Age-Dependent Effects of Hippocampal Neurogenesis Suppression on Spatial Learning

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ABSTRACT: Reducing hippocampal neurogenesis sometimes, but not always, disrupts hippocampus-dependent learning and memory. Here, we tested whether animal age, which regulates rate of hippocampal neurogenesis, is a factor that influences whether deficits in spatial learning are observed after reduction of neurogenesis. We found that suppressing the generation of new hippocampal neurons via treatment with temozolomide, an antiproliferation agent, impaired learning the location of a hidden platform in the water maze in juvenile mice (1–2 months old) but not in adult mice (2–3 months old) or middle-aged mice (11–12 months old). These findings suggest that during juvenility, suppression of neurogenesis may alter hippocampal development, whereas during adulthood and aging, pre-existing neurons may compensate for the lack of new hippocampal neurons. © 2012 Wiley Periodicals, Inc.

KEY WORDS: hippocampus; dentate gyrus; memory; development; aging

INTRODUCTION

The dentate gyrus of the hippocampus is a brain region in which new neurons continue to be generated throughout the lifespan (Christie and Cameron, 2006; Ming and Song, 2011). Because of the critical role of the hippocampus in cognitive function (Eichenbaum, 2004; Wang and Morris, 2010), the potential contribution of newly generated neurons to learning and memory has been the focus of numerous studies (reviewed in Deng et al., 2010). These studies, however, have produced conflicting results, with some reporting that reduction of neurogenesis impairs hippocampus-dependent learning and memory (e.g., Winocur et al., 2006; Imayoshi et al., 2008; Garthe et al., 2009) and others reporting that reduction of neurogenesis has little or no effect (e.g., Meshi et al., 2006; Hernandez-Rabaza et al., 2009; Jaholkowski et al., 2009). Whether a disruption in learning and memory is observed following a reduction in hippocampal neurogenesis is likely determined by an interplay of factors, including the number of neurons targeted (Ko et al., 2009), the maturational stage of the targeted neurons at the time of testing (Farioli-Vecchioli et al., 2008), the type of behavioral task used to assess learning and memory (Shors et al., 2002), and whether the targeted neurons are removed before or after learning (Arruda-Carvalho et al., 2011).

Age is a strong regulator of hippocampal neurogenesis (Lazarov et al., 2010) and thus is another factor that may influence whether the suppression of neurogenesis produces impairments in learning and memory. After the initial formation of the dentate gyrus during embryonic and early postnatal periods (Altman and Bayer, 1990), the continued generation of new neurons declines steadily with age, with relatively high rates of neurogenesis in juvenility, moderate rates in adulthood, and low rates in old age (Seki and Arai, 1995; Kuhn et al., 1996; Ben Abdallah et al., 2010). Here, we predicted that the effect of reducing neurogenesis on spatial learning would be more pronounced in younger animals, when large numbers of new neurons are being generated and incorporated into developing hippocampal circuits. We suppressed neurogenesis in juvenile (1–2 months old), adult (2–3 months old), and middle-aged (11–12 months old) mice by treatment with temozolomide (TMZ), which alkylates the DNA of dividing cells thereby leading to apoptotic cell death. We found that TMZ treatment impaired learning to locate a hidden platform in the water maze in juvenile mice but not in adult or middle-aged mice, suggesting that the likelihood of detecting disruptions in learning and memory after suppression of neurogenesis depends on animal age.

METHODS

Mice

Mice (129Svev × C57Bl/6, F1 cross) were bred at the Hospital for Sick Children and kept on a 12 h light/dark cycle (lights on at 0700 h) with free access to food and water. All procedures were approved by the Animal Care Committee at The Hospital for Sick Children.

TMZ Treatment

Starting at 1 (juvenile), 2 (adult), or 11 (middle-aged) months of age, mice were treated with TMZ

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using a previously established protocol (Garthe et al., 2009). Mice were given four rounds of TMZ (Sigma); each round occurred 1-week apart and consisted of one injection per day (25 mg kg^{-1}, i.p.) for 3 consecutive days (juvenile $n = 16$ (4M, 12F), adult $n = 17$ (4M, 13F), middle-aged $n = 14$ (5M, 9F)). Control mice were injected with vehicle (10% DMSO in 0.9% saline) on an identical schedule (juvenile $n = 15$ (3M, 12F), adult $n = 16$ (4M, 12F), middle-aged $n = 13$ (5M, 8F)).

