

Neurogenesis Response of Middle-Aged Hippocampus to Acute Seizure Activity

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Abstract

Acute Seizure (AS) activity in young adult age conspicuously modifies hippocampal neurogenesis. This is epitomized by both increased addition of new neurons to the granule cell layer (GCL) by neural stem/progenitor cells (NSCs) in the dentate subgranular zone (SGZ), and greatly enhanced numbers of newly born neurons located abnormally in the dentate hilus (DH). Interestingly, AS activity in old age does not induce such changes in hippocampal neurogenesis. However, the effect of AS activity on neurogenesis in the middle-aged hippocampus is yet to be elucidated. We examined hippocampal neurogenesis in middle-aged F344 rats after a continuous AS activity for >4 hrs, induced through graded intraperitoneal injections of the kainic acid. We labeled newly born cells via daily intraperitoneal injections of the 5'-bromodeoxyuridine (BrdU) for 12 days, commencing from the day of induction of AS activity. AS activity enhanced the addition of newly born BrdU+ cells by 5.6 fold and newly born neurons (expressing both BrdU and doublecortin [DCX]) by 2.2 fold to the SGZ-GCL. Measurement of the total number of DCX+ newly born neurons also revealed a similar trend. Furthermore, AS activity increased DCX+ newly born neurons located ectopically in the DH (2.7 fold increase and 17% of total newly born neurons). This rate of ectopic migration is however considerably less than what was observed earlier for the young adult hippocampus after similar AS activity. Thus, the plasticity of hippocampal neurogenesis to AS activity in middle age is closer to its response observed in the young adult age. However, the extent of abnormal migration of newly born neurons into the DH is less than that of the young adult hippocampus after similar AS activity. These results also point out a highly divergent response of neurogenesis to AS activity between middle age and old age.

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Introduction

Production of new neurons through proliferation of neural stem/progenitor cells (NSCs) and neuronal differentiation of newly born cells occurs all through life in the hippocampus of nearly all mammals [1–4]. A large body of research in animal models suggests that a significant fraction of newly born neurons (i.e. dentate granule cells) integrate into the hippocampal circuitry and participate in functions such as learning, memory and mood [5–12]. However, the extent of hippocampal neurogenesis varies conspicuously in response to alterations in the dentate gyrus (DG) microenvironment. For example, increased levels of NSC mitogenic factors (such as fibroblast growth factor-2 [FGF-2], brain-derived neurotrophic factor [BDNF], insulin-like growth factor-1 [IGF-1] and vascular endothelial growth factor [VEGF]), enhanced physical activity, caloric restriction, environmental enrichment, and increased neural activity considerably enhance the extent of hippocampal neurogenesis [13–25]. In contrast, aging is a negative regulator of hippocampal neurogenesis, which is typified by an increased quiescence of NSCs likely due to age-related adverse changes in their microenvironment

([26–30]; however, see [31]). Thus, the DG microenvironment appears to be one of the major factors that regulate the extent of hippocampal neurogenesis.

The young hippocampus typically responds to insults such as stroke and acute seizure (AS) activity with considerable enhancements in NSC mitogenic factors and increased hippocampal neurogenesis, which likely reflect a compensatory plasticity in response to injury [32–39]. However, it is unclear whether such injury-induced increases in hippocampal neurogenesis are beneficial or detrimental to the hippocampal function. While studies in certain brain injury models suggest that such plasticity is valuable for diminishing impairments in cognitive function after brain injury [40–42], studies in seizure models suggest that such plasticity (particularly the abnormal migration and synaptic connectivity of newly born neurons) contributes to epileptogenesis and the development of chronic epilepsy [43; however, see 44, 45]. On the other hand, the aged hippocampus responds to insults such as stroke with either mild or no increase in neurogenesis [34]. Furthermore, the aged hippocampus fails to increase the extent of neurogenesis in response to AS activity because of predominantly glial differentiation of newly

