showed behaviors resembling seizures described by Racine Scale stages 1-3. Motor alterations of these groups were observed only when recordings were analyzed in slow speed. In addition, behavioral manifestations as, rhythmic head movements, sudden head movements, whole body movements and immobility were also observed in both, SE and NSE groups. We concluded that slow speed analysis of motor alterations was essential for the observation of NSE findings, which suggests that possibly many behaviors may be underestimated in epilepsy experimental research.

Keywords: Pilocarpine • Non-SE • Female rats • Video-monitoring • Slow speed analysis

Introduction

Pilocarpine (Pilo) experimental model of epilepsy was first described in male rats by Turski et al., and in female rats by Amado and Cavelheiro [1,2]. Behavioral, pathologic and electroencephalographic characteristics found in male and female epileptic rats induced to Pilo resemble those observed in Temporal Lobe Epilepsy (TLE) patients, making this a very useful experimental model of study [3].

Over the years, many experimental research groups around the world have devoted to the study of epilepsy, but only few of them have reported the number or percentage of non-SE animals (NSE) obtained in their experiments. Previous studies from our laboratory, in which female rats were treated with Pilo, showed that 40-50% of them did not have SE (data not published). Other works obtained 29% of NSE male rats after using Pilo and 54-88% of NSE female rats depending on estrus cycle day [4,5]. Despite the common and variable occurrence of NSE rats in this model of epilepsy, the discard of this animals or the use as controls are not unusual among researchers [6]. However, few studies have been done toward the comprehension of such animals.

A study showed that 50% of NSE male rats induced to Pilo lost cells from the entorhinal cortex, and 17%, of them showed mossy fiber sprouting. This work showed that functioning abnormalities of mossy cells may arise without status epilepticus and may be related to sprouting. Other group observed SRS in NSE male rats under telemetry, 6-8 months after Pilo administration. Authors also reported commitment of hippocampus, piriform cortex and thalamus in MRI analysis, 1 year after Pilo injection [6].

Female's estrus cycle regularity was affected by brain seizures after animals received Pilo injection. Brain seizures interferes in the normal physiological function of hypothalamic-pituitary axis, altering the production and release of estrogens and progesterone from the ovaries, which in turns affect female's cyclicity (Amado and Cavelheiro, 1998). A higher neuronal brain excitability was observed in castrated SE female rats after Pilo administration. The removal of female hormones due to castration lead to a smaller latency to the first seizure and a higher number of seizures after Pilo administration [7].

The study of the occurrence of SRS in the Pilo model's chronic phase of castrated and non-castrated SE and NSE female rats may contribute to the understanding of the influence of females' hormones in the neuronal excitability in this epilepsy model.

Considering that, the main purpose of this work was: a) to investigate the possible occurrence of SRS in castrated and non-castrated female rats that presented and did not present behavioral SE after Pilo administration; and, b) to access female's estrus cycle regularity in order to have and indirect information about the occurrence of seizures, since animals may show brain seizures without behavioral manifestations.

It is expected the occurrence of SRS in the model's chronic phase of SE and NSE castrated and non-castrated animals, with a higher number of seizures in the castrated-SE group. Alteration in females' cyclicity of non-castrated-SE and -NSE females is also expected with a higher number of seizures in castrated SE behavioral animals.

Materials and Methods

Animals

Female Wistar adult rats, weight range 200–230 g, n =137, with regular estrus cycle, housed under environmentally controlled conditions in a light/dark cycle (12/12h), temperature 21±2°C and granted free access to food and water, were used. Adequate measures were taken to minimize animal’s pain or discomfort. All experimental protocols were approved by the ethics committee of the UNIFESP (#0211/12).

