Video Article

Isolation and Expansion of Adult Canine Hippocampal Neural Precursors

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Abstract

The rate of neurogenesis within the adult hippocampus has been shown to vary across mammalian species. The canine hippocampus, demonstrating a structural intermediacy between the rodent and human hippocampi, is therefore a valuable model in which to study adult neurogenesis. *In vitro* culture assays are an essential component of characterizing neurogenesis and adult neural precursor cells, allowing for precise control over the cellular environment. To date however, culture protocols for canine cells remain under-represented in the literature. Detailed here are systematic protocols for the isolation and culture of hippocampal neural precursor cells from the adult canine brain. We demonstrate the expansion of canine neural precursor cells as floating neurospheres and as an adherent monolayer culture, producing stable cell lines that are able to differentiation into mature neural cell types *in vitro*. Adult canine neural precursors are an underused resource that may provide a more faithful analogue for the study of human neural precursors and the cellular mechanisms of adult neurogenesis.

Video Link

The video component of this article can be found at https://www.jove.com/video/54953/

Introduction

Regional variations in the rate of neurogenesis have been observed along the dorsoventral axis of the rodent hippocampus[1,2]. Furthermore, the rates of hippocampal neurogenesis also show distinct inter-species variation, with precursor cell turnover in the subgranular zone shown to be significantly lower in adult humans than in rodents[3-5]. Inter-species differences in hippocampal structural anatomy may be relevant here, as it has been postulated that neural stem cell distribution along the murine ventricular neuraxis may be influenced by cephalic flexures during embryological development[6]. To date, the rodent brain remains the most popular system in which to study adult neurogenesis. However, the brain of the domestic dog (*Canis familiaris*), with a size and structural organization intermediate between that of humans and rodents[7], represents a valuable yet highly underused animal model. The canine hippocampus in particular embodies this structurally intermediate nature[8-10] and can provide a unique perspective on intrinsic variations in neural precursor cell populations. With many closer parallels to the human brain, the canine model may also offer insight into the biology of adult human neurogenesis.

In vitro culture assays have become a key tool for the study of neural precursors and the cellular and biomolecular processes of adult neurogenesis. The neurosphere assay and adherent monolayer culture represent the two predominant systems for expanding neural precursor cells *in vitro*[11-13]. Protocols for brain extraction, hippocampal microdissection or neural precursor culture assays have been well documented for the rodent model[14-16]. However, for the adult canine brain they remain comparatively few[17,18], focused instead on fetal or neonatal tissue[19-21].

In our published study[7] we investigated regional variations in neurogenesis and neural precursor cell populations across the dorsoventral axis of the adult canine hippocampus. Although highly dependent on breed, adulthood in canines is reached between 1 and 3 years of age. Here, we present detailed methods for the extraction, isolation and culture of neural precursor cells from the canine hippocampus. We provide systematic protocols for the expansion of neural precursor cells as both floating neurospheres and as an adherent monolayer culture, and for their subsequent differentiation into mature neural cell types.

Protocol

In accordance with New South Wales, Australia law, *post mortem* brain tissue was acquired from adult dogs euthanized for reasons unrelated to the study.

1. Preparation of Culture Medium

1. Prepare a 0.1% gelatin solution by adding 0.1 g of gelatin powder to 100 ml of distilled water and agitating at 37 °C until dissolved. Sterilize the solution by UV irradiation for 15 min. This media can then be stored at 4 °C for up to 1 month.