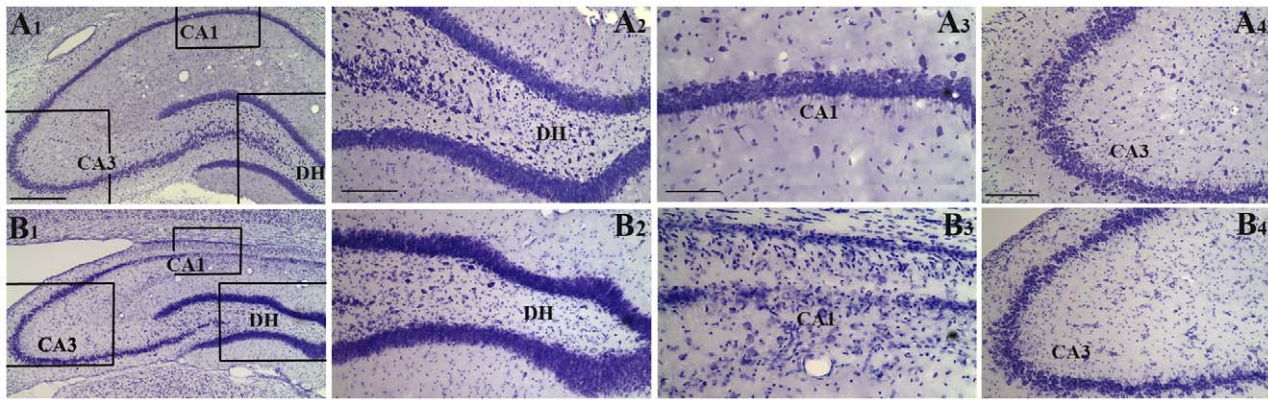


Figure 1. Comparison of the hippocampal cytoarchitecture via Nissl staining between an intact middle-aged animal (A1) and a middle-aged animal that underwent acute seizure (AS) activity (B1). The hippocampal section illustrated in B1 is visualized at 13 days after kainic acid induced AS activity. Figures A2–A4 and B2–B4 show magnified views of marked regions of the DH (A2, B2), the CA1 subfield (A3, B3) and the CA3 subfield (A4, B4) from figures A1 and B1 respectively. Note that considerable neurodegeneration is apparent in the DH (B2), the CA3c subregion (B2) and the CA1 cell layer (B3) of the middle-aged animal that underwent AS activity. Scale bar, A1, B1 = 500 μ m; A2, A4, B2, B4 = 200 μ m; A3, B3 = 100 μ m.
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born cells in the aged DG after AS activity [46]. Moreover, the loss of neurogenic response in the aged hippocampus to AS activity is typically associated with severe memory impairments and chronic epilepsy [47]. Thus, the neurogenic response of the hippocampus diverges considerably between the young adult age and the old age. Additionally, the loss of neurogenic response may be contributing to the severe cognitive dysfunction and chronic epilepsy observed in the aged population after AS activity.

The neurogenic response of the middle-aged hippocampus to AS activity is currently unknown. This is an important issue because the age-related increased quiescence of NSCs, decreases in the basal concentration of NSC proliferation factors, and diminished neurotrophic response after injury, become apparent as early as middle age in the hippocampus [29,30,48,49]. Considering the above, it is of interest to ascertain whether the response of hippocampal neurogenesis to AS activity in middle age is akin to its response observed in the young adult age or to what was seen in the old age. We investigated the extent and pattern of hippocampal neurogenesis after a continuous AS activity (stages III–V seizures) for over 3 hrs in the middle-aged (12-months old) F344 rats. The seizures were induced through 2–4 graded intraperitoneal injections of the kainic acid (KA). Newly born cells in the DG were labeled via daily intraperitoneal injections of the 5'-bromodeoxyuridine (BrdU) for 12 days, commencing from the day of AS activity. Newly born cells and neurons in the subgranular zone (SGZ) and the granule cell layer (GCL) were quantified at 24 hours after the last BrdU injection (equivalent to 13 days post-injury) using BrdU and doublecortin (DCX) immunostaining, BrdU-DCX dual immunofluorescence and confocal microscopy, and the optical fractionator cell counting method [50,51]. Furthermore, to ascertain the extent of aberrant migration of newly born neurons, numbers of ectopically placed newly born DCX-positive (DCX+) neurons were measured in the dentate hilus (DH).

Results

AS Activity and Hippocampal Neurodegeneration with KA Injections

Acute seizure activity was induced in middle-aged (12-months old) F344 rats through 2–4 graded intraperitoneal injections of the excitotoxin KA (3 mg/Kg body weight [b.w.]/hour). Seizures

were observed for over 4 hours after 2–4 injections of KA. The behavioral seizures that manifested as unilateral forelimb clonus (stage III seizures), bilateral forelimb clonus (stage IV seizures), or bilateral forelimb clonus with rearing and falling (stage V seizures) were quantified in seven animals. The average numbers of seizures varied from 7 seizures (Mean \pm SEM, 7.0 ± 0.3) in the first hour, ~ 12 seizures (11.9 ± 0.6) in the second hour, ~ 19 seizures in the third hour (18.8 ± 0.92) and ~ 20 seizures (19.6 ± 1.5) in the fourth hour after the onset of seizure activity. The average duration of individual seizures was ~ 26 seconds (26.3 ± 1.0) in the first hour, ~ 44 seconds (43.5 ± 1.1) in the second hour, ~ 49 seconds (49 ± 1.3) in the third hour, and ~ 53 seconds (53.1 ± 2.4) in the fourth hour after the onset of seizure activity. Kainic acid induced AS activity caused significant neurodegeneration in the hippocampus and extrahippocampal regions when examined 13 days after AS activity. In the hippocampus, bilateral neuronal cell loss was evident in the DH, CA1 pyramidal cell layer and CA3c subregion of the CA3 pyramidal cell layer (Fig. 1 [B1–B4]), in comparison to the intact age-matched hippocampus (Fig. 1 [A1–A4]). However, the dentate granule cell layer did not display any apparent loss of neurons (Fig. 1 [B1, B2]). Thus, the extent of hippocampal neurodegeneration after KA-induced AS activity in middle-aged animals appeared akin to what was reported earlier for young adult and aged animals [46,52].

Addition of Newly Born Cells to the SGZ-GCL after AS Activity

Addition of newly born cells to the SGZ-GCL over a period of 12 days after AS activity was examined via BrdU immunostaining at 24 hours after the last of twelve daily BrdU injections ($n = 5$ /group). This demonstrated newly generated cells in the SGZ-GCL throughout their antero-posterior extent (Fig. 2 [A1–B2]). In comparison to the SGZ-GCL of age-matched intact control animals, SGZ-GCL of animals that underwent AS activity exhibited a clear increase in the density of newly born BrdU-positive (BrdU+) cells (Fig. 2 [A1–B2]). The degenerated regions (the DH and the CA3 pyramidal cell layer) also showed a large number of BrdU+ cells (Fig. 2 [B1]). Quantification of the numbers of BrdU+ cells revealed enhanced addition of newly born cells to the SGZ-GCL after AS activity (5.6 folds increase, $p < 0.001$, Fig. 2 [C1]).

