Differential neurogenesis in the adult rat dentate gyrus: an identifiable zone that consistently lacks neurogenesis

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Abstract
The dentate gyrus continues to produce new neurons in adult rodents. The possibility of differential regulation of neurogenesis within regions of the dentate gyrus is largely unexplored, despite several other aspects of this phenomenon being well characterized in a large number of studies. In this report, we describe an area located at the anterior pole of the dentate gyrus that consistently lacks neurogenesis. This neurogenically quiescent zone invariably lacks expression of the neuroblast marker doublecortin (DCX), bromodeoxyuridine and Ki-67, though DCX expression can be elicited in response to a combined paradigm of environmental enrichment and wheel running. We propose that this region may provide a valuable model system to discern the factors that regulate the process of neurogenesis.

Introduction
Neurogenesis in adult mammals is well characterized in at least two brain regions: the subventricular zone in the lateral ventricles, and the subgranular zone (SGZ) of the hippocampal dentate gyrus (for a recent review, see Ming & Song, 2005). The process of neurogenesis in the dentate gyrus is of particular interest, given this structure’s role in various types of learning and memory (Shors et al., 2002; Snyder et al., 2005; Aimone et al., 2006). However, the majority of reports examining dentate neurogenesis have treated the dentate gyrus as a whole, with little attention paid to potential differential regional regulation.

In the current study, we have examined the differential distribution of doublecortin (DCX)-expressing cells within the dentate gyrus of normal adult rats. More specifically, we describe an identifiable area associated with the granule cell layer that consistently lacks DCX-expressing cells, which we refer to here as the 'neurogenically quiescent zone' (NQZ). In addition, we also present evidence that more general markers of actively cycling cells [bromodeoxyuridine (BrdU) and Ki-67] are consistently absent from this area, despite the presence of stem-like cells. We believe that the NQZ may provide a stable, highly consistent molecular microdomain that will be valuable in elucidating the regulatory mechanisms of neurogenesis.

The results of this study have been previously reported in abstract form (Melvin & Sutherland, 2006).

Materials and methods
Reagents and suppliers
All animals were obtained directly from Charles River Laboratories (Wilmington, MA, USA). Euthansol was obtained from Schering-Plough (Pointe Claire, QC, Canada). BrdU was purchased from Sigma (product # B5002; St Louis, MO, USA).

Primary antibodies were as follows: rat anti-BrdU (BU1/75, product # OBT0030, Oxford Biotechnology, Oxfordshire, UK); goat anti-DCX (product #sc-8066, Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit anti-Ki-67 (product #NCL-Ki-67p, Novocastra, Newcastle-upon-Tyne, UK); guinea pig anti-glia fibrillary acidic protein (GFAP; product #31223-200, Advanced Immunochemicals; Long Beach, CA, USA); mouse anti-nestin (product # 556309, BD Pharmingen; San Jose, CA, USA).

Secondary antibodies were as follows: Alexa Fluor 488 chicken anti-rat (product #A21470, Molecular Probes, Eugene, OR, USA); biotin-SP-conjugated donkey anti-goat (product #705-065-147; Jackson ImmunoResearch, West Grove, PA, USA); Alexa Fluor 488 donkey anti-rabbit (product #A21206, Molecular Probes); Alexa Fluor 488 donkey anti-mouse (product # A-21202, Molecular Probes); biotin-SP-conjugated donkey anti-guinea pig (product #706-065-148; Molecular Probes); streptavidin Alexa Fluor 568 (product #S11226, Molecular Probes).

Experimental subjects and injections
A total of 10 3–3.5-month-old male Long–Evans Hooded rats were used in this study. Five of the animals received a single intraperitoneal injection of 150 mg/kg of BrdU, mixed at a concentration of 50 mg/mL in 0.9% sterile saline with heating. Upon sufficient cooling, animals were injected. All animals received injections between 11.00 and 12.00 h. As animals were housed in pairs, one of each (randomly selected) was injected with BrdU, while the corresponding cage-mate served as a saline control. All animals were perfused 3 h after BrdU injection by an injection of sodium pentobarbital (150 mg/mL). All procedures were conducted in accordance with the University of Lethbridge and Canadian Council on Animal Care (CCAC) animal welfare committee guidelines.
Environmental enrichment and wheel running

