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Cell Differentiation and Proliferation During Brain Development

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CELL DIFFERENTIATION AND PROLIFERATION DURING BRAIN DEVELOPMENT

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Abstract
During brain development, cells can proliferate and differentiate into either neuronal or glial cells. Neuronal cells are capable of sending signals, while glial cells function to provide nutrients and structural support. The ratio of these cell types can give insight into brain function in comparative studies among species and in studies of phylogenesis and pathology. In order to complete this project in neuroscience, a new technique called the isotropic fractionator was utilized to estimate a neuronal vs. non-neuronal cell count of the rodent brain. Brains ranging from age postnatal day 1 to postnatal day 23 were dissected into 4 regions for analysis: cerebellum, cerebral cortex, midbrain, with specific focus on the olfactory bulb. Nuclei from each region were isolated and stained for analysis using fluorescent microscopy. Peak increase in cell counts began postnatal day 5 and continued until postnatal day 17. Results indicate each region contains a higher proportion of neuronal cells compared to non-neuronal cells.

Introduction

Differentiation

The brain is an organ part of the central nervous system and is involved in all body systems. During brain development, the epithelium of the developing cerebral wall consists of two types of progenitor cells: neuronal and glial cells (Rakic, 1981). Neuronal cells are capable of firing an action potential triggered by a shift in membrane potential. This allows the cells to send a signal to neighboring cells by releasing a neurotransmitter as part of neuronal connectivity. Communication between neurons allows for information to be carried to the proper region of the brain to be processed. Glial cells however, are not capable of firing action potentials, but instead they function as support by creating optimal conditions for the neuronal cells. One way in which they are able to achieve this is by monitoring the concentrations of potassium ions, which affects cell potential and the ability of a neuron to fire an action potential (Horio, 2001). Oligodendrocytes, a type of glial cell, are able to lay down a mixture of proteins and phospholipids called myelin (Purves, 1970). This speeds up the rate of action potentials, allowing signals to be sent faster.

During embryonic development, neural stem cells have the ability to either divide, producing more stem cells, or differentiate to neural progenitor cells, which further differentiate into a specific type of neuron or glial cells. Neurogenesis is the process by which new neurons are formed from neural stem cells (Rusznak, 2016). The process begins in all brain regions during early embryonic development and continues through early postnatal stages.

Only a few neurogenic zones remain active during adulthood: the subgranular zone in the hippocampus, and the subventricular zone of the ventricles. The subgranular zone generates granule cells, a type of neuron found in the cerebellum (Ming et al., 2011). The subventricular zone is where the neurons generated migrate to the olfactory bulb to become interneurons.

Neuronal progenitor cells are also able to differentiate into glial cells, but the time in which the fate of a progenitor cell is determined remains unknown (Rakic, 1981).

Throughout life, neurogenesis occurs in the basal layer of the epithelium to generate new sensory neurons. The number of cells added daily to the olfactory bulb ranges from 10, 000 to 80,000 (Kaplan et al., 1985). Research using adult rodents has shown that the size of the olfactory bulb does not significantly change throughout life (Biebl et al., 2000). This implies that the continuous neurogenesis is accompanied by cell loss. In normal healthy adult animals, when the neurons reach the end of their lifespan of 1-3 months, they undergo apoptosis and are replaced by new neurons (Ekberg, 2012). Since neurons experience a 1-3% daily turnover rate, variations in cell count may occur between animals.

In the subventricular zone, radial glia-like cells, a type of progenitor cell, divide and become transient amplifying cells (Rusznak, 2016). These cells generate neuroblasts, an embryonic cell from which nerve cells are developed. A chain of neuroblasts is formed, and it is able to migrate to the olfactory bulb through a tube formed by astrocytes, a type of glial cell. After reaching the olfactory bulb, the immature neurons detach and migrate toward glomeruli where they differentiate into different types of interneurons. The majority of the neuroblasts become periglomerular while the rest develop into juxtaglomerular neurons (Ming et al., 2011). This process is continuously repeated, as olfactory neurons have a lifespan of approximately 1-3 months (Ekberg, 2012).

Glial cells are generated through the process of gliogenesis. Neuroepithelial progenitor cells in the neural tube and forebrain transform into radial glia, the primary type of progenitor cell. After radial glial cells generate neurons, a signal is produced to redirect differentiation toward astrocytes or oligodendrocyte precursor cells (Kriegstein, 2009). It is unclear how a

progenitor cell decides to become an astrocyte or oligondendrocyte, but astrocytes begin to appear around embryonic day 18 and oligodendrocytes are first seen postnatally. The typical gestation period for a rodent is 19-22 days. Astrocytes, the first glial cell to be developed, have been observed to hold the ability to be converted into intermediate progenitors which can then become neuroblasts by transcription factor SOX2 (Rusznak, 2016). A transcription factor is a protein that regulates the conversion of DNA into RNA. Molecules of RNA synthesize proteins, which determine a cell's function. Certain astrocytes express SOX2 and it can be induced by mitogenic and gliogenic signals. The newly generated neuroblasts can generate mature neurons when supplied with both brain-derived neurotrophic factor and the protein noggin, or with valproic acid alone. One reprogrammed astrocyte is capable of producing multiple functional neurons (Rusznak et al., 2016). A glial cell's ability to change its fate illustrates the high plasticity of the brain. Due to the ability of a glial cell to transform into a neuronal cell, I hypothesize that this occurs during development, contributing to a higher ratio of neuronal to glial cells.

The fate of a progenitor cell toward becoming a neuronal or glial cell may be determined by the cell's intrinsic properties or by external physiological conditions. During development, the brain is able to differentiate into the two cell types through the use of signaling molecules. These signals include extrinsic chemicals such as ciliary neurotrophic factor and leukemia inhibitor factor (Mayer, 1994). Studies have shown that transcription factors are able to bind to a repressive protein that represses gliogenic gene transcription, preventing the formation of glial cells. Vice versa, certain proteins are able to inhibit neurogenesis while gliogenesis is being promoted (Miller et al., 2007). Because neurogenesis occurs first, I predict to find a higher ratio of neuronal to glial cells during development.

Olfactory Bulb

This study focuses on the olfactory bulb. Olfaction is a critical sensory system to rodents during development. Rodents are not able to see until approximately postnatal day 13, meaning they must rely on their sense of smell for finding food, finding their mother, and overall navigation. Other brains regions will be analyzed for comparison, but I am most interested in olfactory bulb development because of its critical role in early life stages. Previous studies have not focused on the olfactory bulb.