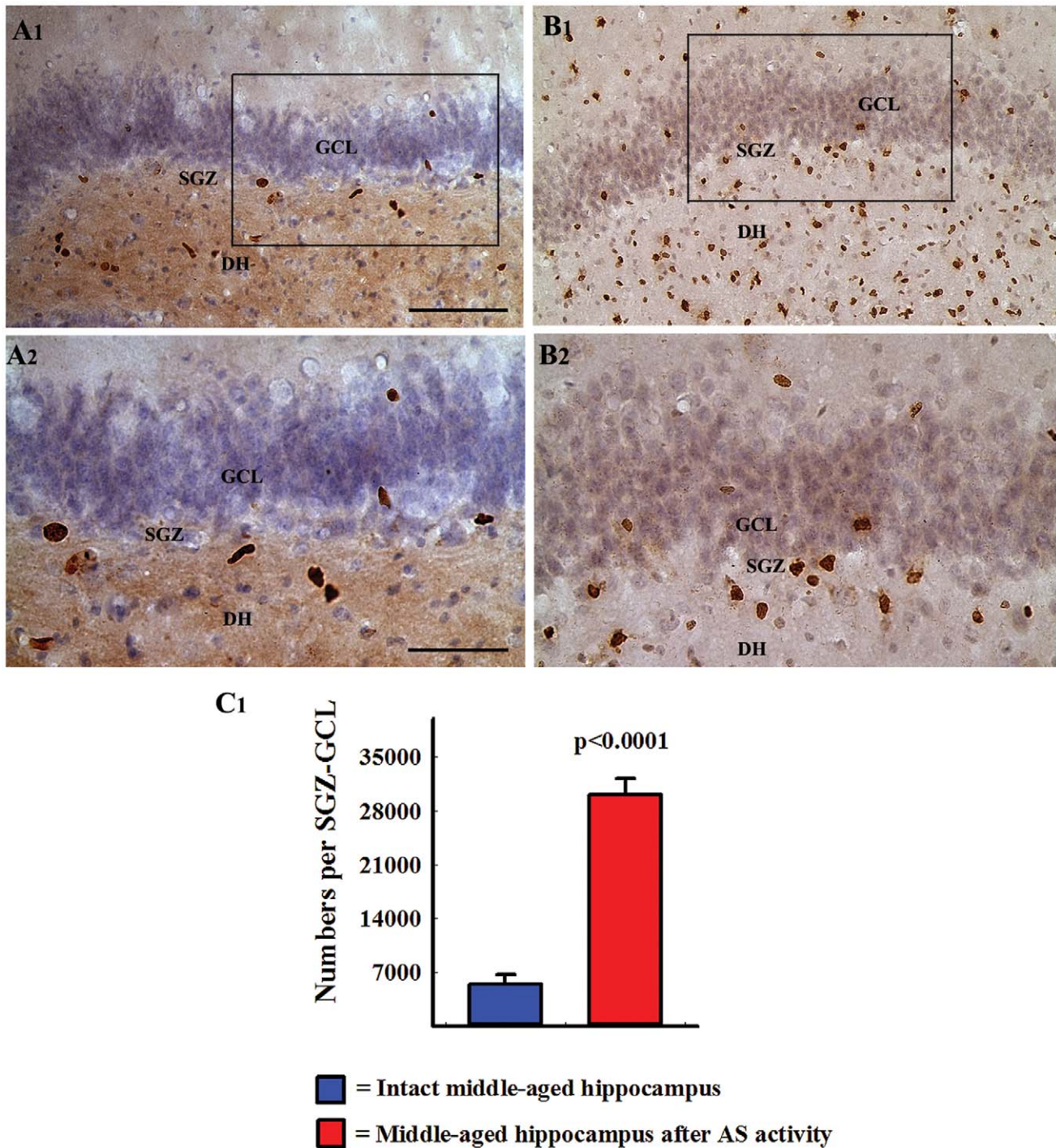


Figure 2. Comparison of the distribution of newly generated cells in the dentate gyrus (DG) at 24 hours after 12 daily injections of 5'-bromodeoxyuridine (BrdU) between an intact middle-aged animal (A1) and a middle-aged animal that underwent acute seizure (AS) activity (B1). The cells are visualized through BrdU immunostaining and hematoxylin counterstaining. A2 and B2 are magnified views of marked regions from A1 and B1. Note that the density of newly born cells in the subgranular zone-granule cell layer (SGZ-GCL) increases in the middle-aged rat after AS activity (B1, B2), in comparison to the age-matched naïve rat (A1 and A2). Also note that the dentate hilus (DH) shows increased density of BrdU immunopositive cells in the rat that underwent AS activity (B1). Scale bar, A1 and B1 = 100 μ m; A2 and B2 = 50 μ m. The bar chart in C1 compares absolute numbers of newly born cells added over a period of 12 days to the SGZ-GCL between intact middle-aged rats and middle-aged rats that underwent AS activity ($n = 5/\text{group}$). Note that, AS activity considerably increases the numbers of newly born cells in the SGZ-GCL of middle-aged rats.

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Extent of Differentiation of Newly Born Cells in the SGZ-GCL into Immature DCX+ Neurons after AS Activity

Dual immunofluorescence for BrdU and DCX and confocal microscopic analyses revealed neurons among newly born BrdU+ cells in the SGZ-GCL (Fig. 3 [A1–C1]). The BrdU expression was found in the nucleus whereas DCX staining was observed in the soma and dendrites of newly born neurons (Fig. 3 [A1–C1]). Doublecortin, an excellent marker of immature neurons in the DG, is typically expressed within 3 hours after birth in neuronally committed newly born cells [53], and serves as a marker for at least two weeks after birth [50]. Quantification of BrdU+ cells expressing DCX revealed that AS activity in middle-aged animals reduced the fraction of newly born cells that differentiate into neurons ($n=5/\text{group}$). The overall neuronal differentiation of newly born cells was reduced to $29.0 \pm 1.5\%$ after AS activity from $74 \pm 1.9\%$ observed in intact animals ($p < 0.0001$, Fig. 3 [D1]).

Extent of Initial Neurogenesis after AS Activity

Using data such as the total number of BrdU+ newly born cells and the percentage of newly born cells that differentiate into DCX+ neurons, we measured the extent of neurogenesis in the SGZ-GCL after AS activity ($n=5/\text{group}$). The extent of hippocampal neurogenesis was clearly increased after AS activity (2.2 folds increase, $p < 0.0001$, Fig. 4). Thus, despite a reduced conversion of newly born cells into neurons, AS activity enhanced the extent of neurogenesis in the SGZ-GCL of middle-aged animals through a much greater increase in the production of new cells.

