Published in final edited form as:

Neurosci Lett. 2009 April 10; 453(3): 233–237. doi:10.1016/j.neulet.2009.02.035.

Age dependent mortality in the pilocarpine model of status epilepticus

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Abstract

Status epilepticus (SE) is an acute neurological emergency associated with significant morbidity and mortality. Age has been shown to be a critical factor in determining outcome after SE. Understanding the causes of this increased mortality with aging by developing an animal model to study this condition would play a major role in studying mechanisms to limit the mortality due to SE. Here we employed pilocarpine to induce SE in rats aged between 5 to 28 weeks. Similar to clinical studies in man, we observed that age was a significant predictor of mortality following SE. While no deaths were observed in 5-week old animals, mortality due to SE increased progressively with age and reached 90% in 28-week old animals. There was no correlation between the age of animals and severity of SE. With increasing age mortality occurred earlier after the onset of SE. These results indicate that pilocarpine-induced SE in the rat provides a useful model to study age-dependent SE-induced mortality and indicates the importance of using animal models to elucidate the mechanisms contributing to SE-induced mortality and the development of novel therapeutic interventions to prevent SE-induced death.

Keywords

Status epilepticus; Sprague-Dawley rats; Pilocarpine; Age; Mortality; Development; Senescence

Status epilepticus (SE) is a major neurological emergency associated with significant morbidity and mortality [7,8,11]. Clinical studies from our group and others have demonstrated that SE-

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induced mortality occurs as the result of the effects of prolonged seizures on the body [15, 27]. Human data has demonstrated a role for hemodynamic and cardiac factors in the precipitation of death following SE [2] and that age is a critical factor in determining the outcome after SE [16,26,27]. Clinical studies have shown that following SE, the adult population has a much higher mortality than the younger population [27]. Thus, it is important to develop animal models that can be used to elucidate mechanisms underlying age-dependent mortality following SE and to develop potential therapeutic strategies to prevent death.

This study evaluated the effects of age as a contributing factor to the mortality following pilocarpine-induced SE in the rat and determined if SE in animals manifests an age-dependency as observed in the human. Our results show that age is a significant predictor of mortality following SE. This model could be extremely useful for deciphering the pathophysiological mechanisms underlying SE-induced mortality in man and highlights the need to develop animal models to study mortality from SE.

All animal use procedures were in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by Virginia Commonwealth University's Institutional Animal Care and Use Committee. Animal procedures were carried out in a manner to minimize any pain and suffering. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) ranging in age from 5- to 28-weeks were housed two per cage on a 12-h light/dark cycle and provided with food and water *ad libitum*. All animals were handled identically and provided with enrichment materials using standard procedures in our animal care facility. The results presented in this study were obtained by a retrospective analysis of data obtained over a four year period with a pooled n= 218 animals to evaluate the incidence of age-dependent mortality due to SE. The widely used pilocarpine-induced SE model in rat was employed using previously established methods in our laboratory [9]. Pilocarpine is a chemoconvulsant that initiates seizures [28] via activation of muscarinic M1 receptors [10]. Upon induction of seizures, their maintenance is dependent upon other mechanisms; mainly activation of the NMDA receptor by release of the excitatory transmitter glutamate [23]. To minimize peripheral effects of M1 receptor activation, rats were first administered methyl-scopolamine (1 mg/kg, i.p.) (Sigma, St. Louis, MO, USA) 30 min before pilocarpine (375 mg/kg, i.p.). Onset of SE was determined by the presence of continuous class 4–5 level seizures as assessed using a modified Racine scale [20]. **Racine scale assessments were objectively evaluated by a minimum of three trained observers. Each observer independently scored the SE and following the experiment the scores were averaged for each animal. Thus, each final Racine score for an individual animal was based on the average of a minimum of three observations.** We have had considerable experience in evaluating behavioral seizures in rats using this procedure. Surviving animals undergoing SE for a duration of 60 min were rescued by administration of three consecutive injections of diazepam (5 mg/kg, i.p.) (VCU Health Systems Pharmacy, Richmond, VA, USA) at 1, 3 and 5 h following SE onset to terminate seizures. No major differences were noted to diazepam response between age groups used in our study. Animals were constantly observed for mortality. Surviving animals were supplemented with saline and lactose solution and were transferred to cages and returned to the vivarium and underwent continued observation for mortality. We used at least 10 animals per age group. Data were expressed as percent mortality. Significance was tested following a multi regression analysis employing a pair-wise comparison with a Wald Chi-square test using SAS 9.1.3 (Statistical Analysis Software, Cary, NC) and was plotted using Sigmaplot 8.0 (SPSS, Chicago, IL). Survival curves were examined using Kaplan-Meier Survival Analysis.