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2. Prepare a 3:1 ratio of Dulbecco's Modified Eagle Medium (DMEM) and nutrient mixture F12 by adding 167 ml of F-12 to 500 ml of DMEM (4.5 g/L D-glucose). Add 6.7 ml of Penicillin/Streptomycin solution (for a 1% final concentration). This media can be stored in the dark at 4 °C for up to 1 month.
3. Prepare 500 ml of serum media by combining 440 ml of DMEM (4.5 g/L D-glucose), 5 ml of L-alanyl-L-glutamine dipeptide (200 mM), 5 ml of Penicillin/Streptomycin and 50 ml of fetal bovine serum (FBS). This media can be stored in the dark at 4 °C for up to 1 month.
4. Complete growth medium consists of Neural Stem Cell (NSC) Basal Medium and Proliferation Neural Supplement-A (NS-A), 2% FBS, 2 µg/ml heparin, 20 ng/ml epidermal growth factor (EGF) and 10 ng/ml basic fibroblast growth factor (bFGF). Make this media fresh, immediately prior to brain dissection, and warm to 37 °C in a water bath.

2. Brain Extraction

1. Begin extraction within 6 hr *post mortem* by sterilizing the dorsal surface of the dog's head using povidone-iodine.
2. Using a scalpel, make a longitudinal incision along the sagittal midline, across the entire dorsal surface of the cranium, to expose the underlying masseter muscles. The length of this cut will depend on the species and age of the dog.
3. At the rostral and caudal borders of the cranium, make additional 5 - 10 cm perpendicular cuts on both sides, passing behind the eyes and ears respectively, to allow the skin to be fully reflected.
4. Using a scalpel, separate the masseter muscles from their attachment to the sagittal crest of the skull and scrape any remaining connective tissue from the cranium.
5. Using an oscillating bone saw cut the cap of the skull in a circumferential line. Using a probe or scalpel sever any remaining attachments with the dura, and then carefully remove the skull cap to expose the dorsal surface of the brain.
   Caution: The thickness of the bone varies at different regions in the skull, so caution must be exercised to not penetrate too deep and damage the underlying brain.
6. Sever the spinal cord by inserting a scalpel between the upper cervical vertebrae.
7. With one hand to gently lift the brain, use a scalpel to carefully free the brain from the cranial fossae and sever the connecting nerves and blood vessels located on its ventral side. Gently lift the brain out of the skull and into a container of DPBS.
   Caution: The large olfactory bulbs of the canine brain may extend deep into the anterior cranial fossa. Care must be taken when removing the brain so as to preserve these structures.

3. Hippocampal Dissection

1. Rinse the brain in Dulbecco's phosphate buffered saline (DPBS) to remove any blood and place it dorsal side down onto a 150 mm Petri dish.
2. Slowly bisect the brain longitudinally through the mid-sagittal plane using a wet brain knife and a single slice that utilizes the full length of the blade. Do not apply additional downward pressure, or use a sawing motion, as this may damage the tissue.
3. Placing each hemisphere medial surface up, dissect out the hippocampus using a scalpel and fine forceps, and transfer it to a 35 mm tissue culture dish.

4. Isolation of Neural Precursor Cells

1. Mince the tissue samples using a scalpel blade into approximately 1 mm^3 pieces.
2. Transfer the tissue into a 15 ml tube, add 2 ml of 0.1% Trypsin EDTA, and incubate for 7 min in a water bath at 37 °C.
3. Halt the enzyme reaction by adding 4 ml of serum media to the tube. Pellet the suspension by centrifugation at 100 x g for 7 min and remove the supernatant.
4. Resuspended the pellet in 300 µl of DPBS, and mechanically dissociated by gently pipetting up and down, using a 1,000 µl pipette tip, until a smooth homogenate forms.
5. Add 14 ml of serum media to the tube and pass the cell suspension through a 40 µm cell strainer. Pellet the suspension by centrifugation at 100 x g for 7 min, remove the supernatant, and resuspend in complete growth medium.

5. Neurosphere Culture

1. From the cell suspension obtained at isolation, count the number of viable cells using Trypan blue dye exclusion and a hemocytometer.
2. Dilute the cell suspension in complete growth medium and seed at 1 x 10^5 cells/cm^2 into uncoated wells of a 6 well plate.
3. Incubate at 37 °C, 5% CO_2 and > 95% humidity for 14 - 28 days, replace 50% of the growth media every 7 days. Measure the diameter of the neurospheres regularly. If allowed to grow beyond 100 µm in diameter, cell death and spontaneous differentiation can occur within the neurospheres.

