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Original Article

Acute Seizures in Old Age Leads to a Greater Loss of CA1 Pyramidal Neurons, an Increased Propensity for Developing Chronic TLE and Severe Cognitive Dysfunction

Bharathi Hattiangady[1], Ramkumar Kuruba[1] and Ashok K. Shetty[*]

Medical Research and Surgery Services, Veterans Affairs Medical Center, Durham, NC 27705.
Division of Neurosurgery, Department of Surgery, Duke University Medical Center, Durham, NC 27710.

[1]The first two authors contributed equally to this work

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ABSTRACT: The aged population displays an enhanced risk for developing acute seizures (AS). However, it is unclear whether AS in old age would elicit a greater magnitude of hippocampal neurodegeneration and inflammation, and an increased predilection for developing chronic temporal lobe epilepsy (TLE) and cognitive dysfunction. Therefore, we addressed these issues in young-adult (5-months old), aged (22-months old) F344 rats after three-hours of AS activity, induced through graded intraperitoneal injections of kainic acid (KA), and terminated through a diazepam injection. During the three-hours of AS activity, both young adult and aged groups exhibited similar numbers of stage-V motor seizures but the numbers of stage-IV motor seizures were greater in the aged group. In both age groups, three-hour AS activity induced degeneration of 50-55% of neurons in the dentate hilus, 22-32% of neurons in the granule cell layer and 49-52% neurons in the CA3 pyramidal cell layer without showing any interaction between the age and AS. However, degeneration of neurons in the CA1 pyramidal cell layer showed a clear interaction between the age and AS (12% in the young adult group and 56% in the aged group), suggesting that an advanced age makes the CA1 pyramidal neurons more susceptible to die with AS activity. The extent of inflammation measured through the numbers of activated microglial cells was similar between the two age groups. Interestingly, the predisposition for developing chronic TLE at 2-3 months after AS activity was 60% for young adult rats but 100% for aged rats. Moreover, both frequency & intensity of spontaneous recurrent seizures in the chronic phase after AS were 6-12 folds greater in aged rats than in young adult rats. Furthermore, aged rats lost their ability for spatial learning even in a scrupulous eleven-session water maze learning paradigm after AS, in divergence from young adult rats which retained the ability for spatial learning but had memory retrieval dysfunction after AS. Thus, AS in old age result in a greater loss of hippocampal CA1 pyramidal neurons, an increased propensity for developing robust chronic TLE, and a severe cognitive dysfunction.

Key words: Chronic epilepsy; cognitive dysfunction; epilepsy in the elderly; hippocampal pyramidal neurons; learning and memory; neurodegeneration; neuroinflammation; spontaneous recurrent seizures; status epilepticus; temporal lobe epilepsy

Aging induces numerous changes in the hippocampus, a brain region that is prone to acute seizures (AS) or status epilepticus (SE) and is believed to be the site that generates most spontaneous recurrent seizures (SRS) in the chronic phase after an episode of AS or SE. The changes in the aged hippocampus include decreased concentrations of vital neurotrophic factors that play neuroprotective roles, enhanced levels of glucocorticoids

***Correspondence should be addressed to:** Ashok K. Shetty, M.Sc., Ph.D., Division of Neurosurgery, DUMC Box 3807, Duke University Medical Center, Durham NC 27710. E-mail: Ashok.Shetty@Duke.Edu
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and molecules that induce oxidative stress, and diminished concentration of endogenous antioxidants [1-7]. The other changes in the aged hippocampus comprise decreased numbers of GABA-ergic inhibitory inter-neurons that control the activity of excitatory principal neurons [8-10], the possible aberrant mossy fiber innervation of granule cell dendrites in the inner molecular layer enhancing the excitatory tone of the dentate gyrus (DG) [11, 12] decreased frequency of spontaneous inhibitory postsynaptic potentials, and an increased propensity for large amplitude prolonged excitatory postsynaptic potentials following disinhibition [13]. The above changes likely contribute to the perceived increases in the susceptibility of the aged population for developing AS [14-19].

While many instances of AS in the elderly population occur after insults such as stroke, a degenerative disease, tumor, head injury and infections, the cause of AS in this population is unknown in nearly 50% of cases [20, 21]. Previous studies in animal models also suggest that aged animals exhibit increased seizure susceptibility and an enhanced injury after seizures [22-28]. This was mainly evidenced by the observation that latency to onset of any given behavioral manifestation (e.g. stages III to V seizures) after an excitotoxin such as kainic acid (KA) administration is consistently shorter in aged rats [25] and aged rats exhibit intense behavioral motor seizures with a dose of KA that fails to elicit behavioral motor seizures in young adult rats [27]. Thus, the concept that the aged brain exhibits an increased susceptibility for developing AS is well supported from multiple previous studies. However, it is unknown whether a certain period of AS activity would trigger a greater magnitude of neurodegeneration and inflammation in the aged hippocampus than that observed in the young adult hippocampus. Moreover, it is unclear whether AS in the old age would lead to an increased propensity for developing chronic temporal lobe epilepsy (TLE), an increased intensity of spontaneous recurrent seizures (SRS) in the chronic phase of TLE, in comparison to the effects of AS in the young adult age. Additionally, it is unknown whether AS in old age would induce a greater cognitive dysfunction than AS in the young adult age. Comprehending these issues are important for developing apt therapeutic neuroprotective interventions shortly after AS because ~50% of elderly patients who develop chronic epilepsy typically have a history of an initial precipitating injury such as AS [29, 30].

Therefore, in this study, we compared the above issues between young-adult (5-months old) and aged (22-months old) F344 rats after three-hours of AS activity, induced through graded intraperitoneal injections of kainic acid (KA) and terminated through a diazepam injection. The extent of neurodegeneration in both age groups was quantified at 12 days after AS via an exhaustive stereological counting of surviving neurons that are positive for the neuron-specific nuclear antigen (NeuN) in different hippocampal cell layers. The hippocampal inflammation was quantified through stereological counting of activated microglial cells identified through immunohistochemical staining for the ED-1 antigen. The propensity for developing chronic epilepsy after AS and the intensity of chronic TLE was assessed through measurement of the frequency, intensity and duration of stages III-V SRS in the 2nd and 3rd month after AS. The ability for spatial learning and memory function was investigated in both groups in the 4th month after AS via a water maze test (WMT) and by comparing their learning and memory indices with age-matched naïve control groups.

MATERIALS & METHODS

Animals, and induction & management of acute seizure activity

The animals used in this study were obtained from the National Institutes of Aging colony of F344 rats maintained at Harlan Sprague-Dawley (Indianapolis, IN). Four major groups of rats were used for this study, which comprise intact young adult rats (4-5 months old), intact aged rats (22 months old), young adult rats receiving graded intraperitoneal injections of KA, and aged rats receiving graded intraperitoneal injections of KA. The animals were individually housed in an environmentally controlled room (~23°C) with a 12:12-hour light-dark cycle, and were given food and water ad libitum. All experiments were performed as per the animal protocol approved by the animal studies subcommittee of the Durham Veterans Affairs Medical Center and institutional animal care and use committee of the Duke University Medical Center.

For induction of AS and SE, rats received intra-peritoneal injections of KA every hour until they developed SE. The initial doses of KA were given at 3.0 mg/Kg bw [31-33]. However, when rats began to display stage III seizure activity (characterized by unilateral forelimb clonus), the subsequent KA injection was reduced to a dose of 1.5 mg/Kg bw. The KA injections were terminated when rats displayed either a state of continuous stage IV seizures (typified by bilateral forelimb clonus with signs of rearing) or a first stage V seizure (characterized by bilateral forelimb clonus with rearing and falling) followed by continuous stages III-V seizures for over 10 minutes. Seizures were scored for three hours after the onset of SE as per the modified

Racine's scale [34-36]. Thus, the onset of SE in this study was defined as the occurrence of continuous stage IV seizures for over 10 minutes or the occurrence of a first stage V seizure followed by continuous stages III-V seizures for over 10 minutes. Both young adult and aged animals were allowed to have multiple stages III-V seizures for 3 hours after the onset of SE, following which all motor seizures were terminated with a single dose of diazepam (at 5 mg/Kg bw). All rats that underwent SE were given a moistened rat chow and a 10 ml injection of lactated Ringer's every day for 3-5 days after the SE. To avoid confounds, rats that did not exhibit stages III-V seizures in each of the three hourly windows after the onset of SE were excluded from further analyses in this study. The numbers of seizures during the 3-hours of AS activity were compared between the young adult and aged groups using an unpaired and two-tailed t-test or a nonparametric Mann-Whitney U-test (i.e. when standard deviations of the seizure scores significantly differed between the two age groups).

