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Injury-induced neurogenesis: consideration of resident microglia as supportive of neural progenitor cells

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Abstract

The induction of neurogenesis in the adult subgranular zone (SGZ) by injury is often accompanied by changes in the extracellular environment that can have significant impacts on neural progenitor cells (NPC). We examined the induction of neurogenesis in the SGZ at 72 hrs following an injection of the hippocampal toxicant, trimethyltin (TMT; 2mg/kg, ip) inducing apoptosis in dentate granule neurons. BrdU+ incorporation during the active period of neuronal death indicated NPC proliferation and migration of newly generated cells into the granule cell layer (GCL). BrdU+ cells were transiently in contact with process bearing microglia within the inner SGZ layer. Contact with GFAP+ astrocyte processes occurred once cells were within the GCL. A small percentage of the BrdU+ cells within the SGZ region showed immunoreactivity for tumor necrosis factor (TNF) p75 receptor (TNFp75R). In mice deficient for TNFp75R, TMT injection produced an equivalent level of dentate granule cell death however; BrdU+ cells were localized at the SGZ as compared to the presence of cells within the GCL in the WT mice dosed with TMT. These data suggest that cells generated by NPCs in the SGZ induced with a focal lesion to the dentate granule neurons of adolescent mice maintain the capacity to utilize the neuroinflammation and microglia responses within their environment for migration into the GCL.

Keywords

microglia; neural progenitor cell; subgranular zone; inflammation; neurogenesis

It is now accepted that the adult brain maintains neurogenic capability in discrete germinal regions, the subgranular zone (SGZ) of the dentate gyrus (DG) within the hippocampus and in the subventricular zone (SVZ) of the lateral ventricle. The SGZ, located between the glia-rich hilus and the tightly-packed neurons of the granule cell layer (GCL), is the result of a continued presence of a relatively small number of proliferating cells derived from the cells of the hippocampal pseudostratified ventricular epithelium during embryogenesis. Neurons generated from hippocampal neural progenitor cells (NPCs) migrate primarily to the inner third of the GCL. Once there, they assume the nuclear and cytoplasmic morphology of surrounding dentate granule neurons, express biochemical markers of immature and mature neurons (Kempermann et al., 2004) and become incorporated into hippocampal-dependent declarative memory networks (van Praag et al., 2002; Kee et al., 2007).

The specialized SGZ microenvironment or “niche” for NPCs is comprised of astrocytes, microglia, and a limited population of oligodendroglia. In particular microglia within the neurogenic “niche” have been identified as putative regulators of adult neurogenesis (Monje

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et al., 2002; 2003; Ekdahl et al., 2003; Barkho et al., 2006; Butovsky et al., 2006). For example, down-regulation of NPC proliferation and neuronal differentiation has been attributed to injury-induced microglia release of pro-inflammatory cytokines, interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) (Monje et al., 2002; 2003; Ekdahl et al., 2003; Cacci et al., 2008). Acute brain insults, e.g., stroke and status epilepticus (SE), can induce a neuroinflammatory and microglia response but they also trigger increased neurogenesis. In addition, the survival, migration, and differentiation of NPCs into neurons can be directed by microglia (Aarum et al., 2003).

One of the primary microglia-secreted pro-inflammatory cytokines is TNF α . *In vitro* studies demonstrate a detrimental effect of TNF α on NPC survival and differentiation (Cacci et al., 2005; Liu et al., 2005). TNF α initiates its multiple effects on cell function by binding to two distinct cell surface receptors, a 55kDa type-1 receptor (TNFp55R) and a 75kDa type-2 receptor (TNFp75R). TNFp55R contains a cytoplasmic sequence identifying an intracellular death domain required for transduction of apoptotic signals (Micheau and Tschopp, 2003; Thorburn, 2004). In contrast, TNFp75R activation primarily initiates trophic/ protective actions (Shen et al., 1997; Yang et al., 2002). Cultured NPCs from human fetal brain express TNF α , as well as, both TNFp55R and TNFp75R (Klassen et al., 2003; Sheng et al., 2005). NPCs isolated from the mouse striatum express TNFp55R (Ben-Hur et al., 2003). Iosif et al (2006) demonstrated an increase in NPC proliferation in mice lacking TNFp55R, while mice lacking TNFp75R showed a minor decrease, suggesting a regulatory component of the TNF α signaling pathway. Given that microglia are the primary source of TNF α within the brain, the expression of TNFRs on NPCs provides a possible mechanism by which, upon stimulation, microglia influence injury-induced neurogenesis.