TMZ reduces neurogenesis by attaching a methyl group to guanine and adenine DNA residues. Because DNA repair mechanisms, such as the enzyme O^6-methylguanine-DNA methyltransferase (MGMT), act to remove the methyl groups from the purine bases (Jacinto and Esteller, 2007), this methylation is transient and should not normally affect transcription. However, when DNA is under replication, base methylation results in a mismatch, leading to fragmented DNA and apoptotic cell death (Friedman et al., 2000). Although TMZ has acute effects on non-neuronal progenitor cells (e.g., hematopoietic progenitors), the spaced nature of the treatment protocol allows for recovery from these nonspecific effects and prevents long-term changes in general health, locomotor function, and exploratory activity in mice (Garthe et al., 2009). In humans, clinical trials have shown TMZ to be an effective component of chemotherapy for brain tumors among children (Bagatell et al., 2011), adults (Stupp et al., 2002), and the elderly (Wick et al., In press), suggesting no age-related differences in drug absorption or distribution.

Water Maze

Four weeks after the start of TMZ or vehicle treatment, mice were trained in the hidden platform version of the Morris water maze (Morris, 1981). A circular plastic pool (120 cm diameter, 50 cm height) was filled with water (~26°C) to a depth of 40 cm. Water was made opaque by the addition of nontoxic paint. A circular escape platform (10-cm diameter) was submerged 0.5 cm below the water surface in the center of one of the pool quadrants. The pool was surrounded by curtains, located at least 1 m from the pool wall, that were painted with distinct geometric cues. Mice received three training trials per day for 6 days. Trials started when mice were released into the pool, facing the wall, from one of four possible points. A different release point was used for each trial on each day, and the order of release points varied pseudorandomly across days. Trials ended when mice reached the platform or 60 s elapsed. If a mouse failed to find the platform, it was guided by the experimenter. Before training on Days 1, 3, and 5, mice received a 60-s probe test with the platform removed from the pool. A final probe test was given on Day 7. Swim paths were recorded by an overhead video camera and tracked using automated software (Watermaze 3.0, Actimetrics). The dependent measure during training was latency to reach the platform. The dependent measures during probe tests were number of crosses over the platform location (10 cm diameter), percentage of time spent in a circular zone (15 cm radius) centered on the platform location, average proximity to the platform location (Gallagher et al., 1993), and percentage of time engaged in thigmotaxic behavior (swimming within 5 cm of the pool wall). Density plots depicting areas of the pool visited more frequently during the probe tests were generated using a custom program (Matlab 2010, Mathworks) developed in our laboratory.

BrdU Injection and Immunohistochemistry

One day following the last TMZ or vehicle injection, mice received a single injection of 5-bromo-2′-deoxyuridine (BrdU, 200 mg kg^{-1}, i.p., Sigma) dissolved in phosphate-buffered saline (PBS). Twelve days later, mice were anesthetized with chloral hydrate and perfused transcardially with PBS followed by 4% paraformaldehyde (PFA). Brains were postfixed overnight in PFA and transferred to 30% sucrose. Coronal sections (40 μm) were cut along the entire anterior–posterior extent of the dentate gyrus using a cryostat. Sections were treated with 1 N HCl at 45°C for 30 min, 1% H_2O_2 for 15 min, and 0.2 M glycine in PBS for 10 min. To quench age-related autoflourescence, sections were also treated with 0.15 mM $CuSO_4$ for 1 h (Schnell et al., 1999). Sections were incubated with the primary antibody (rat anti-BrdU, 1:1,000, Accurate Chemicals) overnight and the secondary antibody (Alexa-488 goat anti-rat, 1:1,000, Invitrogen) for 2 h. Antibodies were diluted in blocking solution containing 2.5% bovine serum albumin, 5% goat serum, and 0.3% Triton X-100 dissolved in PBS. Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 1:10,000, Sigma) and mounted on slides with Permafluor antifade medium (ThermoScientific). Quantification of $BrdU^+$ cells was performed for all middle-aged mice and a random subset of juvenile (TMZ $n = 6$, vehicle $n = 6$) and adult (TMZ $n = 7$, vehicle $n = 6$) mice using a Nikon epifluorescent microscope. $BrdU^+$ cells in the subgranular zone were manually counted in every fourth section, and an estimate of the total number of $BrdU^+$ cells per dentate gyrus was obtained by multiplying the number of cells counted by 4.

Statistical Analysis

BrdU data were analyzed using unpaired t tests. Behavioral data were initially analyzed using ANOVA with age (juvenile, adult, middle-aged) and treatment (vehicle, TMZ) as between-subject factors and day as a within-subject factor. Because of the presence of higher-order interactions, separate ANOVAs with treatment as a between-subject factor and day as a within-subject factor were performed within each age, followed by unpaired t tests or Tukey's post hoc tests. Because both male and female mice were used, we initially included sex as a factor in the ANOVAs; no significant effects involving sex were found, therefore this factor was dropped from analysis.