Animal perfusions and tissue processing

Twelve days after the 3-hour AS activity, subgroups of rats from both young adult and aged groups were fatally anesthetized with isoflurane and perfused transcardially with 4% paraformaldehyde solution. The rats from control groups (intact young adult and intact aged groups) were also similarly perfused. The brains were dissected out, post-fixed in 4% paraformaldehyde for 16 hours at 4^0C and cryoprotected in 30% sucrose solution in phosphate buffer (PB). Thirty-micrometer thick cryostat sections were cut coronally through the entire septo-temporal axis of the hippocampus and collected serially in 24-well plates filled with PB. Every 20th section through the entire hippocampus was then selected in each of the animals and processed for identification of surviving neurons via NeuN immune-histochemistry. Another series of sections (every 20th) were processed for visualization of activated microglial cells through ED-1 immunohistochemistry.

Immunohistochemistry

The sections were rinsed in 0.1M phosphate buffered saline (PBS, pH= 7.4), treated with a PBS solution containing 20% methanol and 3% hydrogen peroxide for 20 minutes and washed thrice in PBS. The sections were next treated with 10% normal horse serum in PBS containing 0.1% Triton-X 100 for 30 minutes and then incubated overnight in respective primary antibody solutions prepared in PBS containing 0.1% Triton-X 100. For NeuN immunostaining, a monoclonal anti-NeuN raised in mouse (1:1000; Millipore) was used. Whereas, for ED-1 immunostaining, a monoclonal anti-rat CD68 raised in mouse (1:500, Serotec) was employed. Following incubation in the primary antibody solutions, the sections were processed using the avidin-biotin complex method using an elite mouse ABC kit (Vector Labs). Briefly, the sections were rinsed in PBS, treated with a biotinylated horse anti-mouse IgG solution (Vector labs) for 60 minutes, washed in PBS, and incubated with the ABC reagent (Vector labs) for 60 minutes. The immunoperoxidase reaction was visualized using diaminobenzidine (DAB) as a chromogen (Vector Labs). The sections were then washed thoroughly and mounted on gelatin-coated slides. The ED-1 immuno-stained sections were counterstained with hematoxylin to visualize the different hippocampal cell layers. The sections were next dehydrated, cleared and cover slipped with Permount.

Measurement and analyses of the numbers of NeuN+ neurons and ED-1+ activated microglial cells

The NeuN+ neurons in different cell layers of the hippocampus were counted in every 20th section through the entire septo-temporal axis of the hippocampus, in every animal belonging to each of the four major groups (n = 4/Group). The hippocampal cell layers for NeuN+ neuron counting comprised the dentate hilus, the dentate granule cell layer and the CA1 & CA3 pyramidal cell layers. Furthermore, the ED-1+ activated microglial cells in different regions of the hippocampus (such as the DG and the CA1 & CA3 subfields) were similarly counted in every 20th section through the entire septo-temporal axis of the hippocampus, in every animal belonging to the young and aged groups that underwent 3-hours of AS activity (n = 4/Group). An optical fractionator method using the StereoInvestigator system (Microbrightfield Inc.) was used for counting of NeuN+ neurons and ED-1+ cells. The StereoInvestigator system comprised a color digital video camera (Optronics Inc., Muskogee, OK) interfaced with a Nikon E600 microscope. Cells that are positive for NeuN/ED-1 in each cell layer/region were counted from 50-300 frames chosen via systematic random sampling scheme in every 20th section using the 100X oil immersion lens. The systematic random sampling procedure provides an unbiased and efficient sampling technique. In this scheme, once the region of interest is marked with a contour, sampling sites are evenly distributed throughout the region of interest. The distribution pattern of the sampling sites in each tissue section is systematic, as the distance from each sampling site to the next is the same. The placement of this pattern

of sampling sites is the random component. However, the counting frame size varied depending upon the cell layer or the region analyzed. For NeuN+ neuron counts, a frame size measuring 20 X 20 µm was used for the granule cell layer and the CA1 and CA3 pyramidal cell layers and a frame size measuring 40 X 40 µm was used for the dentate hilus. On the other hand, for ED-1+ cell counts, a frame size of 40 X 40 µm was used for all three regions (the DG and the CA1 and CA3 subfields).

A detailed methodology employed for cell counts are described in our previous reports [37-39]. To begin with, the contour of cell layers (such as the dentate hilus, the dentate granule cell layer and the CA1 & CA3 pyramidal cell layers) or subfields (such as the DG and the CA1 and CA3 subfields) were delineated in every section using the tracing function of the StereoInvestigator. Following this, the optical fractionator component was activated and the number and location of counting frames and the counting depth for each section was determined by entering parameters such as the grid size, the thickness of the top guard zone (4 µm) and the optical dissector height (8 µm). A computer driven motorized stage then allowed the section to be analyzed at each of the counting frame locations. All NeuN+/ED-1+ cells that were present within the 8µm section depths in each location were counted. The StereoInvestigator program then calculated the total number of NeuN+/ED-1+ cells per each cell layer/region by utilizing the optical fractionator formula, N = 1/ssf.1/asf.1/hsf.EQ-. The abbreviation ssf represents the section sampling fraction, which was 20 in this study as every 20th section was sampled; asf symbolizes the area sampling fraction, which is calculated by dividing the area sampled with the total area of the respective cell layer or subfield (i.e. the sum of cell layer/subfield areas sampled in every 20th section); hsf stands for the height sampling fraction, which is calculated by dividing the height sampled (i.e. 8 µm in this study) with the section thickness at the time of analysis (i.e. 20 µm in intact control animals and 15µm in animals that underwent 3 hours of AS activity); EQ- denotes the total count of particles sampled for the entire cell layer or the subfield.

All data for individual groups are presented as means ± standard errors (SEM). The data on NeuN+ cell counts in intact young adult rats (n=4), young adult rats that underwent 3-hours of AS activity (n=4), intact aged rats (n=4), and aged rats that underwent 3-hours of AS activity (n=4) were compared to determine the possible interaction between Age and AS activity on the extent of neurodegeneration. For this, values from the above four groups were analyzed using two-way ANOVA with Bonferroni post-tests. Specifically, we examined whether the effects of 3-hours of AS activity on the survival of NeuN+ neurons in different hippocampal cell layers differ between the young adult and aged groups. The ED-1+ cell counts were compared between the young adult and aged groups (n=4/group) that underwent 3-hours of AS activity using an unpaired and two-tailed t-test or a nonparametric Mann-Whitney U-test (when standard deviations significantly differed between the two groups).

Assessment of chronic epilepsy and quantification of SRS

In the 2nd and 3rd month after 3 hours of AS activity, the frequency and intensity of SRS in additional subgroups of both young adult and aged rats (n=5/group) were scored in two 4-hr sessions per week (8 hours/week, 32 hours/month, 64 hours total for the two months). The investigators who scored the SRS were unaware of the identity of animals (young versus aged) as random code numbers were assigned to these rats. The scoring comprised quantification of the frequency of all (stages III-V) SRS, frequency of stage-V seizures and the duration of individual SRS. The various parameters of SRS were compared between the young adult and aged groups using an unpaired and two-tailed t-test or a nonparametric Mann-Whitney U-test (i.e. when standard deviations of the seizure scores significantly differed between the two age groups).

Analyses of spatial learning and memory function using a WMT

Animals belonging to all four groups (young adult control rats, young adult rats that underwent 3-hours of AS activity, aged control rats and aged rats that underwent 3-hours of AS activity; n=5/group) were trained to find the platform submerged in water using spatial cues [40]. This training was done in the 4th month after 3-hours of AS activity. The water maze tank was filled with room temperature water to 35 cm height and extra-maze cues were placed on the walls of the room. Rats were first trained to find a circular platform submerged in water within one of the four quadrants using spatial cues. The movement of rat in the water maze tank was continuously video-tracked and recorded using the computerized ANY-Maze video-tracking system. The training comprised 11 sessions over 6 days (one session on day 1 and 2 sessions/day on days 2-6) with four trials per session. This is an extensive spatial learning protocol in which both young adult and aged controls exhibit ability for spatial learning and hence serves as a useful learning protocol for identifying changes in learning ability after 3-hours of AS activity.