Pilocarpine administration

Induction to TLE was made in 86 regular female rats (in the estrus day of estrous cycle) and 48 castrated females. To prevent peripheral cholinergic pilocarpine effects, subcutaneous methylscopolamine (1mg/kg, Sigma) was administered 30 min before 4% Pilo (370mg/kg, Sigma). All females (SE and NSE) received Diazepam (1mg/kg, ip, Santsa) and Tiopental (30mg/kg, ip, Cristália) 4 hours after Pilo administration. Female rats were considered SE when uninterrupted seizures were observed for 4 hours. Animals who showed interrupted seizures, meaning that started and stopped minutes later, were considered NSE. Seventy-two hours after Pilo injection, 9 SE and 14 NSE rats were castrated.

Groups

Experimental groups in this work were, NSE: female rats that did not present behavioral SE (4h of uninterrupted seizures) after Pilo administration; NSE-Cast: NSE females castrated 72h after Pilo; and, Cast-NSE: females castrated 6 days before Pilo administration and did not develop behavioral SE. As control groups: SE: females that showed behavioral SE after Pilo injection; SE-
Cast: SE females castrated 72h after Pilo; Cast-SE: females castrated 6 days before Pilo administration and presented behavioral SE; and, CTL: control group, females that received saline instead of Pilo.

**Analyzed parameters**

In all groups it was observed: development or not of behavioral SE after Pilo; latency to behavioral SE; mortality; regularity of estrous cycle in non-castrated female groups (SE and NSE), frequency of spontaneous seizures in three SE groups; identification and percentage of seizures-like behaviors in three NSE groups; and, identification and description of other behavior patterns for all experimental groups.

**Seizure Classification**

Female behaviors from all groups were analyzed considering the 10 stages of seizures described by Michael et al., [8]: 1- facial movements only; 2- stage 1 and head nodding; 3- stage 2 and forelimb clonus; 4- stage 3 and rearing; and 5- stage 4 and falling; stage 6, multiple stage 5 seizure; stage 7, jumping; stage 8- running and jumping; stage 9- stage 8 followed by tonic-clonic seizure; and, stage 10- multiple stage 9. Behaviors observed in this work that did not match those described above are presented in the results section.

**Estrous cycle access**

Vaginal smearing was collected 2-3 weeks/month during one year follow-up experiment from 6 SE, 18 NSE and 3 control female rats. Smears were taken between 8:00-9:00 a.m. using a plastic pipette tip filled with 10ul of saline solution, by inserting the tip (not deeply) into the rat vagina. Unstained material was observed under a light microscope, where the types of cells indicated the phase of estrous cycle, as described by previous studies [All external stimuli that could possibly alter rats’ cyclicity were avoided [9,10].

**Castration**

Castration was performed in female rats: a) at 6 days before Pilo injection, since findings show a significant decrease in blood sexual hormone concentration with systemic repercussions 4 days after castration [11]. Additional reports confirm the effects of castration 6 days after the procedure [12] or, b) at 72h after Pilo injection, since studies in our laboratory show that females have a better recovery after this period. After anesthesia with ketamine (75mg/kg, ip., Syntec) and xylazine (12mg/kg, ip., Syntec), females had their abdomen shaved and sterilized with iodopovidone. An incision of 1.5cm was made in skin and musculature in the lower midline, bilateral ovaries were identified and removed, and opened tissues were sutured. Ibuprofen (20mg/kg, Medley) was given by mouth once, 3-h after animals recovered from anesthesia, and added to bottle water (12mg/kg/day per rat) for 5 days to avoid pain. No female rats were lost due to the surgery.

**Video-Monitoring**

After recovering from Pilo injection, females were taken to video-monitoring room, which is equipped with IR-light video cameras coupled to a recording system, VD16E480C model, Intelbras (Brazil). Each group was video-recorded 14 days’ month starting 2-3 weeks after Pilo or saline injection and followed for the 12th month. Due to the complexity in obtaining SE-Cast females, this group were video-recorded from 1st-4th map (month after Pilo) only. For a better analysis of animal motor alterations, recordings were analyzed in periods as follow: 1st-4th, 5th-8th and 9th-12th map. CTL group was not video-recorded. Video-recordings obtained were analyzed in fast speed (the maximum system speed) by two researchers blinded for the experimental condition.