RESULTS

Suppression of Hippocampal Neurogenesis

As expected (Seki and Arai, 1995; Kuhn et al., 1996; Ben Abdallah et al., 2010), juvenile mice had more new hippocampal

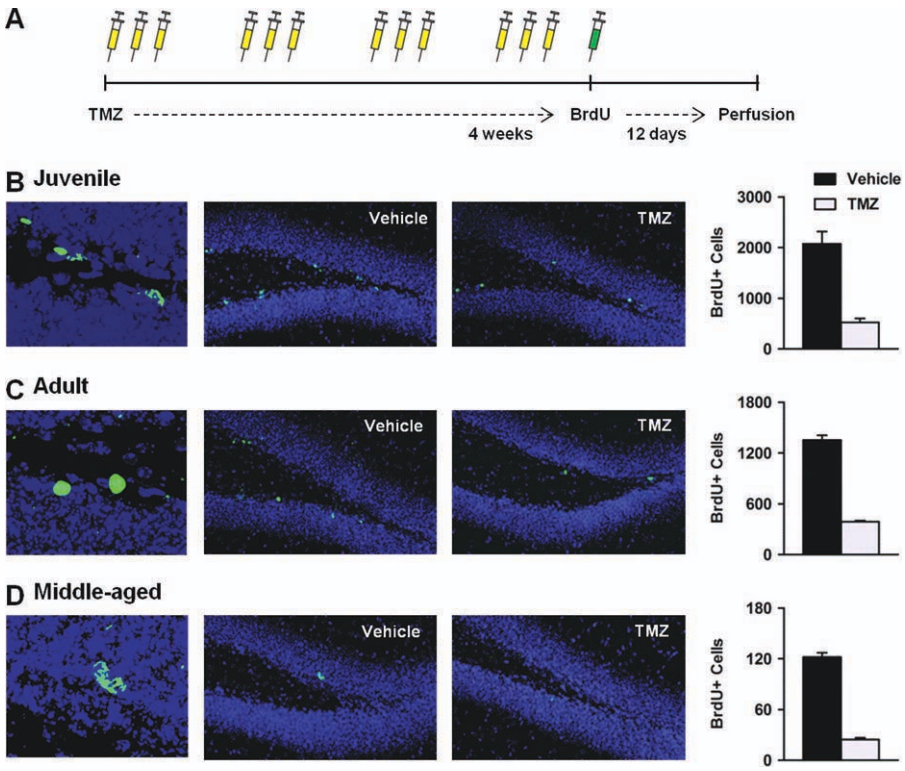

FIGURE 1. TMZ treatment. Four weeks of TMZ treatment (A) reduced the number of BrdU$^+$ cells in the subgranular zone of the dentate gyrus in (B) juvenile, (C) adult, and (D) middle-aged mice. Left panels: high-magnification image of BrdU$^+$ cells; middle panels: low-magnification image of BrdU$^+$ cells in vehicle-treated mice; right panels: low-magnification image of BrdU$^+$ cells in TMZ-treated mice. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

neurons compared to adult mice, and middle-aged mice showed a marked decline in hippocampal neurogenesis (Figs. 1B–D). Despite these age differences, treatment with TMZ (Fig. 1A) effectively suppressed neurogenesis in all age groups (juvenile: $t_{10} = 6.14$, $P < 0.001$, adult: $t_{11} = 7.14$, $P < 0.001$, middle-aged: $t_{27} = 16.96$, $P < 0.001$), reducing the number of BrdU$^+$ cells in the subgranular zone of the dentate gyrus by 70–80% (Figs. 1B–D).

Water Maze: Age-Dependent Effects

Following TMZ treatment, juvenile (Fig. 2A), adult (Fig. 3A), and middle-aged (Fig. 4A) mice were trained to locate a hidden platform in the water maze. To assess the development of spatial learning, no-platform probe tests were conducted at regular intervals throughout training (Days 1, 3, 5, and 7). Overall, we found differences among age groups in water maze performance during training trials (age main effect, latency: $F_{2,85} = 2.85$, $P < 0.001$) as well as during the no-platform probe tests (age main effect, crosses: $F_{2,85} = 18.46$, $P < 0.001$, zone: $F_{2,85} = 13.12$, $P < 0.001$, proximity: $F_{2,85} = 5.92$, $P = 0.004$, speed: $F_{2,85} = 6.70$, $P = 0.002$; age $\times$ day interaction, crosses: $F_{6,255} = 2.42$, $P = 0.027$, zone: $F_{6,255} = 2.70$, $P = 0.015$, proximity: $F_{6,255} = 3.12$, $P = 0.006$, speed: $F_{6,255} = 3.72$, $P = 0.001$).