Each trial lasted 90 seconds and the inter-trial interval was 120 seconds. The location of the platform remained constant across all trials and days. In each session of training comprising four trials, the animal was released into the pool at each of the four quadrant edges with the animal's head facing the wall of the water maze tank. These start positions are chosen in a pseudorandom fashion across trials. Once the animal reached the platform, it was allowed to remain on the platform for approximately 30 seconds. If the animal failed to reach the platform within the trial ceiling, the experimenter gently guided the animal through the water and placed it on the platform where it remained for 30 seconds. The mean latency to reach the platform (and the swim path length) in different sessions was measured and the overall learning ability in all groups was assessed through repeated measures ANOVA followed by Newman-Keuls multiple comparisons post hoc test. The overall improvement in learning over the training sessions in all groups was also determined by comparing the latencies to reach the platform between the first and last sessions.

Twenty-four hours after the last learning session, rats were subjected to a 45-second memory retrieval (probe) test. For this, the platform was removed and rats were released from the quadrant opposite to the original position of the platform. The total dwell time in the quadrant where platform was originally placed (also termed as "platform quadrant") during the 45-second probe test was measured and used as an index of memory retrieval ability. To determine the possible interaction between the age and AS activity on memory retrieval function in the probe test, dwell times in the platform quadrant from the four groups were analyzed using two-way ANOVA with Bonferroni post-tests. Specifically, we examined whether the effects of 3-hours of AS activity on memory retrieval function differ between the young adult and aged groups. Additionally, we also measured whether age alone impairs memory retrieval function in the intact control groups.

RESULTS

Induction of SE as a function of age

The induction of SE in young adult rats required an average dose of 5.5 mg of KA (Mean ± SEM = 5.5 ± 0.6 mg) which is equivalent to an average of ~4 hourly injections of KA at 3 mg/Kg bw. In contrast, the induction of similar SE in aged rats required an average dose of only 2.38 mg of KA (2.38 ± 0.2 mg) which is equivalent to ~2 hourly injections of KA at 3 mg/Kg bw. Thus, the dose of KA required to elicit SE in aged rats is 57% less than the dose of KA required to elicit SE in young adult rats ($p<0.01$). Interestingly, the average dose of KA that elicited SE in aged rats was insufficient to promote SE induction in young adult rats (data not shown), suggesting that aged rats clearly have an increased susceptibility for developing SE and AS activity. This finding is consistent with the results of previous studies on KA-induced AS and SE in aged rats [25, 27].

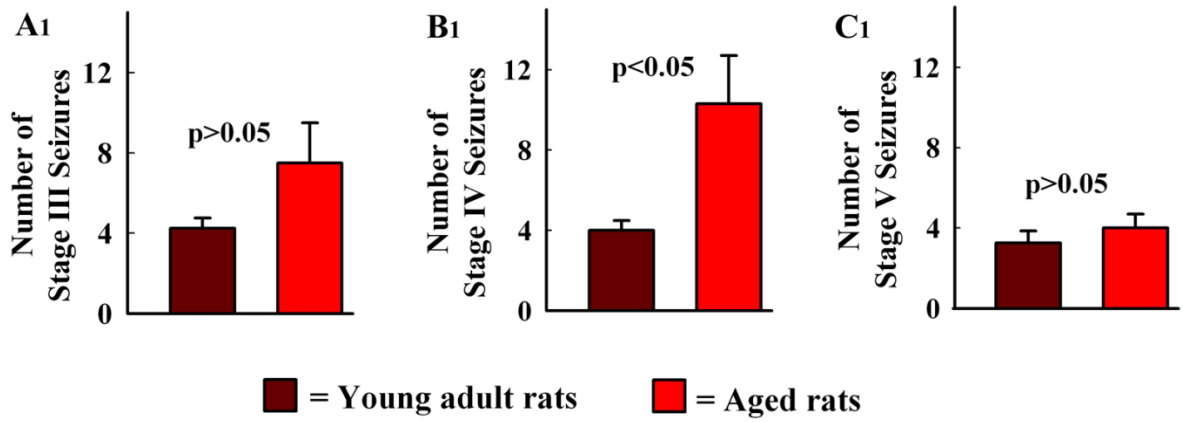

Figure 1 – Comparison of the numbers of stages III-V seizures during the three hours of acute seizure (AS) activity between the young adult and aged rats. Note that there are no significant differences between the two age groups for the numbers of stage III seizures (A1) or stage V seizures (C1). However, the numbers of stage IV seizures (B1) are greater in aged rats than in young adult rats ($p<0.05$).

Extent of AS activity after the induction of SE

We compared the numbers of all stages III-V seizures during the 3-hours of AS activity between the two age groups to ascertain the difference in AS activity after the induction of SE. This analysis demonstrated no significant differences between the two age groups for the numbers of stage III seizures (the milder form of motor seizures, Fig. 1[A1]) or stage V seizures (the most severe form of motor seizures, Fig. 1 [C1]). However, the numbers of stage IV seizures (an intermediate type between the stages III and V seizures) were greater in aged rats than in young adult rats ($p<0.05$; Fig. 1 [B1]). Thus, a much lower dose of KA is capable of not only eliciting SE but also maintaining a greater extent of overall AS activity in aged rats. However, the numbers of most severe form of seizures (i.e. stage V seizures) were comparable between the two age groups after SE induction.

