Research report

Pilocarpine-induced status epilepticus increases cell proliferation in the dentate gyrus of adult rats via a 5-HT$_{1A}$ receptor-dependent mechanism

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Abstract

The dentate gyrus continues to produce granule neurons throughout life. Mossy fibers, the axons of granule neurons, undergo atypical sprouting in both clinical and experimental mesial temporal lobe epilepsy. Mossy fiber sprouting (MFS) has been hypothesized to underlie the network reorganization that is thought to produce spontaneously recurring seizures, possibly via the formation of new recurrent excitatory circuits. Hippocampal neurogenesis may be a critical step in the development of MFS, given that it is enhanced by at least 2-fold in the aftermath of pilocarpine-induced status epilepticus. Since it is known that serotonin (5-HT) 1A receptor activation also increases granule cell genesis in the dentate gyrus in rats, and reciprocally, that blockade of this receptor decreases it, we examined whether 5-HT$_{1A}$ receptor blockade would prevent the seizure-induced enhancement of neurogenesis. The ability to block seizure-induced neurogenesis would provide a test for its role in the network reorganization, especially in regards to MFS, which might underlie seizure development. In the present study, it was found that blockade of the 5-HT$_{1A}$ receptor before and after pilocarpine treatment prevented seizure-induced hippocampal cell proliferation and survival, and, its prevention by chronic treatment with a 5-HT$_{1A}$ receptor antagonist (WAY-100,635) did not prevent the development of MFS or spontaneously recurring seizures. Taken together, these results suggest that 5-HT$_{1A}$ receptor activation is a critical step in the activation of seizure-induced cell proliferation and survival in the dentate gyrus, however, not for the onset of spontaneously recurrent seizures and MFS.
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1. Introduction

Mesial temporal lobe epilepsy (MTLE) is a debilitating clinical disorder with a distinct histopathology in the hippocampus and neighboring regions [44]. The pilocarpine model of epilepsy in rodents has been used in the study of MTLE, since it also produces spontaneously recurring limbic seizures and extensive damage to the temporal lobe [11,37] Furthermore, the damage produced in the hippocampal formation after pilocarpine treatment is quite similar to that seen in MTLE, including neuron loss in the pyramidal cell layers and hilar regions [1,14,19,42], gliosis [6,33,40], granule cell layer delamination [24,25,45], and mossy fiber axon sprouting [2,26,37].

One of these changes, mossy fiber sprouting (MFS), has several features which suggest that it may be important for the development of experimental epilepsy, and perhaps MTLE in humans. In MFS, granule cells of the dentate gyrus undergo axon collateral sprouting into the inner molecular layer [2,32] resulting in the formation of new axodendritic granule cell-granule cell synapses [43]. Functional studies have shown that MFS results in the forma-

tion of new recurrent excitatory circuits which may increase the overall excitability in the dentate gyrus [27,58,60], and could be a critical step in the development of epilepsy.

Another property of the dentate gyrus that may further implicate it in the development of epilepsy, is its continual production of granule neurons through adulthood. Under normal conditions, new neurons originate from dividing progenitor cells in the subgranular zone and migrate to the granule cell layer [9,30,31]. Following seizures, neurogenesis is substantially increased, with at least some of these new neurons migrating ectopically [45]. Seizure-induced progenitor cell division occurs after brief seizures [5,55], and following more prolonged seizures, such as status epilepticus [39,45]. When considered in the context of epilepsy and network reorganization in the hippocampus, seizure-induced neurogenesis may give rise to MFS, either solely, or in tandem with preexisting mossy fibers, or, it may itself be critical for the development of epilepsy, independently from any role in MFS.

The putative link between seizure-induced neurogenesis and MFS was addressed in a previous study [47], where it was shown that the abolishment of cell proliferation with whole brain X-irradiation, applied before and after pilocarpine-induced status epilepticus, did not prevent MFS. However, the interpretation of these studies is compromised by the global effects that X-irradiation may produce, including alterations in gene expression [16,17], and the death of other types of proliferating cells.

In the present study, our objective was to examine the effects of preventing seizure-induced neurogenesis on the development of rodent epilepsy (using the pilocarpine model). It has been shown that drugs which elevate synaptic levels of 5-HT [28,29,36], and a specific 5-HT$_{1A}$ receptor agonist [20,29], all increase cell proliferation and survival in the dentate gyrus of adult rats. Reciprocally, brain 5-HT depletion substantially decreases hippocampal neurogenesis [7,8], as does systemic administration of specific 5-HT$_{1A}$ receptor antagonist drugs [28,29,52]. It has also been shown that 5-HT levels are elevated in the hippocampal formation during and following seizure induction [10,12,61], and that 5-HT$_{1A}$ receptors are present in the dentate gyrus granule cell layer and hilar border [18]. This provided the rationale for our hypothesis that seizure-induced neurogenesis might be activated through the 5-HT$_{1A}$ receptor. Therefore, we assessed whether continuous 5-HT$_{1A}$ receptor antagonist treatment would prevent seizure-induced neurogenesis, MFS, and spontaneous limbic seizures, using the pilocarpine model of epilepsy. To this end, we examined 5-bromo-2′-deoxyuridine (BrdU) labeling of mitotically active cells, Timms staining for mossy fiber axons, and behavioral monitoring for stage 5 spontaneously recurring seizures, according to the scale of Racine [49]. Some of these results appeared in previous abstracts [50,51].

2. Materials and methods

2.1. Animal treatments

Male Sprague–Dawley rats (Charles River, 200 g, 50 days old) were used for all experiments. All procedures were conducted in accordance with the Princeton University Institutional Animal Care and Use Committee. Food was removed on the night before the induction of SE with pilocarpine (325–350 mg/kg, i.p.). All animals received methylatropine (5 mg/kg, i.p.) 30 min before pilocarpine administration to limit peripheral cholinergic effects. Animals were monitored throughout SE induction, and seizure severity was assessed according to the scale of Racine [49]. Animals that did not show obvious signs of SE were not used, since at least 1 h of SE is required to develop SRS in the pilocarpine model of epilepsy [35]. SE occurred within 15–60 min and was characterized by continuous motor-limbic seizures accompanied by intermittent rearing and falling and occasional wild running spells. After 3 h of SE, animals were lightly anesthetized with a pentobarbital (10 mg/kg, i.p.)/diazepam (5 mg/kg, i.m.) combination. In order to administer WAY-100,635 (N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-(2-pyridinyl) cyclohexanecarboxamide trihydrochloride) a 5-HT$_{1A}$ receptor antagonist, continuously for 14 days, Alzet model 2002 miniosmotic pumps (Alza) were implanted subcutaneously (4 mg/kg per day×14 days) in the upper region of the back. Control rats received saline instead of WAY-100,635. For several days following SE, animals were injected subcutaneously with lactated Ringer's solution daily and provided with moist rodent chow until they fully recovered.