For a total of 20 days, rats were placed in daily alternating conditions of environmental enrichment and wheel running. Rats were group housed (n = 6) in the enriched environment for 24 h, and then were transferred individually to wheel running cages for the ensuing 24 h period. The enriched environment consisted of a circular tub with a diameter of 1.5 m and height of 60 cm. A wire mesh cover was placed over the top of the tub from which several bungee cords were attached. A variety of toys and different lengths of polyvinyl chloride tubing were present in the tub, and these were rearranged every 24 h in an effort to encourage exploration. Each of the wheel running cages (Mini Mitter, Bend, OR, USA) contained a running wheel attached to a counter in order to monitor running activity. All rats had ad libitum access to food and water during the course of the experiment. Animals were subsequently perfused within 2 h of the last day of exposure.

Perfusions, histology and immunohistochemistry

After a lethal injection of sodium pentobarbital (150 mg/mL), animals were transcardially perfused with 150 mL of 0.1 M phosphate-buffered saline (PBS), pH 7.4, followed by 200 mL of 4% paraformaldehyde in 0.1 M PBS. Brains were removed and postfixed in 4% paraformaldehyde in PBS for 24 h at 4 °C. This solution was then replaced by 30% sucrose in PBS containing 0.02% sodium azide and, when the brains sunk, they were cut at 40 μm into either a 1/5 or 1/7 section sampling fraction on a freezing sliding microtome (American Optical, model #860; Buffalo, NY, USA). With each brain, the collection of coronal sections started at a random point before the beginning of the dentate gyrus, and was sectioned exhaustively through its entire rostral-caudal axis. Sections were collected into PBS containing 0.02% sodium azide and stored at 4 °C until processed.

Immunohistochemistry was conducted as free-floating sections, using 0.1 M PBS with 0.3% Triton X-100 as a diluent in all cases. Incubation times were 24 h for all primary antibodies and secondary antibodies, and 1 h for tertiary reagents. Incubations were carried out at room temperature on a rotating table. Every immunohistochemical procedure included labelling for DCX to serve as a reference for the location of the NQZ. The NQZ is operationally defined in this study as the contiguous area along the dorsal blade that, from the point where the two blades meet and extending laterally, lacked DCX + cell bodies.

To determine the frequency with which Ki-67 + cells were found within the NQZ, one series from each animal was triple-labelled with rabbit anti-Ki-67 (1 : 1000) and goat anti-DCX (1 : 500), using Alexa-488-conjugated donkey anti-rabbit (1 : 250) and a biotinylated donkey anti-goat (1 : 6000) antibodies as secondary reagents; DAPI was used as a counterstain to delineate the granule cell layer. Streptavidin-conjugated Alexa 568 (1 : 500) was subsequently used to detect DCX.

To detect the presence of BrdU, the tissue was processed through several DNA denaturing steps in order to retrieve the BrdU epitope. Briefly, the tissue was first exposed to a solution of 2 × saline sodium citrate buffer in 50% formamide at 65 °C, followed by two rinses in 2 × saline sodium citrate buffer alone at room temperature. Sections were then placed into 2 N HCl at 37 °C for 30 min. After several rinses in PBS over approximately 1.5 h, the tissue was then placed into rat anti-BrdU (1 : 100) and goat anti-DCX (1 : 500) primaries. Following primary incubations, the tissue was rinsed three times in PBS, and placed into Alexa Fluor 488 chicken anti-rat (1 : 600) and biotin-conjugated donkey anti-goat (1 : 6000). Sections were rinsed again, and placed into streptavidin-conjugated Alexa 568 (1 : 500) before mounting.

Sections were mounted out of PBS and overslipped with a glycerol-based anti-fade reagent (9.8% polyvinyl alcohol, 2.5% 1,4-diazabicyclo[2.2.2]octane, 24% glycerol in 0.1 M Tris-HCl, pH 8.3; all obtained from Sigma). Signals were subsequently analysed under appropriate filters using a Zeiss Axioskop2 MotPlus microscope or a Nikon C1 confocal microscope where appropriate. Control experiments included the incubation of sections in the absence of primary antibodies. All images were captured using a QImaging Retiga EXi CCD camera (Burnaby, British Columbia, Canada).