There are two main organs that control odor perception in vertebrates: the olfactory epithelium and the vomeronasal organ (Menini, 2010). These organs transmit odor recognition signals to parts of the initial olfactory processing center in the brain, the olfactory bulb. The process of olfaction occurs when an odorant molecule enters the nasal cavity of an animal. After entering the cavity, the molecules are absorbed into a layer of mucus that is secreted by the Bowman's glands in the olfactory epithelium (Hayden et al., 2014). The odorant is transported by odor-binding proteins to the dendrites of the olfactory sensory neurons. Dendrites are the part of the neuron that receive impulses from other cells and transmit the signal to the cell body. The odor ligand interacts with an olfactory receptor at the dendrite, resulting in a change in shape and the subsequent conversion of guanosine diphosphate (GDP) to guanosine triphosphate (GTP)(Menini, 2010). When bound by ligand, this olfactory receptor is able to activate adenylyl cyclase which catalyzes the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). An increase in cAMP causes ion channels to open, allowing sodium and calcium ions into the cell causing a membrane depolarization. The action potential in the axons of the olfactory sensory neurons transmit a signal though the foramina of the cribriform

plate to the olfactory bulb (Hayden et al., 2014). The olfactory bulb projects sensory information to the primary olfactory cortex. The mediodorsal thalamic nucleus receives input from the primary olfactory cortex and relays it to the orbitofrontal cortex. The orbitofrontal cortex is a part of the brain that is involved with association learning (Courtiol et al., 2015).

Studies have shown that the olfactory epithelium is divided into four distinct zones containing a mosaic of neurons that express the same receptor gene. These different zones project information to different parts of the olfactory bulb. One part of each zone projects axons to the medial part of the bulb while the other projects axons to the lateral side of the bulb (Ressler et al., 1993). Once in the olfactory bulb, olfactory sensory neuron axons terminate within glomeruli. Each sensory neuron projects to only a single glomerulus, but each glomerulus receives input from about 1000-2000 sensory neurons. Axons of sensory neurons that express the same type of odorant receptor are found to converge on the same glomerulus. Glomeruli that hybridize the same receptor probe are located at bilaterally symmetrical positions in the two olfactory bulbs, which suggest a highly organized pattern of sensory information. (Ressler et al., 1993).

The neurons in the olfactory bulb are categorized based on the layers in which they are found. The axons of olfactory sensory neurons create synapses in the glomerular layer. There are several thousand glomeruli at the surface of the rodent olfactory bulb, and each glomerulus represents a single odorant receptor (Mori et al., 1999). This allows for odor discrimination. The neurons surrounding glomeruli are called juxtaglomerular cells of which there are three types: periglomerular, tufted, and short axon cells (Hayar et al., 2004). The mitral cell layer contains the largest type of neuron in the olfactory bulb, the mitral cell. These cells receive synaptic inputs from the axons of olfactory sensory neurons and make reciprocal synapses with the dendrites of

the periglomerular cells. Beneath the mitral cell layer lies the granule cell layer, containing the granule cells which are unique due to their lack of axons (Nagayama et al., 2014).

External tufted cells are types of juxtaglomerular cells that fire action potentials, receive monosynaptic inputs from olfactory neurons, and establish excitatory synaptic connections on periglomerular and short axon cells (Hayar et al., 2004). External tufted cells have a primary dendrite that branches out within a single glomerulus, periglomerular cells have short thin dendrites within a restricted portion of a glomerulus, and short axon cells have dendrites in several neighboring glomeruli (Nagayama et al., 2014). The arrangement of synapses causes rhythmic bursts of action potentials in external tufted cells which in turn causes bursts of excitatory postsynaptic potentials or excitatory postsynaptic currents in periglomerular and short axon cells of the same glomerulus. The synchronized activity of the external tufted cells of each glomerulus could provide a synchronized burst onto mitral cells which are the output neurons of the olfactory bulb (De Saint Jan et al., 2009). Therefore, it is believed that the external tufted cells are involved in the coordination of glomerulus activity.

Olfactory ensheathing cells are the type of glia present in the olfactory system. Unlike other types of glia, these cells are able to migrate from periphery into the central nervous system. Olfactory ensheathing cells also differ from other glia by encasing large bundles of unmyelinated axons rather than myelinated (Purves, 1970). The olfactory ensheathing cells are thought to promote axon growth by providing a cellular substrate containing molecules that facilitate axonal adhesion and extension by expressing growth-promoting agents. The olfactory sensory axons and the olfactory ensheathing cells fuse with the outer surface of the telecephalon which stimulates it to form the olfactory bulb (Eckberg et al., 2011).

Cerebellum

The cerebellum is the part of the brain that is responsible for sensory-motor processing. It is composed of a layer of neurons called Purkinje cells, sandwiched between layers of granule cells, another type of neuron. Purkinje cells release a neurotransmitter called gamma-aminobutyric acid (GABA) which exerts an inhibitory action on neurons to reduce signaling. Formation of Purkinje cells has been found to be complete by embryo day 16 and is followed by the formation of glial cells (Buffo, 2013). Purkinje cells are present at birth but do not start growing until postnatal day 7 through postnatal day 30 (Pouzat, 1997). The growth of these cells can contribute to the peak increase in brain mass/volume that occurs from postnatal day 7-10 (Semple et al., 2013). Purkinje cells send signals to nuclei at the base of the cerebellum which relay the information to the thalamus. Other types of neurons such as basket cells and stellate cells are found within this layer. The granular layer contains Golgi cells, a type of inhibitory interneuron. During postnatal day 1 and postnatal day 14 of rodents, basket and stellate cells arise from progenitor cells in the white matter and reach their final location during postnatal day 7 and postnatal day 21 (Pouzat, 1997). The main glial cells of the cerebellum are oligodendrocytes, astrocytes, and Bergmann glia (BG). (Buffo, 2013)

Cerebral Cortex

The cerebral cortex is the part of the brain that is responsible for higher brain functions such as consciousness, perception and attention. It is a thin layer of gray matter that covers the rest of the brain. Gray matter is tissue that contains nerve cell bodies and dendrite branching (Shipp, 2007). The cortex contains six layers and is divided into 4 regions based on function: the frontal lobe, the parietal lobe, the temporal lobe, and the occipital lobe. The six layers can be further divided into three parts. First is the supragranular layers which permits communication

between regions of the cortex. Next is the internal granular layer which is responsible for connections to the thalamus, which receives sensory input. There are three main types of neurons within the cortex: the pyramidal cell, the stellate cell, and Martinotti cells (Abeles, 1991). The pyramidal neuron is most prominent, which are excitatory neurons that are capable of long-distance axonal projections. Research has found that the cortex reaches about 90% of its adult weight by postnatal day 20 (Semple et al., 2013). This is the major reason why I focused on differentiation and proliferation of brain cells from postnatal days 1-23.

Midbrain

The midbrain is the region of the brain located beneath the cerebral cortex that acts as a relay for sensory information and is involved with controlled body movements. The tegmentum and tectum are parts of the midbrain responsible for visual and auditory reflexes. It also contains the thalamus, which is the part of the brain responsible for sending sensory stimuli to the cerebral cortex. The main type of neurons found in this region are dopaminergic neurons, which are the main source of dopamine in the central nervous system (Hegarty et al., 2013). Dopamine is a neurotransmitter that helps to regulate movement and attention. Progenitor cells become dopaminergic neurons through protein signaling. The transcription factors Nurr1, Lmx1b, and Pitx3 activate the synthesis of dopamine and cause dopaminergic cells to proliferate during development (Chinta et al., 2004).