6. Neurosphere Passage

1. To passage as a floating culture, when the neurospheres reach approximately 100 µm in diameter, combine all the neurosphere containing media into a single tube. Pellet by centrifugation at 100 x g for 7 min and remove the supernatant.
2. Resuspend the pellet in 1 ml of 0.1% Trypsin EDTA and incubate for 7 min in a water bath at 37 °C. Gently pipette up and down 100 times, using a 200 µl pipette tip, to ensure dissociation of the neurospheres.
3. Halt the enzyme reaction by adding 5 ml of serum media to the tube and centrifuge at 100 x g for 7 min.
4. Remove the supernatant, resuspend the cell pellet in 1 ml complete growth medium and count the number of viable cells using Trypan blue dye exclusion and a hemocytometer.
5. Dilute the cell suspension in complete growth medium and seed at 1 x 10^5 cells/cm^2 into uncoated wells of a 6 well plate. Incubate at 37 °C, 5% CO_2 and >95% humidity for 14 - 28 days, replacing 50% of the growth media every 7 days.

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7. Adherent Culture of Neural Precursor Cells

1. Add a sufficient volume of 0.1% gelatin solution to cover the surface of a tissue culture dish and cure in an incubator at 37 °C for 1 hr prior to processing the neurospheres for adherent culture.
2. From the floating neurosphere culture, combine all media into a single tube. Pellet the neurospheres by centrifugation at 100 x g for 7 min and remove the supernatant.
3. Resuspend the pellet in 1 ml of 0.1% Trypsin EDTA and incubate for 7 min in a water bath at 37 °C. Gently pipette up and down 100 times, using a 200 µl pipette tip.
4. Add 5 ml of serum media to halt the enzyme reaction and centrifuge at 100 x g for 7 min. Discard the supernatant and resuspend the cell pellet in 1 ml complete growth medium and count the number of viable cells using Trypan blue dye exclusion and a hemocytometer.
5. Dilute the cell suspension in complete growth medium, remove the gelatin from the tissue culture flasks and seed the cells at 1 x 10^4 cells/cm^2.
6. Incubate the cells at 37 °C, 5% CO_2 and > 95% humidity. Replace the media with fresh, warmed, complete growth media every three days until the culture reaches approximately 80% confluence (after approximately 7 days).

8. Neural Colony Forming Assay

1. Combine Neural Colony Forming Cell (NCFC) Assay media components: NCFC serum free medium, Proliferation NS-A, and collagen solution (3 mg/ml). Supplement this media with 2 µg/ml heparin, 20 ng/ml EGF, and 10 ng/ml bFGF.
2. Following hippocampal tissue dissociation resuspend the cells in neural colony forming assay medium, pre-warmed to 37 °C in a water bath. Seed the cells at a clonal density of 1.1 x 10^5 cells/ml into a 35 mm culture dish.
3. Incubate the cells in this semi-solid collagen matrix at 37 °C, 5% CO_2 and > 95% humidity. Culture for 28 days, replacing 50% of the growth media every 7 days.

9. Differentiation of Neural Precursor Cells

1. Add a sufficient volume of 5 µg/cm^2 laminin solution to cover the surface of a tissue culture dish and cure in an incubator at 37 °C for 24 hr before processing the cells for differentiation.
   Note: Remove the laminin solution and rinse the coated surface twice in DPBS immediately prior to seeding.
2. When passaging, seed the cell suspension at 1 x 10^4 cells/cm^2 in DMEM-F12 (3:1) media (pre-warmed to 37 °C) supplemented with 20 ng/ml EGF and 40 ng/ml bFGF onto the laminin coated culture dish.
3. Incubate the cells at 37 °C, 5% CO_2 and > 95% humidity and allow cells to proliferate for 3 days.
4. To differentiate the adherent neural precursor cells, after 3 days replace the media with differentiation media containing DMEM-F12 (3:1) media pre-warmed to 37 °C, supplemented with 10 ng/ml brain derived neurotrophic factor (BDNF).
5. During differentiation replace 50% of the media every 3 days. Differentiation occurs gradually over approximately 21 - 28 days.
   Caution: Once the cells have begun to differentiate, only replace 50% of the media at one time as exposure to air can have a detrimental effect on the health of the cells.