Twelve hours recordings from 7 NSE, 4 Cast-NSE and 6 NSE-Cast females randomly chosen were watched again in slow speed (the same speed in which movements occur), after fast speed analysis. Since electroencephalography (EEG) could not be performed in this work, manifestations that resemble seizures in these groups were referred here as seizure-like behavior.

**Statistical Analysis**

Results are shown in terms of mean ±SD, mean ± SE and the 95% of confidence interval (CI). Differences were considered significant at p <0.05 for all analyses. Statistical differences were evaluated using T-Student test for latency to SE, Mann-Whitney was used for latency to first motor alteration, Chi-square was applied for mortality caused by tonic seizure and in the course of SE and, Kruskal Wallis for intragroup and among groups analysis. Statistical procedures were performed using GraphPad Software Prism 4.0.

**Results**

Groups

Forty NSE and 30 SE rats out of 86 regular females were obtained after Pilo administration in the estrus day of the cycle (16 died due to tonic seizure (Figure 1). Fourteen out of 40 NSE females were castrated 72h after Pilo injection giving rise to NSE-Cast group. One NSE and 1 NSE-Cast rat died, resting respectively 25 and 13 females in each group (Figure 1). Nine out of 30 SE females were castrated 72h after Pilo originating SE-Cast group (1 died). Thirteen out of 21 SE rats obtained died 24-72h after Pilo administration. Thus, 8 females remained in the SE group and in SE-Cast as well. From the 48 females castrated 6 days before Pilo administration, 22 did not show behavioral SE, while 16 died. Ten castrated females died after drug injection due to tonic seizure and 8 out of 16 Cast-Cast died 24-72h after Pilo. Thus, Cast-NSE and Cast-SE group had respectively 22 and 8 females each. Three regular female rats constituted the control group during 1-year experiment.

**Latencies after Pilo Injection**

Females that developed behavioral SE after Pilo showed quietness followed by masticatory automatisms, salivation, head nodding and body shakes. Limbic behaviors repeated every 3-8min until behavioral SE was observed. Latency to first motor alteration (Mann-Whitney Test) in non-castrated was smaller than in castrated females, values were respectively 4,38±14,52 min (9,45 ± 2,27 min mean(SE) and 12,71-19,96 min (16,33 ± 2,77min mean(SE), confidence interval (CI) (95%) was considered, p<0.05 (Figure 2A). Castrated females showed more aggressive behaviors as tonic and tonic-clonic seizures than non-castrated animals. Latency to SE of CTL-Student Test showed smaller latency to non-castrated (CI: 20,41-26,44 min; 23,43 ± 1,23min meanSD) than to castrated (CI:24,48-31,52 min; 28 ± 1,48min meanSD), p<0,05, CI (95%) was considered for both groups (Figure 2B). Values concerning mortality of castrated and non-castrated animals was not significant. Castrated and non-castrated females that did not develop behavioral SE showed inconsistent seizures, up to stage 5 from Racine (Racine, 1972), with marked beginning and end.

**Estrous Cycle**

Five out of 6 (83%) SE females showed irregularity from 1st-4th map, and all of them were irregular from 5th-12th months of experiment. In NSE females, irregularity was observed in 6 of 18 (33%) rats up to 3map, 16 of 18 (89%) from 5th-8th map and 9 of 14 females (64%) from 9th-12th months of experiment. Irregularity was observed in terms of constant estrus at vaginal smearing. CTL female group showed only few periods of irregularity during the experiment.