We evaluated the effects of pilocarpine induced SE in animals at 5, 7, 10, 15, 18 and 28 weeks of age. The animals were monitored for seizure severity (Racine score and electrophysiology) and for mortality during SE for up to 48-h following onset of SE (Fig 1 and Table 1). Forty-eight hours following onset of SE is a time point whereby SE-induced mortality has reached

a plateau in all age groups studied under our experimental conditions. We choose 5-weeks as our first age group because the validity of modeling SE in rats in the first two weeks after birth is questionable due to the immature hypothalamo–pituitary–adrenal axis [21]. Thus, the 5-week time point allowed for full maturation of the stress system and also represents a time point before puberty is achieved in rats. The 28-week time point was an age where the rat is adult by human standards and we observed maximal mortality with this model of SE. The selected dose of pilocarpine (375 mg/kg, i.p.) is commonly used in our laboratory and others. Previous studies have evaluated different doses of pilocarpine and demonstrated that pilocarpine at 375 mg/kg can evoke maximal electrographic seizure activity during SE (reviewed in: [5]).

Electrophysiological studies were conducted using standard procedures [22]. Animals were monitored throughout the SE using a video EEG monitor (BMSI 5000, Nicolet) and spike frequency and pattern characterized using Insight II (Persyst Development Corporation). Spike frequency was recorded using 6–18 s epochs. During each pattern spike frequency was quantified by measuring an average for each channel. Final results were then averaged for each postnatal age. Animals were allowed to progress for at least 20 min to ensure good SE recording. In agreement with our previous studies [3,18,19,22], electrographic analysis of pilocarpine-induced seizure activity in rats revealed that there was no significant difference in average time from first seizure to SE with age. The electrographic spike patterns (Fig. 1A) were not different for animals in different age groups during SE (5-wk, 15-wk and 28-wk). Spike frequency during SE did not increase across the age groups used in our study. The electrographic analysis was consistent with the Racine data (Table 1). Regression analysis of seizure severity indicated that there was no correlation between age and the severity of SE ($r^2 = 0.43$, Fig. 1B).

In a multivariate logistic regression analysis (Fig. 2), age was found to be a significant predictor of mortality due to pilocarpine induced SE ($p < 0.0001$). No deaths were observed in 5-week old animals, while in 18-week old animals mortality increased to 80% and peaked at 90% for the 28-week old rats. The odds ratios for mortality dramatically increased for aging animals. Multivariate statistical analysis demonstrated that the odds of mortality due to SE in the adult group (15, 18 and 28 weeks) were 11.3 times the odds of mortality in the younger group (5, 7 and 10 weeks) (95% CI: 22.34–95.72). In order to quantify the correlation between mortality after SE and age, a regression analysis was also performed. The percent mortality correlated significantly with the age of the animal ($r^2 = 0.89$).