Isotropic Fractionation

Quantitative approaches have been used to study brain composition, development and function. Data can be used to make comparisons between species and between normal and diseased conditions. Previous studies have utilized stereological methods to create an unbiased three-dimensional interpretation of two-dimensional cross sections of tissues. The optical

fractionator is a technique designed to quantify neurons by applying an optical dissector to randomly selected samples. The optical dissector is a three-dimensional probe attached to a microscope which allows for cells to be counted. Estimates are made based on calculations of density, which makes the results dependent on volume (Korbo et al., 1990). Because a measurable volume is needed, this method is challenging for regions that are not well defined and especially during early mammalian development when distinct regions/structure have not been established.

Isotropic fractionation is a new method designed to quantify cells in a shorter amount of time (Herculano-Houzel et al., 2005). Unlike the stereological methods, undefined brain regions are able to be studied by transforming the structure into a homogenous suspension with a known volume, containing nuclei that can be stained and counted. The results are independent of density, which allows the data gathered from this method to be used in comparative studies. While this technique has not yet been widely used, it has provided significant data in studies on primate brain composition (Collins et al., 2010), adult rodent brain cell density (Herculano-Houzel et al., 2006), and even in human Alzheimer's patients (Andrade-Moraes et al., 2013). This study will utilize the isotropic fractionation method in order to further investigate the composition during brain development of rodents.

4′,6-diamidino-2-phenylindole (DAPI) is a DNA specific fluorescent probe. The dye enters a permeated cell and makes its way into the minor grooves of DNA. The nitrogen of DAPI forms a bond with the oxygen of the DNA nucleotides. DAPI favors regions high in the nucleotides adenine and thymine due to the narrower groove allowing for tighter fit, electrostatic factors, and steric advantage which allows the molecule to sit deeper (Kapuscinski, 1995). This causes brighter fluorescence than in guanine-cytosine regions. DAPI is excited by wavelength

405nm and emits a wavelength around 460nm, which corresponds to blue on the visible light spectrum (Figure 1). Using fluorescent microscopy, the presence of blue light will allow us to collect a total cell count, based on the cell theory that all cells contain a single nucleus housing DNA.

Antibodies are proteins that are able to recognize and bind to a specific molecule on the surface of a cell called an antigen. A secondary antibody recognizes the antigen on the primary antibody and assists with detection due to the presence of an attached fluorescent signal. Neuronal cells can be distinguished from glial cells through the use of a specific primary antibody and a fluorescent secondary antibody. The protein NeuN (neuronal nuclei), has only been found in nervous tissue, primarily in the nucleus bound to the matrix (Mullen et al., 1992). NeuN is formed in neuroblasts during embryogenesis and remains in the neurons throughout development. Studies have found that possible functions for this protein include regulating cellular processes, such as alternative splicing and presentation of the neuronal phenotype (Gusel'nikova et al. 2015). Anti-NeuN is a primary antibody that detects and directly binds to the protein, although the exact binding site remains unclear (Mullen et al., 1992). Because Anti-NeuN is not fluorescent, a secondary antibody containing the dye cyanine 3 is added, and its function is to bind to the primary antibody to aid in detection, sorting, and purification of the target molecule, NeuN. Cyanine 3 is excited by wavelength 532nm and emits a wavelength of approximately 570nm, which corresponds to an orange color on the visible light spectrum. The cyanine 3 attached to the end of the secondary antibody tags the neuronal cells for us, not only making it visible under the microscope, but allowing for the distinction of neuronal cells (Figure 1). Cells that are NeuN-negative cannot be identified however, as they could potentially be either glial cells, epithelial cells, or neurons that express very low levels of NeuN.

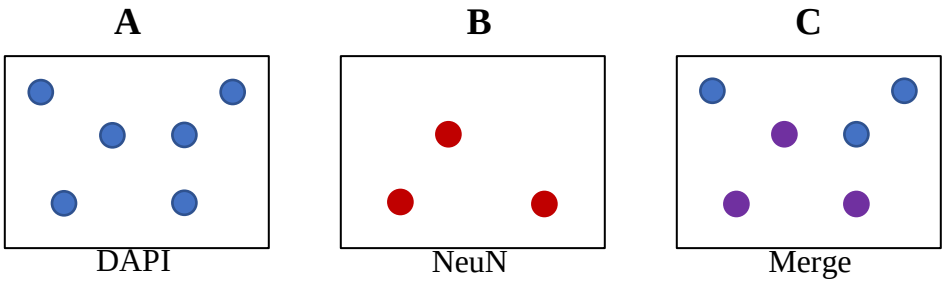

Figure 1. The total number of neurons is estimated by determining the number of DAPI-stained nuclei (A) that are also NeuN positive(B). Purple indicates the presence of a neuronal cell(C).

Rodents are typically used as the model organism in neuroscience. Rats became the primary model organism studies due to their short gestation period of 19-22 days, relatively large size of their brains, knowledge of their entire genetic makeup, and their physiology patterns. Both human and rodent brains, especially rats, share similar structure and connectivity (Ellenbroek, 2016) This has allowed researchers to use rodents to study mechanisms of events such as development, diseases or brain injury and evaluate potential therapeutic approaches. In particular, activity patterns in the olfactory bulb in rats have been used to compare human perception of smell. Researchers used different odorants and observed neurons interactions to study perceptual similarities between rats and humans. Similarities between the two were noted, but there were also many differences (Soh et al., 2014). Despite the differences between the two, the use of rodents has allowed scientist to create a basis for a model system to understand sensory perception.

In mammals, reptiles, birds, and amphibians, the first step of brain development is the formation of the neural tube. This occurs around gestation day 10 in rats which corresponds to gestational day 24-28 in the human brain (Semple et al., 2013). Historically, differences in brain development among species were assessed by weighing the brain tissue. Studies have found that

the rat brain reaches about 90% of its weight when it begins weaning, typically occurring around postnatal day 20. This corresponds to a 2-3-year-old human. The peak brain growth spurt is observed between postnatal days 7-10 in rodents and around birth in humans (Semple et al., 2013). Therefore, I predict to observe a peak increase in cell count in rats of this age, indicating the highest rates of neuro/gliogenesis. Results of this study can be used for comparative studies and can be used to predict development patterns in other species.

In this experiment, I predict that there will be a higher proportion of neuronal cells to glial cells due to the ability of glial cells to transform into neuronal cells (Rusznak et al., 2016). Furthermore, I hypothesize that the olfactory bulb will contain a higher proportion of neuronal cells due to the fact that olfaction is the only sense that does not need to travel through the thalamus (Courtiol et al., 2015). As a result, there must be a higher proportion of neuronal cells in order for a strong signal to be sent. I predict to see a peak increase in neuro/gliogenesis during postnatal days 7-10, as this has been found to be when the peak growth spurt occurs (Semple et al., 2013).