2.2. Experimental procedures

2.2.1. Experiment 1: 3 hours into SE

Cell proliferation and cell death in the dentate gyrus were examined immediately following 3 h of SE in three groups: PILO ($n=4$); PILO+WAY ($n=5$); CONTR ($n=5$). Animals in the PILO+WAY group were injected subcutaneously with WAY-100,635 (5 mg/kg) 30 min prior to pilocarpine treatment, and in 90-min intervals thereafter (for a total of three injections). After 2 h of SE and 1 h before sacrifice, rats were injected intraperitoneally (i.p.) with BrdU (200 mg/kg, dissolved in 0.9% NaCl and 0.007 N NaOH) (Sigma, St. Louis, MO) in order to label mitotically active cells in S-phase [38,41].

2.2.2. Experiment 2: 14 days post-SE

Cell proliferation and MFS in the dentate gyrus were assessed at 14 days post-SE in PILO ($n=3$), CONTR ($n=5$) and following continuous WAY-100,635 administration (PILO+WAY; $n=4$). On the 14th day, BrdU (200 mg/kg, i.p.) was administered 2 h before perfusion in

order to label proliferating cells. Brains were processed for BrdU immunohistochemistry, and the Timms staining procedure for heavy metals (the mossy fiber axon pathway is distinguished by an unusually high content of zinc).

2.2.3. Experiment 3: 4 weeks post-SE

Cell survival, MFS in the dentate gyrus, and spontaneously recurring seizures (SRS) were assessed after acute and chronic WAY-100,635 administration 14 days after SE in three groups: PILO ($n=5$); PILO+WAY ($n=6$); CONTR ($n=6$). Rats were implanted with minipumps (loaded with saline or WAY-100,635) at days 1 and 14, in order to deliver WAY-100,635 continuously for the 4-week duration of the experiment. BrdU (50 mg/kg, i.p.) was administered once daily, on days 3–7 post-SE. The choice to administer single daily injections of BrdU during this period was based on the initial report that cell proliferation in the dentate gyrus is maximally elevated on days 3 and 7 following pilocarpine-induced SE [45]. Some rats were also video-monitored (PILO, $n=5$; PILO+WAY, $n=6$) for SRS for 12 h daily, starting at 4 days post-SE. Brains were processed for BrdU immunohistochemistry and Timms staining.

2.2.4. Experiment 4: 8 weeks post-SE

MFS and SRS were assessed after a 4-week period of WAY-100,635 (4 mg/kg per day, s.c.) administration followed by a 4-week observational period in the three groups: PILO ($n=12$); PILO+WAY ($n=12$); CONTR ($n=10$). After 4 weeks of recording (day 57), the rats were perfused, and the brains were processed for BrdU immunocytochemistry and Timms staining.

2.3. Seizure monitoring

In order to assess whether rats developed epilepsy following pilocarpine treatment, the PILO and PILO+WAY groups were observed for stage 5 spontaneously recurring seizures (SRS), via recording with a wide-angle lens video camera-VCR system from 07:00 to 19:00 h daily, corresponding to the light portion of the L-D cycle. The decision to record only the diurnal period was based on previous reports that in the pilocarpine model SRS are most prevalent in the light period [11,34]. Video monitoring was conducted by placing 12 cages ($n=6$ for each group) sideways on a cage rack so that all animals could be recorded and scored on a single video record. Observations were made on a wide screen TV and SRS scored by an observer unaware of the experimental conditions. Criteria used for scoring SRS were similar to stage 5 kindled seizures, defined as at least one sequence of rearing and falling with rolling over on side accompanied by chewing, facial automatisms, and generalized clonic

seizures [49]. Stage 5 SRS lasted anywhere from 15 to 60 s. The choice to set stage 5 seizures as the threshold for scoring SRS was done so partly out of expedience (i.e., monitoring 12 rats for 12 h daily), and based on previous literature [11,37]. Hence, measuring only stage 5 SRS was expected to be a measure of, but not an exhaustive account of, SRS frequency since: (1) SRS similar to stage 3–4 kindled seizures was noted in many of the animals, and (2) nonconvulsive seizures were ignored, and would require EEG recording to observe. This threshold also allowed a clear distinction for SRS, since classification of the less severe SRS (especially stage 1–2 SRS) was nearly impossible to observe.

2.4. Tissue processing

Animals were deeply anesthetized with pentobarbital and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.2), and also perfused with 0.37% sodium sulfide solution prior to fixation for subsequent Timms staining. Brains were post-fixed from 12 to 36 h in the same fixative, and then cryoprotected with a 30% sucrose–0.1 M PB solution. Frozen brains were sectioned (40 μm) coronally throughout the septotemporal extent of the hippocampus with a sliding microtome. BrdU immunocytochemistry was performed according to a previously reported protocol [21]. Sections were incubated with 0.3% H_2O_2 in phosphate-buffered saline (PBS) for 20 min and mounted onto Vectabond-coated slides (Vector Laboratories, Burlingame, CA) and dried under an air stream for several hours. In order to permeabilize the tissue for enhanced antibody penetrations, the slides were incubated with 0.1% trypsin in Tris buffer (pH 7.4) for 10 min, rinsed twice with PBS (pH 7.4), incubated for 30 min with 2 N HCl, rinsed three times in PBS (pH 6.0), incubated for 20 min in 3% normal horse serum in PBS (pH 7.4), and incubated with anti-BrdU (mouse monoclonal, 1:250; Novocastra Laboratories, UK) in PBS with 0.5% Tween-20 overnight at 4 °C. Subsequently, the sections were rinsed five times in PBS and processed using the avidin–biotin horseradish peroxidase method (Vectastain Elite ABC, Vector). Sections were incubated in secondary biotinylated antibody (goat-anti-mouse, 1:200) with normal serum in PBS for 90 min, incubated in avidin–biotinylated peroxidase substrate in PBS for 90 min, with three PBS rinses in between, and then reacted in 3,3′-diaminobenzidine and H_2O_2 for 2–10 min. Specificity of antibody labeling was confirmed by treatment of control sections the same as described above, but without primary antisera. The Timms staining procedure was carried out according to the method of Danscher [15,48]. All slide-mounted sections were lightly counterstained for Nissl (neuronal cell body stain) using cresyl violet, dehydrated, cleared, and coverslipped under Permount.

