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How Can We Coax The Brain To Self-Repair?

Christina Welch and Barbara Murdoch
Dept. of Biology, Eastern Connecticut State University,
Science Building, Room 353, 83 Windham St., Willimantic, CT 06226

Abstract

When someone experiences a stroke, their brain performance is affected as critical blood supply is cut off, thus resulting in loss of function as the neurons die. It was once believed that if you damaged or injured your brain it could not self-repair. However, as the field of science advances this theory is being challenged. We now know there are discrete regions of the nervous system that can replace cells that are lost or damaged. One example is the tissue that is responsible for our sense of smell, called the olfactory epithelium. When the olfactory epithelium repairs itself through cell replacement, its function is likewise restored. This is how we are able to maintain a memory of the smells we have encountered over our lifetime. By applying regenerative mechanisms from the olfactory epithelium to the brain, functional recovery following a stroke may allow people to regain partial brain function and cognition. By studying the olfactory epithelium in chicken embryos, our goal is to identify the cells that are capable of cell replacement, termed regeneration. We use antibodies, like those found in your immune system, to apply fluorescent tags specifically to the cells thought to regenerate. A laser confocal microscope is used to visualize the cells labeled with fluorescent tags and produces a digital image that allows one to determine the location and abundance of the labeled cells. This research will contribute to the overall understanding of embryonic development in the olfactory epithelium and the long-term goal of identifying how neural tissue can regenerate to replace lost neurons.

Introduction

When someone experiences a traumatic brain injury or a stroke their performance is affected as critical blood supply is cut off. This consequently results in a loss of function as cells within our nervous system die. These unique cells are called neurons, which are responsible for communications throughout the brain and body. When communication within the brain is affected, the individual may experience physical and cognitive deficits throughout their nervous system. The field of biology has been working for many years to better understand and develop mechanisms for the replacement of these damaged cells. There are specialized cells in the human body that have the ability to differentiate into and replenish several different cell types, these cells are termed stem cells (Panchision, 2006). Most of the research in the nervous system surrounding stem cells has been due to their two distinct properties: (1) Their ability to self-renew and (2) Their ability to differentiate into a variety of diverse cell types. Stem cells function as a repair system within the body, by replacing lost or damaged cells (Reynolds and Weiss, 1992). Each individual stem cell has the capacity to differentiate into a specific type of cell with a unique function (Stem Cell Basics, 2009).

It is believed that all cells in our nervous system begin as stem cells and progress in development (Okano, 2010). Stem cells in turn, give rise to an intermediate phase termed progenitor cells where they take on a more committed role toward differentiation. A cell is considered differentiated when it can only produce one specific tissue or cell type. Stem cells and progenitors can be found throughout the body in almost every tissue unlike a traditional cell that can only replace cells of its own identity. A stem cell is known to be pluripotent, due to its ability to differentiate into one of the 200 cells in the human body. However, stem cell and progenitor cell production can vary dependent on the location or tissue type to which they are found. This can be seen when comparing blood stem cells which are easily replaced as they are located in our bone marrow, compared to stem cells that are located in our brain which once they are lost, for the most part, cannot be regenerated. Stem cells are located in specific environments called niches. These environments are so important to the cell, because they coerce the stem cells to their final cell fate and identity (Maldonado-Soto et al., 2014). Stem cells are continually being researched due to their possible therapeutic potential that could help in the replacement of lost or damaged cells, especially throughout the nervous system.

The nervous system is a complex network throughout the body and is responsible for the interactions and communications between organs. There are three main cell types that are produced in the brain as a part of the nervous system: Astrocytes, neurons, and oligodendrocytes. Each of these cell types plays an important role within the human body, and when they are damaged or lost the body consequently suffers through loss of function and perhaps decline in cognition. Although the brain cannot readily regenerate these lost cells, there are however, discrete regions of the nervous system that can replace these cells types to a limited extent (Pachision, 2006). One example of this is the tissue that is responsible for our sense of smell, called the olfactory epithelium (Fig. 1). The olfactory epithelium is an epithelial tissue that is a part of our nervous system- a neuroepithelium. The olfactory epithelium has the ability to continually regenerate new neurons in a process known as neurogenesis (Schwob, 2002). Neurons located in the olfactory epithelium are sensory neurons that receive signals from the environment and send these signals to the central

nervous system where they are perceived as the sense of smell (Brookes, 2010). New neurons in the olfactory epithelium are produced in response to the loss of neurons, to help an organism maintain their sense of smells throughout their lifetime. When the olfactory epithelium repairs these lost or dead cells through cell replacement, its function is likewise restored. By applying regenerative mechanisms from the olfactory epithelium to the brain, there is a potential that functional recovery following a stroke may allow individuals to regain partial brain function and cognition.