An apparent sensitivity and vulnerability of dentate granule neurons is demonstrated across a wide spectrum of hippocampal injuries thus, various models have been developed to examine injury-induced stimulation of hippocampal neurogenesis. For example, following acute ischemic insult, a significant amount of dentate granule cell apoptosis occurs that is accompanied by an increase in proliferation of new cells in the SGZ and migration to the GCL within 7-10 days (Jin et al., 2001). In rodent models of temporal lobe epilepsy, an increased rate of neurogenesis occurs in the hippocampus within a week of seizure activity (Bengzon et al., 1997; Gray and Sundstrom, 1998; Parent and Murphy, 2008). Traumatic brain injury results in apoptosis of DG neurons within 6 hr of injury (Clark et al., 1997; McCullers et al., 2002) and neurogenesis at 7 days (Lu et al., 2007; Yu et al., 2008). Systemic exposure to the organometal, trimethyltin (TMT) induces active apoptosis of dentate granule neurons between 6 and 72 hr; however, contrary to the other injury models, this results in a concurrent rapid and robust proliferation of NPCs in the SGZ between 2-5 days of insult (Harry and Lefebvre d'Hellencourt, 2003; Harry et al., 2004; Corvino et al., 2005; Ogita et al., 2005). This activity occurs during the peak level of microgliosis and elevations in IL-1 α and TNF α (Brucoleri et al., 1998; Brucoleri and Harry, 2000; Harry and Lefebvre d'Hellencourt, 2003; Harry et al., 2008a). Both Ogita et al., (2005) and Harry et al., (2004) reported that the newly generated cells matured into dentate neurons within 10 days suggesting proliferation and survival within a high inflammatory environment. As this would be inconsistent with the proposed detrimental effect of microglia activation on NPC proliferation, we examined one aspect of the gliotic response, the morphological phenotype and location of glia in contact with cells migrating from the SGZ. We now provide data suggesting that an interaction between glia and proliferating cells within the hippocampus contributes to NPC proliferation and migration of generated cells following injury.

Materials and Methods

Animals

Twenty-one day old CD-1 male mice (Charles River Labs, Raleigh, NC) and an additional cohort of mice, TNFp75R^{-/-} (C57BL/6-*Tnfrsf1b*^{tm1Mwm}) and C57BL/6J (WT) (Jackson Labs; Bar Harbor, ME) were examined. Mice were administered a single intraperitoneal (i.p.) injection of 2 mg/kg trimethyltin hydroxide (TMT; 2ml/kg) or saline (n=10). Previous work demonstrated that the peak time of NPC proliferation occurs within the first 24-96 hrs post-injection (McPherson et al., 2003). Thus, based upon this temporal pattern, mice were injected with bromodeoxyuridine (BrdU; 50 mg/kg i.p.) at the time of TMT dosing and at 12 hr intervals for a total of 6 injections. The multiple injections of BrdU at 12 hr intervals allowed for incorporation during discrete intervals within the peak time of proliferation and would generate a gradient of BrdU+ cells and their migration into the blades of the dentate. A relationship with glia could then be examined relative to distance from the SGZ within the same animal.

Animals were individually housed in a dual corridor, semi-barrier animal facility (21° ± 2°C; 50% ± 5% humidity; 12-hr light/dark cycle). Food (autoclaved NIH 31 rodent chow) and deionized, reverse osmotic-treated water were available *ad libitum*. At 72 hrs post-TMT, mice were deeply anesthetized with CO₂ and decapitated. Brains were dissected and bisected in the midsagittal plane. One hemisphere was immersion fixed in 4% paraformaldehyde (PFA)/0.1M phosphate buffer (PB; pH 7.2) overnight (ON), processed for paraffin embedding, and 10 µm serial sections cut through the hippocampus. Sentinel animals recorded negative for pathogenic bacteria, mycoplasma, viruses, ectoparasites, and endoparasites. All experiments were conducted according to an animal use protocol approved by NIEHS/NIH Animal Care and Use Committee.