2.5. Data analysis

In all analyses conducted, slides were coded, so that the person conducting the analysis was unaware of the case and treatment condition, and the code was not broken until the analysis was completed. For counting BrdU-labeled cells, a modified version of the stereological optical fractionator method [59] with Image Pro software (Media Cybernetics, Bethesda, MD) was used on BrdU immuno-peroxidase-stained sections. For every 12th section of the dentate gyrus, numbers of BrdU-labeled cells were determined at $\times 400$ and $\times 1000$ using an Olympus BX-60 light microscope. For all BrdU-immunolabeling experiments, cells were counted in the subgranular zone and granule cell layer (SGZ/GCL). The total volumes for the dentate gyrus and SGZ/GCL were determined using Cavalieri's principle with cross-sectional areas obtained with the ImagePro software program. No change in volume in the GCL or dentate gyrus was observed in any of the experiments conducted. Hence, these data were expressed as estimates of the total number of BrdU-labeled cells. Indices of cell death were obtained by counting pyknotic cells in the granule cell layer of Nissl-stained sections as described above. Pyknotic cells were characterized by the absence of a nuclear membrane and cytoplasm, accompanied with condensed, darkly stained spherical chromatin [22]. Statistical comparisons for total cell counts were performed by a one-way ANOVA, followed by a post hoc Tukey HSD, and values were represented in terms of mean$\pm$S.E.M. for each experimental condition. SRS totals were analyzed using a Student's t-test, and also represented as mean$\pm$S.E.M for each experimental condition. Non-parametric statistical approaches (e.g., Randomization, χ^2, Mann–Whitney U) were also performed on the SRS data, since these data violated the assumptions required for parametric statistical analysis. However, non-parametric analyses did not change the statistical significance of the SRS results.

3. Results

3.1. Cell proliferation in the DG at 3 h into and 14 days following SE

Since 5-HT release is increased in the hippocampus during limbic seizures [10,12,61], first, we wanted to assess whether this effect might stimulate progenitor cell division in the dentate gyrus, during the SE event itself, and whether 5-HT$_{1A}$ receptor antagonist administration would prevent this effect. After 3 h of pilocarpine-induced SE (with BrdU given after 2 h of SE), the majority (~80%) of proliferating cells were found in the SGZ and innermost portion of the GCL, with the remaining cells in the hilus. Most BrdU-immunolabeled cells were spheroid-shaped, approximately 10 μm in diameter (Fig. 1). SE produced a

2-fold increase in the number of BrdU-labeled cells in the SGZ/GCL in PILO rats ($n=4$; 5679$\pm$534; $P<0.05$) compared to saline-treated CONTR rats ($n=5$; 2913$\pm$281). Many BrdU-labeled cells in PILO rats were observed were in aggregates of two to five cells, located principally in the SGZ (Fig. 1). A third group of rats treated with PILO and WAY-100,635 (PILO+WAY: $n=5$; 2594$\pm$162; $P<0.05$) showed a total absence of the seizure-induced increase of cell proliferation relative to PILO rats ($F(2,10)=24.071$; $P=0.0002$; Fig. 2). Behaviorally, the PILO+WAY rats did not demonstrate any attenuation in SE, in latency to onset of SE nor seizure intensity, relative to saline vehicle-treated PILO rats. At 14 days following SE (with BrdU given 2 h before sacrifice), the PILO-treated rats still revealed a significant 5-fold increase ($F(3,6)=13.410$; $P=0.0061$) in the number of BrdU-labeled cells in the SGZ/GCL (PILO: $n=3$; 10 056$\pm$747) relative to CONTR rats ($n=3$; 3788$\pm$885; $P<0.05$), and PILO-treated rats receiving WAY-100,635 (PILO+WAY: $n=3$; 5104$\pm$1050; $P<0.05$; Fig. 3). The same BrdU-labeling profile was observed at 14 following SE as after 3 h of SE, in that the majority (~75%) of proliferating cells were confined to the SGZ and innermost portion of GCL, with the rest localized to the hilar region.

Previous reports have shown that granule cell death may stimulate progenitor cell birth in the dentate gyrus (cf. Ref. [21]). In order to address the issue that 5-HT$_{1A}$ receptor antagonist treatment might be decreasing progenitor cell division indirectly, by exerting a neuroprotective effect on granule cells in the dentate gyrus, Nissl-stained pyknotic cell profiles (Fig. 1) were counted in the GCL in CONTR, PILO, and PILO+WAY rats. After 3 h of SE, PILO-treated rats showed an 8-fold increase ($F(2,10)=29.153$; $P=0.0001$) in the total number of pyknotic cells (PILO: $n=4$; 3663$\pm$195; $P<0.05$) relative to saline-treated CONTR rats ($n=4$; 411$\pm$51). However, the number of pyknotic cells examined in PILO-treated rats was not reduced by WAY-100,635 treatment (PILO+WAY: $n=5$; 3055$\pm$429; $P=0.46$; Fig. 4). Nissl-stained pyknotic cells were often observed in close proximity to BrdU-labeled cells in the SGZ (Fig. 1). Pyknotic cells appeared to be more evenly distributed throughout the GCL compared to the BrdU-labeling, which was almost entirely in or adjacent to the SGZ.