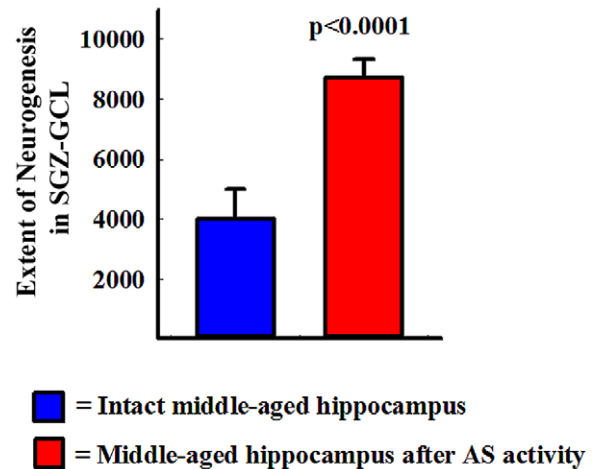


Figure 4. Comparison of absolute numbers of newly born neurons added over a period of 12 days to the subgranular zone-granule cell layer (SGZ-GCL) between intact middle-aged rats and middle-aged rats that underwent acute seizure (AS) activity. The extent of neurogenesis was calculated by using absolute numbers of newly born cells and percentages of newly born cells that differentiate into neurons in both groups. Note that, AS activity considerably increases the extent of neurogenesis in the SGZ-GCL of middle-aged rats ($n=5/\text{group}$). doi:10.1371/journal.pone.0043286.g004

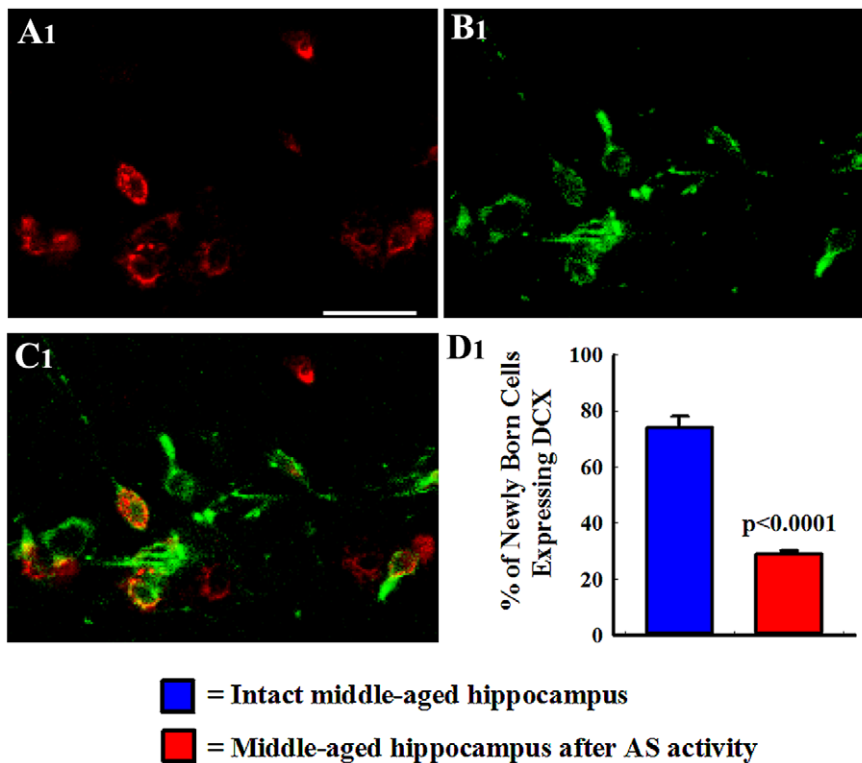


Figure 3. Neuronal differentiation of newly born cells in the subgranular zone-granule cell layer (SGZ-GCL). Analyses were performed at 24 hours after the last of twelve daily injections of 5'-bromodeoxyuridine (BrdU) and visualized through BrdU and doublecortin (DCX) dual immunofluorescence and confocal microscopy. Figures A1–C1 show examples of newly born (BrdU+) cells (red nuclei) that are positive for DCX (green fluorescence in the cytoplasm of the soma and dendrites). The bar chart in D1 compares percentages of BrdU+ cells that express DCX ($n=5/\text{group}$). Note that AS activity diminishes the overall neuronal differentiation of newly born cells. Scale bar, A1–B3 = 20 μm . doi:10.1371/journal.pone.0043286.g003

Numbers of DCX+ New Neurons after AS Activity

We examined DCX+ newly born neurons in the SGZ-GCL and the DH as an additional measure of the status of DG neurogenesis after AS activity (Fig. 5 [A1–D1]; $n=5/\text{group}$). An increased density of DCX+ newly born neurons was observed after AS activity in the SGZ-GCL (Fig. 5 [B1, B2]), in comparison to their counterparts in age-matched intact control animals (Fig. 5 [A1, A2]). The DH showed DCX+ newly born neurons in both intact and post-seizure conditions (Fig. 5 [C1, D1]). However, the density of such neurons was greater after AS activity, reflecting an increased abnormal migration of newly born neurons with AS activity (Fig. 5 [D1]). Most DCX+ newly born neurons that migrated into the DH exhibited horizontally oriented dendrites or dendrites that projected mostly towards the DH (i.e. basal dendrites; Fig. 5 [C1, D1]). In addition, some newly born neurons located in the SGZ-GCL exhibited long basal dendrites (Fig. 5 [C1, D1]), as reported previously for young adult animals after AS activity [54,55]. We separately measured the numbers of DCX+ neurons in the SGZ-GCL and the DH (Fig. 6 [A, B]). This demonstrated that AS activity increased the numbers of newly born DCX+ neurons in the SGZ-GCL by 2.6 folds ($p<0.0001$, Fig. 6 [A]). This level of enhancement is akin to the 2.2 folds increase observed in the extent of neurogenesis described above. This is not surprising because our previous study has shown that a vast majority of DCX+ neurons represent neurons that were born during the 12 days prior to euthanasia [50]. The DH exhibited 2.7 folds increase in the number of DCX+ neurons ($p<0.01$, Fig. 6 [B]). When numbers of newly born neurons in the SGZ-GCL and the DH were combined, the DG displayed 2.6 folds increase in numbers of newly born neurons after AS activity ($p<0.0001$; Fig. 6 [C]). However, percentages of total numbers of DCX+ neurons that migrate into the DH were comparable (17%) between intact rats and rats that underwent AS activity because AS activity proportionately increased the numbers of newly born neurons in both SGZ-GCL and the DH. Thus, the overall neurogenic response of the middle-aged hippocampus to AS activity appeared closer to the response of the young adult hippocampus than the response of the aged hippocampus to AS activity reported earlier [35,37,46].