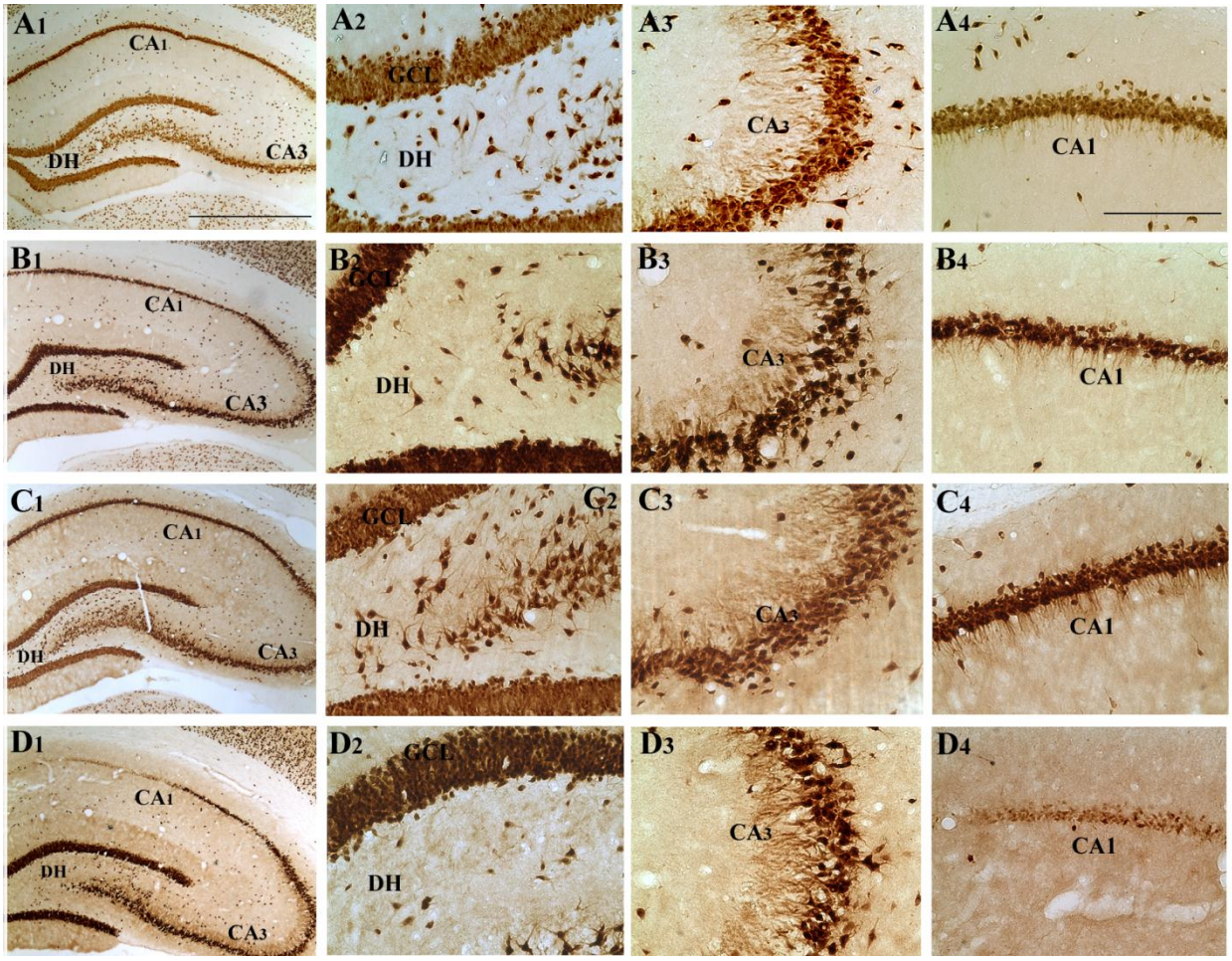

Figure 2 – Comparison of the cytoarchitecture of the hippocampus through NeuN immunostaining between a young adult naïve control rat (A1), a young adult rat at 12 days after three hours of acute seizure (AS) activity (B1), an aged naïve control rat (C1), and an aged rat at 12 days after three hours of AS activity (D1). Scale bar, A1-D1 = 500 µm. A2, B2, C2 & D2 illustrate magnified views of the dentate hilus from A1, B1, C1 and D1. Note the loss of dentate hilar neurons in both young adult and aged rats after AS activity (B2, D2) in comparison to age-matched controls (A2, C2). A3, B3, C3 & D3 show enlarged views of the CA3 pyramidal cell layer from the four groups. Note a reduced packing density of neurons in both young adult and aged rats after AS activity (B3, D3), in comparison to respective age-matched controls (A3, C3). A4, B4, C4 & D4 show magnified views of the CA1 pyramidal cell layer from the four groups. In comparison to age-matched controls (A4, C4), thinning of the CA1 pyramidal cell layer is evident in the young adult rat that underwent AS (B4) and a considerable loss of CA1 pyramidal neurons is obvious in the aged rat that underwent AS (D4). Scale bar, A2-D2, A3-D3, A4-D4 = 100 µm.

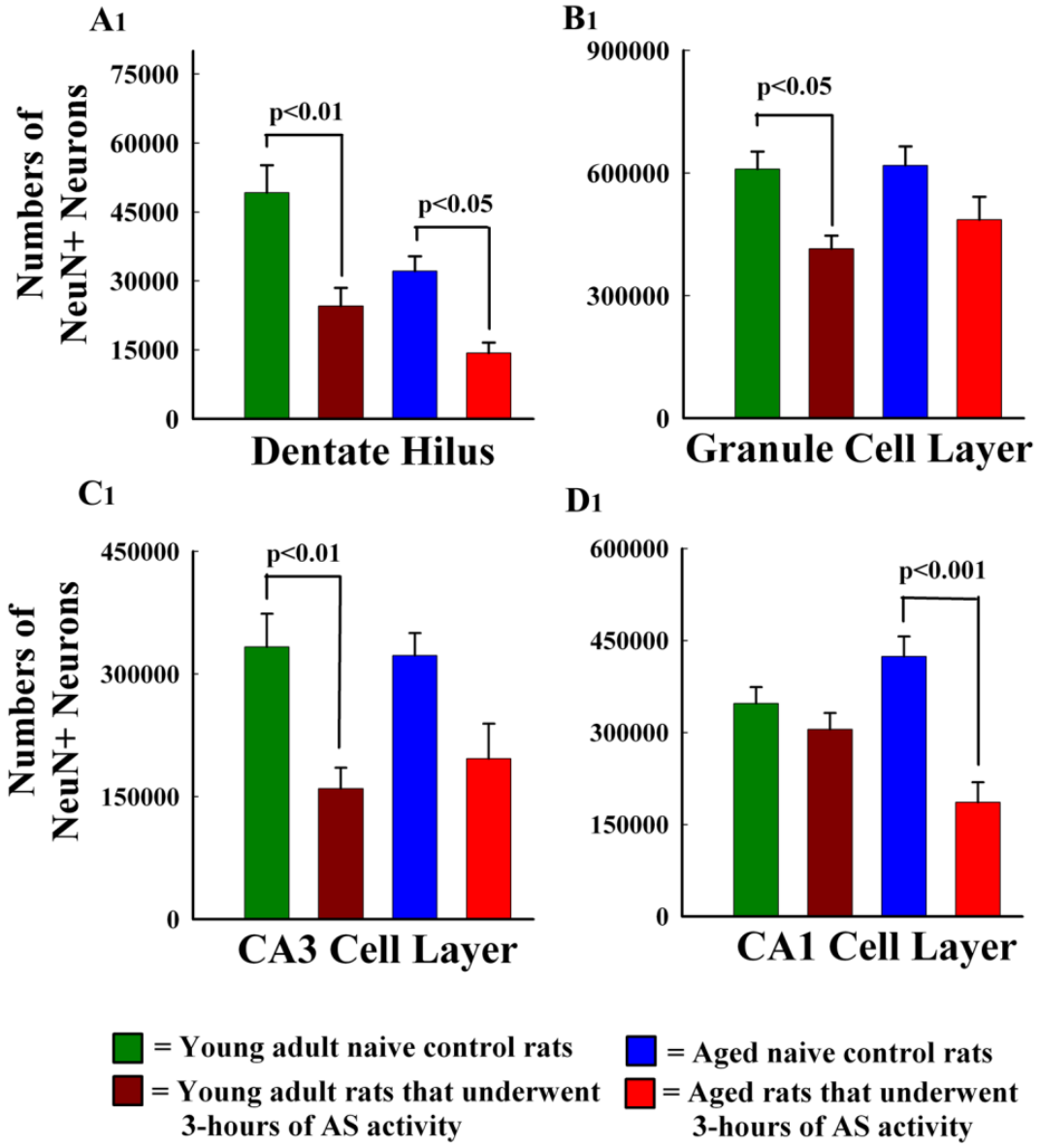

Figure 3 – Comparison of the numbers of NeuN+ neurons between different groups for the dentate hilus (A1), the dentate granule cell layer (B1), the CA3 pyramidal cell layer (C1), and the CA1 pyramidal cell layer (D1). Two-way ANOVA revealed that the loss of neurons after AS between the two age groups was comparable for the dentate hilus (50-55% reduction, $p<0.001$; A1), the dentate granule cell layer (22-32% reduction, $p<0.01$; B1) and the CA3 pyramidal cell layer (49-52% reduction, $p<0.01$; C1). Hence, there was no interaction between the age and AS for the loss of neurons in these regions. In contrast, there was an interaction between age and AS activity for the CA1 pyramidal cell layer (D1) because AS activity induced a differential loss of CA1 pyramidal neurons between young adult rats (12% reduction) and aged rats (56% reduction). Bonferroni post-tests revealed that AS-mediated loss of neurons was significant for the dentate hilus ($p<0.01$), the granule cell layer ($p<0.05$) and the CA3 pyramidal cell layer ($p<0.01$) of young adult rats (A1, B1, C1), and the dentate hilus ($p<0.05$) and the CA1 pyramidal cell layer ($p<0.001$) of aged rats (A1, D1).

Hippocampal neurodegeneration after 3 hours of AS activity

To assess the extent of neurodegeneration after 3-hours of AS activity, we performed NeuN immunostaining in serial sections through the entire hippocampus and quantified the numbers of surviving NeuN+ neurons in different cell layers of the hippocampi from both age groups using the optical fractionator method at 12 days after AS activity. Figure 2 illustrates NeuN+ neurons in distinct cell layers of the hippocampus from different groups. To determine the possible role of interaction between age and AS activity in the overall neuro-degeneration, two-way ANOVA analysis was performed for NeuN+ neuron numbers from the four groups (young adult controls, young adults with AS activity, aged controls, aged with AS activity). In the dentate hilus, there was no interaction between the age and AS activity ($F=0.7$, $p>0.05$) because AS activity induced a similar loss of neurons in both groups (50-55% reduction, $F=27.1$, $p<0.001$)). Bonferroni post-tests revealed that AS-mediated loss of neurons was significant for both young adult ($p<0.01$) and aged ($p<0.05$) groups (Fig. 3 [A1]). However, the number of neurons that was left in the dentate hilus after AS activity in aged rats was 42% less than young adult rats that underwent AS activity. This discrepancy is because aging itself had reduced the numbers of neurons in the dentate hilus by 35% (Fig. 3 [A1]).