Methods

Brains:

The use of rats for experimentation has been approved by Otterbein University Animal Care and Use Committee. An adult rat gave birth to twelve pups. Starting postnatal day 1, one rat pup was anesthetized with isofluorane every other day (postnatal day 1, day 3, day 5, etc.) The toe pinch method was used to ensure the rats were completely unconscious. An incision was made into the chest cavity to expose the heart. Using a needle attached to a syringe, a 1X phosphate buffer saline (PBS) solution was injected into the left ventricle to clear out the blood from the brain. A small cut was made into the right atrium to allow for blood drainage. Once the blood ran clear,

the perfusate was switched with a chilled 4% paraformaldehyde fixative solution. After fixation was complete, indicated by the liver changing from a dark red to a pale yellow, the brain was excised and stored in 4% paraformaldehyde to preserve the tissue.

Isotropic Fractionator:

Total brain mass and volume were measured for each brain. Every other brain (postnatal day 1, day 5, day 9, etc). was dissected into regions of interest: olfactory bulb, cerebellum, cerebral cortex, and remaining areas. Tissue was placed into a dissociation solution containing 1% Triton 1-X and 40mM sodium citrate. Nuclei were exposed by grinding the tissue using a Tenbroeck tissue homogenizer to disrupt the cell membranes. A 40 microliter aliquot was removed and mixed with 5mg/mL 4′,6-diamidino-2-phenylindole (DAPI) . While using the fluorescent microscope (100X), the nuclei were diluted using PBS to ensure proper density for counting. Following the DAPI addition the suspension was spun down, and nuclei were incubated at room temperature under agitation with anti-NeuN primary antibody for 2+ hours followed by another incubation with cyanine-3 conjugated goat secondary antibody for 2+ hours. Nuclei were spun down and washed using PBS. A 10 microliter sample of the solution was collected and placed in a Neubauer chamber. Using the fluorescent microscope (100x magnification) a total cell estimate was determined by counting the number of nuclei with a blue color (DAPI). The percentage of neuronal cells was determined by counting the number of cells with an orange/red color (anti-NeuN/anti-mouse cy3) (Figure 2). The total number of cells and neurons in the suspension was obtained by multiplying the average number of nuclei per microliter by the total volume. A second round of cell counts was completed by taking an additional aliquot from the same homogenous solution and repeating the analysis.

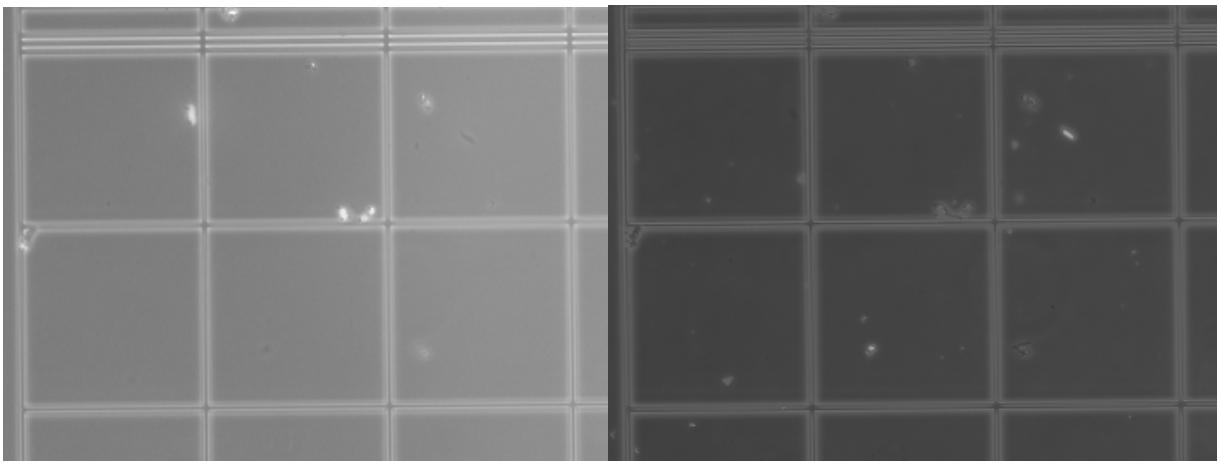

Figure 2 Examples of cells from the postnatal day 9 olfactory bulb placed on Neubauer chamber under fluorescent microscope. Nuclei circled in blue represent(left) stained by DAPI to obtain a total cell count. Nuclei stained blue and also stained red by anti-NeuN/anti-mouse cy3 are circled in purple(right) to represent the overlap.

Results

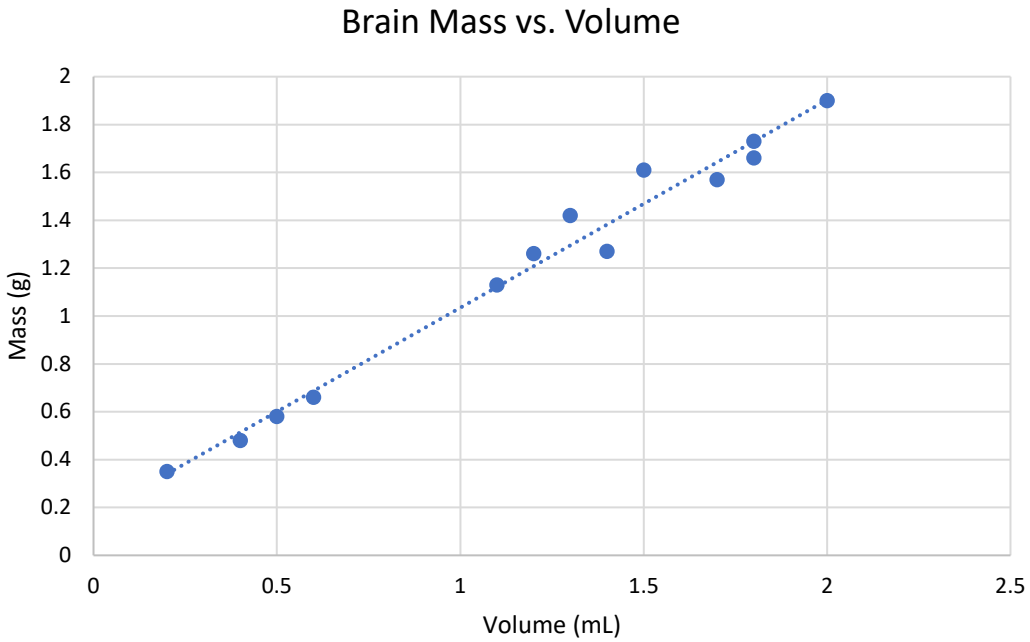

Figure 3 The total brain mass compared to the brain volume (n=1 per data point). Slope represents brain density.