Quantification

Dentate gyrus volumes were calculated using the Cavalieri method (Schmitz & Hof, 2005). Briefly, all images were captured using DAPI to delineate the granule cell layer. For the 1/7 section sampling fraction, an average of 18 sections was obtained through the entire dentate gyrus, and an average of 27 sections for the 1/5 section sampling fraction. After capturing an image of each section under 2.5 × magnification, a systematic sampling grid with an area per point of 0.02 mm² was randomly thrown over each image, and the number of points hitting the granule cell layer and hitting the NQZ were counted. Grids were generated using ImageJ software (http://rsb.info.nih.gov/ij/). An unbiased estimate of volume was calculated according to the following formula:

\[
V_{est} = \sum_{i=1}^{n} P \times a(p) \times T
\]

where \(\sum P\) is the sum of points counted across all sections, \(a(p)\) is the area per point of the grid, and \(T\) is the distance between sections. The total volume of the dentate gyrus, as well as the volume and volume fraction of the NQZ, were calculated.

The precision of these estimates was estimated by calculating the coefficient of error (CE) for each brain using the method of Gundersen (Gundersen et al., 1999; Bermejo et al., 2003). The mean CE across all animals was calculated as the square root of the sum of squared CEs, and was 4.7%.

The number of BrdU + cells was analysed by taking one series from each animal (n = 4), and double-labelling for DCX and BrdU. The number of BrdU + cells was counted in each section under 40 × magnification (NA 0.75), excluding the uppermost focal plane to minimize the number of double counts that occur as a result of split nuclei at the cut surface (Kronenberg et al., 2006). The number of cells counted was then multiplied by the inverse of the respective section sampling fraction to obtain an estimate of the total number of BrdU + cells per dentate gyrus.

To determine the total number of cycling cells in the dentate gyrus, we concurrently detected the presence of DCX, Ki-67 (an endogenous marker expressed in all phases of the cell cycle; Cooper-Kuhn & Kuhn, 2002) and DAPI on another series of sections from all animals (including those animals given BrdU; total n = 10). Statistical analysis (Student’s t-test, with P < 0.05 deemed as statistically significant) determined that there were no differences in terms of the number of Ki-67 cells in saline vs. BrdU-treated animals (data not shown), so the data from both groups were pooled.

In both cases, the numbers of BrdU and Ki-67 + cells were normalized to the total volume (minus the volume of the NQZ) of each brain in order to estimate an average density of each cell type. The average number of expected cells of each type was then scaled to the volume of the NQZ to discern the number of immunoreactive cells expected to be present in this region.
Results

Characterization of the NQZ

Our data indicate the presence of an identifiable subregion in the dentate gyrus that is consistently devoid of neurogenesis. This region, characterized by its lack of DCX expression, is located at the rostral portion of the dentate in coronal sections, and is largely restricted to the dorsal blade of the dentate gyrus (the area between the arrows in Fig. 1). When every seventh section is analysed in the coronal plane,

![Image of sections showing the NQZ](image)

**Fig. 1.** The NQZ depicted in sections of the anterior dentate gyrus cut in the coronal plane. (A–C) DAPI-stained sections (240 μm apart), showing the location of the granule cell layer. (A'–C') The pattern of DCX immunoreactivity in the same sections. The NQZ is the area found between the arrows. Scale bar: 500 μm.

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The NQZ is present generally in about two sections (Fig. 2). This region is present in sections that extend from approximately 2.28 mm posterior to Bregma to 3.00 mm posterior to Bregma (Paxinos & Watson, 2005). As more posterior sections were analysed, the NQZ appears to progressively ‘fill in’ with DCX + cells in a lateral-to-medial gradient (note the arrows in Fig. 1), after which DCX + cell bodies were present relatively homogeneously across both blades of the dentate gyrus. The average volume of this region was 0.036 mm³ (SEM ± 0.004), which represents approximately 1.5% of the total volume of the dentate gyrus.

The NQZ lacks cell proliferation
Along with an absence of DCX + cells, there is an absence of cell proliferation in general. We used both Ki-67 and a single BrdU injection with short-term survival to detect acute proliferation throughout the entire dentate gyrus. In order to compare the average number of proliferating cells in the NQZ with that of other areas of the dentate, we counted the number of BrdU and Ki-67 + cells in the entire dentate gyrus outside the NQZ. The average volume of this region was 0.036 mm³ (SEM ± 0.004), which represents approximately 1.5% of the total volume of the dentate gyrus.