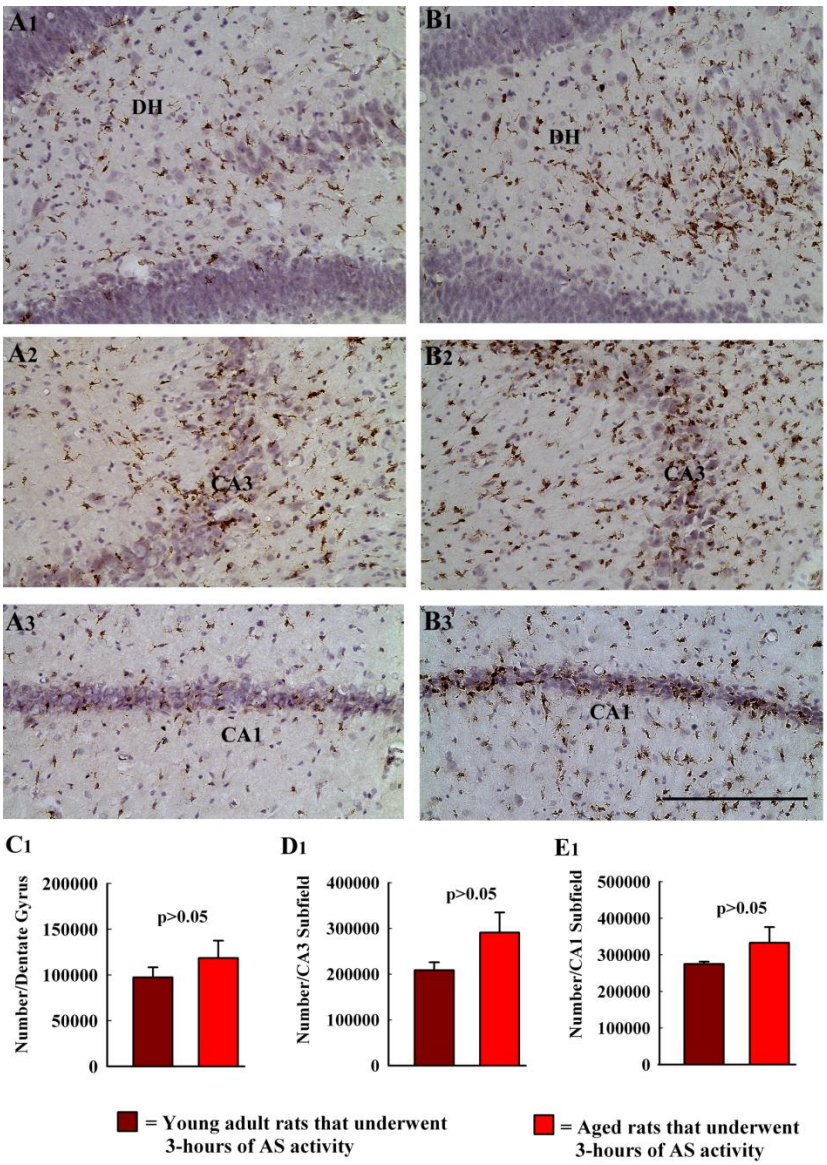

Figure 4 – Comparison of the density and morphology of activated microglial cells (identified through ED-1 immunostaining and hematoxylin counterstaining) in different regions of the hippocampus between the two age groups at 12 days after three hours of AS activity. A1, A2 & A3 respectively illustrate ED-1+ cells (in brown color) in the dentate gyrus (DG), the CA3 subfield and the CA1 subfield of a young adult rat that underwent AS. Whereas, B1, B2 & B3 respectively show ED-1+ elements in the DG, the CA3 subfield and the CA1 subfield of an aged rat that underwent AS. Note that ED-1+ cells in different hippocampal regions of the aged rat (B1-B3) have more complex processes than their counterparts in the young adult rat (A1-A3). Scale bar, A1-B3 = 100 µm. The bar charts in C1, D1 & E1 respectively compare the numbers of ED-1+ activated microglial cells in the DG, the CA3 subfield and the CA1 subfield between the two age groups at 12 days after three hours of AS activity. Note that, the numbers are statistically comparable between the two groups for all regions of the hippocampus.

In the dentate granule cell layer, there was also no interaction between the age and AS activity (F=0.5, p>0.05) because AS activity induced loss of neurons in both young adult and aged groups (22-32% reduction, F=12.1, p<0.01). Bonferroni post-tests revealed that AS-mediated loss of neurons was significant for the granule cell layer in young adult rats (p<0.05, Fig. 3[B1]). The CA3 pyramidal cell layer also showed the same trend. There was no interaction between age and AS activity (F=0.5, p>0.05) because AS activity induced loss of neurons in both young adult and aged groups (49-52% reduction, F=18.4, p<0.01). Bonferroni post-tests revealed that AS-mediated loss of neurons was significant for the CA3 pyramidal cell layer of young adult rats (p<0.01, Fig. 3 [C1]). As a consequence of the above changes and because aging itself did not decrease the numbers of dentate granule cells or CA3 pyramidal neurons, the numbers of neurons that were left in the dentate granule cell layer or CA3 pyramidal cell layer after AS activity in aged rats were comparable to numbers in the young adult rats that underwent similar AS activity.

Contrastingly, in the CA1 pyramidal cell layer, there was a clear interaction between age and AS activity (F=10.7, p<0.01) because AS activity induced a differential loss of CA1 pyramidal neurons between young adult rats (12% reduction, p>0.05; Fig. 3 [D1]) and aged rats (56% reduction, p<0.001; Fig. 3 [D1]) though aging itself did not decrease the numbers. Because of this differential loss, the overall number of neurons in the CA1 pyramidal cell layer of aged rats after AS activity was 39% less than the number in the CA1 pyramidal cell layer of young adult rats that underwent similar AS activity. Thus, advanced age makes the CA1 pyramidal neurons more susceptible to die with AS activity.

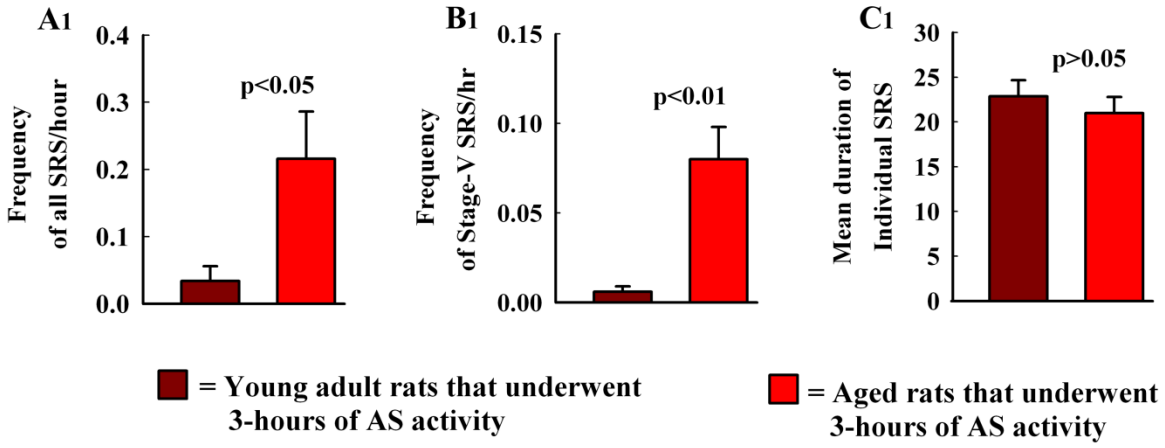

Figure 5 – Comparison of the frequency and intensity of spontaneous recurrent seizures (SRS) between the young adult rats and aged rats in the 2nd and 3rd months after three hours of acute seizure (AS) activity. Figures A1 & B1 compare the frequencies of all SRS (A1) and the stage V seizures (B1; the most severe form of SRS) between the two age groups. Note that in aged rats that underwent three hours of AS activity, the overall frequency of SRS is 6.4 folds greater (p<0.05) and the frequency of stage V seizures is 12.6 folds greater (p<0.01), in comparison to young adult rats that underwent three hours of AS activity. However, the duration of individual SRS between the two age groups was comparable (C1).