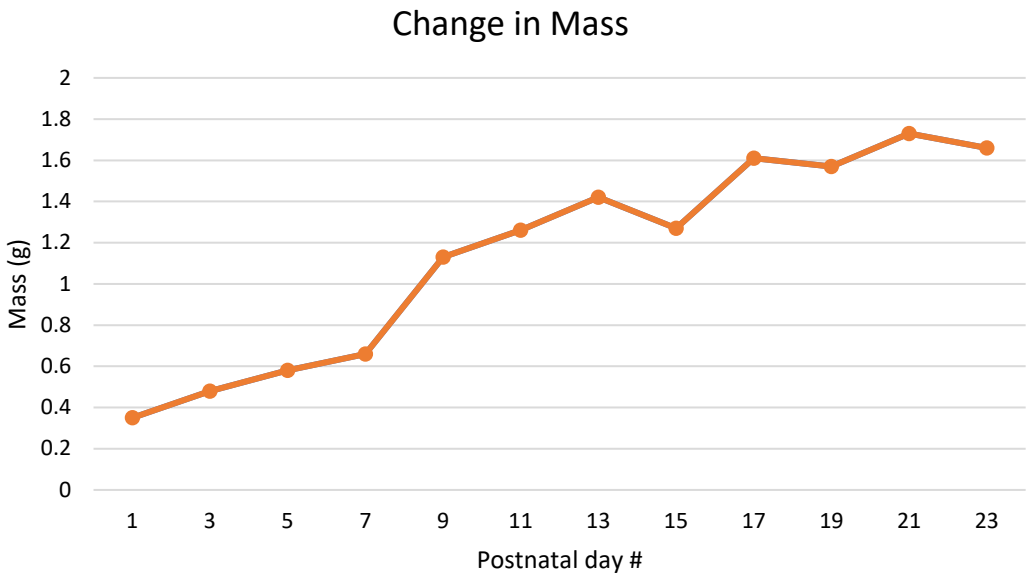

Figure 4 Changes in total brain mass during development (n=1 per data point). A peak change occurred during postnatal days 7-9. Overall, brain mass increased linearly over time.

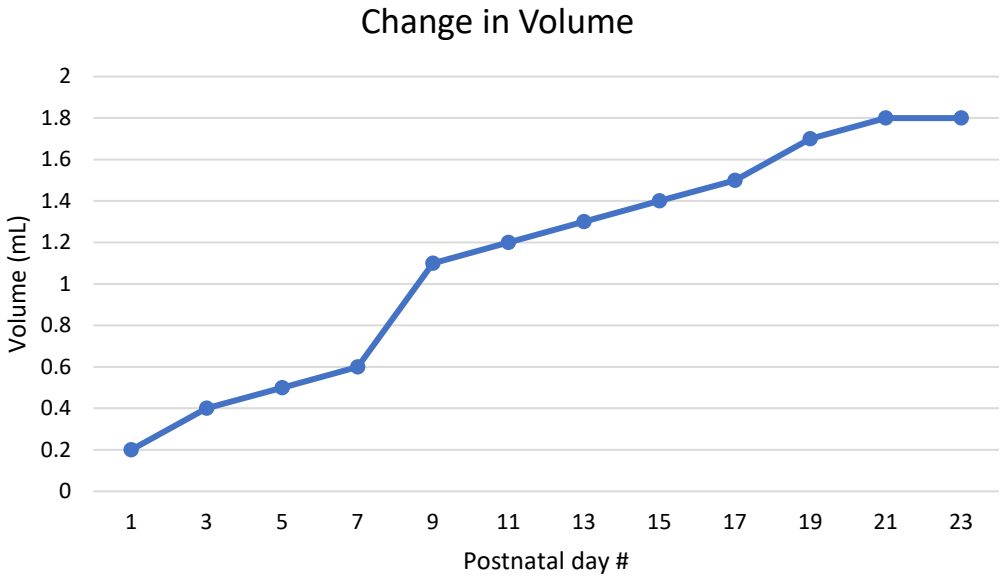

Figure 5 Changes in total brain volume during development (n=1 per data point). A peak increase occurred during postnatal days 7-9, similar to changes in brain mass. Overall, brain volume increased linearly over time.

We found that brain mass and volume are directly proportional (Figure 3). The peak increase in brain mass and volume occurred between postnatal days 7-9 (Figure 4+5). These

results agree with previous studies showing the peak growth spurt to occur during postnatal day 7-10 (Semple et al., 2013). Beginning at postnatal day 5 we observed large increases in neuronal cells for all regions except the olfactory bulb, which we can expect to account for the changes in mass and volume. (Figure 7). The brains began to level off to adult volume by postnatal day 21. This is consistent with previous research stating that the rodent brain reaches 90-95% of adult weight by postnatal day 20 (Semple et al., 2013).

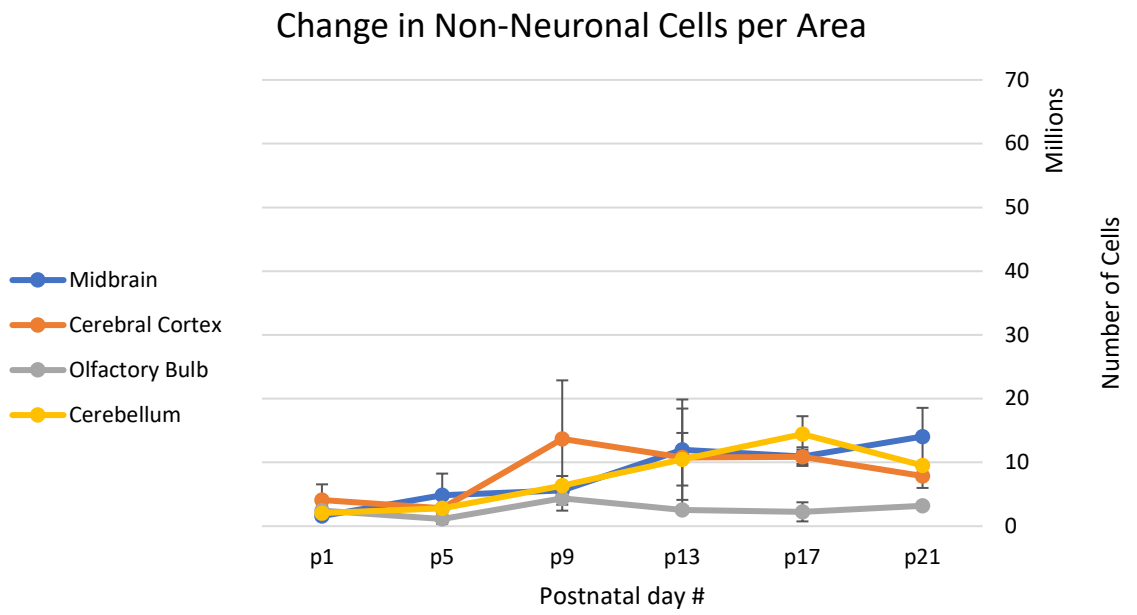

Figure 6 Estimated non-neuronal cell counts for each region using fluorescent microscopy. Error bars indicate standard error calculated between 2 sets of data from the same brain (n=1 per data point). Decreases at postnatal day 21 can be explained by noted damage to tissue during excision.