Representative Results

Through the use of *in vitro* neural precursor assays, neurogenesis and neural precursor cell populations were characterized and compared across the dorsoventral axis of the adult canine hippocampus. Neural precursor cells derived from isolated hippocampal tissue formed floating neurospheres within 14 days of isolation, reaching a diameter of 100 µm by 28 days of culture. Neurospheres derived from dorsal and ventral isolates showed no difference in mean size, and following enzymatic dissociation into single cells, could be passaged as floating neurosphere cultures. Secondary floating neurospheres from both hippocampal regions were able to form within 5 days from passage. When seeded at 1 x 10^4 cells/cm^2 onto 0.1% gelatin coated culture flasks, neural precursor cells were also able to proliferate as adherent monolayers (**Figure 1A**). No morphological differences were observed between dorsal and ventral hippocampal neural precursor cells and both adherent cultures were able to undergo over 10 population doublings without any observed slowing in passage-time (**Figure 1B**). Nestin and Sox2 neural stem cell gene expression in adherent culture was not significantly different across the hippocampal dorsoventral axis (**Figure 2**). More extensive characterization of gene and protein expression, confirming the neural precursor identity of these cells, is reported in our published data[7].

Using an adapted Neural Colony Forming assay[4,6] we assessed neural precursor cell frequency in order to quantify potential differences in neural precursor cell populations across the dorsal and ventral subregions of the canine hippocampus. Cells were seeded at clonal density in a semi-solid collagen matrix that precludes cell fusion, and cultured for 28 days (**Figure 3A**). Within subject comparisons revealed a significantly greater frequency of larger spheres per unit area in dorsal (7.3 ± 2.0) compared to ventral hippocampal-derived cultures (3.6 ± 1.7; n = 5; repeated measures ANOVA $F = 96.8$; $p = 0.001$; **Figure 3B**).

Under differentiation conditions, adherent canine neural precursor cells showed progressive alterations in gross morphology, developing longer and more elaborate processes. Protein expression for neuronal (βIII-tubulin) and glial (Glial fibrillary acidic protein; GFAP) markers also increased following differentiation (**Figure 4**). No significant difference observed in the number of positively labeled cells between dorsal and ventral isolates. Our previously published data corroborates these protein expression changes, demonstrating up-regulation of their associated genes along with down-regulation of neural precursor genes over the 28 day differentiation period[7].

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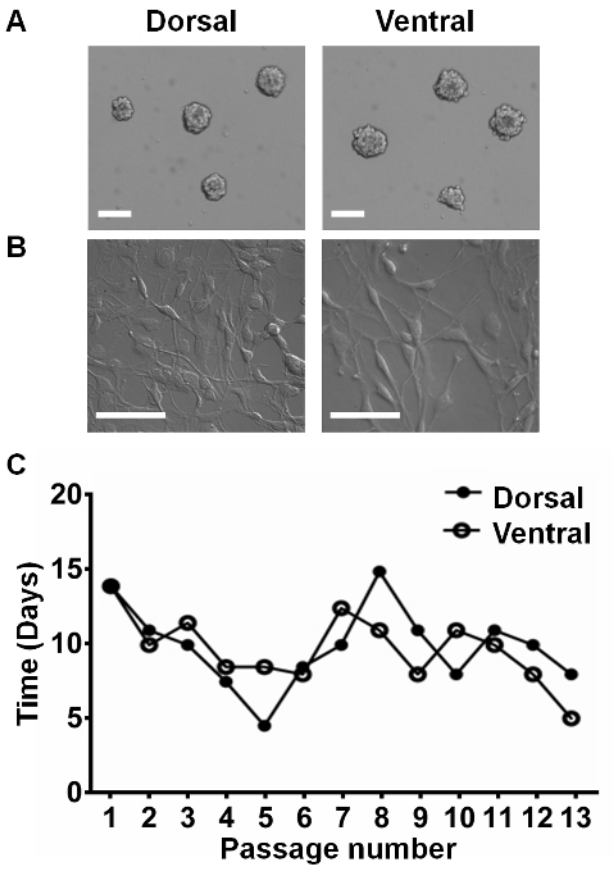