Hippocampal inflammation after 3 hours of acute seizure activity

Both young adult and aged rats exhibited large numbers of activated microglial cells immunopositive for ED-1 in all subfields of the hippocampus when examined at 12 days after 3-hours of AS activity (Fig. 4). Qualitative observation of ED-1 immunoreactive elements suggested that the aged rats exhibit a greater level of inflammation than young adult rats after AS activity (Fig. 4 [A1-B3]). However, stereological quantification revealed no differences in numbers of ED-1+ activated microglial cells between the young adult and aged groups for any subfields of the hippocampus (Fig. 4 [C1-E1]. Based on this, it appeared that a greater extent of ED-1 immuno-reactivity in aged rats is due to an increased complexity of processes of ED-1 positive microglial cells than their counterparts in young adult rats (Fig. 4 [A1-B3]). Thus,

the extent of inflammation after AS activity was similar between the young adult and aged rats in terms of the numbers of activated microglial cells but appeared somewhat greater in aged rats in terms of the complexity of the processes of activated microglial cells.

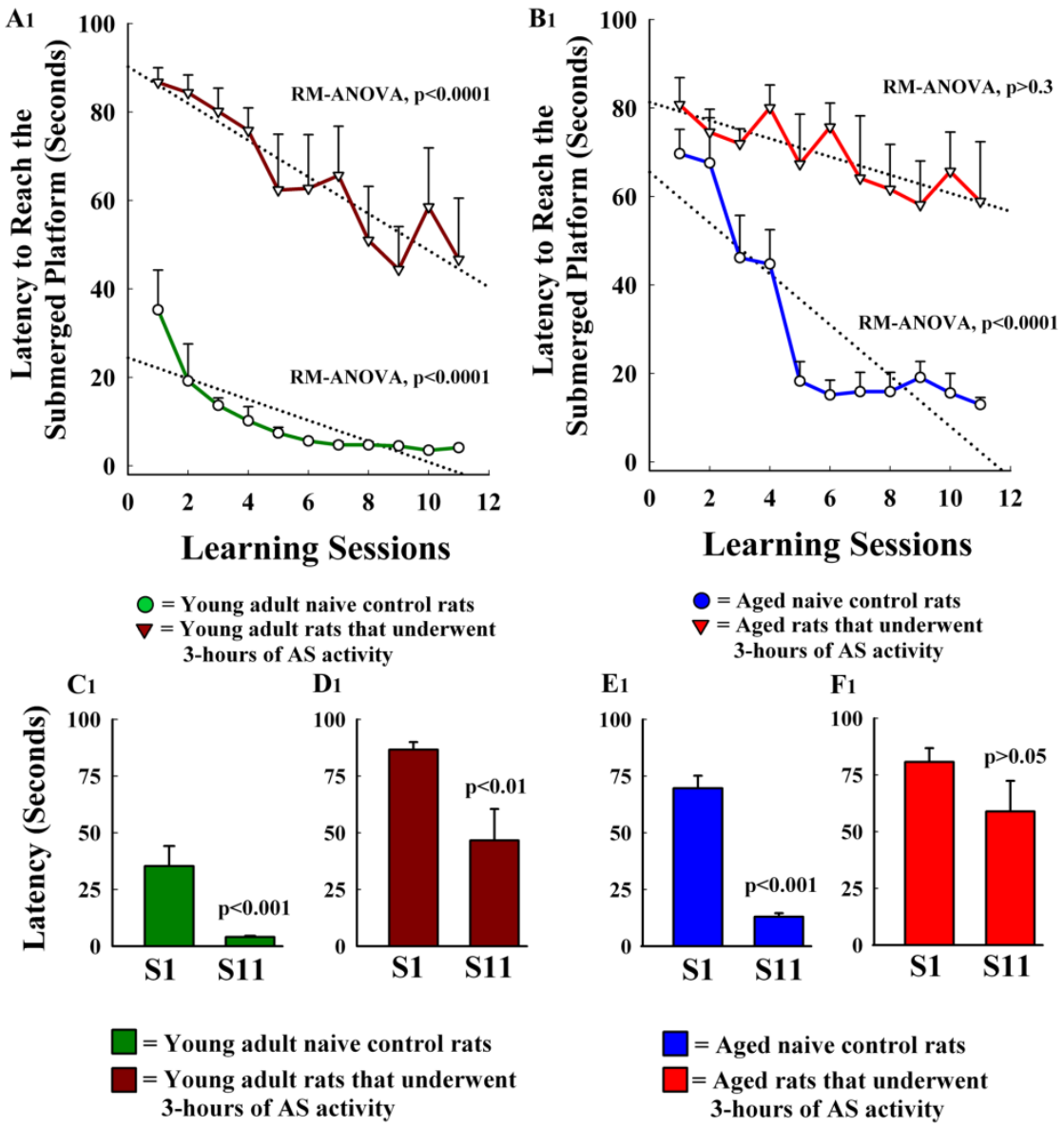

Figure 6 – Comparison of the spatial learning function in a water maze test between young adult naïve control rats & young adult rats at 3 months after acute seizure (AS) activity (A1), and between aged naïve control rats & aged rats at 3 months after AS activity (B1). Note that, repeated measured ANOVA showed capability for spatial learning in the young adult control group (the green colored learning curve in A1), the aged control group (the blue colored learning curve in B1) and the young adult group that underwent 3-hours of AS activity (the maroon colored learning curve in A1). The Newman-Keuls post-tests revealed a significant decrease in the latency to reach the submerged platform between the first and eleventh sessions in all of these groups (C1, D1 & E1). However, the aged group that underwent 3-hours of AS activity exhibited inability for spatial learning (the red colored learning curve in B1). There was also no significant reduction in the latency to reach the hidden platform between the first and eleventh sessions (F1). Thus, among the four groups of rats, only the aged rats that underwent 3-hours of AS activity exhibited a clear inability for spatial learning.

Propensity for developing chronic TLE and the extent of SRS in the chronic phase after AS

Measurement of SRS (stages III-V motor seizures) in the 2nd month after 3-hours of AS activity revealed SRS in 60% young adult rats (i.e. in 3 out of 5 rats measured) and 100% of aged rats (i.e. in 5 out of 5 rats measured), suggesting that aged rats exhibit a greater propensity for developing chronic TLE after 3-hours of AS activity. Quantification of SRS in the 3rd month after AS activity also revealed the same trend. Comparison of the frequency of all SRS observed for the entire recording period of 64 hrs (i.e. in the 2nd and 3rd months after AS activity) revealed that the frequency of SRS in aged rats is 6.4 folds greater than young adult rats ($p<0.05$; Fig. 5 [A1]). Comparison of stage V seizures (the most severe form of SRS) during this period demonstrated that the frequency of stage V seizures in aged rats is 12.6 folds greater than young adult rats ($p<0.01$; Fig. 5 [B1]). The duration of individual seizures in rats that had SRS was however comparable between the two age groups ($p>0.05$, Fig. 5 [C1]). Thus, 3-hours of AS activity in the aged group not only results in the manifestation of SRS in all rats by the 2nd month after AS activity but also leads to a robust chronic TLE characterized by a greater frequency and intensity of SRS than young adult rats.

Extent of spatial learning and memory impairments after 3 hours of acute seizure activity

Both young adult and aged rats were analyzed for spatial learning and memory function using a WMT which comprised a robust learning paradigm of 11 training sessions in the 4th month after 3-hours of AS activity. Similar WMT was also performed on age-matched naïve control rats for comparison. To ascertain the ability for spatial learning in each group, we performed repeated measures ANOVA with learning sessions (n=11) as the repeated measure and the latency to reach the submerged platform as the dependent variable. The Student Newman-Keuls test was employed for post-tests. This analyses revealed a clear capability for spatial learning in the young adult control group ($p<0.0001$, Fig. 6 [A1]), the aged control group ($p<0.0001$, Fig. 6 [B1]) and the young adult group that underwent 3-hours of AS activity ($p<0.0001$, Fig. 6 [A1]). The post-tests also revealed a significant decrease in the latency to reach the submerged platform between the first and eleventh sessions. The reduction was 88% in the young adult control group ($p<0.001$, Fig. 6 [C1]), 81% in the aged control group ($p<0.001$, Fig. 6 [E1]) and 46% in the young adult group that underwent of 3-hours of AS activity ($p<0.01$, Fig. 6 [D1]). However, the overall learning in the young adult group that underwent AS activity was impaired in comparison to the young adult control rats ($p<0.001$, two-way repeated measures ANOVA).