Discussion

This study provides novel evidence that NSCs in the SGZ of the middle-aged hippocampus retain their ability to enhance neurogenesis in response to AS activity. Our earlier study, by comparing the response of NSCs to AS activity in the young adult and aged hippocampi, has suggested that the ability of NSCs to enhance neurogenesis in response to AS activity is lost with aging [46]. However, the current results demonstrate that major modifications in neurogenesis response to AS activity do not occur at least until the middle age. Interestingly, the overall increase observed in normal neurogenesis in response to AS activity in the middle-aged hippocampus matched the extent of increase seen earlier in the young adult hippocampus after similar AS activity [46]. However, this reaction greatly differed from the lack of neurogenesis response observed in the aged hippocampus after AS activity [46].

Effect of Aging on AS Activity Mediated Normal Hippocampal Neurogenesis

A normal pattern of hippocampal neurogenesis involves continuous addition of newly born neurons to the GCL through proliferation of hippocampal NSCs in the SGZ, and neuronal differentiation and migration of newly born cells into the GCL.

This pattern of neurogenesis in the young or adult hippocampus is highly sensitive to AS activity, which has been authenticated by the observation of increased addition of newly born neurons to the GCL by hippocampal NSCs in several animal models of epilepsy [35–39,55]. Our earlier study performed in young adult F344 rats demonstrated that AS activity enhances the addition of newly born cells to the SGZ-GCL by 2.3 folds but not in the aged (24-month old F344 rat) hippocampus [46]. However, it was unclear whether the loss of neurogenesis response to AS activity would occur as early as middle age or just restricted to the very old age. The current study addressed this issue and demonstrates that NSCs in the SGZ of the middle-aged hippocampus retain their ability to enhance normal neurogenesis in response to AS activity. These results highlight that major alterations in neurogenesis response to AS activity do not occur at least until the middle age. The extent of neuronal differentiation of newly born cells after AS activity in the middle-aged hippocampus (29%) was however midway between the extent seen in the young adult hippocampus (68%) and the aged hippocampus (9%) after similar AS activity [46]. Nevertheless, the rate of conversion of newly born cells into neurons was adequate for enhancing the overall extent of neurogenesis by 2.2 folds because the overall addition of new cells to the SGZ-GCL exhibited 5.6 folds increase after AS activity. The results also imply that age-related changes in the amount of neurogenesis in response to AS activity are not due to a lack of proliferation of NSCs with aging but rather due to changes in the neuronal differentiation of newly born cells, which is likely a consequence of adverse changes in the microenvironment after AS activity with aging. Interestingly, such adverse changes in microenvironment in response to AS activity appear to occur progressively during the course of aging.

The precise role of increased extent of normal neurogenesis after AS activity is yet to be elucidated. However, a previous study shows that newly born neurons added to the GCL after AS-activity have reduced excitability than newly born neurons that are added to the GCL under normal conditions [44]. This has resulted in a suggestion that newly added granule cells to the GCL after AS activity attempt to ease the AS-activity induced hyperexcitability in the DG [44]. From this perspective, increased level of normal pattern of neurogenesis after AS activity appears beneficial for reducing the DG hyperexcitability and spontaneous recurrent seizures observed in the chronic phase after AS activity. Furthermore, recruitment of greater numbers of newly born granule cells into the hippocampal circuitry may also reduce the overall cognitive dysfunction in the chronic phase after AS activity because newly born granule cells that integrate into the hippocampal circuitry are believed to strengthen the learning and memory function [7–12]. If these premises are accurate, one would then expect the aged animals to display greater numbers of spontaneous seizures and severe cognitive dysfunction than young adult animals after similar AS activity because AS activity has no effect on normal neurogenesis in the aged hippocampus but greatly increases normal neurogenesis in the young adult hippocampus [46]. Indeed, our recent study shows that similar levels of AS activity leads to an increased frequency and intensity of spontaneous recurrent seizures and greater cognitive dysfunction in aged rats than in young adult rats [47]. Thus, it is likely that increased levels of normal hippocampal neurogenesis after AS activity are beneficial for reducing both spontaneous recurrent seizures and cognitive dysfunction emerging in the chronic phase after AS activity. However, this compensatory reaction occurring from the plasticity of NSCs in response to seizures is not sufficient for