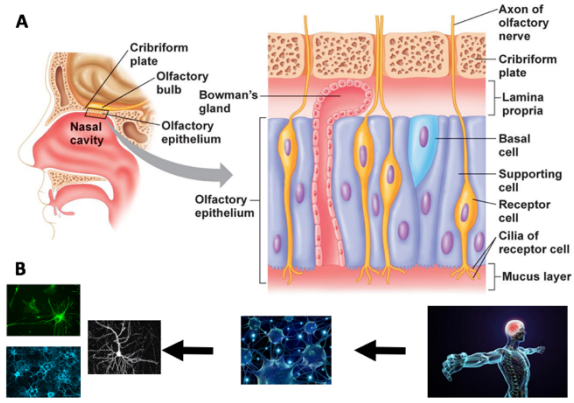

Figure 1. (A) Illustration of the olfactory epithelium and its location within the human body. (B) We work to develop a better understanding of how specific cells are developed within the body to help and aid in development.

Most of the current knowledge and research on neurogenesis and development in the olfactory epithelium has been performed in model organisms such as rodents (Murdoch and Roskams, 2007). However, the model organisms used in this research are chicken embryos, due to the similarities and parallels they have with human tissue and development (Bahr, 2008). By using tissue from chick embryos, it allows for a more precise and comprehensive study of the changes in development of the olfactory epithelium over an extended period of time. The specific stages in embryonic development that are of greatest interest, are days 3.5, 9, and 14, as determined by Hamburger and Hamilton's stages of embryonic chick development (Hamburger and Hamilton, 1992).

The main objective of this research is to determine the identity of regenerating cells that may produce all cells. In order to do this, it is crucial to understand how the mechanisms that govern neurogenesis can be used to repair neuronal loss and their potential for therapeutic medicine. By employing the use of quantitation software, we can compare the results across different stages of development to understand where during development regenerating cells can be identified. Further research will look into what signals are responsible for and affect cell differentiation within the niche of a stem cell (Kawauchi, 2004). In order to achieve this objective, it was necessary to test the premise that once when a cell is differentiated into a specific cell type, like a neuron, it cannot continue dividing. I performed an *in vivo* assay for chick neurogenesis to follow and study cellular development in the olfactory epithelium.

Materials and Methods

Tissue Preparation

Fertilized chicken embryos were incubated until the desired day of embryonic development, (embryonic day 9 and 14, E9 and E14, respectively). The chick embryos were dissected from the eggs and transferred into sterile phosphate buffer saline (PBS) to be washed. The embryos were carried through a series of washes in PBS and transferred to 4% paraformaldehyde in PBS overnight at 4°C to fix the tissue. In order to preserve and prepare the tissues for cryosectioning, embryos were cryoprotected at 4°C in 10%, 20%, and 30% sucrose/PBS solutions, for 24 hours each. After embryos were carried through the sucrose solutions, tissue was embedded in Tissue-Tek medium for optimal

cutting temperature (OCT), to be able to section the tissue at 12μm on the HM 550 cryostat. Tissue sections were mounted onto Superfrost plus glass slides (Fisher), which are required for the sustained tissue adhesion throughout the immunostaining and when being stored at -20°C, until time for further analysis.

Immunofluorescence and Immunohistochemistry

Frozen tissue sections were thawed and microwaved on high in 0.01M citric acid twice for 5 minutes, followed by a 5-minute wash in PBS. To assure permeability, tissue sections were placed in 0.1% Triton X-100/PBS/4% bovine serum for 30 minutes, to help block non-specific binding of antibodies in the cells. Primary antibodies were incubated overnight at 4°C at concentrations specific to individual antibodies. The antibodies used were proliferating cell nuclear antigen (PCNA; Sigma) 1:500, and NCAM (Developmental Studies Hybridoma Bank) 1:500.

The next day, tissue sections were washed twice for 5 minutes in PBS, and secondary antibodies were prepared. The secondary antibodies used were Alexafluors 488 or 568 raised in donkey or goat (Invitrogen Molecular Probes) 1:200. The secondary antibodies were added in PBS/2% bovine serum and were placed on the tissue sections for 45 minutes at room temperature. Sections were then washed twice in PBS for 5 minutes before incubation for 10 minutes with diaminopyridine imidazole (DAPI; 0.5 ug/ml). Following DAPI staining, tissue sections were placed in PBS for a 5-minute wash before the tissue sections were mounted with Prolong Gold and coverslips were added.

Image Analysis

All tissue sections were imaged and visualized with an Olympus Fluoview FV1000 confocal microscope with FV10-ASW software and compiled using Adobe Photoshop 7.0.

Quantification of cells

The percentage of PCNA-positive cells was determined by counting 3 sections under 400x magnification. The percentage of antigen-positive cells was determined by the formula: Total number of antigen positive cells/total number of cells (DAPI-stained nuclei) x 100. Neuronal cell bodies were used to accurately count neurons and help us differentiate between a dividing cell and a neuron.