Clinical studies have demonstrated that SE is associated with high mortality and deaths can occur even after the control of SE [8,26]. Thus, we evaluated the time of death after SE in the various age groups. There were no deaths during SE and for up to 36-h following the termination of SE in the 5 and 7-week old animals. The mortality (3.44%) observed in the 7-week old animals occurred almost 2-days following SE. For the 15-week old animals greater than 90% mortality occurred by 24-h whereas the 18 and 28-week animals rarely survived past 12-h following the termination of SE (Fig. 3). With increasing age there was a decreased survival time following SE (Fig. 4). The Log-Rank analysis revealed a significant difference between survival curves for the different age groups ($p < 0.001$). The median survival times for the 28, 18 and 15-week age groups were 1.0 ± 0.19 , 4.5 ± 2.4 and 14 ± 2.6 h post-SE onset respectively, and were all significantly different from the three younger groups (10, 7 and 5-week) when evaluated using Holm-Sidak pairwise multiple comparison ($p \leq 0.002$). No significant differences in survival curves were found amongst the younger groups (10, 7 and 5-week). These results indicated that increasing age was associated with both an increase in mortality and decrease in survival time following pilocarpine-induced SE.

This paper presents evidence that pilocarpine-induced SE in rats can be used as a reliable model to study the relationship between age and mortality. There is an abundance of clinical evidence

that suggests children exhibit low mortality and morbidity following SE [16], but the aging population is much more vulnerable to death due to SE [26] and that mortality may involve cardiovascular dysfunction [2]. However, it has been shown that it is difficult to study the cause of mortality in humans [2]. The pilocarpine induced SE model in rodents has been widely used as a model of SE and has been shown to be very similar to the human clinical condition [2]. Drugs that are used clinically to control SE in humans exhibit similar pharmacological effects in the pilocarpine model of SE [2]. Our observations using the pilocarpine model to explore the age dependence of mortality due to SE further demonstrates a significant association with the human condition.

The 28-week (adult rat) age was chosen as the older age, since we observed maximal response (mortality) at this time point with the conditions of SE-induction in this study. Previous behavioral studies indicate that this species reach the geriatric phase of life between 18–24 months [1]. Thus, the conditions of the pilocarpine model used in this study produced maximal mortality at adult aged rats as opposed to that observed clinically in the elderly population. Although rats were more sensitive to SE at a younger age than humans, this may in part be accounted for by the type of SE induced by pilocarpine versus the natural causes of SE in humans [12] or to other underlying differences. Age-dependent alterations in benzodiazepine efficacy could potentially confound mortality observations; however, since many of the older animals in our study died before diazepam injections, any possible age-dependent sensitivity to benzodiazepines is likely not contributing to the mortality observed here. In addition, a dose-dependent effect of pilocarpine on seizure severity has been reported [5]. We have performed a dose response analysis of pilocarpine induced SE (325–425 mg/kg at 10 weeks of age) and found only a slight dose-dependent increase in mortality (data not shown). These results indicate that at this age the dose of pilocarpine can have some effect on mortality and further studies are warranted to investigate the dose-dependent effects on a broader age-range. Possibly modifying experimental conditions or utilizing other methods of SE-induction would allow for the development of models that have the same age dependency seen in humans.

Systemic administrations of kainic acid or fluorothyl are other methods for induction of SE in rodents that are associated with lesser overall mortality but still display an age-dependent increase in mortality following SE [6,14,25,29]. However, compared to pilocarpine-induced SE, differences exist in terms of seizure severity and intensity of regional CNS damage [4]. The purpose of this study is to demonstrate that animal models may be useful to investigate the high mortality of SE associated with age. Further studies with the pilocarpine and other animal models of SE may offer important insights into the pathophysiology of mortality with aging in SE.

Previous studies using the rat pilocarpine-induced SE model have observed age dependent effects of SE on behavioral outcome, histological changes and electrophysiological parameters [3,13,18,19,22,24]. However, there are conflicting data regarding the age-dependent susceptibility of animals to SE. While some studies have shown increased mortality after SE in rats aged between P1–P17 [3], others have shown a lower mortality in this same age-group [19]. The majority of studies have characterized effects of SE on rats ranging in age from P1 to around P90 [3,18,19,22], but have not evaluated the effects of SE over an extended age range that includes older-aged rats (28-week old). In addition, these studies lacked a statistical evaluation of the effects of age on mortality in SE. To our knowledge this study provides the first demonstration using multivariate statistical analysis of an effect of age in the rat pilocarpine model of SE. It is important to evaluate the effects of SE across the age spectrum in an animal model and compare these results to the findings in the human population [7,8, 11].