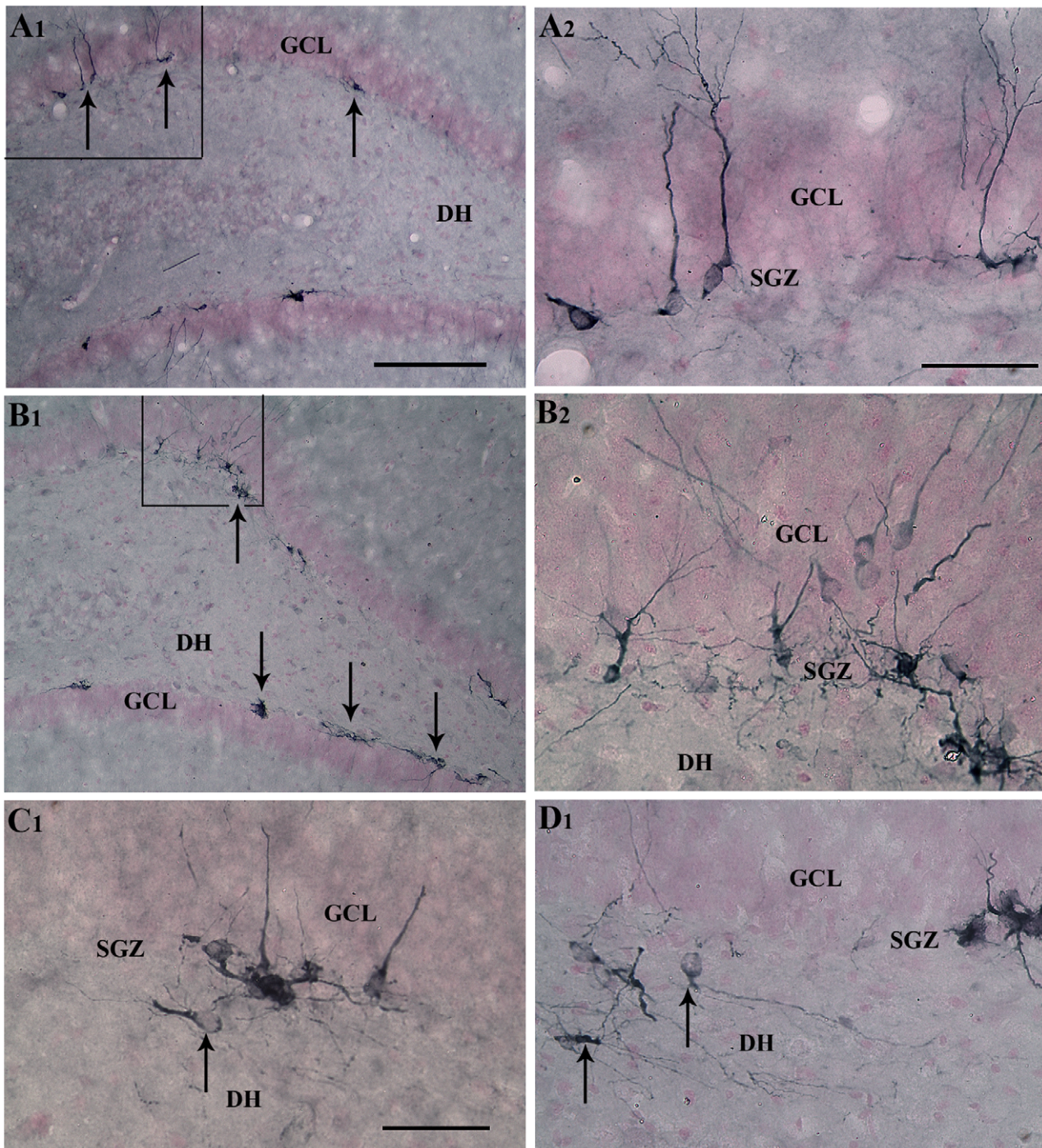


Figure 5. Comparison of the distribution of newly born neurons in the subgranular zone-granule cell layer (SGZ-GCL) between an intact middle-aged animal (A1) and a middle-aged animal that underwent acute seizure (AS) activity (B1). The sections were visualized through doublecortin (DCX) immunostaining. A2 and B2 are magnified views of marked regions from A1 and B1. Arrows in A1 and B1 denote clusters of newly born neurons. Note that the overall density of newly born neurons increases in the middle-aged rat after AS activity (B1, B2), in comparison to the age-matched naïve rat (A1, A2). Figures C1 and D1 show ectopically placed newly born neurons in the dentate hilus (DH; arrows) of an intact middle-aged rat (C1) and a middle-aged rat that underwent AS activity (D1). Scale bars, A1, B1 = 200 μ m; A2, B2, C1, D1 = 50 μ m. doi:10.1371/journal.pone.0043286.g005

blocking the evolution of AS activity induced initial hippocampal insult into chronic epilepsy in young adult animals [47]. This is likely due to the emergence of multiple other epileptogenic changes after the initial hippocampal insult [54,56–63].

Effect of Aging on AS Activity Mediated Abnormal Hippocampal Neurogenesis

Abnormal pattern of hippocampal neurogenesis is characteristically seen after AS activity or brain injury [35,64,65]. This is typified by aberrant migration of significant fractions of newly