Results

The goal of this experiment was to identify specific cells that are capable of regeneration, and understand the communication networks that will coax regenerating cells to produce new neurons. In order to achieve these goals, it was necessary to first understand chick development and cell morphology within the olfactory epithelium. Through the imaging of the tissues sections with the confocal microscope, we were able to identify the different cell types and begin observing differences in pattern and location of these cells.

The first objective of this experiment was designed to validate the known premise that neurons do not regenerate and to confirm that our reagents and systems were functioning properly. The three different colors, represent the three different antibody fluorescent tags used to identify the different cell types as seen in Figure 2. To test for the presence of dividing cells, an antibody was used that was specific for proliferating cell nuclear antigen, PCNA (Strzalka and Ziemienowicz, 2011). PCNA is a known antibody marker that has been used in the mouse olfactory epithelium as well to reveal and identify progenitors (Murdoch and Roskams, 2007; Murdoch and Roskams, 2008). To stain for and detect neurons, a neural cell adhesion marker, NCAM was used (Jørgensen, 1995). NCAM is believed to be

significant in neuronal emergence and remodeling. As well a general marker for nucleic acid, DAPI, was used to label all cells present.

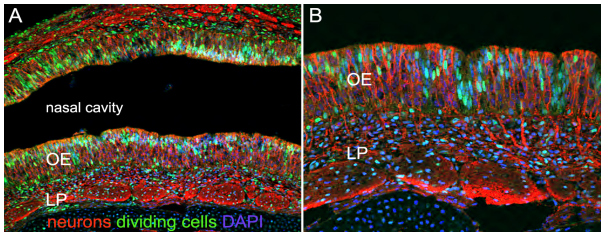

Figure 2. Antibody staining can distinguish the different cells types present in the olfactory epithelium of chick embryos at E14. Different antibodies were used to stain i) dividing cells (green), ii) neurons (red). Blue was a stain used to label all cell nuclei.

We wanted to answer the question if PCNA, our antibody marker for progenitors (dividing cells), could distinguish from non-dividing cells. In order to accomplish this, fluorescent immunohistochemistry was used with the confocal microscope. By increasing our magnification to focus in on a specific region of the OE, we could examine the specific location of dividing cells at the single cellular level (Fig. 3). These antibodies additionally allowed us to determine the identity of the cells. If certain cells were identified we would expect to either see them labeled with PCNA or NCAM, but not both, as to the best of our knowledge, neurons (NCAM+ cells) do not divide. We designed this to test the premise that once when a cell is differentiated they cannot continue dividing, therefore once when a neuron is developed we should not find a dividing cell present. As anticipated, PCNA was not detected in the cell nuclei of neuronal cell bodies, indicating that neurons were not dividing (Fig. 3A,B).

We focused on specific cells in particular regions and where there were pockets of progenitors, we saw an absence of NCAM+ neurons (Fig. 3C,D). This validated in our experiments, the premise that neurons do not divide. This has been confirmed at the earlier stages of development in chick embryos in other scientific literature and reviews (Purves et al., 2001). Overall the results in our chick tissue indicated that PCNA successfully labeled dividing cells, while NCAM was able to identify the neurons present.

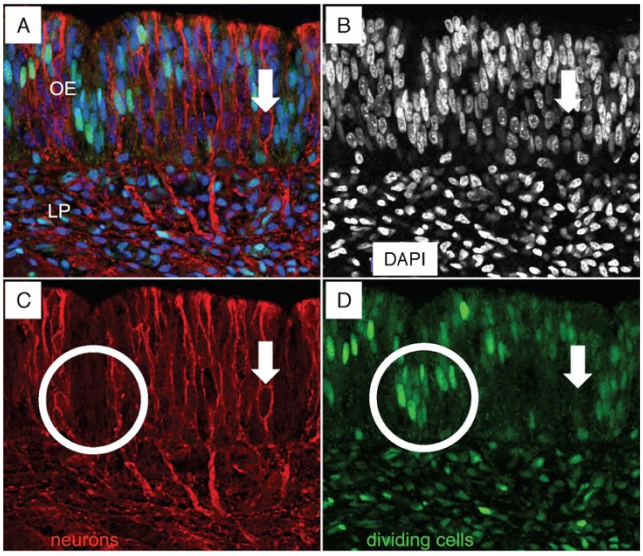

Figure 3. A-D) Increased magnification to focus in on individual cells and groups of cells to determine the identify of specific cells. This confirmed previous results from an earlier stage of development. Neurons do not divide in the OE.