**Findings from SE, Cast-SE and SE-Cast groups**

All females from SE, Cast-SE and SE-Cast groups became epileptic since the 1st map. Video-recordings from these groups were watched in fast speed, which showed that seizures persisted for the 12 months observation period. Seizures of these animals reached stage 8, as described by Michael et al., [8], however more than 50% were among stage 5-7. The same animals were observed along the 1-year experiment, however few animals had to be removed from video-monitoring room during this period to give place to other researchers. A double checking analysis of all seizures were done in slow speed for details description, which showed that seizures were accompanied of a variety of different manifestations as, isolated head movements, whole body movements and, immobility. These behavioral manifestations were classified in: 1) Head Rhythmic (HR) manifestations: identified as oscillatory head movements that included, side-to-side, up-and-down and back-and-forth; 2) Head Sudden (HS) manifestations: movements usually with high amplitude as, head projection and/or retraction, drop, version (sometimes accompanied of neck and/or trunk (hyper)extension); 3) Whole Body (WB) manifestations: involved head, trunk and limbs as follow, shaking, fast walking in circles, 90-360° spin and imbalance; and, 4) Immobility (IM) manifestation: the absence of movements with eyes open (staring eyes). Authors observed the presence of seizures and behavioral manifestations in all SE groups, along the one-year observation period (Table 1). Statistically significant difference concerning the number of seizures were observed between SE and SE-Cast groups from 1st-4th map, p<0,0036 (Figure 3). No differences were found in the number of the behavioral manifestations showed by SE groups.

Seizures were frequently accompanied of the behavioral manifestations in the SE group from 5th-8th map and 9th-12th map. In these periods, 69% and 100% of seizures showed WB, and, HS and IM, respectively. In the SE-Cast it was also frequent with seizures, from 1st-4th map, which were accompanied of WB; and / or, IM manifestations. On the other hand, in SE females from 1st-4th map and Cast-SE group from 1st-4th map and 5th-8th map, behavioral manifestations with seizures were less frequent. The period, 9th-12th map, of Cast-SE group showed only seizures alone, as shown in Table 1.

Among SE females, the occurrence of IM was commonly seen during seizures stage 3-5, and lasts from 10 seconds to 5 minutes. Behavioral manifestations also occurred alone- without seizures, in less than 10% of SE, Cast-SE and SE-Cast groups. HS and WB manifestation were the most identified as, sudden
Figure 1. Distribution of females among groups.

Figure 2. Latency in non-castrated and castrated groups. A. Latency to first motor alteration with Mann-Whitney test B. Latency to SE with T-Student test. Values are shown by mean ± SE.

Table 1: Seizures and behavioral manifestations in SE, SE-Cast and Cast-SE groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Period (map) and n of rats</th>
<th>Number of seizures</th>
<th>Number and % of seizures with manifestations</th>
<th>Behavioral Manifestation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE (n=8)</td>
<td>1st-4th (n=8)</td>
<td>132</td>
<td>5 (4%)</td>
<td>WB: spin</td>
</tr>
<tr>
<td></td>
<td>5th-8th (n=4)</td>
<td>148</td>
<td>102 (69%)</td>
<td>HS: neck extension; and/or, IM</td>
</tr>
<tr>
<td></td>
<td>9th-12th (n=1)</td>
<td>4</td>
<td>4 (100%)</td>
<td>HS: neck and trunk hyperextension;</td>
</tr>
<tr>
<td>Cast-SE (n=4)</td>
<td>1st-4th (n=4)</td>
<td>47</td>
<td>3 (6%)</td>
<td>WB: shaking and/or, IM</td>
</tr>
<tr>
<td></td>
<td>5th-8th (n=3)</td>
<td>127</td>
<td>6 (6%)</td>
<td>HR: up and down head oscillation; and/or, IM</td>
</tr>
<tr>
<td></td>
<td>9th-12th (n=2)</td>
<td>31</td>
<td>0 (0%)</td>
<td>IM</td>
</tr>
<tr>
<td>SE-Cast (n=3)</td>
<td>1st-4th (n=3)</td>
<td>5</td>
<td>5 (100%)</td>
<td>WB: shaking; and/or, IM</td>
</tr>
</tbody>
</table>

Note: SE: epileptic; Cast-SE: castrated and then epileptic; SE-Cast: epileptic and then castrated; n: number; map: months after pilo; %: percentage; HR: head rhythmic manifestation; HS: head sudden manifestation; WB: whole body manifestation; IM: immobility.