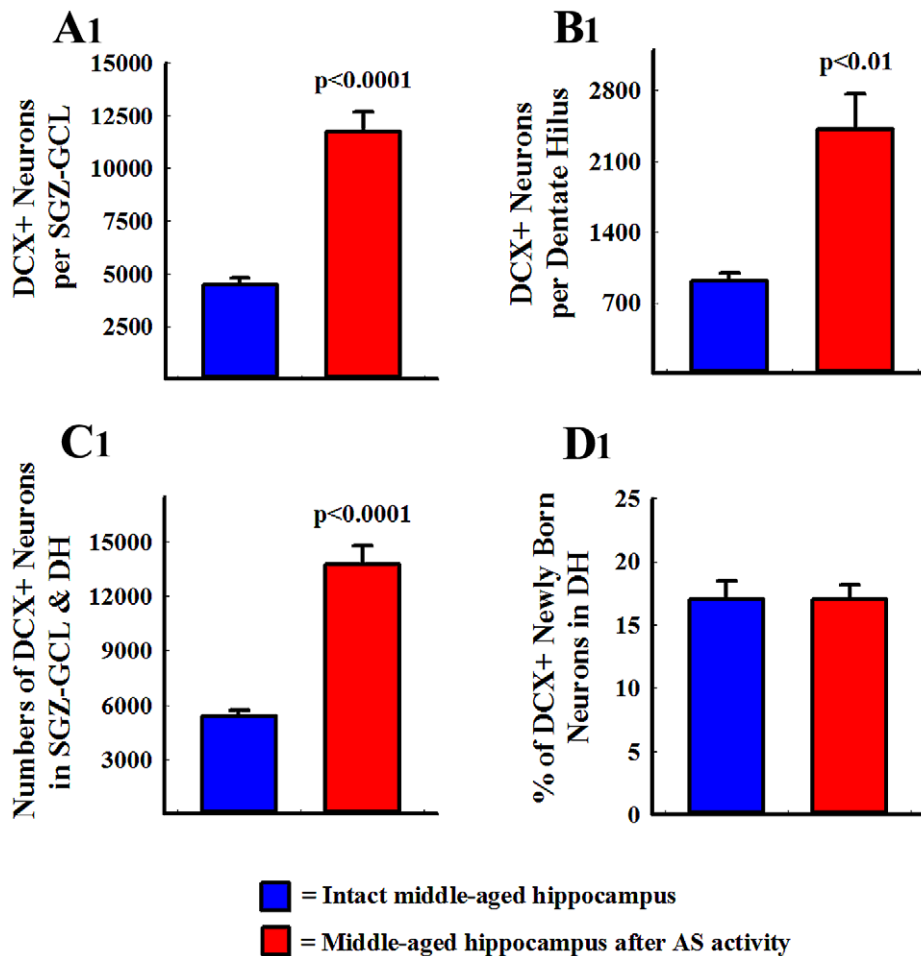


Figure 6. Comparison of the numbers of doublecortin-positive (DCX+) newly born neurons between intact middle-aged rats (n = 5) and middle-aged rats (n = 5) that underwent acute seizure (AS) activity in the subgranular zone-granule cell layer (SGZ-GCL; A1), the dentate hilus (DH; B1), the SGZ-GCL + DH (C1). The numbers of newly born neurons are proportionately increased after AS activity in the SGZ-GCL (A1) and the DH (B1). The bar chart in D1 compares percentages of DCX+ neurons that are located in the DH between the two groups. Note that, the percentage of DCX+ neurons that migrate into the DH are comparable between intact middle-aged rats and middle-aged rats that underwent AS activity.

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born neurons into the DH, which is predominantly conspicuous in the early phase after a brain insult. In this study, AS activity increased abnormal neurogenesis by 2.7 folds in the middle-aged hippocampus, which closely parallels 2.2 folds increase in normal neurogenesis described above. This implies a balanced increase in the numbers of newly born neurons in the GCL and the DH after AS activity in middle-aged animals. This pattern is different from what was seen in the young adult hippocampus but closer to the situation in the aged hippocampus, as AS activity increases abnormal neurogenesis by 10 folds in the young adult hippocampus and 1.7 folds in the aged hippocampus [46]. There is a strong suggestion in the current literature that aberrant integration of newly born granule cells in the DH contributes to the development of spontaneous recurrent seizures in the chronic phase of epilepsy [43,65–71]. Based on the above, one would expect the aged and middle-aged animals to display lower numbers of spontaneous recurrent seizures than young adult animals after similar AS activity because AS activity minimally increases the actual numbers of abnormally displaced granule cells in the DH of the middle-aged and aged hippocampi but greatly increases the population of such neurons in the young adult hippocampus [46].

However, comparison of spontaneous recurrent seizures between aged and young adult animals after AS activity in a recent study reveals that the opposite is true [47]. Thus, a cause-effect relationship between abnormal neurogenesis and incidences of spontaneous seizures seems unlikely. A study in a kindling model of epilepsy also supports this conclusion [45]. Nevertheless, based on the aberrant integration of newly born neurons that migrate into the DH [65–71], some contribution of abnormal neurogenesis to spontaneous recurrent seizures cannot be ruled out particularly in the young adult hippocampus.

Plasticity of NSCs in the Middle-aged Hippocampus to Different Types of Insults

Inability of NSCs in the aged hippocampus to up-regulate normal neurogenesis following various types of insults is well documented in most studies of aged animals and human samples ([34,38,46,72,73]; but see [74]). In contrast, the response of NSCs in the middle-aged hippocampus to various insults is mostly positive in terms of enhancing neurogenesis, be it a model of stroke [75], hippocampal deafferentation [51] or seizures (the current study). However, a few studies have also suggested inability of

NSCs in the middle-aged hippocampus to up-regulate neurogenesis in response to excitotoxic cell death of CA3 pyramidal neurons [38] and chronic cerebral hypoperfusion [76]. This implies that NSC plasticity in the middle-aged hippocampus depends on the type of hippocampal injury. Considerable damage to the middle-aged hippocampus, particularly a significant loss of efferent target neurons of the newly generated neurons such as CA3 pyramidal neuron loss occurring with excitotoxic injury, is associated with loss of NSC plasticity [38]. On the other hand, insults involving a moderate or no loss of CA3 pyramidal neurons such as after AS activity, stroke, and deafferentation stimulate NSC plasticity and enhance neurogenesis in the middle-aged hippocampus [51, 75 and the current study]. Differential response of neurogenesis in the middle-aged hippocampus to brain insults may also be due to diverging response of neurotrophic factors that positively regulate neurogenesis such as BDNF, FGF-2 and VEGF to various types of hippocampal injury. This possibility is also supported by the earlier findings that CA3 region excitotoxic injury in the middle-aged hippocampus does not increase the concentration of BDNF to levels seen in the young adult hippocampus after similar injury [49].

Materials and Methods

Animals

Middle-aged (12-months old) animals obtained from the National Institutes of Aging colony of F344 rats (Harlan Sprague-Dawley, Indianapolis, IN) were used in this study. A week after arrival, animals were classified into two groups: a control group ($n = 5$) where animals were maintained without any intervention, and a seizure group where animals received graded intraperitoneal injections of KA ($n = 8$). All experiments were performed as per the animal protocol approved by the animal studies subcommittees of the Durham Veterans Affairs Medical Center and the Central Texas Veterans Health Care System.