Histology

To confirm the level of dentate granule cell death at 72 hrs post-TMT, one randomly selected paraffin embedded section from each brain was subjected to terminal deoxynucleotidyl transferase-mediated dUTP-biotin *in situ* end labeling (TUNEL; ApopTag®, Intergen, Purchase, NY) and visualized by horseradish peroxidase-conjugated digoxigenin antibodies (diluted 1:1000 in PBS) and 3,3'-diaminobenzidine (DAB) substrate. A second randomly selected paraffin embedded section from the hippocampus of each mouse (n=10) was transferred to 0.01 M citrate buffer (pH 6.0), subjected to heat-induced epitope retrieval (HIER) using a decloaking chamber (Biocare Medical, Walnut Creek, CA), blocked with 10% normal goat serum/1% BSA in phosphate buffered saline for 30 min, then incubated with rabbit polyclonal anti-active caspase 3 (AC3: 1:1000, 18 h, 4°C, Chemicon Intl., Temecula, CA) and visualized with goat anti-rabbit IgG AF 488 (1:1000; Molecular Probes, Inc., Eugene, OR). Neurons were labeled with Neurotrace® blue fluorescent Nissl stain (1:500, 1hr, RT; Molecular Probes). To examine the localization of BrdU+ cells or the proximity of microglia or astrocytes with such cells, one initial section of the hippocampus was randomly selected and then every 6th section was collected for a total of 10 sections of the hippocampus per mouse (n=10). Based upon the work of Widera et al. (2006) showing that cyclin D1 plays a crucial role in the proliferation of NSCs induced by TNF-α, we included cyclin D1 as an additional marker. This selection process was repeated for microglia and astrocyte staining, and for co-staining of BrdU or cyclin D1 and glia. Rehydrated sections were subjected HIER in 0.01 M citrate buffer (pH 6.0), and blocked with either a MOM™ immunodetection kit (Vector Labs, Burlingame, CA) or 10% normal goat serum/1% BSA/ PBS for 30 min. Sections were incubated 18 hr at 4°C, with rat anti-BrdU (1:10,000, Chemicon, Temecula, CA) or with mouse anti-cyclin D1 (1:200; Zymed Labs, Inc., San Francisco, CA). Astrocytes were labeled by rabbit polyclonal anti-glial

fibrillary acidic protein (GFAP; 1:200, 1 hr, 24°C, Dako Corp, Carpinteria, CA). Microglia were identified by morphological criteria of size and ramification of cells stained with a rabbit polyclonal antibody to ionized calcium-binding adaptor molecule 1 (Iba-1; 1:500; 1 hr, 24°C; Wako Chemicals, Richmond, VA). TNF receptor localization was detected with rabbit polyclonal anti-TNFR1 (p55; 1:500, #CSA-815, Stressgen, Ann Arbor, MI) or polyclonal goat anti-mouse TNFR2 (p75; 1:500, #AF426PB, R&D Systems, Minneapolis, MN). Antibody specificity was verified by Western blotting (Harry et al., 2008b). Staining was visualized with IgG Alexa Fluor® (1:1000, Molecular Probes). Coverslips were mounted with Prolong® Antifade Reagent with or without the nuclear stain, DAPI (Molecular Probes).

Microscopy

Digital images were acquired using a SpotRT™ cooled, charged-couple device camera (Diagnostic Instruments, Sterling Heights, MI) on a Leica DMRBE microscope (Wetzlar, Germany) equipped with epifluorescence and Z-control and Metamorph™ (Universal Imaging Co., Downingtown, PA). Contact between glia and BrdU+ cells was further examined via deconvoluted z-stack images and nearest neighbor correction. Co-localization images and representative contact images were confirmed by confocal microscopy.