Figure 1: *In Vitro* Expansion of Adult Canine Hippocampal Neural Precursor Cells. Adult canine hippocampal neural precursor cells isolated from the dorsal and ventral hippocampal regions are expanded as **(A)** floating neurospheres or as **(B)** an adherent monolayer. **(C)** As an adherent monolayer these neural precursor cells could undergo passage over 10 times without significant decline in doubling speed. n = 5; scale = 50 µm. Modified from published figure[7]. Please click here to view a larger version of this figure.

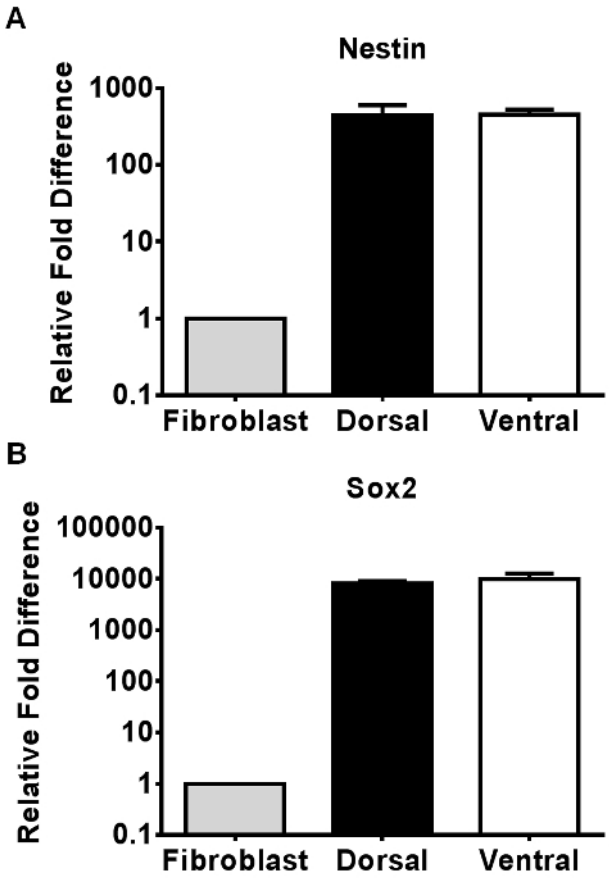

Figure 2: Gene Expression in Proliferating Adult Canine Hippocampal Neural Precursor Cells. Expression of neural stem cell genes **(A)** Nestin and **(B)** Sox2 was confirmed in adult canine hippocampal neural precursor cells under adherent proliferative culture conditions. Expression of these genes was equivalent in both the dorsal and ventral hippocampus derived cells. Adult canine fibroblasts were used as a control line to compare differences in relative gene expression. n = 5; error bars = SEM. Modified from published figure[7]. Please click here to view a larger version of this figure.