Induction of AS Activity in Middle-aged Rats

Acute seizure activity in middle-aged rats was induced through graded intraperitoneal (i.p.) injections of KA, as described in our previous studies [46,47]. Intraperitoneal injections of KA were administered to animals every hour at a dose of 3.0-mg/Kg b.w. until they developed continuous AS activity or status epilepticus (SE). Induction of continuous AS activity in middle-aged rats required 2–4 hourly injections of KA. Thus, each animal received a total KA dose of 6–12.0 mg/Kg b.w. Seizures were scored as described in our earlier report [46]. The stages III–V motor seizures were typified by unilateral forelimb clonus with lordotic posture (stage III), bilateral forelimb clonus and rearing (stage IV) and bilateral forelimb clonus with rearing and falling (stage V). The onset of continuous AS activity was defined as the first stage IV or V seizure that did not decline after several minutes. All animals receiving the above doses of KA exhibited continuous stages III–V motor seizures for >4 hours after the onset of AS activity. We quantified the number of motor seizures (stages III–V) and the duration of individual seizures for each hour during the period of SE. Rats that underwent AS activity were given moistened rat chow and a 5–10 ml injection of lactated Ringer's solution every day for 3–5 days. Out of 8 rats that underwent AS activity, two rats died during the survival period after the induction of AS activity and one rat was excluded as it exhibited seizures for less than three hours. The remaining 5 rats were used for further studies as described below.

Labeling of Newly Born Cells in the DG and Tissue Processing

Rats belonging to both control group and the group that underwent AS activity received daily i.p. injections of BrdU (Sigma, St Louis, MO) for 12 consecutive days, at a dose of 100-mg/kg b.w. In rats that underwent AS activity, daily BrdU injections commenced on the day of AS activity and ended on post-seizure day 12. Rats were fatally anesthetized with isoflurane and perfused with 4% paraformaldehyde at 24 hours after the last of twelve daily BrdU injections and brains were collected. The brains were next post-fixed in 4% paraformaldehyde for 16 hours at 4°C and cryoprotected in 30% sucrose solution in phosphate buffer (PB). Thirty-micrometer thick cryostat sections were cut coronally through the entire antero-posterior axis of the hippocampus and collected serially in PB. Every 15th section through the hippocampus was selected in each of the animals and processed for Nissl staining to visualize the hippocampal cytoarchitecture as well as the extent of hippocampal injury after AS activity. Nissl staining was accomplished through histological processing of serial sections mounted on glass slides for hydration, cresyl violet treatment, dehydration and clearing.

Measurement of Numbers of BrdU+ Cells and DCX+ Neurons in the DG

A set of serial sections (every 15th) through the entire septo-temporal axis of the hippocampus were selected in each animal belonging to the two groups and processed for BrdU immunostaining using a monoclonal antibody to BrdU (Roche; Indianapolis, IN). Another series (every 15th) of sections from animals were processed for DCX immunostaining using a polyclonal antibody to DCX (Santa Cruz Biotechnology; Santa Cruz, CA) using the avidin-biotin complex method, as described in our earlier reports [27,28,50]. The BrdU+ cells in the dentate SGZ (two-cell thick region from the inner margin of the dentate GCL) and the GCL were counted in every 15th section through the entire antero-posterior extent of the hippocampus in every animal belonging to the two groups ($n = 5$ /group). Furthermore, DCX+ neurons in the SGZ-GCL and the DH were counted separately in animals belonging to the two groups. The StereoInvestigator system (Microbrightfield Inc., Williston, VT), consisting of a color digital video camera (Optronics Inc., Muskogee, OK) interfaced with a Nikon E600 microscope was used for counting of cells [27,28,51]. In brief, cells were counted from 50–400 randomly and systematically selected frames (each measuring 40×40 μm) in every selected section using a 100X oil immersion objective lens. The numbers and densities of counting frames were determined by entering grid size in the optical fractionator component of the StereoInvestigator system. For cell counts in every chosen section, the contour of SGZ-GCL area was first delineated using the tracing function. The optical fractionator component was then activated, and numbers and locations of counting frames and the depth for counting was determined by entering parameters such as grid size, thickness of the top guard zone (4 μm) and the optical dissector height (i.e. 8 μm). A computer driven motorized stage then allowed the section to be analyzed at each of the counting frame locations. All cells that came into focus in the middle 8-μm section thickness were counted if they were entirely within the counting frame or touching the upper or right side of the counting frame. The StereoInvestigator program then calculated the total number of BrdU+/DCX+ cells per each chosen region (SGZ-GCL or the DH) by utilizing the optical fractionator formula, as described in our earlier reports [27,28,51].

Analyses of Neuronal Fate-choice Decision of Newly Born Cells

For measuring the percentages of newly born cells (BrdU+ cells) that differentiated into DCX+ neurons in the SGZ-GCL, we processed serial sections from animals belonging to both groups for DCX and BrdU dual immunofluorescence, as described in our earlier reports [28,51]. Cells that exhibited BrdU and DCX co-expression were identified using a confocal microscope. Fractions of BrdU+ cells that expressed DCX were quantified by examination of individual BrdU+ cells via one-micrometer thick optical Z-sections sampled from different regions of the SGZ-GCL.

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Statistical Analyses

For every parameter, the average value was first calculated separately for each animal before the means and standard errors were determined for the total number of animals included per group. The values (Mean \pm S.E.M.) from two groups of animals were compared using a two-tailed, unpaired Student's t-test.

Author Contributions

Conceived and designed the experiments: AKS. Performed the experiments: MSR BH BS. Analyzed the data: AKS BH MSR. Contributed reagents/materials/analysis tools: BS MSR BH. Wrote the paper: AKS.

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