Three distinct sections (region of interest: ROI) of the hippocampal dentate granule layer (DGL) were identified at increasing distances from the SGZ with the assumption that this would represent cells at different stages of maturation and migration. ROI-1) a 2-cell width at the inner blade considered the SGZ; ROI-2) the next consecutive 5-6 neuron width, and ROI-3) the outer granular neuronal region of the dentate blade. The identification of BrdU+ NPC derived cells adhered to exclusion criteria of a minimum of 8 µm diameter. These criteria were selected for two reasons. One was the fact that mature dentate granule neurons were undergoing apoptosis and we wanted to ensure that we did not include apoptotic neurons (< 6 µm) that may have incorporated BrdU. The second was based upon the fact that the early time interval of between 1 and 3 days allowed for the examination of proliferating cells but not for uniform co-labeling with markers of cell fate determination. As we previously reported, the immature neuronal markers of doublecortin and Prox-1 were not increased in this model until 4-7 days post-TMT (Harry et al., 2004; McPherson et al., 2003) and, at these earlier time points, we observed the NPC marker, nestin (data not shown) indicative of non-differentiated progenitor cells. According to design considerations for sampling of rare events, the total number of BrdU+ cells, BrdU+ cells in contact with microglia, and BrdU+ cells in contact with astrocytes was determined for each region. Again a selection criterion was required as, by 72 hrs post-TMT, amoeboid microglia were actively phagocytizing dying neurons. Thus, contact between BrdU+ cells and process bearing ramified microglia was recorded. If the microglia cell fully encircled the BrdU+ cell or demonstrated an amoeboid like morphology it was excluded. If the BrdU+ cell maintained a diameter >8 µm and a ramified microglia (nucleus size of maximum 2 µm) was in contact it was included. 2 independent trained investigators blind to the experimental conditions conducted evaluations.

Statistical analysis

Statistical significance was determined with Student's t-test. All data is presented as mean +/- SD. All statistical significance levels were set at $p < 0.05$.

Results

TMT induced histopathology

The extent of NPC proliferation in the SGZ is dependent upon the severity of damage. In addition, the level and stage of microgliosis is also dependent upon the timing and severity of neuronal death. These two features can significantly confound the interpretation of any data with regards to neurogenesis or the impact of a microglia response. The TMT mouse model is highly reproducibility in severity and timing of neuronal death across individual mice. Consistent with previous studies, acute exposure to TMT resulted in clinical signs of seizure activity between 18-30 hr and apoptotic death of dentate granule cells as identified by TUNEL (Fig. 1A,B) and AC3 (Fig. E,F) characterized by nuclear pyknosis and karyolysis.

In the normal CD-1 mouse hippocampus, GFAP+ astrocytes are observed primarily at the borders of the densely packed GCL. Consistent with radial astrocyte morphology, cell bodies were observed along the inner blade with fibrous astrocyte processes extending into the GCL (Fig. 1C). Similar to what has been previously reported (Harry et al., 2008a,b), after an injection of TMT, GFAP immunoreactivity was elevated and astrocytes showed a slight thickening of the processes within the GCL (Fig. 1D). In the normal hippocampus, microglia display very thin, ramified processes and normally, are not evident within the densely packed dentate GCL but rather seen within the hilus and molecular layer (Fig. 1G). Consistent with our previous work, within the GCL, heterogeneity in microglia responses was observed at 72 hrs. Within proximity to dying neurons, microglia showed an amoeboid morphology characteristic of a phagocytic phenotype. In addition, increase staining was also observed for ramified process-bearing microglia also showing heterogeneity in morphology (Fig. 1H).

Induction of NPC proliferation in the SGZ

The BrdU dosing regimen allowed for a gradient of labeled cells within the GCL, representing different stages of generation and migration. Figure 1A represents the mouse SGZ. In the normal adolescent hippocampus, while higher than what is observed in the adult mouse, BrdU+ cells were sparse and restricted to the inner blade/SGZ layer of the dentate (Fig. 2A). Consistent with our previous work (Harry et al., 2004; McPherson et al., 2003), the increased number of BrdU+ cells in the TMT dosed mice was distributed across the full width of the upper and lower blades of the dentate gyrus (Fig. 2B). The upper and lower blades of the GCL were demarcated into 3 distinct regions of interest (Fig. 2C). Region of interest (ROI) 1 was inclusive of the SGZ, ROI-2 represented the middle 3rd of the GCL, and the outer 30% would represent the requirement for replacement of dentate granule neurons across the full width of the GCL. When we examined the distribution of BrdU+ cells across the GCL as a function of identified ROIs (Fig. 2C), the BrdU+ cells in the control hippocampus were located within the ROI-1 representing the SGZ. Following TMT, a significant increase in the number of BrdU+ cells within ROI-1 was detected as well as the increased presence of BrdU+ cells within both ROI-2 and -3 consistent with the visual images of a more uniform distribution across the GCL. In previous work, we confirmed this proliferation with multiple markers such as PCNA and Ki-67 and demonstrated that the cells matured into dentate granule neurons by subsequent co-localization of staining with NeuN at 10-14 days (Harry et al., 2004). As an additional marker of cell proliferation, the cell cycle protein, cyclin D1 is present in the adult SGZ (Heine et al., 2004) and plays a role in the proliferation of NPCs induced by TNF α and signaling via IKK/NF- κ B (Widera et al., 2006). Cyclin D1 expression was increased, but to a significantly lesser extent than BrdU, after TMT (McPherson et al., 2003). Figure 3 illustrates the co-localization of cyclin D1 and BrdU in the GCL. We estimated that 80% of the cyclin D1+ cells co-stained with BrdU.