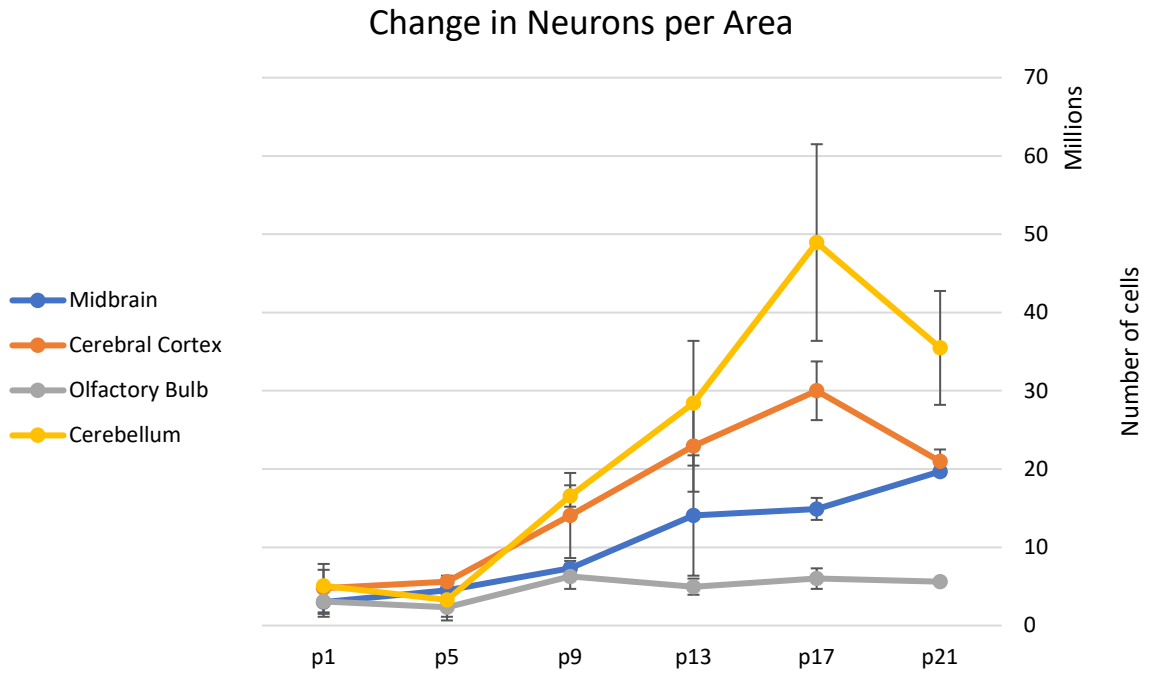

Figure 7 Estimated neuron count for each region using fluorescent microscopy. Error bars indicate standard error calculated between 2 sets of data from the same brain (n=1 per data point). Decreases at postnatal 21 can be explained by noted damage to tissue during excision.

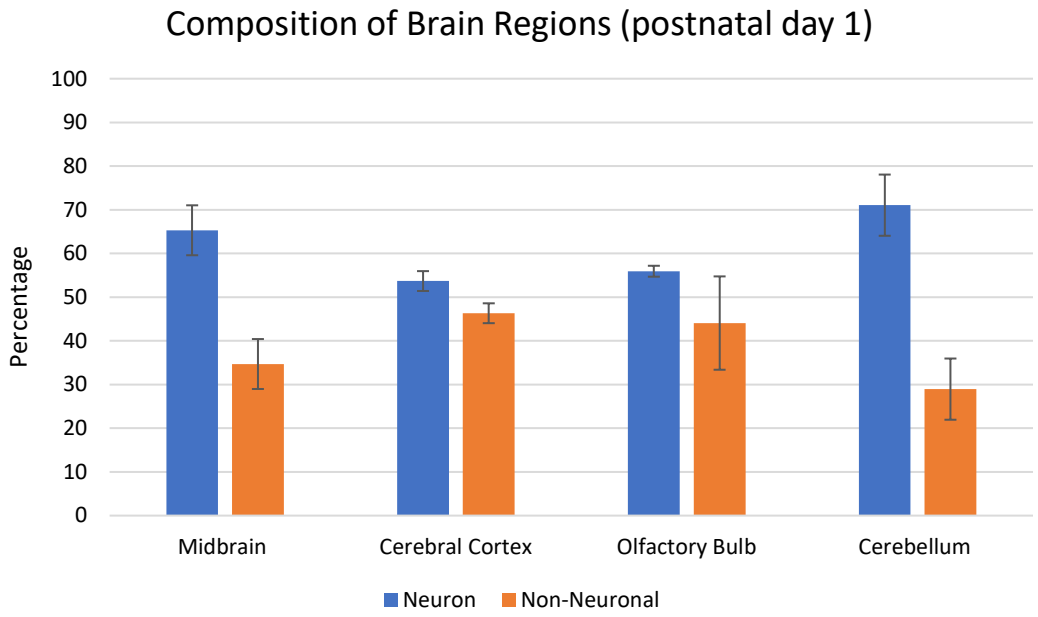

Figure 8 Percentage of neurons compared to non-neuronal cells in each area of interest at postnatal day 1. Error bars represent the standard area between 2 samples of the same brain (n=1).

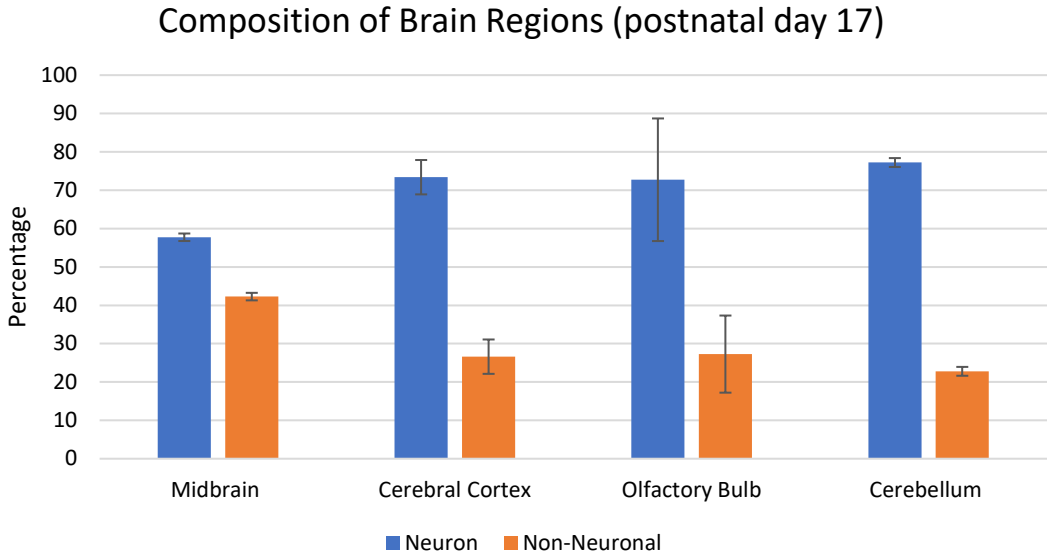

Figure 9 Percentage of neurons compared to non-neuronal cells in each area of interest at postnatal day 17. Error bars represent the standard area between 2 samples of the same brain (n=1).