Fig. 2. The location and size of the NQZ in coronal sections with respect to the entire granule cell layer of the dentate gyrus. In every seventh section in one representative animal, the area (denoted by the # of points) of the granule cell layer is provided from anterior to posterior (white bars). In addition, the area occupied by the NQZ is also shown (black bars). Note the relative area occupied by the NQZ relative to the entire granule cell layer, and its relative position within the dentate gyrus.

![Fig. 2](image1)

The number of BrdU + cells expected to be present in the NQZ, given the average density of BrdU + cells in all other areas outside the NQZ, though an average of 29 cells would be present in the NQZ given its volume, none was ever seen. (B) The number of Ki-67 + cells expected in the NQZ, given the density of Ki-67 + cells throughout the rest of the dentate gyrus. Though 66 were expected, only two were ever seen in the NQZ, both of which were in one animal out of 10 (see Results for an explanation).

The NQZ contains stem-like cells
In order to confirm that the NQZ contained cells capable of supporting neurogenesis, we sought to detect stem-like type I cells by using GFAP and nestin immunohistochemistry. Type I cells that give rise to DCX + cells in the dentate gyrus express both GFAP and nestin, have a radial morphology with a process that extends through the granule cell layer, and a roughly triangular-shaped cell body (Fukuda et al., 2003; Namba et al., 2005). Qualitative analysis of sections labelled with GFAP and nestin indicated that cells with features consistent with type I cells were indeed present in the NQZ.

![Fig. 3A](image2)

![Fig. 3B](image3)

The number of BrdU + cells expected to be present in a volume of dentate equal to the size of the NQZ is 29. Across four animals, however, we observed none. With respect to Ki-67, we expected to observe approximately 66 cells, but we observed that there were essentially none (Fig. 3B). It should be mentioned that we found potentially two Ki-67 + cells in the region of the NQZ, both of which were in the same animal. However, it is possible that these cells may in fact be more properly classified as hilar cells with respect to their position, as the definition of the SGZ at the light microscopic level is rather loose (i.e. two–three nuclear diameters below the granule cell layer; Kempermann, 2005).
Environmental enrichment and wheel running elicits neurogenesis in the NQZ

It has been noted in several studies that both environmental enrichment and wheel running can result in an increase in neurogenesis in the dentate gyrus (Kempermann et al., 1998; Nilsson et al., 1999; van Praag et al., 1999). To this end, we exposed rats to alternating conditions of environmental enrichment and wheel running for 20 days. As a result of this exposure, DCX + cell bodies were found to be present in the NQZ (Fig. 5, arrows).

Discussion

We find that there is a region of the adult dentate gyrus that consistently lacks neurogenic features. In stark contrast to most of the dentate gyrus, markers of proliferation (acute BrdU and Ki-67) and a specific phenotypic marker associated with developing neuroblasts (DCX) are absent. Though we do not contend that this is the only area of the dentate that does not undergo neurogenesis, it is of particular interest because its presence in normal adult animals is invariant and its location is anatomically identifiable between animals. We also

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demonstrate that the lack of neurogenesis is not likely to be due to the absence of stem-like type I cells, as GFAP+ /nestin + cells with the correct morphology are seen in this region. These results suggest that there may be a quiescent population of stem-like cells in the NQZ.

**Heterogeneity in dentate gyrus proliferation**

The dentate gyrus is often assumed to be a relatively homogenous structure throughout its extent (Scharfman et al., 2002). As such, relatively few papers have been published showing neurogenic regional variability within the dentate gyrus. In fact, Rao & Shetty (2004) indicated that the distribution of DCX + cells does not vary along the anteroposterior axis of the dentate gyrus. Kempermann et al. (2003) reported that there were differences in BrdU incorporation between the dorsal and ventral blades, though no differences were seen between hemispheres. Importantly, this component of the study did not look at neurogenesis specifically, as BrdU incorporation on its own is not indicative of a neuronal phenotype. In addition, Silva et al. (2006) conducted a topographical analysis of both neurogenesis and apoptosis in 1- and 3-month-old rats. A general gradient of proliferation was reported to exist in 1-month-old animals, from the lateral aspect of the dorsal blade to the middle portion of the ventral blade; such regional differences were largely absent, however, in 3-month-old animals, similar to the age that was used in the current study. This study also reported variations in the numbers of proliferating cells at different rostrocaudal levels as well.