3.2. Four and 8 weeks following SE

At 4 weeks following SE, examination of cell survival in the dentate gyrus (one BrdU injection daily, from days 3 to 7 post-SE) revealed a 4-fold increase ($F(2,9)=26.844$; $P=0.0002$) in the total number of BrdU-labeled cells in the GCL in PILO ($n=5$; 6300$\pm$617) relative to CONTR ($n=3$; 1299$\pm$275; $P<0.05$) and PILO+WAY rats (2880$\pm$876; $P<0.05$; Fig. 5). At 4 weeks, the PILO+WAY group was showing slightly larger numbers of BrdU-labeled cells compared to CONTR, but this was not
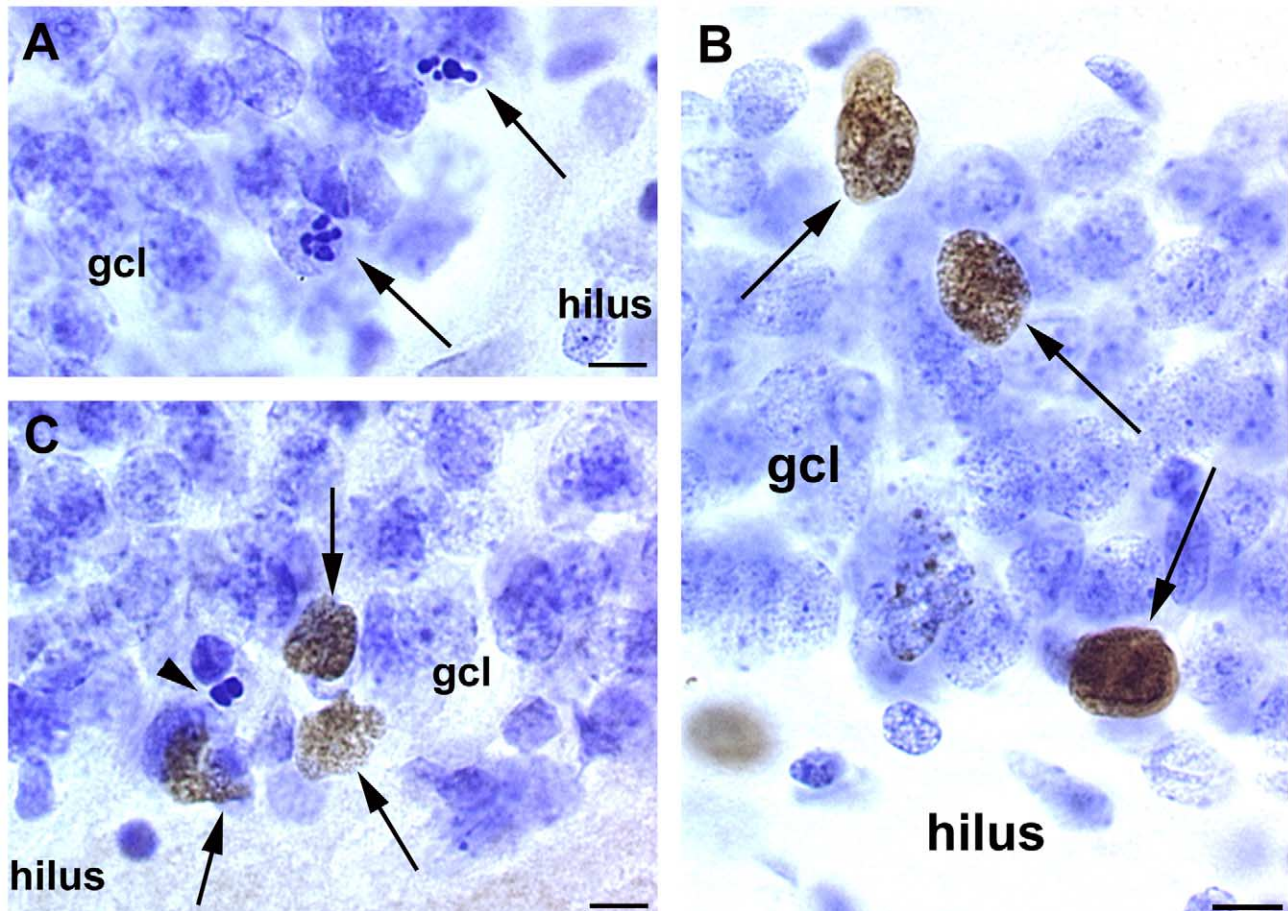
Fig. 1. Photomicrographs of BrdU-labeled cells counterstained with cresyl violet. These cells were increased in number following pilocarpine-induced SE. (A) Two pyknotic cell morphologies (arrows) are present in the SGZ of a PILO+WAY. Note that there are no proliferating cells observed in the vicinity of dying cells, as compared to the PILO case (C). (B) Several BrdU-labeled cells are present in this PILO rat (arrows). Note the granule cell progenitor morphology. (C) An example of a pyknotic cell (arrowhead) that was observed to be in close proximity to proliferating cells (arrows). Again, note the progenitor cell morphology of the BrdU-labeled cells. Proliferating cells were often found to be in the vicinity of dying cells in PILO rats at 3 h SE. gcl, granule cell layer. Scale bars: 10 μm (A–C).

statistically significant ($P=0.1921$). There were numerous BrdU-labeled cells scattered throughout the GCL and hilus in PILO and PILO+WAY rats. Many of these cells were located more superficially in the GCL, indicating that these cells may have migrated following their last division.

MFS was assessed in all groups at 4 and 8 weeks post-SE, in order to determine whether preventing seizure-induced granule cell genesis would result in any blockade of MFS. At both time points, extensive Timms staining was observed in the inner molecular layer of PILO rats compared to CONTRs, and this effect was not prevented in the PILO+WAY group (Figs. 5 and 6). Extensive MFS into the inner molecular layer was seen in both PILO and PILO+WAY rats (Figs. 5D,F and 6D,F).

Since the latency to onset of spontaneously recurring seizures (SRS) in the pilocarpine model is 4–44 days [11,34], it was assessed whether 5-HT$_{1A}$ receptor antagonist treatment, and subsequent blockade of seizure-induced granule cell genesis, would affect the development or total number of observed stage 5 SRS. PILO+WAY rats moni-

tored from 14 to 28 days following SE showed a relative decrease in the total number of SRS ($n=5$; 2.4 ± 1.2) compared to PILO rats ($n=5$; 4.6 ± 2.2). However, this difference was not statistically significant ($P=0.41$; Fig. 6). Two PILO and two PILO+WAY rats never displayed any behavioral signs of epilepsy throughout the duration of the behavioral monitoring. At 8 weeks following SE (rats behaviorally monitored from days 28 to 56), PILO rats ($n=12$; 18.8 ± 5.6) appeared to show about a 2-fold greater stage 5 SRS compared to PILO+WAY rats ($n=10$; 9.3 ± 4.0). Once again, the increase was not statistically significant ($P=0.18$). Although the 8-week experiment was designed to assess SRS past the latent period of 4–44 days of the pilocarpine model, one PILO and three PILO+WAY rats never demonstrated any stage 5 SRS.

It was predicted that preventing seizure-induced neurogenesis might result in a delayed onset or reduction in MFS or SRS PILO+WAY relative to PILO rats. At 4 weeks, three of the PILO+WAY rats and five PILO rats that were monitored for SRS were also processed for
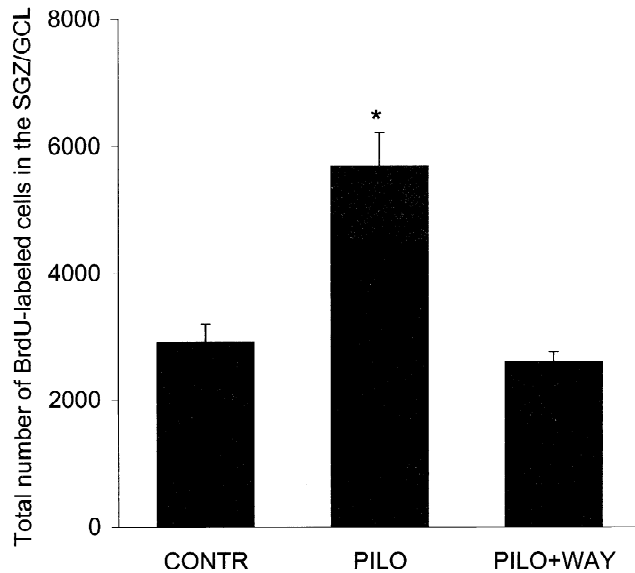
Fig. 2. Stereological estimates for the total number of proliferating cells in the SGZ/GCL at 3 h into SE. Bars represent mean+S.E.M. The asterisk indicates a significant difference from CONTR ($P<0.05$).