Figure 3. Difference in the number of seizures between SE and SE-Cast groups from 1st-4th map, p=0.0036.
Table 2. Seizure-like behavior and behavioral manifestations in NSE, Cast-NSE and NSE-Cast females (based on 12h-slow speed observation).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Period (map)</th>
<th>Number of seizure-like and stage</th>
<th>Number and % of seizure-like with manifestations</th>
<th>Behavioral Manifestation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSE (n=7)</td>
<td>1&lt;sup&gt;st&lt;/sup&gt;-4&lt;sup&gt;th&lt;/sup&gt; (n=3)</td>
<td>8 / 2-3</td>
<td>8 (100%)</td>
<td>HS: head version, neck extension and/or, WB: shaking</td>
</tr>
<tr>
<td>Cast-NSE (n=4)</td>
<td>1&lt;sup&gt;st&lt;/sup&gt;-4&lt;sup&gt;th&lt;/sup&gt; (n=2)</td>
<td>8 / 2-3</td>
<td>8 (100%)</td>
<td>HS: head and trunk version and/or, neck extension and hyperextension and/or, IM</td>
</tr>
<tr>
<td>NSE-Cast (n=6)</td>
<td>5&lt;sup&gt;th&lt;/sup&gt;-8&lt;sup&gt;th&lt;/sup&gt; (n=2)</td>
<td>3 / 2-3</td>
<td>3 (100%)</td>
<td>HS: head and trunk version and/or, head and trunk extension</td>
</tr>
<tr>
<td></td>
<td>5&lt;sup&gt;th&lt;/sup&gt;-8&lt;sup&gt;th&lt;/sup&gt; (n=2)</td>
<td>3 / 2-3</td>
<td>3 (100%)</td>
<td>HS: head version and/or, head and trunk extension</td>
</tr>
</tbody>
</table>

Note: NSE: non-epileptic; Cast-NSE: castrated and non-epileptic; NSE-Cast: non-epileptic and then castrated; n: number; map: months after pilo; WB: whole body manifestation; IM: immobility.

neck extension followed by imbalance or by neck version; and, shaking and spin, respectively. The videos of manifestations described in this work are available in the supplemental material.

Findings from NSE, Cast-NSE and NSE-Cast

Video-recording analysis of NSE, Cast-NSE and NSE-Cast groups were initially done in the fast speed, but did not evidence any motor alteration. This negative result raised the question whether the fast speed analysis could hinder the observation of female’s manifestations, which possibly were hidden by fast speed. To answer this, 12h-recordings (3 hours per night) of 4 following nights, were reassessed. Dark period was chosen because animals are more active on this period [13]. For that, 7 NSE, 4 Cast-NSE and 6 NSE-Cast females, from all observation periods, were re-analyzed in slow speed. Due to this approach, these animals could not be followed along the experiment.

Reassessment of video-recordings in slow speed confirmed the presence of behaviors that resemble seizures stage 1-3 of. Seizure-like behaviors were observed alone and also accompanied of the behavioral manifestations, but no statistically difference were found among the three groups.

All seizure-like behaviors identified in NSE and NSE-Cast females on the period between 1<sup>st</sup>-4<sup>th</sup>map were followed by one or more behavioral manifestations. The most observed in these groups were HS and WB manifestations; and, HS manifestations respectively. NSE-Cast group also had all seizure-like behaviors from 5<sup>th</sup>-8<sup>th</sup>map and 9<sup>th</sup>-12<sup>th</sup>map accompanied by the same HR and HR manifestations. Different from that, were for NSE females between 5<sup>th</sup>-8<sup>th</sup>map and 9<sup>th</sup>-12<sup>th</sup>map, which had up to 25% of all seizure-like behaviors accompanied of behavioral manifestations. These females showed HS and WB manifestations in the intermediate observation period and the same WB manifestation (walking in circles) in the last period (Table 2).