Given the short half-life (approx. 1 hr) at the end of mitosis of proteins like Ki-67 and the cyclins, versus the long-term expression of BrdU, would be consistent with the absence of a reverse association. However, staining for cyclin D1 provided us an additional marker for proliferating cells in the SGZ. Consistent with our previous work (McPherson et al., 2003), we found no co-localized staining between cyclin D1 or BrdU and GFAP or Iba-1 (data not shown).

Glia cells in contact with proliferating cells

Given the neurogenesis previously reported for TMT-induced hippocampal damage occurring within a high pro-inflammatory cytokine environment, we examined the proximity and contact relationship between glia and proliferating cells within the peak interval of induction by the TMT insult. The BrdU dosing regimen allowed for a 3-day interval of labeling capturing cells across a range of migration distances throughout the width of the GCL. Given that an active process of neuronal death and microglia activation was ongoing during the peak time of proliferation, it was necessary to set morphological criteria to distinguish between microglia of different morphological phenotypes. For inclusion in counting, BrdU+ cells were required to be $> 8 \mu\text{m}$ in diameter and the microglia cells to be of a ramified process bearing morphology. Figure 4 A (microglia) and B (astrocytes) provides representative images of cells in contact that meet the criteria for inclusion in the quantitation. Based upon our previous work with this model, any expression of active caspase 3 will be accompanied by a microglia cell that has shifted its morphological phenotype to amoeboid; while microglia with ramified processes, as represented in Figure 4B, are associated with neuronal survival (Kraft et al., 2009). Once these morphological criteria were established for use at the light microscopic level, 2 investigators blind to the experimental conditions examined the sections for contact between BrdU+ cells and microglia or astrocytes. Given the changes that occur in the shape of the hippocampus as the brain is sectioned, we ensured that equivalent sized regions across sections from all animals were used for quantitation. Contact between microglia and BrdU+ cells was greatest within the inner third of the blade and less in the outer ROI-3 layer (Fig. 4C). Contact with GFAP+ astrocytes was minimal within the inner ROI-1 section and became more pronounced in ROI-2 and 3. While we cannot rule out contact with fine non-GFAP+ astrocytic processes, the GFAP+ contacts detected were less in the inner region, ROI-1, as compared to the other two ROIs (Fig. 4C).

BrdU+ cells in SGZ express TNFp75R

Our previous work demonstrated by *in situ* hybridization, that both process-bearing and amoeboid microglia upregulated expression of TNF α in the hippocampus following TMT (Brucoleri et al., 1998). In addition, the induction of NPC proliferation within the SGZ occurs during a period of peak elevation of mRNA levels for TNF α and receptors (Lefebvre d'Hellencourt and Harry, 2005; Harry et al., 2008b). Based upon earlier reports suggesting that the effect of TNF α on NPC could be dependent on receptor expression (Ben-Hur et al., 2003; Klassen et al., 2003; Iosif et al., 2006), we determined the expression of TNFRs by BrdU+ cells in the SGZ following TMT. Within the SGZ and ROI-1 of the dentate gyrus, a limited number of BrdU+ cells were found to express TNFp75R at 72 hr (Fig. 5). We did not detect such cells in the other two ROIs. We were unable to detect TNFp55R expression in BrdU+ cells at this time; however, this does not rule out an earlier or transient expression similar to what we have previously described for the expression in AC3+ neurons (Harry et al., 2008b). We then examined the injury and induced neurogenesis in mice deficient for TNFp75R. Consistent with our previous reports (Harry et al., 2008b); the absence of this receptor did not diminish the severity of the neuronal death following TMT nor modify the astrocyte or microglia response (data not shown). An increase was observed in the number of BrdU+ cells along the SGZ in both WT and TNFp75R deficient

mice relative to saline controls. At the time point examined, BrdU+ cells could be seen within the GCL in the WT mice (Fig. 6A) while, in the TNFp75R deficient mice these cells remained within ROI-1 (Fig. 6B). We did not determine the number of BrdU+ cells generated.