BrdU-immunolabeling and Timms staining. The PILO+ WAY rats did not show any prevention of SRS and MFS, even though there was a blockade of the seizure-induced increase in cell survival. Nor was there any trend observed between total SRS of MFS into the inner molecular layer of the dentate gyrus and BrdU-labeling within PILO and PILO+WAY groups. In the 8-week experiment, no attempts were made to correlate BrdU-labeling with MFS or SRS, and MFS into the inner molecular layer was present
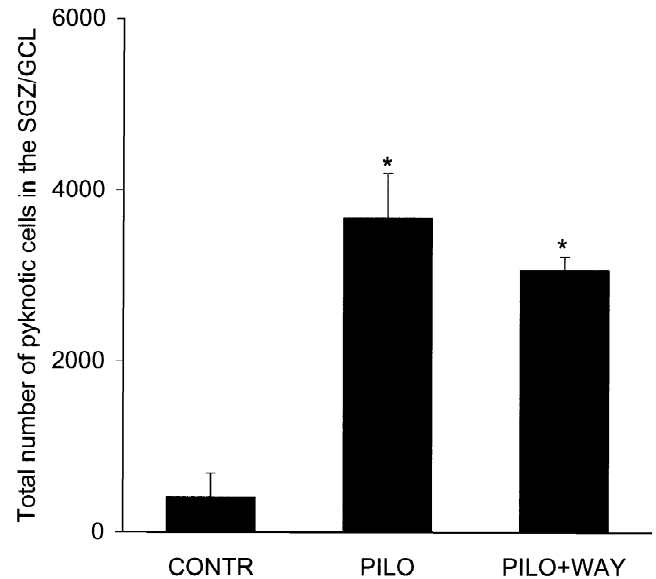

Fig. 4. Stereological estimates for the total number of Nissl-stained pyknotic cell bodies in the SGZ/GCL of the dentate gyrus at 3 h into SE. Bars represent mean+S.E.M. Asterisks indicate significant difference from CONTR ($P<0.05$).

in all animals that underwent behavioral monitoring, regardless of the individual variations in total stage 5 SRS.

4. Discussion

Using the pilocarpine model of epilepsy in rats, the present studies were designed to investigate the possible role for seizure-induced, hippocampal neurogenesis in the
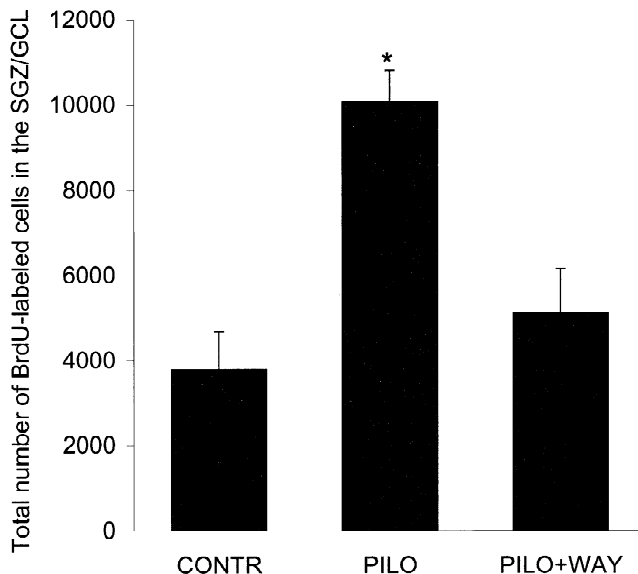

Fig. 3. Stereological estimates for the total number of BrdU-labeled proliferating cells in the SGZ/GCL at 14 days post-SE. Bars represent mean+S.E.M. The asterisk indicates a significant difference from CONTR ($P<0.05$).
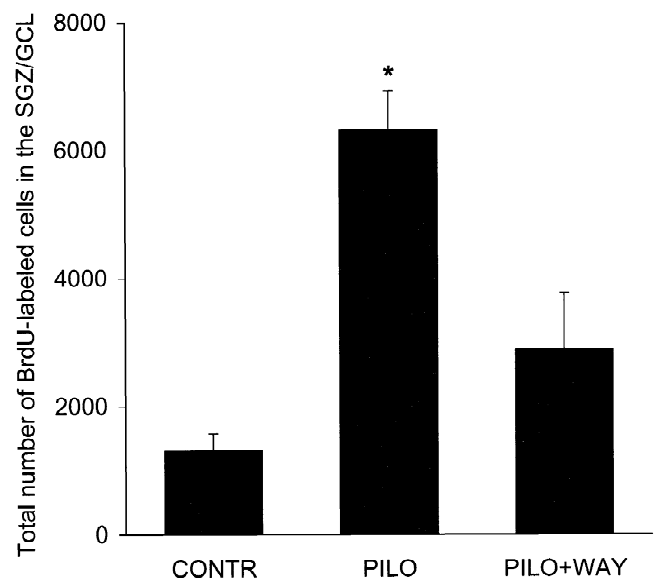