Consistent with the age-related impairment in spatial learning reported in previous studies (Barnes, 1979; Gallagher et al., 1993), we found that middle-aged mice performed worse than juvenile and adult mice both during training trials (middle-aged vs. juvenile, latency: $P = 0.003$; middle-aged vs. adult, latency: $P < 0.001$) and during probe tests (middle-aged vs. juvenile, crosses: $P < 0.001$, zone: $P = 0.029$, speed: $P = 0.001$; middle-aged vs. adult, crosses: $P < 0.001$, zone: $P < 0.001$, proximity: $P = 0.049$). Also, we found that adult mice performed better than juvenile mice during training (latency: $P = 0.022$) and probe tests (zone: $P = 0.034$, proximity: $P = 0.004$).

Water Maze: Treatment-Dependent Effects

Although all groups of mice showed improvements in performance across days (day main effect, latency: $F_{5,425} = 82.82$, $P < 0.001$, crosses: $F_{3,255} = 101.68$, $P < 0.001$, zone: $F_{3,255} = 107.91$, $P < 0.001$, proximity, $F_{3,255} = 104.99$, $P < 0.001$, thigmotaxis: $F_{3,255} = 299.60$, $P < 0.001$, speed: $F_{3,255} = 4.66$, $P = 0.003$), TMZ-treated mice generally showed poorer performance compared to vehicle-treated mice (treatment main effect, crosses: $F_{1,85} = 5.77$, $P = 0.018$, zone: $F_{1,85} = 4.47$, $P = .037$; treatment $\times$ age $\times$ day interaction, thigmotaxis: $F_{6,255} = 2.83$, $P = 0.011$).