Discussion

Under normal conditions, adult-generated hippocampal cells migrate primarily to the inner third of the granular cell layer where they assume the nuclear and cytoplasmic morphology of surrounding neurons, express biochemical markers of immature and mature neurons (Kempermann et al., 2004), extend axonal projections to form synapses with CA3 pyramidal cell neurons (Markakis and Gage, 1999), and become incorporated into hippocampal-dependent declarative memory networks (van Praag et al., 2002; Kee et al., 2007). When significant damage occurs to the dentate granule cell population, NPCs are exposed to an environment of elevated pro-inflammatory cytokines including TNF α ; yet, NPC proliferation is induced and migration of the generated cells is stimulated. We now provide data demonstrating a differential response and location of microglia within the GCL with injury that may indicate the contribution of cell-cell interactions between the two cell populations during the proliferation and migration process.

While numerous studies have documented adverse effects of microglia and their secreted factors on NPC proliferation, others have suggested that these factors can be stimulatory to the repair and generation of NPCs within the brain (Aarum et al. 2003; Ekdahl et al. 2003). Thus, it is not unlikely that an injury in a young animal that initiates a microglia response would also initiate a neurogenic response. For example, previous work with adrenalectomy-induced apoptotic dentate granule cell death suggested that microglia activation contributed to an environment conducive to neurogenesis (Battista et al., 2006). In the case of TMT, the stimulation of NPCs occurs during the active period of neuronal death and elevated TNF α levels (Harry et al., 2008a). Additionally, a developmental ontogeny for responses to TNF α has been suggested due to the high expression of TNF α within the embryonic brain (Yamasu et al., 1989; Mehler and Kessler, 1997). It has been suggested that the effect of TNF α on NPCs depends upon localized receptor expression (Ben-Hur et al., 2003; Klassen et al., 2003; Iosif et al., 2006). There is a limited amount of information regarding the expression of TNF α or TNF receptors (TNFR) on NPCs. Klassen et al. (2003) demonstrated TNF α expression on NPC derived from neonatal rat hippocampus and in cultured NPCs from human fetal brain. The cultured human NPCs were found to express both TNFp55R and TNFp75R (Klassen et al., 2003). Progenitor cells isolated from mouse striatum were found to express TNFp55R (Ben-Hur et al., 2003). In the current *in vivo* study, we demonstrate the expression of TNFp75R on BrdU+ cells in the SGZ following TMT, suggestive of localization on hippocampal NPCs. However, at the time point examined, we did not observe the expression of TNFp55R. It is possible that this is not necessarily reflective of a general lack of expression but rather that the expression of TNFp55R is transient and occurs at a different time. In our previous work, we demonstrated a transient expression of TNFp55R followed by the expression and internalization of TNFp75R in dentate granule neurons undergoing early stages of apoptosis that could be as short as 6 hrs (Harry et al., 2008b). Thus, further examination along a temporal sequence would be required to determine receptor expression. To determine if the role for cyclin D1 in the TNF α signaling via IKK/NF κ B identified by Widera et al. (2006) was also at play in the current model, additional studies are needed to examine the co-localization of cyclin D1, BrdU, and TNFp75R as the action may require concurrent expression or not utilize the TNFp75R. Although, given the relatively short half-life of cyclin D1 and the transient expression of TNFp75R this may be difficult to quantify. In fact, in the one time point examined in the TNFp75R knockout mice, we were unable to detect a sufficient number of cyclin D1 cells

for analysis. The work of Iosif et al. (2006) showed an increase in cell proliferation with or without stimulation in mice lacking TNFp55R, while mice lacking TNFp75R showed no change in basal proliferation and a minor decrease in NPC proliferation following SE. Our findings are somewhat consistent with these results in that, at the early 72 hr time point, BrdU+ cells were localized at the SGZ layer in mice deficient for TNFp75R and there was no indication of migration into the dentate blades. However, with similar severity levels of dentate granule cell death, migration was observed in the wildtype mouse within the first 72 hrs. We did not determine if the actual number of NPCs was increased. Further studies are required to determine if this represents a long-term deficit or only a delay in migration and the role that TNFR signaling may play in this process. Whether these differences are directly due to the absence of TNFp75R or the result of the production of other pro-inflammatory cytokines cannot be determined in the current study. In previous work (Harry et al., 2008b), we reported that at the dose level used in the current study, mRNA levels for various pro-inflammatory cytokines in the hippocampus was similar to the wildtype. However, at a lower dose level, when there was no elevation in the wildtype mouse, mRNA levels were elevated for MIP1 α and IL-1 α in the TNFp75ko mice. Thus, it is likely that other unique changes are occurring in the TNFp75ko mouse following TMT that may have a significant impact on the NPCs.