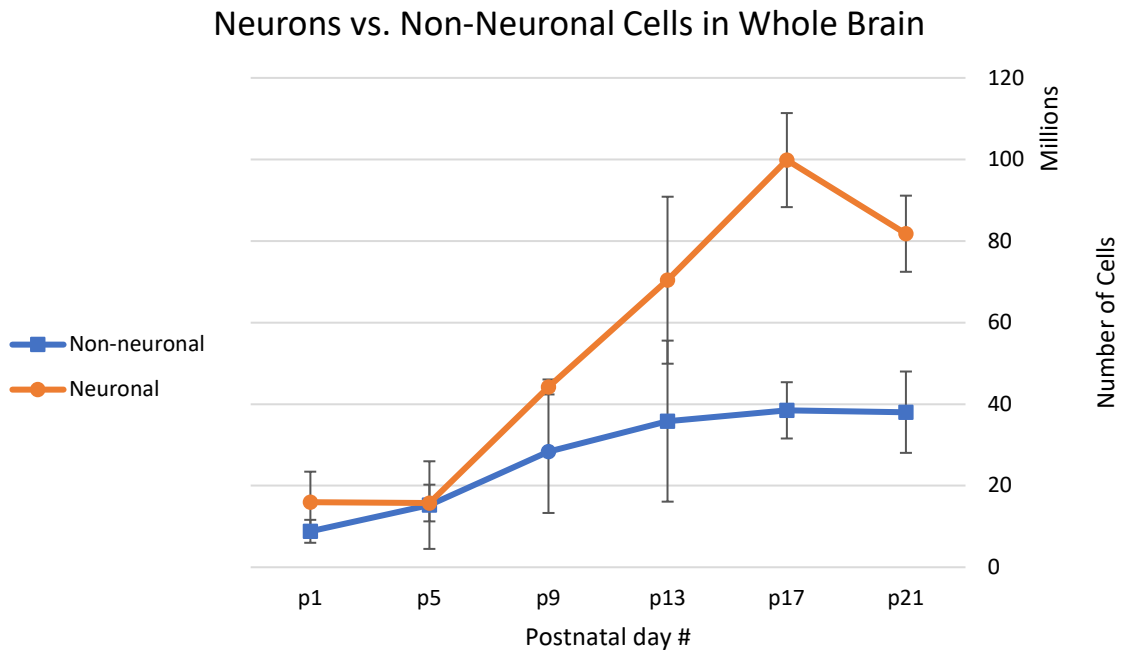

Figure 10 Changes in neurons compared to changes in non-neuronal cell count in all 4 regions combined during development. Error bars indicate standard error calculated between 2 sets of data from the same brain (n=1 per data point). Decreases at postnatal day 21 can be explained by noted damage to tissue during excision.

Table 1 Average total cell counts compared to neuronal cell counts using fluorescent microscopy.

Age	Total Cells	Neurons	% Neurons
Adult	115562500 ± 27437500	88468750 ± 26781250	76.55 ± 5.30
p21	119812500 ± 19312500	81787500 ± 9337500	68.26 ± 3.30
p17	138318750 ± 18431250	99843750 ± 11531250	72.18 ± 1.31
p13	106218750 ± 40218750	70387500 ± 20475000	66.27 ± 6.79
p9	72543750 ± 16856250	44212500 ± 1837500	60.95 ± 12.29
p5	30993750 ± 15243750	15750000 ± 4500000	50.82 ± 13.82
p1	24750000 ± 10312500	15937500 ± 7500000	64.39 ± 4.20

Overall, there was a higher proportion of neuronal cells to glial cells in the entirety of the brain. This specific finding agrees with the results of other isotropic fractionator experiments but opposes that of experiments using stereological methods which report a higher proportion of

glial cells (Herculano-Houzel, 2005). Stereological methods rely on density, and therefore can only quantify discrete brain regions. As a result, these methods may not accurately account for cells in regions that are not well defined. My results showed that there is consistently a higher proportion of neuronal cells during the postnatal period, as it increased from 64% at p1 to 72% at postnatal 17 (Table 1). While the adult rat yielded a total of 76.55% neuronal cells compared to the 60% previously found, I cannot conclude that this is the true proportion as there are multiple reasons to consider for cell loss. Possible reasoning includes loss of tissue during brain excision, and loss of cells between washes.

I found that the cerebellum contained the highest proportion of neuronal cells, followed closely by the cerebral cortex (Figure 7). This is expected of the cerebellum but not of the cerebral cortex as others have shown find the cerebellum to have approximately 3 times as many neurons as the cortex (Herculano-Houzel, 2005). My results are likely due to both neuronal and non-neuronal cell loss from lack of complete homogenization. As I predicted, the olfactory bulb also had a higher percentage of neuronal cells but did not experience large increases in total cells (Figures 6+7).

Discussion

I find it significant that the brains experienced a large increase in cell counts beginning postnatal day 5. It illustrates a critical period that exists during brain development. For future studies, it would be beneficial to analyze a sample each day from multiple subjects until the data begins to level off in order to gain a better understanding of important benchmarks. Interestingly, the cerebellum experienced a large increase in cells starting on postnatal day 13. This was the day in which the rodents first opened their eyes. I believe this occurs because the animal becomes capable of receiving more sensory input, requiring more neurons to receive the signals,

and more glial cells to support the increase in neurons. The cerebellum is the region responsible for sensory motor processing, and therefore at postnatal day 13 it has to coordinate movements based on visual stimuli. Each region experienced a significant increase in both total cells and neurons, except for the olfactory bulb. Overall the total amount of cells remained steady in the olfactory bulb, but the composition changed from about 50% neurons to about 70% (Figures 8+9). This could be as a result of glial cells converting to a progenitor cell which allows it to go through the process of neurogenesis to switch into a neuronal cell. During this process, transcription factors inhibit gliogenesis (Miller et al., 2007). As a result of a steady number of total cells, I can conclude that a rodent is born with majority of its olfactory cells, due to olfaction being the critical sensation for survival until postnatal day 13.

Isotropic fractionation made it possible to estimate cell counts for regions not well defined, especially in the newborn brains that are lacking in structure. This method relies on creating a completely homogenous solution that exposes intact nuclei for staining. Using the Tenbroeck homogenizer, I was unable to obtain a perfectly homogenous cell suspension. This resulted in cells clumping together which made it difficult to estimate an accurate number. Only distinct nuclei within clumps were accounted for. Furthermore, the lack of a completely homogenous solution made the aliquot used in the hemocytometer more likely to be misrepresentative of the entire solution, creating a need for multiple trials. Due to time restrictions, I was only able to achieve 2 trials of cell counts, which resulted in a high variance. For future trials it may be beneficial to try a different method of homogenization such as using a sonic dismembrator or to change the dissociation solution.

The use of fluorescent microscopy allowed me to identify cells through the use of dyes with different emission wavelengths. DAPI binds to DNA, found in all cells, and emits a blue

fluorescence. However, DAPI is susceptible to photoconversion, resulting in a green fluorescence (Kapuscinski, 1995). This was observed in some samples, but they were not counted for in order to prevent a false positive. As a result, a lower yield of countable nuclei was obtained. In the future it may be beneficial to use less DAPI or Hoescht 33342, another type of DNA that experiences less photoconversion compared to DAPI. The other dye used, cyanine 3, was attached to an antibody that targets the protein NeuN. However, previous studies have found that the mitral cells in the olfactory bulb and the Purjinke cells in the cerebellum do not express NeuN (Mullen et al., 1992). Due to this, I have reason to believe that the total neurons for these regions are actually higher than the results we obtained.