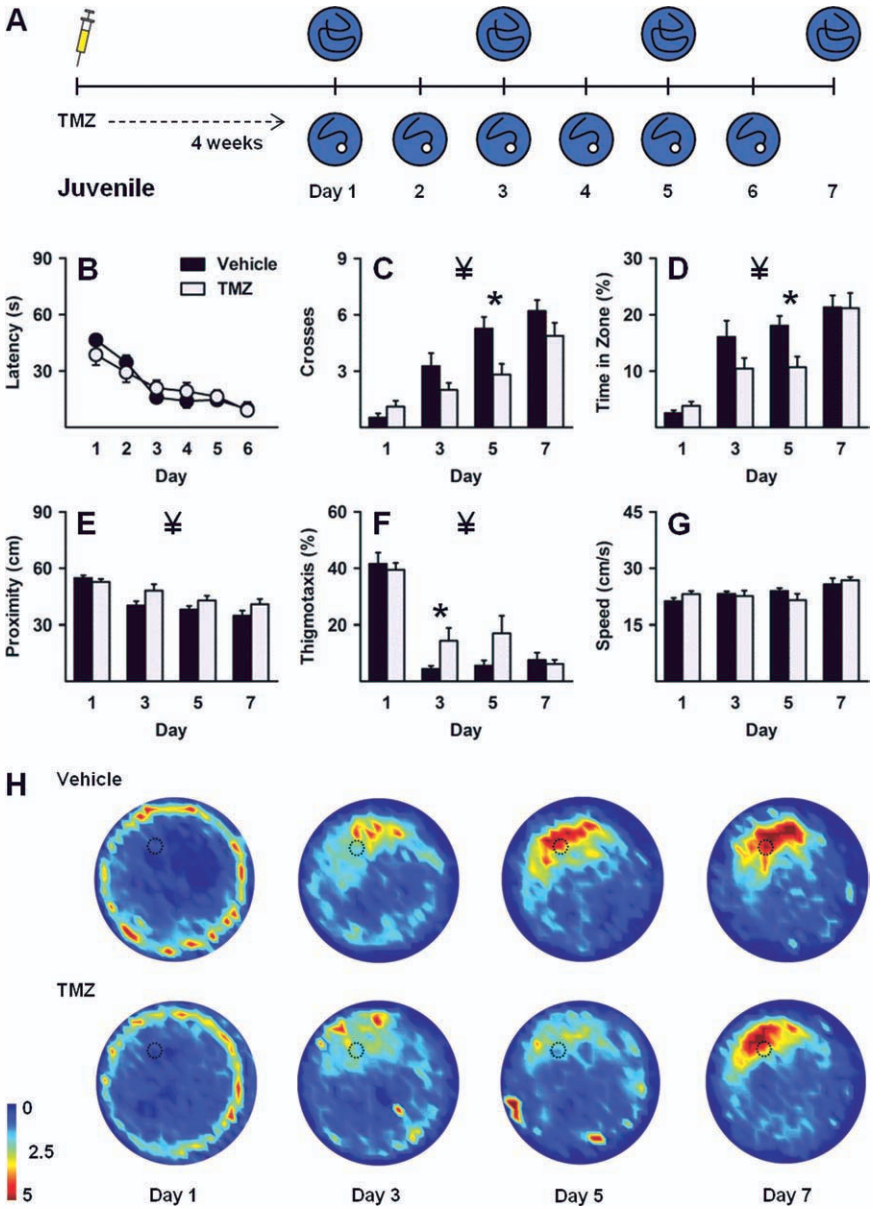

FIGURE 2. Juvenile mice. After TMZ treatment (A), juvenile mice were trained to locate a hidden platform in the water maze (bottom row of circles), with no-platform probe tests conducted at regular intervals throughout training (top row of circles). (B) There was no difference between groups in latency to reach the platform during training. During probe tests, however, TMZ-treated mice showed less precise searching compared to vehicle-treated mice considering (C) number of platform crosses, (D) time spent in a circular zone centered on the platform location, (E) average proximity to the platform, and (F) time engaged in thigmotaxic behavior. (G) Both groups showed equivalent swim speed. (H) Density plots showing where mice concentrated their search for the platform during the probe tests, with color scale representing average number of visits per mouse per 5 × 5 cm^2 area. ¥ denotes treatment main effect or treatment × day interaction, $P < 0.05$. * denotes TMZ vs. vehicle, $P < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Analysis of performance within each age group, however, revealed that the effect of TMZ treatment on spatial learning was more pronounced in juvenile mice compared to adult and middle-aged mice. Among juveniles, TMZ-treated mice learned the platform location more slowly than vehicle-treated mice, crossing the platform location fewer times (Fig. 2C; treatment main effect, $F_{1,29} = 6.33$, $P = 0.018$; treatment × day interaction, $F_{3,87} = 3.15$, $P = 0.029$; Day 5, $t_{29} = 2.90$, $P = 0.007$), spending less time in a zone centered on the platform location (Fig. 2D; treatment × day interaction, $F_{3,87} = 2.87$, $P = 0.041$; Day 5, $t_{29} = 2.90$, $P = 0.007$), and swimming further from the platform location (Fig. 2E; treatment main effect, $F_{1,29} = 5.49$, $P = 0.026$). Interestingly, TMZ-treated mice also spent more time swimming along the walls of the pool (Fig. 2F; treatment × day interaction, $F_{3,87} = 2.84$, $P = 0.042$; Day 3, $t_{29} = 2.05$, $P = 0.050$), suggesting difficulty in