While some animal studies have reported an age-dependent increase in oxidative stress as a critical factor underlying neuronal death following SE [17], the rapid physiological changes that result in death during or following SE remain poorly understood. This has been partly due to variations in different animal models of SE such as type of the chemoconvulsant used, differential response (no SE or very high mortality), strain/species of animal and route of chemoconvulsant administration. These issues have been reviewed in a recent article by Avoli's group [5]. Although pilocarpine-induced SE is a well established and widely used model for SE and AE, further studies to investigate mortality associated with SE utilizing additional models such as kainic acid or continuous hippocampal stimulation are warranted.

The results of this paper indicate that pilocarpine-induced SE in the rat provides a useful model to study death from SE. The findings show an age-dependent increase in mortality following pilocarpine-induced SE. This can be a very valuable tool to conduct controlled studies to identify the mechanisms mediating mortality and allow for the development of therapeutic interventions to prevent death due to SE.

Acknowledgments

NINDS Grants RO1NS051505 and RO1NS052529, and award UO1NS058213 from the NIH CounterACT Program through NINDS to RJD supported this study. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the federal government. We thank our research colleagues Drs. Dawn Carter and Katherine Falenski for their critical suggestions. We also thank Dr. Viswanathan Ramakrishnan for his help with the statistical analysis. We thank Elisa Attkisson for her help with these studies.

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Figure 1. Severity of SE following pilocarpine administration in animals of different ages. **A.** Electrographical traces were obtained and analyzed as described in methods section. No significant differences in the spike frequency or EEG pattern were observed for a. 5-wk, b. 15-wk and c. 28-wk old animal. **B.** The severity of pilocarpine-induced seizures was quantified using a modified Racine scale (0; no response – 6; death) by averaging scores from at least 3 **independent** observers (mean $\pm$ SEM). Linear regression analysis of seizure severity and age revealed that there was no correlation between Racine scale score and animal age ($r^2=0.43$). (n= 10, 29, 69, 90, 10 and 10 animals for 5, 7, 10, 15, 18 and 28 week age-group respectively).

Figure 2. Age-dependent mortality in the pilocarpine model of SE. Deaths due to SE increased progressively with age and development. Linear regression analysis gave $r^2 = 0.89$. Dotted lines represent 95% confidence interval limits.

Figure 3.

Distribution of time of death following pilocarpine induced SE. **A.** No mortality is observed for 5-week old animals. **B.** Mortality due to SE in the 7-week group is observed exclusively 48-h following SE. **C.** For the 10-week group the deaths are distributed across the time points with 50% of mortality occurring during the first 12-h following SE. **D.** By 15-weeks of age, 67% of deaths occurred in the first 12-h following SE. **E.** The 18-week age group demonstrated 75% mortality in the first 12-h following SE-onset with 25% of those occurring during the 60 min SE episode. **F.** In the 28-week age-group, 100% of mortality occurred within 12-h following SE with 55% of those occurring within 60 min of onset (during SE).

Figure 4.

Survival curves following pilocarpine induced SE. Animals were observed for 48-h following time of SE-onset and mortality was recorded where applicable. Data from all age groups were then analyzed using Kaplan-Meier Survival Analysis and plotted out as percent survival over time. Log-rank test revealed a significant difference between survival curves across all age groups ($p \leq 0.001$).

Table 1

Electrographic spike frequency and behavioral Racine scores for different age groups of rats following pilocarpine-induced SE.

Age (weeks)	Spike frequency (Hz)	Racine Score (mean $\pm$ SEM)
5	7.85 $\pm$ 0.61	5.0 $\pm$ 0.0
7	9.02 $\pm$ 0.93	3.93 $\pm$ 0.07
10	9.08 $\pm$ 0.10	4.0 $\pm$ 0.0
15	9.07 $\pm$ 0.42	3.8 $\pm$ 0.06
18	8.85 $\pm$ 0.72	5.2 $\pm$ 0.08
28	7.95 $\pm$ 0.80	6.0 $\pm$ 0.0