As previously stated, I cannot be sure that the percentages for each region are representative of the true proportion of cells. A sample size of 1 per data point is too small to determine accurate percentages as animals in the same litter vary in size and do not grow at the same rate. There is also variation between litters because animals have different genetic compositions, causing variation in size and gene expression which ultimately determines a cell's function. However, I found that the brain has an overall higher percentage of neurons which is consistent with previous studies using multiple animal trials (Herculano-Houzel, 2005). My results showed a higher percentage of neurons in all 4 regions, while studies on the adult brain have found that only the cerebellum and olfactory bulb contain more neuronal cells than non-neuronal cells (Herculano-Houzel, 2005). With improvement to my methods and more animal trials, differentiation and proliferation that takes place during development can be accurately determined. The use of DAPI and primary antibody NeuN only allows us to determine neuronal vs. glial or epithelial cells. This project can be taken further to identify the types of neuronal and glial cells found by looking at electrical properties and gene expression. Cell counts from

isotropic fractionation can then be utilized in studies to observe brain evolution anatomy and brain function.

Appendix

ATP- adenosine triphosphate

Cy3- indocarbocyanine

cAMP- cyclic adenosine monophosphate

GDP- guanosine diphosphate

GTP-guanosine triphosphate

DAPI- 4′,6-diamidino-2-phenylindole

DNA- deoxyribonucleic acid

NeuN- neuronal nuclei

RNA- ribonucleic acid

PBS- phosphate buffer saline

References

Abeles M. (1991), Corticonics: Neural Circuits of the Cerebral Cortex.

Buffo A (2013). Origin, lineage, and function of cerebellar glia. *Progress in Neurobiology*. 109:42-63

Courtiol, E., & Wilson, D. A. (2015). The olfactory thalamus: Unanswered questions about the role of the mediodorsal thalamic nucleus in olfaction. *Frontiers in Neural Circuits,9.* doi:10.3389/fncir.2015.00049

De Saint Jan D., Hirnet D., Westbrook GL., Charpak S. (2009)External tufted cells drive the output of olfactory bulb glomeruli. *Journal of Neuroscience.* 29(7):2043-52

Ekberg, J. A., Amaya, D., Mackay-Sim, A., & John, J. A. (2012). The Migration of Olfactory Ensheathing Cells during Development and Regeneration. *Neurosignals,20*(3), 147-158. doi:10.1159/000330895

Ellenbroek B., Youn J. (2016). Rodent Models in neuroscience research: Is it a rat race?. *Disease Models and Mechanisms*.9(10): 1079-1087

Gusel'nikova V., Korzhevskiy D. (2015) NeuN as a neuronal nuclear antigen and neuron differentiation marker. *ActaNaturae* 7(2)

Hayar A., Karnup S., Ennis M., Shipley MT (2004). External tufted cells: a major excitatory element that coordinates glomerular activity. *The Journal of Neuroscience*. 24(30):6676-85

Hayden, S., & Teeling, E. C. (2014). The Molecular Biology of Vertebrate Olfaction. *The Anatomical Record,297*(11), 2216-2226. doi:10.1002/ar.23031

Herculano-Houzel, S., Mota, B., & Lent, R. (2006). Cellular scaling rules for rodent brains. *Proceedings of the National Academy of Sciences of the United States of America*. 103:12138-43

Herculano-Houzel, S., & Lent, R. (2005). Isotropic Fractionator: A Simple, Rapid Method for the Quantification of Total Cell and Neuron Numbers in the Brain. *Journal of Neuroscience*. 25:2518-2521

Horio Y. (2001) Potassium channels of glial cells: distribution and function. *Japanese Journal of Pharmacology.* 87(1): 1-6

Kaplan M.S., McNelly N.A., Hinds J.W. (1994) Population dynamics of adult-formed granule neurons of the rat olfactory bulb. J Comp Neurol. 1985;239:117–25

Kapuscinski J (1995). DAPI: a DNA-specific fluourescent probe. *Biotech & Histochemistry*. 70(5):220-33

Korbo L., Pakkenberg B., Ladefoged O., Gunderson HJ., Arlien-Soborg P., Pakkenberg H. (1990). An efficient method for estimating the total number of neurons in rat brain cortex. *Journal of Neuroscience Methods*. 31(2):93-100

Kriegstein, A. (2009) The Glial Nature of Embryonic and Adult Neural Stem Cells. *Annual Review of Neuroscience.* 32:149-184

Mayer, M. (1994). Ciliary neutrophic factor and leukemia inhibitory factor promote the generation, maturation, and survival of oligodendrocytes in vitro. *Development.* 120(1):143-53

Menini, A. (2010). *The Neurobiology of Olfaction*. Boca Raton: CRC Press/Taylor & Francis.

Ming G., Song H. (2011) Adult Neuorgenesis in the Mammalian Brain: Significant Answers and Significant Questions. *Neuron.* 70(4): 687-702

Mori, K., Nagao, H., & Yoshihara, Y. (1999). The Olfactory Bulb: Coding and Processing of Odor Molecule Information. Science 286:711-5

Mullen R., Buck C., Smith A., NeuN, a neruonal specific nuclear protein in vertebrates. *Development*. 116, 201-211

Nagayama, S., Homma, R., & Imamura, F. (2014). Neuronal organization of olfactory bulb circuits. *Frontiers in Neural Circuits,8*. doi:10.3389/fncir.2014.00098

Pouzat C., Hestrin S. (1997). Developmental Regulation of Basket/Stellate Cell Purkinje Cell Synapses in the Cerebellum. *The Journal of Neuroscience*. 17(23) 9104-9112

Purves, D., Fitzpatrick, D (1970). The Olfactory Bulb. Neuorscience. 2nd edition.

Rakic, Pasko. (1981). Neuronal-glial interaction during brain development. *Trends in Neuroscience*. 21:184-187

Ressler K.J., Sullivan S.L., Buck L.B.(1993) A zonal organization of odorant receptor gene expression in the olfactory epithelium. Cell. 73:597–609

Rusznak Z., Henskens W., Schofield E., Kim W(2016). Adult Neurogenesis and Gliogensis: Possible Mechanisms for Neurorestoration. *Experimental Neurobiology*. 25(3): 103-112

Semple, B. D., Blomgren, K., Gimlin, K., Ferriero, D. M., & Noble-Haeusslein, L. J. (2013). Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Progress in Neurobiology*. 106: 1-16

Shipp, S. (2007) Structure and function of the cerebral cortex. *Primer*. 17(12):443-449

Soh, Z., Saito, M., Kurita Y., Takiguchi N., Ohtake H., Tsuji T. (2014), A Comparison Between the Human Sense of Smell and Neural Activity in the Olfactory Bulb of Rats , *Chemical Senses*. 39:91-105