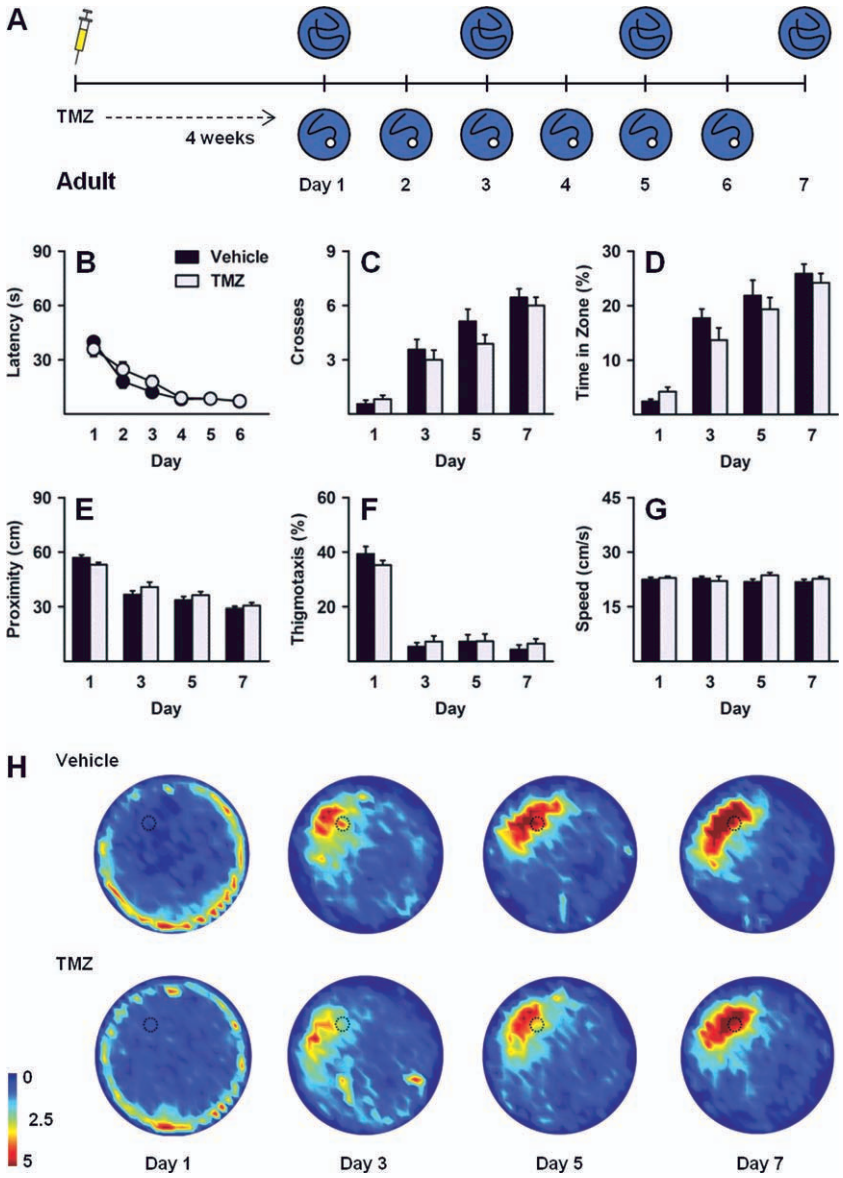

FIGURE 3. Adult mice. After TMZ treatment (A), adult mice were trained to locate a hidden platform in the water maze (bottom row of circles), with no-platform probe tests given at regular intervals during training (top row of circles). There were no differences between TMZ- and vehicle-treated groups in (B) latency to reach the platform during training, (C–F) measures of search precision during the probe tests, or (G) swim speed. (H) Density plots showing where mice concentrated their search for the platform during the probe tests, with color scale representing average number of visits per mouse per 5 × 5 cm^2 area. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

shifting from thigmotaxis to the use of spatial strategies to locate the hidden platform (Garthe et al., 2009). Both groups, however, showed improvements in performance across days (Day main effect within vehicle group, latency: $F_{5,70} = 20.48$, $P < 0.001$, crosses: $F_{3,42} = 22.60$, $P < 0.001$, zone: $F_{3,42} = 18.00$, $P < 0.001$, proximity: $F_{3,42} = 17.01$, $P < 0.001$, thigmotaxis: $F_{3,42} = 51.34$, $P < 0.001$; day main effect within TMZ group, latency: $F_{5,75} = 10.37$, $P < 0.001$, crosses: $F_{3,45} = 11.23$, $P < 0.001$, zone: $F_{3,45} = 15.45$, $P < 0.001$, proximity: $F_{3,45} = 4.49$, $P = 0.008$, thigmotaxis: $F_{3,45} = 17.48$, $P < 0.001$). Swim speed increased across days (Fig. 2G; day main effect within vehicle group, $F_{3,45} = 3.77$, $P = 0.018$; day main effect within TMZ group, $F_{3,45} = 3.98$, $P = 0.013$), but no differences between groups were observed, indicating that TMZ did not disrupt motor function.

In contrast to juvenile mice, we found no significant effect of TMZ treatment on spatial learning in adult mice. Both TMZ- and vehicle-treated mice showed improvements in performance across days (Figs. 3B–F; day main effect within vehicle group, latency: $F_{5,75} = 35.37$, $P < 0.001$, crosses: $F_{3,45} = 24.80$, P < 0.001, zone: $F_{3,45} = 29.97$, $P < 0.001$, proximity: $F_{3,45} = 64.25$, $P < 0.001$, thigmotaxis: $F_{3,45} = 111.93$, $P < 0.001$; day main effect within TMZ group, latency: $F_{5,80} = 17.86$, $P < 0.001$, crosses: $F_{3,48} = 25.37$, $P < 0.001$, zone:

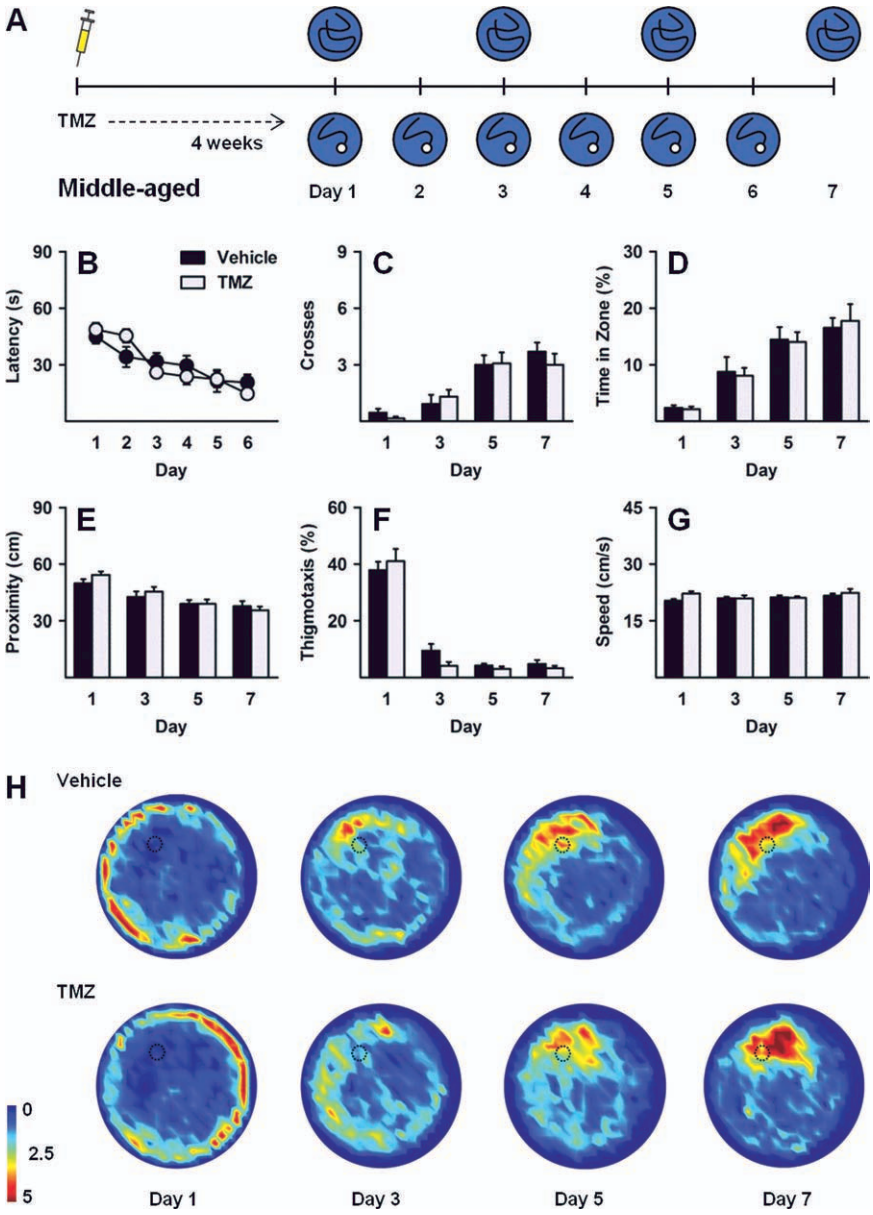

FIGURE 4. Middle-aged mice. After TMZ treatment (A), middle-aged mice were trained to locate a hidden platform in the water maze (bottom row of circles), with no-platform probe tests given at regular intervals during training (top row of circles). There were no differences between TMZ- and vehicle-treated groups in (B) latency to reach the platform during training, (C–F) measures of search precision during the probe tests, or (G) swim speed. (H) Density plots showing where mice concentrated their search for the platform during the probe tests, with color scale representing average number of visits per mouse per 5 × 5 cm^2 area. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

$F_{3,48}$ = 21.44, $P < 0.001$, proximity: $F_{3,48}$ = 34.40, $P < 0.001$, thigmotaxis: $F_{3,48}$ = 69.83, $P < 0.001$) and equivalent swim speed (Fig. 3G).

Likewise, we found no effect of TMZ treatment on spatial learning in middle-aged mice. Both TMZ- and vehicle-treated mice showed improvements in performance across days (Figs. 4B–F; day main effect within vehicle group, latency: $F_{5,60}$ = 4.06, P = 0.003, crosses: $F_{3,36}$ = 17.16, $P < 0.001$, zone: $F_{3,36}$ = 17.06, $P < 0.001$, proximity: $F_{3,36}$ = 6.89, P = 0.001, thigmotaxis: $F_{3,36}$ = 92.76, $P < 0.001$; day main effect within TMZ group, latency: $F_{5,65}$ = 20.56, P = 0.001, crosses: $F_{3,39}$ = 11.58, $P < 0.001$, zone: $F_{3,39}$ = 15.07, $P < 0.001$, proximity: $F_{3,39}$ = 26.29, $P < 0.001$, thigmotaxis: $F_{3,39}$ = 82.41, $P < 0.001$) and equivalent swim speed (Fig. 4G).