Fig. 5. Stereological estimates for the total number of BrdU-labeled cells in the SGZ/GCL at 4 weeks post-SE. Bars represent mean+S.E.M. The asterisk indicates a significant difference from CONTR ($P<0.05$).
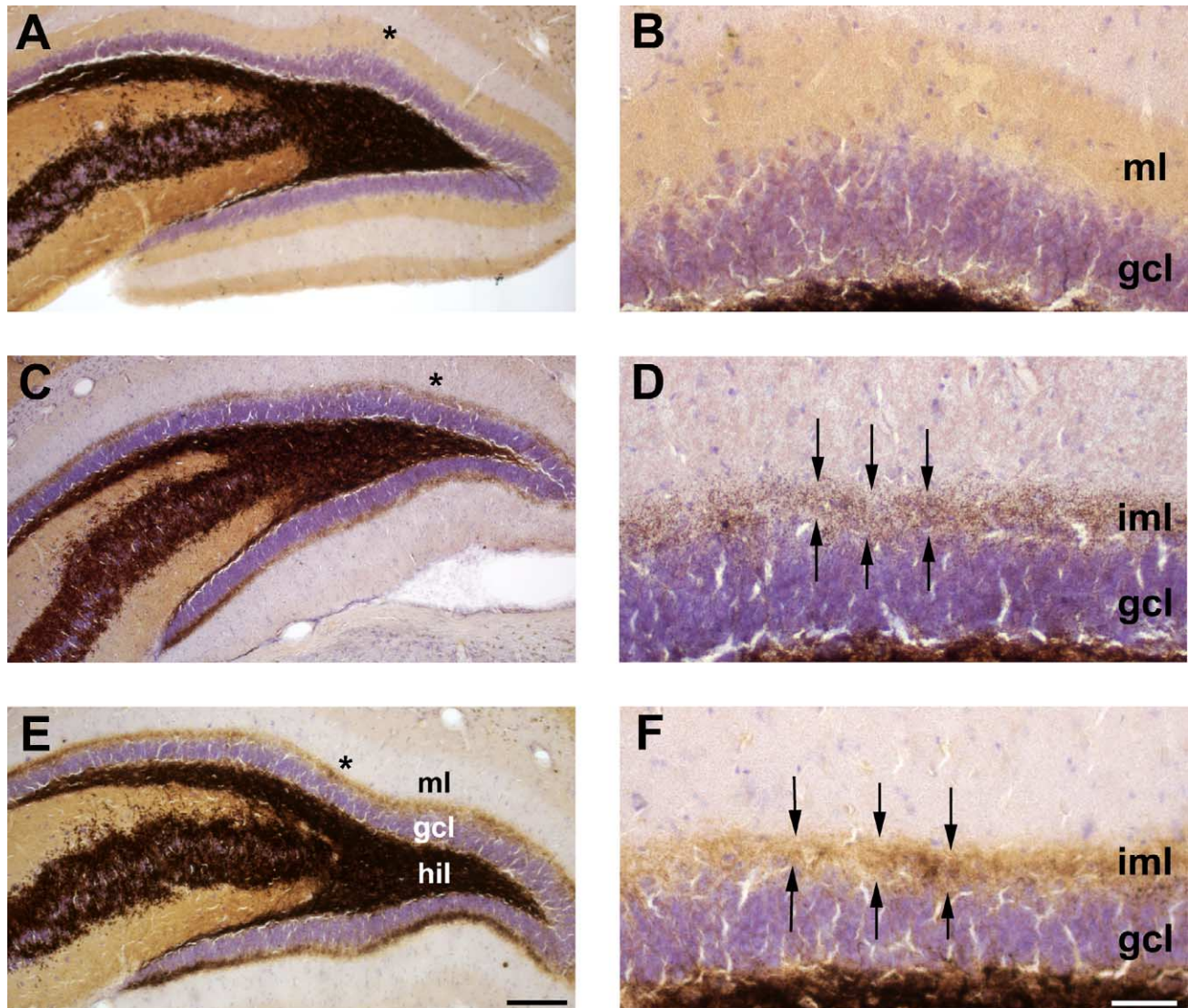
Fig. 6. Photomicrographs of Timms staining and cresyl violet counterstaining in the dentate gyrus at 4 weeks post-SE of CONTR (A,B), PILO (C,D) and PILO+WAY (E,F). Asterisks (A,C,E) in the molecular layer are shown at higher magnification on the right (B,D,F). MFS is present in the inner molecular layer in PILO (C), and PILO+WAY (E), making a halo around the outside of the GCL, whereas it is absent in CONTRs (A). At higher magnification, MFS is present in the innermost portion of the molecular layer (arrows) in PILO (D) and PILO+WAY (F). gcl, granule cell layer; hil, hilus; iml, inner molecular layer; ml, molecular layer. Scale bars: (E) 250 μm (also applies to A,C), (F) 100 μm (also applies to B,D).

development of MFS and SRS. As predicted, 5-HT$_{1A}$ receptor blockade prevented seizure-induced increases in cell proliferation and survival at the three time points examined following pilocarpine treatment. However, this was not sufficient to prevent the development of either MFS or SRS, which suggests that seizure-induced neurogenesis might not be critical for either process. The SRS data were more variable, and it cannot be ruled out that there would not otherwise be a significant difference in SRS between PILO and PILO+WAY animals with increasing the sample size, or by utilizing another model of epilepsy that is less stringent. This is the first study to demonstrate that a critical step in seizure-induced granule cell genesis might be the activation of brain 5-HT$_{1A}$

receptors. This study also confirms a previous report that neurogenesis is not necessary for the development of MFS [45].

4.1. Experimental epilepsies increase neurogenesis in the dentate gyrus

The results in this study suggest that pilocarpine-induced seizures result in a substantial increase in the rate of granule cell genesis in the dentate gyrus. Cell proliferation examined in the dentate gyrus at 3 h and 14 days post-SE showed, respectively, 2- and 5-fold increases in the PILO groups relative to the CONTR groups. A similar (4-fold) increase in cell survival was observed at 4 weeks post-SE.

Together, these results suggest that pilocarpine-induced SE increases granule cell genesis via increasing the rate of cell proliferation. Moreover, the maintained increase in BrdU cell labeling at 4 weeks following pilocarpine treatment suggests that these cells are not simply dying off following division.

Approximately 75% of all BrdU-labeled cells in the dentate gyrus were found to be in the SGZ and innermost portion of the GCL. Upon examination of cell survival, BrdU-labeled cells were distributed throughout the GCL, which suggests that some of them had migrated out from the SGZ following cell division. This is consistent with other reports showing that newly generated granule neurons migrate into the superficial aspect of the GCL following cell division in the SGZ [9,13,56]. Similar to a previous report [45], we observed that PILO rats showed a decrease in BrdU-labeling intensity compared to CONTRs at 4 weeks post-SE (a 3-week interval between BrdU administration and sacrifice), which most likely indicates that these cells underwent further rounds of cell division. Thus it seems unlikely that BrdU-incorporating cells in the dentate gyrus are undergoing DNA repair, which would otherwise be a consideration given the extent of injury produced by pilocarpine treatment and SE.

The nuclear morphologies of BrdU-labeled cells examined were often oval- and spheroid-shaped nuclei, of 10–15 μm in diameter, a size and shape which is indicative of progenitor and granule cell morphology [3,4,9]. BrdU-labeled nuclei of other cells in the SGZ/GCL are either smaller in size (<5 μm), such as microglia, or less spheroidal in shape than granule neurons and their progenitors. With regard to the latter case, radially oriented glial cells have been shown to incorporate BrdU in the GCL, but have an elongated nuclear shape, as described previously [9]. There were also triangular-shaped cells in the SGZ/GCL region that have been reported to incorporate BrdU [45,56]. Our data revealed an occasional BrdU-labeled cell with radial-like glial cell morphology, but neither small nor triangular-shaped BrdU-labeled nuclei were observed. Given the relative paucity of these other nuclear morphologies, it did not warrant undertaking a counting selection strategy for different cell types or phenotypic analysis. In addition to the pilocarpine model [23,45], other forms of experimental epilepsy have also been shown to increase hippocampal neurogenesis, such as electrical kindling [5,39], systemic kainic acid administration [39], intra-amygdala kainic acid administration [23], intra-amygdala kindling [46], and electroconvulsive seizures [55]. There are now reports that have begun to describe some of the functional properties of newly generated granule neurons following pilocarpine-induced SE [53,54]. When the present conclusions are considered in light of previous research, it seems clear that pilocarpine-induced SE is increasing the rate of granule cell genesis in the dentate gyrus, and that these newly generated neurons might have a compensatory or damaging effect in the development of experimental epilepsies.