All studies that have analysed regional variations in neurogenesis have done so by somewhat arbitrarily partitioning the dentate gyrus and underlining SGZ into zones that rely either on overt anatomical boundaries (Kempermann et al., 2003; Silva et al., 2006) or by dividing the region up along its mediolateral extent into equal segments (Silva et al., 2006). It is therefore difficult to extrapolate any qualitative or quantitative comparisons between these previous studies and ours. By virtue of its lack of DCX + cell bodies, it would be of great interest to conduct further analyses of this area in comparison to other areas of the dentate that do exhibit constitutive neurogenesis.

**Regional variations in neurogenesis after injury**

It is interesting to note that some authors have described regional differences in the neurogenic responsiveness of the dentate gyrus after injury. Of particular interest in the current context are two recent studies using different experimentally induced forms of damage. Covolan et al. (2000) reported an increase in the number of BrdU + cells in sections that would contain the NQZ (in sections 2.45 mm posterior to Bregma) after kainate- and pilocarpine-induced seizures. Conversely, Choi et al. (2003), using a transient forebrain ischaemia model, found that the number of BrdU + cells increased in all sectors of the dentate gyrus, except the rostral suprapyramidal blade. This area, according to their stereotaxic coordinates (2.5–3.0 mm posterior to Bregma), would also contain the NQZ. These results, combined with our data showing the presence of stem-like cells in the NQZ, suggest that this area is capable of being stimulated to undergo proliferation under some conditions, but appears to be quiescent under basal circumstances.

**Regulation of neurogenesis in the NQZ**

Several factors are known to enhance neurogenesis in the dentate gyrus, including environmental enrichment (Kempermann et al., 1998; Nilsson et al., 1999) and wheel running (van Praag et al., 1999). To this end, we sought to determine whether similar treatments were capable of inducing neurogenesis in the NQZ. Using a combination of environmental enrichment and wheel running, we provide evidence of the induction of neurogenesis in the NQZ (Fig. 5, arrows).

One question unanswered by this report is the following. Why is this area neurogenically different than other areas of the dentate gyrus? It is certainly possible that this region of the dentate gyrus has some as yet unknown and relatively unique function that distinguishes it from the rest of the dentate but, given the small volume of this structure, this hypothesis would be difficult to test with the current methods typically used in functional neuroscience. Another viable and easily testable theory is that the NQZ may represent a region of the dentate that is one of the first to succumb to the age-related decline in neurogenesis (Kuhn et al., 1996).

Ageing appears to be the most potent endogenous downregulator of neurogenesis (Kempermann, 2005). In this light, it is interesting to speculate that the NQZ described in this paper may be related to this age-related decline in neurogenesis; specifically, this zone may represent one of the first areas to undergo cellular senescence. This idea generates several concrete predictions. If this hypothesis were true, DCX + neuroblasts would be present in every region of the dentate gyrus immediately after its initial formation; the NQZ would then emerge as animals approach 3 months old. The age-related declines in neurogenesis have been suggested to be due to the lack of specific growth factors that normally provide a permissive environment for neurogenesis (Lichtenwalner et al., 2001; Rao et al., 2005; Shetty et al., 2005). Thus, the lack of neurogenesis in the NQZ in animals of this age may be caused by an early decline in some or all of these factors. This further predicts that neurogenesis may be inducible in the NQZ by methods that restore growth factor levels to that area.

In summary, we believe that the identification and characterization of a region that consistently lacks neurogenesis will provide valuable insights into the mechanisms that regulate the generation of new cells in the adult dentate gyrus. Given that the NQZ appears to have stem-like cells but no DCX + neuroblasts, analysing differential gene expression between this and adjacent areas that do exhibit neurogenesis would provide valuable information as to which genes play key roles in the process of neurogenesis. The description of this area may therefore provide an especially valuable model system with which to elucidate the molecular mechanisms that regulate neurogenesis.

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**Abbreviations**

BrdU, bromodeoxyuridine; CE, coefficient of error; DCX, doublecortin; GFAP, glial fibrillary acidic protein; NQZ, neurogenically quiescent zone; PBS, phosphate-buffered saline; SGZ, subgranular zone.

**References**