DISCUSSION

We tested whether the effect of suppressing hippocampal neurogenesis on spatial learning depends on the age of the animal. Although treatment with TMZ caused a similar proportional

reduction in dentate gyrus neurogenesis in juvenile, adult, and middle-aged mice, we found that this reduction of neurogenesis impaired learning of a hidden platform location in the water maze only in juvenile mice and not in adult or middle-aged mice. Similar to a recent report that decreasing neurogenesis using a transgenic approach altered social behavior in juvenile mice but not in adult mice (Wei et al., 2011), these findings indicate that age is a factor that may influence whether the experimental reduction of neurogenesis affects behavior. Specifically, our results suggest that the younger the animal, the more likely that suppression of neurogenesis will produce behavioral disruptions.

The spatial learning impairment in juvenile mice after suppression of neurogenesis could be attributed to the relatively large number of new neurons affected and/or the impact of this missing population of neurons on the ongoing development of hippocampal circuitry. Although all age groups exhibited a similar proportional reduction (~75%) in neurogenesis in the dentate gyrus after TMZ treatment, the high baseline rate of neurogenesis among juveniles meant that this age group showed the largest decrease in terms of the absolute number of cells (i.e., a 1.5- and 10-fold greater decrease in number of BrdU+ cells compared to adult and middle-aged mice, respectively). Moreover, this relatively large reduction of neurogenesis occurred against the backdrop of continuing structural and functional maturation of the hippocampus (Bachevalier and Beauregard, 1993; Dumas, 2005), with ongoing changes in dendritic arborization (Rahimi and Claiborne, 2007), neurotransmission (Nurse and Lacaille, 1999), and spatial firing (Martin and Berthoz, 2002; Langston et al., 2010; Scott et al., 2010) taking place during the first 2 postnatal months. Treatments that reduce neurogenesis during juvenility, therefore, may not only affect a disproportionately large number of new neurons but also alter the initial formation of hippocampal circuitry, leading to observable disruptions in hippocampus-dependent behaviors.

Consistent with some previous studies showing no effect of reducing hippocampal neurogenesis on water maze performance in adult rodents (Shors et al., 2002; Madsen et al., 2003; Raber et al., 2004; Meshi et al., 2006; Saxe et al., 2006; Jaholkowski et al., 2009), we found no spatial learning impairments in adult or middle-aged mice following neurogenesis suppression. Although adult-generated hippocampal neurons transiently show a lower threshold and higher magnitude of synaptic plasticity during their growth (Schmidt-Hieber et al., 2004; Ge et al., 2007), they ultimately converge with their developmentally generated neighbors in terms of electrophysiological properties (Esposito et al., 2005; Ge et al., 2007), morphology (Zhao et al., 2006), connectivity (Laplagne et al., 2006; Toni et al., 2008), and integration into circuits supporting behavior (Stone et al., 2011). This structural and functional similarity between developmentally and adult-generated neurons may permit pre-existing neurons to compensate for the lack of newly generated neurons in the adult brain, without an obvious decrement in learning ability. Once the hippocampus has fully matured, therefore, it may be relatively resilient to perturbations of neurogenesis, which may account for the mild or even nonexistent disruptions in learning and memory after reduction of neurogenesis during adulthood (reviewed in Deng et al., 2010). An alternative possibility, not explored here, is that older mice have a greater array of cognitive strategies [or "cognitive reserve" (Tucker and Stern, 2011)], which might render them less vulnerable to disruptions in brain function.

As animals enter old age, they display declines in both cognitive ability (Barnes, 1979; Gallagher et al., 1993) and hippocampal neurogenesis (Kuhn et al., 1996; Heine et al., 2004), prompting the idea that age-related cognitive deficits may result from a failure in the generation of new hippocampal neurons. This possibility is supported by correlative studies reporting relationships between low rates of hippocampal neurogenesis and poor spatial learning abilities among aged rodents (Drapeau et al., 2003; Driscoll et al., 2006) (but see Bizon and Gallagher, 2003; Merrill et al., 2003; Bizon et al., 2004) and an experimental study showing that running-induced enhancements in hippocampal neurogenesis are associated with improvements in spatial learning in aged rodents (van Praag et al., 2005). Here, we also found that middle-aged mice had fewer new hippocampal cells and poorer water maze performance compared to younger mice, consistent with the idea that age-related declines in hippocampal neurogenesis could lead to deficits in learning and memory. As additional support of this hypothesis, it might be expected that an even greater suppression of hippocampal neurogenesis by pharmacological treatment should exaggerate the cognitive impairment among older animals. However, we observed no further disruption in spatial learning after TMZ treatment in middle-aged mice. These findings suggest that age-related cognitive deficits may not be caused by fading hippocampal neurogenesis but rather by other neurobiological factors that covary with neurogenesis level.

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