4.2. Seizure-induced neurogenesis may have a serotonergic component

The main finding of this study is that 5-HT$_{1A}$ receptor antagonist treatment prevents seizure-induced increases in cell proliferation and survival in the dentate gyrus (Figs. 2, 3 and 5). The notion that 5-HT might play a modulatory role in seizure-induced neurogenesis in the dentate gyrus emerged from a report showing that limbic seizures increased the release of 5-HT in the hippocampal formation [61]. In two subsequent studies, it was found that 5-HT levels were significantly elevated in the dentate gyrus at various times during and following pilocarpine-induced SE [10,12]. More recently, we discovered that the 5-HT$_{1A}$ receptor plays a role in the regulation of cell proliferation in the dentate gyrus [28,29,52]. In an initial report, the drug fenfluramine, which enhances the release of 5-HT at terminal sites, stimulated granule cell genesis [29]. This effect was prevented with 5-HT$_{1A}$ receptor antagonist (WAY-100,635) treatment prior to fenfluramine administration. From this line of reasoning, we expected to see a rapid increase in cell proliferation during a seizure event itself, such as during SRS, and not solely during the aftermath of SE. Indeed, we found that cell proliferation was increased when examined after only 3 h of SE, and that 5-HT$_{1A}$ receptor blockade prevented this effect (Fig. 2). An earlier report did not find any significant increase in neurogenesis when examined at 24 h post-SE [45]. The most likely explanation for this discrepancy is the lack of standardized methods employed in this area of study, for administering BrdU (i.e., dose, time after SE, number of injections), for the choice of seizure model, and the varying quantitative methods employed (e.g., densitometry versus optical fractionator).

Nonetheless, the question still remains of which factors are directly responsible for the enhancement of seizure-induced neurogenesis. Granule cell death (that occurs following seizure activity [5]) might be one of the factors that stimulates cell proliferation in the dentate gyrus [21]. It is quite possible that 5-HT release is only one of several factors involved in this signalling. If 5-HT$_{1A}$ receptor blockade were having a neuroprotective effect on granule cells during seizures, this would provide for one possible explanation for the observed reduction in cell proliferation. We did observe a large increase in cell death in the GCL after 3 h of pilocarpine-induced SE, but did not see any attenuation of this following WAY-100,635 administration (Fig. 4). We also observed a similar trend at 14 days post-SE (data not shown). Therefore, this work cannot further confirm whether cell death has any direct role in stimulating the production of new neurons in experimental

epilepsies, although 5-HT$_{1A}$ receptor activation might be an important link in these phenomena.

4.3. Seizure-induced neurogenesis is not necessary for the onset of MFS

The results from the Timms staining procedure conducted at the 4- and 8-week time points clearly show that administering WAY-100,635 chronically does not prevent the onset or progression of MFS (Figs. 6 and 7). This is the first result that shows a clear dissociation between seizure-induced granule cell genesis and MFS, since this is the first study to prevent only the seizure-induced increase in cell proliferation. Indeed, 5-HT$_{1A}$ receptor antagonist treatment did not completely abolish all cell proliferation in the DG, but held it to CONTR levels. In the 4-week experiment, we observed MFS in every PILO+WAY rat, even though the number of BrdU-labeled cells was significantly reduced compared to PILO rats. At the present time, only one other study assessed the role of neurogenesis for MFS after pilocarpine-induced SE [47]. In that study, rats received brain X-irradiation 1 day before, and 3 days after, induction of SE with pilocarpine. X-irradiation kills proliferating cells in the SGZ of the dentate gyrus [47,57]. They were able to prevent virtually all progenitor cell proliferation by X-irradiation before and after SE, and they found that X-irradiation did not prevent MFS [47]. However, the interpretation of their results is complicated
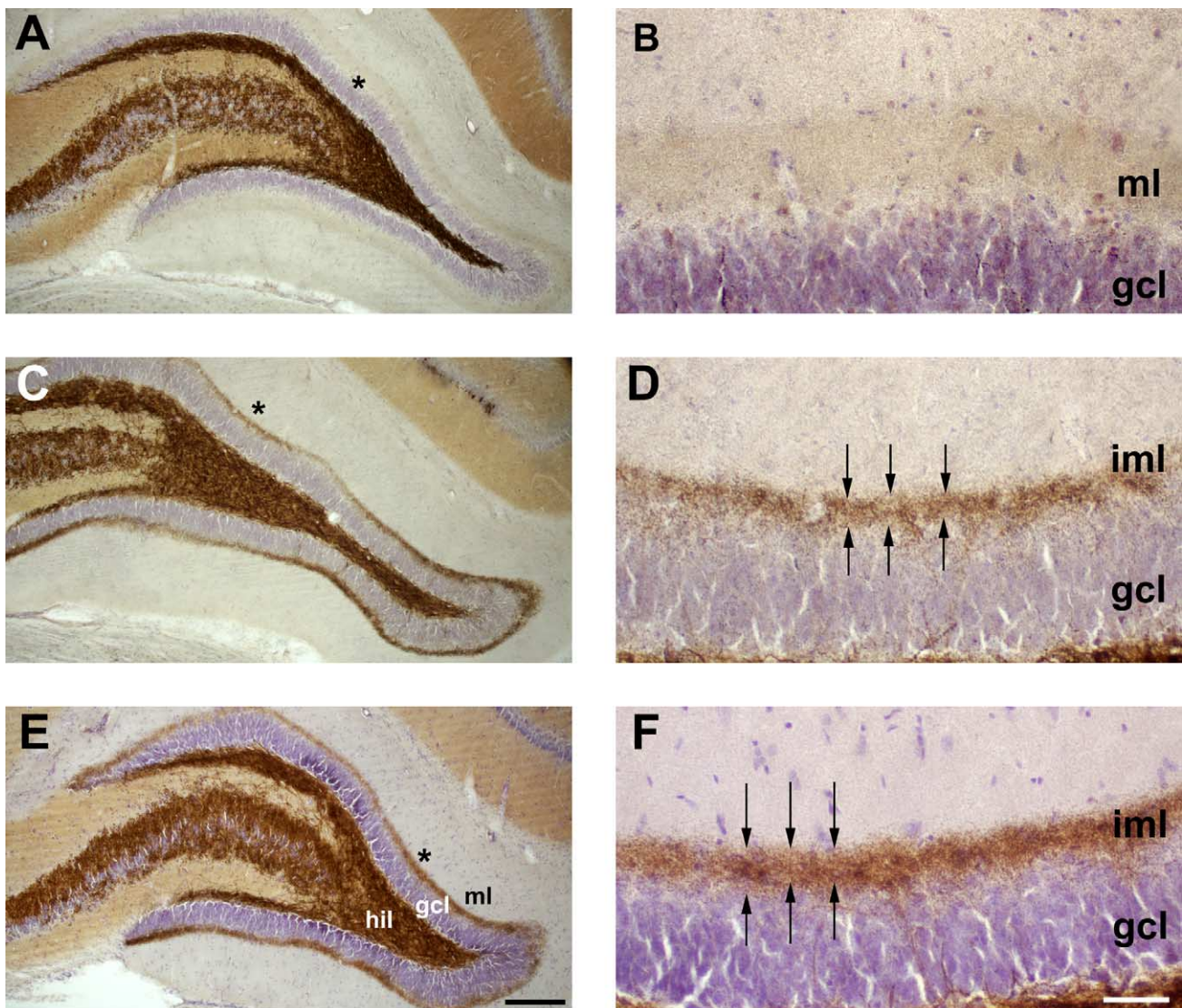