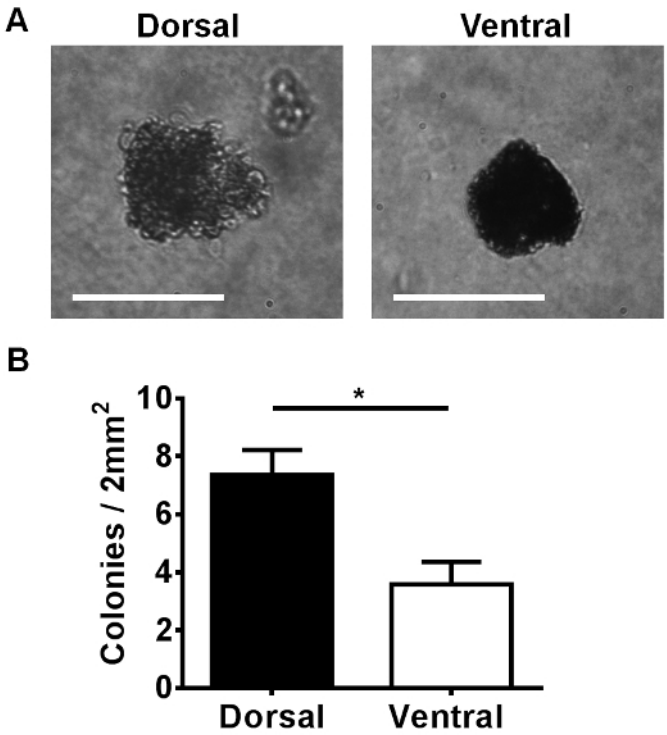

Figure 3: Neural Colony Forming Assay. (A) Adult canine neural precursor cells, seeded at clonal density into a semi-solid collagen matrix, form neurospheres within 28 days of culture. **(B)** Neural precursor cells derived from the dorsal hippocampus generate significantly greater numbers of neurospheres per unit area than those derived from the ventral hippocampus. n = 5; error bars = SEM; scale = 50 µm. Modified from published figure[7]. Please click here to view a larger version of this figure.

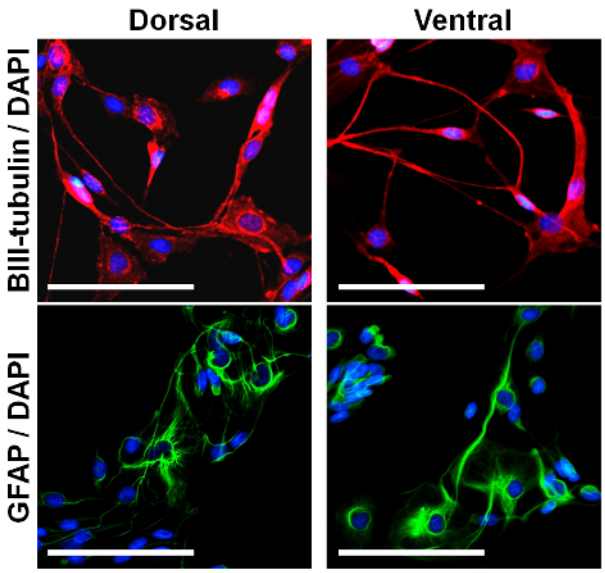

Figure 4: Immunocytochemistry of Differentiated Adult Canine Hippocampal Neural Precursor Cells. When cultured in BDNF for 28 days, adult canine neural precursors from both the dorsal and ventral hippocampus differentiate into mature neuronal (βIII-tubulin positive) and glial (GFAP positive) cell types. n = 5; scale = 50 µm. Modified from published figure[7]. Please click here to view a larger version of this figure.

Discussion

The protocols described here are optimized to maintain favorable culture conditions for maximizing cell viability. The speed and care taken during extraction, isolation and expansion is of critical importance. A critical step for establishing adherent monolayer expansion is the effective dissociation of the primary neurospheres. Following passage, insufficiently dissociated neurospheres may generate secondary floating

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neurospheres. During media change, these neurospheres may be removed, dissociated and reseeded for adherent monolayer passage. Conversely, if post-dissociation cell viability is below 80%, this may be indicative of excessive pipetting during dissociation. Neural precursor cells, and in particular their differentiated counterparts, are sensitive to changes in pH outside of the physiological range, and exposure to air. The observation of sudden widespread cell death may be attributable to these factors. Consequently, a critical step during media changes is for the media to be made fresh each time, and for only 50% of the volume to be replaced. Finally, while mitogenic factors EGF and bFGF support the survival and proliferation of hippocampal neural precursors under serum-free conditions *in vitro*[22,23], the cellular response to these mitogens is highly dependent on the cell developmental age and mitogenic concentration[24,25]. Therefore, ensuring minimal experimental variation in these components is crucial for generating consistent, reproducible cell cultures.