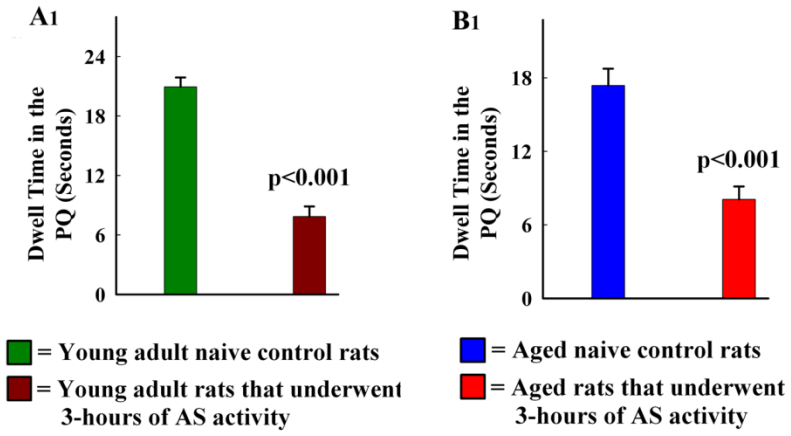

Figure 7 – Comparison of the memory retrieval function between young adult naïve control rats & young adult rats at 3 months after acute seizure (AS) activity (A1), and between aged naïve control rats & aged rats at 3 months after AS activity (B1), as measured through a 45 second probe test conducted at 24 hrs after the last learning session in all four groups. Note that, both young adult control and aged control groups exhibit a clear ability for memory retrieval, which is evidenced by greater dwell times in the platform quadrant (green and blue bars in A1 & B1). In contrast, both young adult and aged rats that underwent AS activity display inability for memory retrieval, which is evidenced by significantly reduced dwell times in the platform quadrant (brown and red bars in A1 & B1).

Contrast to the above three groups, the aged group that underwent 3-hours of AS activity exhibited inability for spatial learning which was evidenced by the repeated measures ANOVA analysis ($p>0.3$, Fig. 6 [B1]). There was also no significant reduction in the latency to reach the hidden platform between the first and eleventh sessions ($p>0.05$, Fig. 6 [F1]). Thus, among the four groups of rats, only the aged rats that underwent 3-hours of AS activity exhibited a clear inability for spatial learning. Inability for spatial learning in this group is neither due to an advanced age nor due to a reduced swim speed with age. This is because age-matched control rats exhibited an excellent ability for spatial learning with the eleven sessions of training paradigm used in this study and the swim speeds (calculated from the distance swam and latency values) were not significantly different between the different groups (data not shown).

The probe test conducted at 24 hrs after the eleventh learning session examined the ability for memory encoding and retrieval in all four groups. Both young adult control and aged control groups exhibited a clear ability for memory retrieval. However, both young adult and aged rats that underwent 3-hours of AS activity displayed inability for memory retrieval. This was evidenced in each of these groups by a considerably reduced dwell time in the platform quadrant (i.e. an area of the quadrant where the platform was placed during learning sessions; Fig. 7). Thus, with 3-hours of AS activity, young adult rats retain their ability for spatial learning in a robust learning paradigm (though the overall learning ability is impaired in comparison to age-matched naïve control rats) but exhibit incapability for retrieving the learned memory at 24 hours after the last learning session. In contrast, with 3-hours of AS activity, aged rats lose their ability for spatial learning even in a robust training paradigm and hence exhibit incapability for memory formation.

DISCUSSION

The results of this study provides a novel evidence that AS activity in the old age results in a much greater brain dysfunction in comparison to the effects of a similar AS activity in the young adult age. This was revealed through a comparative analyses of multiple adverse effects in young adult and aged rats after 3-hrs of AS activity. In comparison to young adult rats that endured AS activity, the aged rats that experienced AS activity exhibited a greater loss of CA1 pyramidal neurons in the hippocampus, an increased proclivity for developing chronic TLE characterized by SRS, an enhanced intensity of SRS in the chronic phase, and a greater impairment in the hippocampal-dependent cognitive function. The issues pertaining to these findings are discussed in the following sections.

Aging and susceptibility to AS

While it is generally believed that the aged population exhibits an increased susceptibility for developing AS, previous studies in animal models have suggested contentious results regarding the effects of advanced age on the vulnerability to AS [41]. Studies on kindling-, maximal electroshocks-, and pentylenetetrazol- induced seizures in certain mouse lines suggested an overall decreased vulnerability to AS with aging [42-45]. However, several other mouse lines exhibited more severe seizure activity in response to KA treatment [28, 46], suggesting that different strains of mice have variable vulnerability to AS in old age. In contrast, evaluation of seizures in rat models after an excitotoxin treatment suggested an increased susceptibility to AS with advanced age [22, 23]. Furthermore, based on multiple age-related changes in brain regions that are prone to seizures (e.g. hippocampus; [47-49]) and an increased hippocampal cell death in the aged brain after an intracerebroventricular excitotoxin administration [24], it appears more likely that advanced age increases the vulnerability to AS induced by excitotoxins. Indeed, a previous study in male F344 rats showed that the latency to onset of any given behavioral manifestation (i.e. stages III to V seizures) after KA administration was consistently shorter in aged rats [25]. Similarly, a study in male Sprague-Dawley rats showed that subcutaneous administration of a single low-dose of KA (5mg/Kg) causes intense behavioral seizures in aged rats but not in young adult rats [27]. Consistent with the above findings in rat models, the current study shows that aged male F344 rats have an increased susceptibility for developing SE and AS activity. This was evidenced by the finding that SE induction in young adult rats requires an average dose of 5.5 mg of KA whereas similar SE induction in aged rats requires only 2.4 mg of KA (i.e. 57% less than the dose required to elicit SE in young adult rats). Additionally, while both young adult and aged groups exhibited similar numbers of stage-V motor seizures during the 3-hrs of AS activity after the induction of SE, the aged group displayed an increased numbers of stage-IV motor seizures. These results underscore that a much lower dose of KA is not only adequate for eliciting SE but also sufficient for maintaining a greater extent of overall AS activity in aged rats.

Thus, studies from rat models clearly show that advanced age is associated with an increased

susceptibility for developing SE and AS activity. While the precise reasons for this increased susceptibility are yet to be ascertained, multiple age-related changes have been suggested to underlie this phenomenon. For example, it has been suggested that an age-related alteration in the limbic and extra-limbic network activity has a role in the increased vulnerability to AS [13, 25]. This hypothesis is supported by several observations. First, advanced age alters the filter function of the DG in a pro-convulsive manner, which is evidenced by a decreased frequency of spontaneous inhibitory post-synaptic potentials and an increased propensity for large amplitude prolonged excitatory postsynaptic potentials following disinhibition [13]. Second, the aberrant mossy fiber innervation of granule cell dendrites observed in the inner molecular layer of the aged hippocampus could contribute to an increased excitatory tone in the DG [11,12]. Third, aging diminishes the numbers of hippocampal GABA-ergic interneurons, which may contribute towards the development of pro-convulsive circuitry in the aged hippocampus [8-10]. Furthermore, it is possible that age-related changes such as: (i) increased numbers of voltage-gated calcium channels [50]; (ii) an altered function of GABA receptors [51]; (iii) an enhanced oxidative stress [27]; and (iv) decreased concentrations of neuroprotective and/or anticonvulsant factors such as the fibroblast growth factor-2, vascular endothelial growth factor, and neuropeptide Y [2, 4] play roles in the increased susceptibility of aged animals to develop AS.