After validating that neurons do not divide, we quantified the proportion of cells in the olfactory epithelium that were PCNA+ progenitors (Fig. 4). A computer program called ImageJ was used to quantitate the dividing cells. When comparing the number of PCNA+ dividing cells to the total number of cells present (Fig. 4), dividing cells represented approximately 48% of all cells at embryonic day 14. In future research, we will be quantitating dividing cells in additional chick embryos to collect more data surrounding day 14 of embryonic chick development, and use this data to see how the quantitation varies across developmental stages.

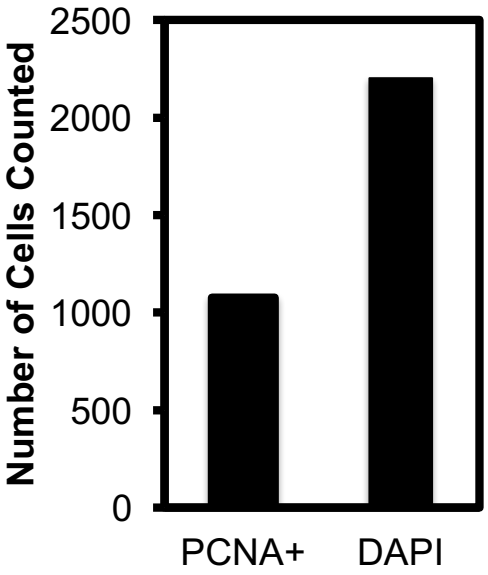

Figure 4. Quantitation of dividing cells labeled with PCNA. Three regions were quantified from each side of the olfactory epithelium in E14 chicks.

Discussion

The regenerative ability of the cells located within the olfactory epithelium is not well understood and is an area demanding research. Our research primarily works to identify cells from chick tissue that have the ability to regenerate across different stages of embryonic development. Our research focused specifically at day 14 of embryonic development, to identify the different cell types present and quantify their abundance. The results indicate that both progenitors and neurons were identified in our tissue sections through our immunofluorescence staining and imaging (Fig. 4). From previous research in the mouse, it has been observed that patterning and location of these cell types change within the olfactory epithelium as development progresses (Murdoch and Roskams, 2008). The changes in cell types across development are due to the regenerative nature and capability of the olfactory epithelium. However, the olfactory epithelium is a region of the peripheral nervous system that is rather difficult to understand, in part because of the challenges with distinguishing embryonic and adult olfactory epithelium progenitors.

Quantifying Cells in the Olfactory Epithelium

Through the research carried out in our experiments, it was determined that 33% of all cells counted at day 14 of embryonic development were detected as PCNA+ dividing cells. This was determined by analyzing three different tissue sections from varying axial levels in the chick head, using a software program to quantify the total number of cells present to compare this to the total number of progenitors counted. Much of the research in this field has been carried out at earlier stages of development, specifically in mouse models (Murdoch and Roskams, 2007; Murdoch and Roskams, 2008). Previous research found that neurogenesis was conserved in both the mouse and chick (Maier and Gunhaga, 2009). However, researchers are still struggling with the exact identity of the cells capable of regeneration in both model organisms.

Studies Across Stages of Development

With the preliminary findings from these experiments, we will look into and study earlier stages of development in more depth, to identify what signals affect cells to progress from a stem cell, into a progenitor, and then into their final cell type, specifically within tissue *in vivo.* From previous research from our lab, we know that the quantitation of specific cell types differs at different stages of development (Abrahams and Murdoch, 2014). We can compare the results from our embryonic day 14 data to results from data collected at an earlier stage of embryonic development at day 9. This prior research while very similar, focused on different axial regions but still examined the percentage of dividing cells present. Abrahams and Murdoch (2014) found that approximately 50% of the total cells were dividing cells at day 9 of development (Abrahams

and Murdoch, 2014). These results show that at an earlier stage in development we see more dividing cells present. This could be explained, that as the embryos progress in development, their cells become more committed and differentiate into neurons or other cell types found in the olfactory epithelium as they do in the brain (Cooper, 2000). There are different mechanisms that affect the number of dividing cells present, and the knowledge of these mechanisms and signals that drive proliferation could be beneficial in understanding the potential therapeutic effects of these cells.

Conclusion:

This research is important as it works to improve the understanding of the regenerative capacity of the brain. As well, helping us develop a better understanding and knowledge of the communication networks that will coax regenerating cells to produce new neurons, ultimately helping us to answer how we can coax the brain to self-repair. It is imperative to note how similar humans are compared to chick embryos, in terms of development and tissue growth. The long-term goal is to understand the communication signals that will coax this repair function on demand. As the nose and brain share many similar cell types, these same mechanisms that allow for self-repair in the nose can be applied to the brain, to one-day help overcome the cellular and cognitive deficits that occur after traumatic brain injuries and reduce the societal toll that these individuals suffer with.

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