For maximum viability, cells should be seeded for culture within 6 hours *post mortem*. However, the extracted brain may be temporarily stored in PBS at 4 °C. This modification may allow for isolation to be delayed by up to 24 hours with minimal reduction in cell yield[26,27]. Growth factor supplementation of the culture media may also be modified to enhance proliferation or to encourage the differentiation of specific neural cell types. Insulin-like growth factor 1 (at 100 ng/ml) has been shown to have a supportive effect on rodent neural stem cells *in vitro*[28,29], while under differentiation conditions pro-neuronal factor BDNF[30] may be used in conjunction with factors such as Interleukin 7 (at 500 ng/ml) or retinoic acid (at 0.1 µM) to induce a bias towards neuronal subtype or glial differentiation[31,32].

The decision on whether to expand neural precursor cells as floating neurospheres or as an adherent monolayer depends largely on the desired downstream applications and culture characteristics. While the neurosphere culture system is simplistic and highly reproducible, it is limited in its ability to generate homogeneous populations of cells. The complex microenvironment within each sphere can promote apoptosis and spontaneous differentiation, encouraging a heterogeneous population[33], while reported fusion of neurospheres that can confound quantification of neural precursor cell populations[34]. Moreover, the repeated mechanical dissociation required for neurosphere passage may also lead to detrimental stress, senescence or even cell death. The use of alternative dissociation enzyme, such as papain, may affect the resultant cell viability[16]. Conversely, the adherent monolayer culture system, with more uniform exposure to environmental mitogens, encourages greater homogeneity in cell type and more symmetrical division. This system has been shown to produce a niche independent population of cells, based on the expression of key stem cell markers[35,36]. This system requires the use of pre-coated culture vessels to promote cellular adherence. The choice of culture substrate should be carefully considered, as it may have significant effects on the differentiation profile of the cultured neural precursors[25,37].

Here we present effective protocols for the isolation, expansion and differentiation of adult canine hippocampal neural precursor cells from fresh *post mortem* tissue. Adult canine neural precursors are under-represented in the literature[18]; with complete protocols for their isolation and expansion, even less so[17]. Our protocols may then serve to increase awareness of this valuable animal model and represent a significant addition to this modest number of studies. Of significance, using our unique method, adult canine hippocampal neural precursors can be successfully expanded more than 10 population doublings. A similar study using adult canine hippocampal neural precursor reported that larger neurospheres, passaged at 100 - 150 µm in diameter, ceased proliferation beyond the fifth generation[17]. As noted in our protocol, excessive neurosphere growth can encourage spontaneous differentiation and apoptosis, which over serial passage may have led to this reduced proliferative ability. We also provide a protocol for adherent monolayer expansion of this cell population, as an alternative to the classical neurosphere expansion system[17-21]. This alternative system affords the user more control over the cellular environment, and significantly broadens the range of downstream applications for these cells, with potential for phenotypic homogenization and directed neuronal differentiation[35,38].

Cultured adult canine hippocampal neural precursor cells have applications across a wide range of cellular and neurobiological research fields. The canine brain possesses a closer homology to the human brain than existing rodent models. As such, adult canine neural precursor cells represent an important, yet understudied, resource. These cells may be used to gain further insight into the mechanisms behind adult neurogenesis in humans, and for the development of regenerative therapies that target this process.

Disclosures

The authors have nothing to disclose.

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