Our current data suggests that, as the BrdU+ cells migrated through the GCL, contact with microglia cells is diminished and contact with GFAP+ astrocytes becomes more prominent. This shift appeared to occur within ROI-2 and 3, which would be at a stage when the cells would be reaching their final migration site. While this may represent a normal shift that would occur under basal neurogenesis, it is difficult to determine given the minimal staining for either microglia or astrocytes within the densely packed GCL of the normal mouse. However, it is apparent that, with the loss of neurons within the GCL, there is a void for which both the astrocytes and microglia can fill as well as removal of the restrictive matrix allowing for cell migration. Thus, while the overall process may be similar the ability to distinguish the cell contacts may not be as robust in the control hippocampus. With the death of dentate granule neurons one would expect a disruption in the integrity of the parenchyma. The associated loss of the cellular matrix may diminish the cellular barriers for soluble factors thus, allowing for glial-glial interactions such as interactions with astrocytes by microglia release of IL-1 (Spranger et al., 1990). We have previously demonstrated elevations in IL-1 by microglia following TMT (Brucoleri et al., 1998). Whether this represents a process unique to the injury or an enhancement of a normal migration of newly generated cells requires additional experimentation.

Microglia effects on granule neuron precursor cells have been previously reported by Morgan et al. (2004) as a mitogenic effect of microglia derived neurotrophic factors. Choi et al. (2008) demonstrated an upregulation of microglia insulin-like growth factor-1 (IGF-1) expression triggering p42/44 mitogen-activated protein kinase (MAPK) activation in NPC of the SGZ resulting in increased proliferation. With TMT injury to the hippocampus, the CA1 pyramidal neurons remain intact. In this case, IGF-1 immunostaining was observed in process-bearing microglia and along GFAP+ processes of astrocytes (Wine et al., 2009). More recently, Thored et al. (2009) reported data suggesting that a long-term accumulation of microglia expressing IGF-1 served in a supportive role for neurogenesis in the subventricular zone following stroke. Thus, further examination of localized changes may help us understand dynamic interactions between microglia and astrocytes with regards to their effects on newly generated cells within the hippocampus. Gaining a better understanding of these events will significantly enhance our ability to identify therapeutic intervention strategies to promote successful brain repair.

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Figure 1.

Representative images of the histopathology of the dentate granule cell layer (GCL) of the hippocampus in control CD-1 male mice (A,C,E,G) and in mice 72-hrs following trimethyltin (2.0 mg TMT/kg bwt, ip) (B,D,F,H).

A-B) Representative images of TUNEL+ cells in (A) control and (B) TMT-dosed mice. A significant number of TUNEL+ cells (brown) were evident within the GCL. Scale bar = 50 μ m.

C-D) Representative DIC images of GFAP+ astrocytes (red) showing thin processes through the GCL in (C) control mice (D) the increased GFAP staining in the GCL of mice dosed with TMT was characterized by thicker astrocyte processes throughout the GCL suggestive of hypertrophy. Scale bar = 25 μ m.

E-F) Representative image of immunostaining for active caspase 3 (AC3; red) with a Nissl (blue) counterstain. (E) control mice displayed minimal staining for AC3 with punctate staining seen only in the blood vessel. In the mice dosed with TMT AC3 staining was evident in neurons showing evidence of collapsed nuclei. Nissl (blue) stained sections of the hippocampus from controls show normal cellular morphology and evidence of dense collapsed cells in the TMT dosed mice. Scale bar = 25 μ m.

G-H) Representative images of process bearing Iba-1+ microglia (red) in (G) control and (H) microglia displaying a thickening and retraction of processes and an amoeboid phenotype in TMT dosed mice. In control mice, microglia are present at both the inner and outer layer of the GCL with processes transverse across the layer. In the TMT dosed mice, round amoeboid microglia can be seen within the GCL. Blue – Dapi counterstain. Scale bar = 25 μ m.