Aging and AS-mediated hippocampal neuro-degeneration

It was not clear from the previous studies whether AS in old age is associated with a greater level of hippocampal neurodegeneration, in comparison to the effects of similar AS in the young adult age. The hippocampus is typically a major focus of AS-induced neuro-degeneration because it is an area of the brain that is most susceptible to damage in response to AS and is believed to be the site that generates most SRS in the chronic phase after AS. A study by Liang et al. [27] compared the extent of hippocampal neurodegeneration in response to a similar dose (5mg/Kg) of KA in young adult and aged rats, a paradigm in which young adult rats did not develop stages III-IV seizures whereas aged rats exhibited stages III-IV motor seizures for 3-4 hours after the KA treatment. Evaluation of neurodegeneration in Nissl stained sections via counts of cells per unit volume showed a significant loss of neurons in the hippocampal CA1 and CA3 pyramidal cell layers of aged rats but not in young adult rats. While this study illustrated that a lower dose of KA can cause both AS and hippocampal neurodegeneration in aged rats, the effects of comparable AS activity on hippocampal neurodegeneration in young adult rats vis-à-vis aged rats could not be ascertained because young adult rats in this study did not exhibit stages III-IV motor seizures [27]. Therefore, in the current study, we compared the effects of 3-hours of AS activity on the extent of hippocampal neurodegeneration between the young adult and aged rats. To measure the absolute numbers of surviving NeuN+ neurons in different cell layers, we counted neurons in serial sections immunostained for the mature neuronal marker NeuN through the entire septo-temporal axis of the hippocampus using the optical fractionator method. Our results demonstrated that 3-hours of AS activity in the young adult and aged rats induced degeneration of 50-55% of neurons in the dentate hilus, 22-32% of neurons in the granule cell layer and 49-52% neurons in the CA3 pyramidal cell layer without showing any interaction between the age and AS. However, the degeneration of neurons in the CA1 pyramidal cell layer showed a clear interaction between age and AS (i.e. 56% in aged rats and 12% in young adult rats), suggesting that an advanced age makes the CA1 pyramidal neurons more susceptible to die with AS activity.

The precise reasons for an increased vulnerability of CA1 pyramidal neurons in the aged brain to AS-mediated death are unknown. However, this may be due an increased level of intracellular calcium in CA1 pyramidal neurons of the aged brain because several studies show that CA1 pyramidal neurons in the aged brain exhibit increased L-type voltage-sensitive calcium channels, elevated intracellular calcium levels, and an altered calcium homeostasis [52, 53]. Furthermore, it is likely that the synaptic connectivity of CA1 pyramidal neurons in the aged brain are altered in a pro-convulsive manner because aging is associated with decreased GABA-B mediated inhibition of the CA1 pyramidal neurons [54, 55] and considerable reductions in both calbindin and parvalbumin expressing GABA-ergic interneurons in the CA1 subfield [10]. Several other age-related changes in the CA1 pyramidal neurons could also be involved, which may include a greater deficit in the concentration of the neuroprotective compound erythropoietin [56] and increased levels of GABA transaminase (a GABA degradation enzyme; [57].

Aging and AS-mediated evolution and intensity of chronic TLE

This study provides a novel evidence that three hours of AS activity is adequate for inducing chronic TLE (typified by SRS) in 100% of aged rats within the 2nd

month after AS, which sharply contrasts with the young adult group where only 60% of rats developed chronic TLE at 2-3 months after AS. This finding suggests that the proclivity for developing chronic TLE after AS is much greater in the aged population than in the young adult population. Furthermore, the fact that 100% aged rats that underwent AS displayed SRS in the 2nd month after AS implies that the latent period for developing chronic TLE after AS is much shorter in the aged population than in the young adult population. Three hours of AS activity also induced a more robust chronic TLE characterized by a greater frequency and intensity of SRS in aged rats, in comparison to young adult rats. However, it remains to be seen whether the young adult rats that were free of SRS at 2-3 months after AS would develop chronic TLE at a much greater delay after AS. Measurement of SRS using continuous electroencephalographic recordings for extended time-points after AS will be needed in the future studies to address this question.

The mechanisms that underlie the AS-mediated increased proclivity/reduced latent period for developing chronic TLE and greater intensity of TLE in the aged group are unknown. However, the following possibilities may have roles in the above phenomena. First, while AS activity induces a similar extent of dentate hilar neuron loss in the young adult and aged groups (in terms of percent loss), the numbers of neurons that are left in the dentate hilus after AS activity are 42% less in aged rats than in young adult rats. This is because the numbers of neurons in the dentate hilus were already reduced by 35% with aging alone. Because most dentate hilar neurons represent GABA-ergic interneurons expressing neuropeptides including the somatostatin and the neuropeptide Y, it is possible that the observed pro-convulsive circuitry in the aged DG [13] becomes convulsive at a faster rate with additional loss of GABA-ergic neurons through AS activity. Second, even though a previous study using a focal hippocampal injury model suggests resistance of the GABA-ergic interneurons to excitotoxic injury in the aged hippocampus [8], it is possible that the overall numbers of surviving GABA-ergic interneurons after AS are greatly decreased in the aged hippocampus, in comparison to the young adult hippocampus after AS. Third, unlike in the young adult rats, AS or focal injury do not increase the addition of new neurons to the granule cell layer in aged rats [33, 58]. Because newly born granule cells that incorporate into the granule cell layer after AS have the ability to reduce the overall excitability of the DG [59], the above loss of plasticity likely contributes towards a faster development of robust convulsive circuitry in the aged DG.

A major role of the aberrant mossy fiber sprouting for the greater intensity for SRS in aged rats that underwent AS activity is unlikely because a previous study has shown a much-dampened mossy fiber sprouting response in aged rats following an excitotoxic lesion in the hippocampus [12]. The role of AS-induced inflammation is also uncertain because the numbers of activated microglial cells in different regions of the hippocampus at 12 days after AS activity were mostly comparable in this study. However, there may be increased levels of oxidative stress as well as inflammation in the epileptogenesis phase (i.e. the latent period between AS and the occurrence of SRS) in the aged group. Thus, increased proclivity and decreased latent period for developing chronic TLE after AS and greater intensity of TLE in the aged group after AS are likely due to multiple factors. Comparative rigorous studies at several time-points after AS activity in the young adult and aged groups are needed in the future to address the above possibilities.

Aging and AS-mediated cognitive dysfunction

Three hours of AS activity did not impair the ability of young adult rats for spatial learning in a dependable water maze paradigm comprising eleven sessions of learning though the overall learning ability was inferior in comparison to age-matched naïve control rats. However, they displayed inability for retrieving the learned memory at 24 hours after the last learning session. In contrast, with 3-hours of AS activity, aged rats completely lost their ability for spatial learning even in the robust training paradigm and hence exhibited incapability for any memory formation with learning. These results suggest that the adverse effects of AS on cognitive function are much severe in the old age than in the young adult age. This could be due to a greater loss of CA1 pyramidal neurons observed in the aged hippocampus after AS and a decreased plasticity of the aged hippocampus to AS or injury. With regard to plasticity, it is known from previous studies that AS activity or focal hippocampal injury in young adult rats increases the concentrations of multiple beneficial neurotrophic factors including the brain-derived neurotrophic factor. However, this kind of neurotrophic response does not occur in aged rats [5].

Furthermore, AS activity or focal hippocampal injury in young adult rats greatly increase the extent of hippocampal neurogenesis [33, 60-62], the substrate believed to be important for functions such as learning and memory [63]. In contrast, a similar AS activity or focal injury fails to enhance hippocampal neurogenesis in aged rats [33, 58]. Increased neurogenesis after AS or

injury in the young adult hippocampus reflects DG plasticity and is likely an attempt from the neural stem cells to self-repair or to reduce the adverse effects of injury on cognitive function. While the newly formed neurons in the DG do not replace the lost CA1/CA3 pyramidal neurons after AS-induced injury, it is likely that the injury-induced increased neurogenesis is involved in reducing the cognitive dysfunction. This is because; the spatial learning and memory function in aged rats mostly predicts the level of DG neurogenesis [64, 65], newly formed neurons in the DG get incorporated into learning & memory circuitry [66], and selective ablation of DG neurogenesis leads to learning & memory dysfunction [63, 67-69] . Moreover, increased DG neurogenesis after injury might strengthen the function of residual CA3 pyramidal neurons in the injured hippocampus via neosynaptogenesis. Since the aged hippocampus fails to increase neurogenesis after injury, it is credible that the overall cognitive dys-function related to injury is much greater in the aged. Additionally, seizures in young adult rats are typically associated with up-regulation of several transcriptional factors such as mRNAs for c-fos and tissue plasminogen activator whereas in aged rats such response is either absent or blunted [70-72]. Thus, a larger loss of CA1 pyramidal neurons and a greatly diminished plasticity in the aged hippocampus in response to AS likely underlie a more severe cognitive dysfunction observed in the aged population after AS.

CONCLUSIONS

Our findings demonstrate that AS in the old age are highly detrimental for brain function. They inflict a much greater loss of hippocampal CA1 pyramidal neurons, increase the likelihood for developing chronic TLE, enhance the intensity of SRS in the chronic phase, and induce a much greater impairment in the hippocampal-dependent cognitive function. Considering these, early application of both anticonvulsant and neuroprotective treatments after the onset of AS is critical for preventing or decreasing the extent of AS-mediated morbidity in the aged population.

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