Fig. 7. Photomicrographs of Timms staining and cresyl violet counterstaining in the dentate gyrus at 8 weeks post-SE of CONTR (A,B), PILO (C,D) and PILO+WAY (E,F). Asterisks (A,C,E) in the molecular layer are shown at higher magnification on the right (B,D,F). MFS is present in the inner molecular layer in PILO (C), and PILO+WAY (E), making a halo around the outside of the GCL, whereas it is absent in CONTRs (A). At higher magnification, MFS is present in the innermost portion of the molecular layer (arrows) in PILO (D) and PILO+WAY (F). gcl, granule cell layer; hil, hilus; iml, inner molecular layer; ml, molecular layer. Scale bars: (E) 250 μm (also applies to A,C), (F) 100 μm (also applies to B,D).

by the global effects that X-ray irradiation may produce, in the absence of SE, including alterations in gene expression [16,17], the destruction of other types of proliferating cells, and reactive gliosis [47]. Despite these potential complications, when their results are examined in context of the present study, neither seizure-induced nor basal neurogenesis appear to be necessary for MFS.

4.4. Seizure-induced neurogenesis and its role in the development of epilepsy

Chronically blocking seizure-induced neurogenesis in the dentate gyrus does not prevent the onset of SRS in the pilocarpine model of TLE. The results from the 4- and 8-week study (Figs. 8 and 9) clearly show that there is no statistically significant reduction of SRS in the PILO+ WAY rats. Nonetheless, it should be noted that the PILO+ WAY rats had fewer stage 5 SRS. An inherent variability associated with the pilocarpine model of experimental epilepsy makes it difficult to draw any strong conclusion with regard to differential treatments designed to show a reduction in seizure frequency. Perhaps a replication of this study using larger numbers of animals per group would compensate for the large variability in SRS. Moreover, the pilocarpine model of epilepsy might either be too severe in its damaging effects on the hippocampal formation and other limbic regions to warrant its application as a model for human temporal lobe epilepsies. A less severe model, or one that selectively induces epileptogenesis in the hippocampal formation might be more appropriate for behavioral assessment of the effects of seizure-induced neurogenesis, and perhaps MFS.
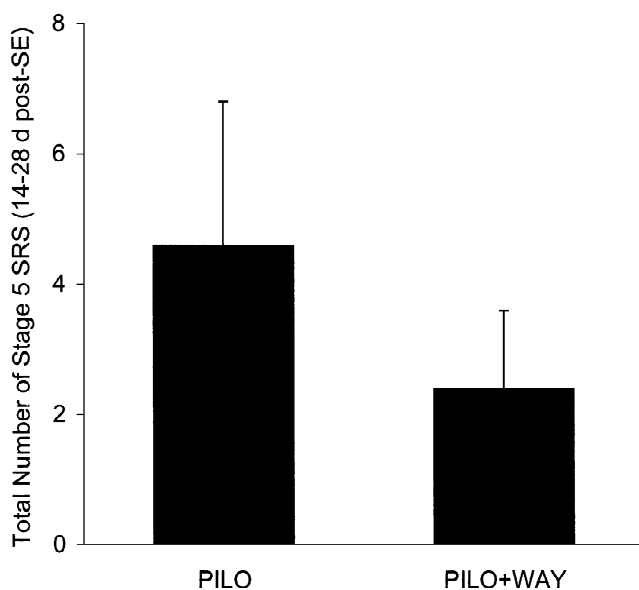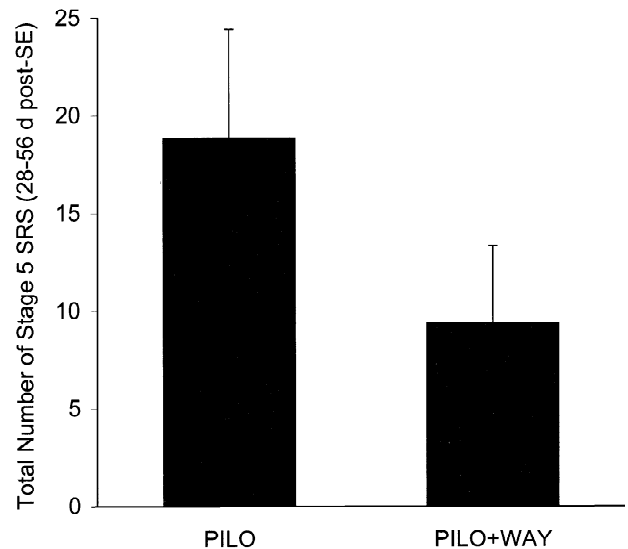
In summary, these findings and others suggest that

Fig. 9. Total number of stage 5 SRS in PILO and PILO+WAY from days 28 to 56 post-SE (8-week experiment). The reduction observed in PILO+WAY was not significantly different from PILO ($P=0.18$).

seizures increase hippocampal granule cell genesis, and our findings implicate 5-HT$_{1A}$ receptor activation as a critical step in this process. This study attempts to manipulate the rate of neurogenesis in order to serve a potentially therapeutic end. Unfortunately, these findings do not address whether seizure-induced neurogenesis is compensatory or augmentative to the ensuing hippocampal damage in this model of epilepsy. Future studies will better address the morphological and functional consequences of granule neurons that are generated following brain injury.

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Fig. 8. Total number of stage 5 SRS in PILO and PILO+WAY from days 14 to 28 post-SE (4-week experiment). The reduction observed in PILO+WAY was not significantly different from PILO ($P=0.41$).

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