Figure 2.

A) Representative subgranular zone (SGZ) location and orientation along the two innermost cell layers (box) of the hippocampal dentate gyrus.

B) Representative image of BrdU+ cells in the control hippocampus and in the hippocampus of mice 72-hrs following trimethyltin (2.0 mg TMT/kg bwt, ip). Mice received 2x day ip injection of BrdU (50 mg/kg body wt) at 12 hr intervals, initiated with the saline or TMT injection, for a total of 6 injections. BrdU+ cells (brown) were located at the SGZ layer in the control mice. In mice injected with TMT, there is significant amount of cell death as evidenced by hematoxylin counter stain indicating loss of cells and presence of dense cells with collapsed nuclei. BrdU+ cells are detected along the SGZ and within the GCL.

C) Schematic of defined regions of interest (ROI) across the blades of the dentate. The upper and lower blades of the GCL were demarcated into 3 distinct regions of interest. ROI-1) a 2-cell width at the inner blade considered the SGZ; ROI-2) the next consecutive 5-6 neuron width, and ROI-3) the outer granular neuronal region of the dentate blade.

D) The mean ($\pm$ SD) number of BrdU+ cells in the SGZ and GCL in control CD-1 male mice and in mice dosed with TMT (10 sections, 10 mice per group). Total number of positive cells determined within each of the regions of interest (ROI) as a distance from the SGZ as described in the Methods section. Sections were counter stained with hematoxylin. Scale bar = 50 μ m. *indicates statistical significance at $p < 0.05$.

Figure 3.

Representative image obtained from the hippocampus of CD-1 male mice dosed with 2mg/kg TMT, ip at PND 21 of the co-localization of BrdU (green) in cells immunopositive for cyclin D1 (red). Merged images demonstrate a co-expression of proteins in cells along the inner border of the GCL. Insets represent the z-stack images of the co-localization. DAPI (blue). Mice received 2x day ip injection of BrdU (50 mg/kg body wt) at 12 hr intervals, initiating with the TMT injection, for a total of 6 injections. Scale bar = 10 μ m.

Figure 4.

Representative images and quantitation of contact between BrdU+ cells and Iba-1+ microglia and GFAP+ astrocytes in the GCL of male CD-1 mice at 72 hrs post-TMT.

A) As apoptosis was an active process at the 72 hr time point following a systemic injection of TMT (2 mg/kg, ip at PND 21), criteria were required to distinguish non-amoeboid, process bearing microglia for contact with BrdU+ cells. Using the “roundedness” as criteria for exclusion we identified Iba-1+ (red) that presented with a process bearing phenotype in contact with BrdU+ cells $>8\ \mu\text{m}$ (green). Scale bar = $10\ \mu\text{m}$.

B) Similar criteria was set for the inclusion of counting GFAP+ astrocytes (green) in contact with BrdU+ cells (red) within the different ROIs in the GCL. Contact could be with either the GFAP+ processes or with the main GFAP+ body of the astrocyte. Scale bars = $10\ \mu\text{m}$.

C) Quantitation of the percentage of BrdU+ cells ($>8\ \mu\text{m}$ diameter) in contact with Iba1+ microglia or GFAP+ astrocytes within each of the ROIs (as described in Methods and in Figure 2) at 72 hrs post-dosing with either saline or TMT (2.0 mg/kg, ip; PND21). Data represents the mean $\pm$ SD. * indicates statistical significance $p<0.05$.

Figure 5. Representative con-focal image of the co-localization of TNFp75R (green) and BrdU (blue) within two cells identified along the inner blade of the GCL (ROI-1). Microglia processes are identified with Iba-1 immunostaining (red). Approximately 15% $\pm$ 4% of the BrdU+ cells at the SGZ co-localized with TNFP75R. Scale bar = 10 μ m.

Figure 6.

Representative image of BrdU+ cells within the SGZ and GCL of (A) WT mice and (B) mice deficient for TNFp75R at 72 hrs post-TMT (2 mg/kg, ip; PND21). BrdU+ cells stained black and were evident within the GCL in WT mice. In the TNFp75R mice BrdU+ cells were localized along the SGZ and not seen within the GCL. Sections were counterstained with Nissl. Scale bar = 50 μ m.