In the initial period observation (1<sup>st</sup>-4<sup>th</sup>map) for the Cast-NSE group, all seizure-like behaviors were followed by HS and IM manifestations. For the next period, HS manifestation was present in 60% of all seizure-like behaviors. The period from 9<sup>th</sup>-12<sup>th</sup>map, was not reassessed in slow speed (Table 2). HS manifestations as, head projection and retraction, head version and shaking were also observed alone—not accompanied of seizure-like behaviors. Videos are available in the supplemental material.

In addition, other manifestations were also observed, although less frequent, in the NSE-Cast-NSE and NSE-Cast groups, which included: sudden abdominal contraction (similar to a hiccup), posterior paws clonus, circling movements, imbalance and tail rigidity.

Discussion

This work was based on a 1-year video-monitoring follow up of castrated and non-castrated SE and NSE female rats that showed 4 classes of behavioral manifestations in the Pilo model’s chronic phase.
Other manifestations, described in animal models and in patients were frequently observed in this work in SE and NSE groups, when slow speed analysis were applied. Up and down head oscillation, an HR manifestation, were observed in SE animals, and resemble head drops movements observed in epileptic patients, which are cortical myoclonus involving neck muscles in the spectrum of myoclonic-astatic epilepsy [18]. This alteration was also described in patients with chorea-acanthocytosis, accompanied of neck extension spasms, which are similar to findings of SE and NSE females that showed neck extension, sometimes followed by neck hypertension and also version, HS manifestations. Spin movements, an HS manifestation, were another manifestation identified in this work in SE groups, and were very similar to the definition of gyRatory seizures, which are described as a rotation around the body axis, of at least 180 degrees, during a seizure [19]. This is observed in 2% of TLE patients, and also in animals [20,21]. GyRatory seizures start with head version followed by body version in 58% of the 2% of TLE patients. These HS manifestations were observed alone or together, in SE and NSE groups as well. Shaking was another HS manifestation commonly observed in SE and NSE females. This was similar to the wet dog shakes observed in other animal models of seizures as, kainic acid, organophosphate pesticide and quinolinic acid. Immobility was observed in SE and also NSE females in this work. This manifestation was previously described in the hyperthermia model of febrile seizures in pups, in which animals were placed in a glass chamber with 41°C of internal temperature for half an hour [22].

Concerning the limitations of this study, EEG application would have confirmed manifestations observed in NSE groups and probably helped in identifying structures involved, but this methodology could not be elected in this work. The estrous cycle and motor findings of NSE rats could not be correlated since data of this group is based on 12h video-recordings. Despite limitations, important motor findings especially concerning the 4 classes of behavioral manifestations in NSE, Cast-NSE and NSE-Cast females could be identified, which suggests that these females were affected by Pilo administration and possibly have developed an epileptic focus [23-25]. Further studies comprising the identification of seizure-like behaviors and manifestations observed in this work, associated to electrophysiological methods, will help identifying structures and pathways involved in the Pilo model, and in directing drug treatments as well.

Conclusion

This work brings important and new evidences to the experimental epilepsy field: 1) behavior manifestations identified in NSE, Cast-NSE and NSE-Cast females are already described in the literature in patients with epilepsy and animal models, suggesting that these animals probably presented partial seizures after Pilo administration; and, 2) slow speed analysis of video-monitoring recordings is essential for the identification of behavioral manifestations, which possibly have been underestimated in experimental epilepsy research.

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Conflict of interest

The authors declare no conflict of interest.

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