Methods in neural cell culture: P19 differentiation

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Division III

May 2014

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A dissertation presented to the School of Natural Science

Hampshire College, Amherst Massachusetts

In partial fulfillment of the requirements for the degree of Bachelor of Arts

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I. Abstract

Models are essential in studying the central nervous system due to its complexity. The efficacy of a protocol is often determined by the gene expression and morphological features of the resultant differentiated cells. I hypothesize that when differentiating the P19 mouse cell line with retinoic acid in two different contexts in monolayer, there will be an observable difference in the neuronal population produced. Using a novel protocol and the one developed by Monzo et al. (2012), we observed the differentiation of cells over a period of 20 days. In initial immunofluorescent staining and gene expression analysis, a distinct difference between the morphological features and gene expressions of the MEM and Monzo cells is evident. Morphology appears to be highly similar between the cells grown in the two differentiation methods for most of the observed period. The gene expression is where the biggest differences lie. The MEM cells retained a proliferative state consistently across the trial period [BC1] while the Monzo cells lost expression over time. Both protocols resulted in neural progenitor cells, and had similar expressions of neuronal markers. The synaptic markers demonstrated that there is increased presynaptic activity in the MEM cells compared to the Monzo cells. I found that there is likely to be a notable difference between cells of the same type and induced with the same chemical cue when they are differentiated using distinct protocols.

II. Acknowledgements

III. Introduction

The human central nervous system (CNS) is immensely complex. The integrated functioning of this complex system gives the CNS a sense of mystery, and along with its relative inaccessibility make its study a major challenge. The normally functioning brain sends and

receives millions of varied signals over the course of a day, triggering additional billions of signals. Trying to determine the basis of a neurodevelopmental or neurodegenerative disorder is practically impossible without removing some of the complexity in the system (Lancaster et al., 2013). This difficulty of studying brain and brainstem as a whole in vivo have led to studies of the brain and central nervous system using model systems (Jucker, 2010), and its component signals and effects throughout the body are only recently being better understood, at least in part by virtue of advances in technology. There have been various models created with this simplification in mind.

In this work, I hypothesize that much can be learned about the formation of neural cells and their connections through a comparative differentiation of stem cells into in vitro neural networks. Some cell types with different differentiation cues have been shown to display differences in gene expression and morphology from the same precursor undifferentiated cells. The same cell type differentiated in differing contexts has resulted in expression of differing gene expression profiles. I propose to differentiate P19 embryonal carcinoma cells into neuronal cells using a novel monolayer differentiation protocol to establish efficiency of differentiation, define the resulting morphological features and gene expression profiles, and to compare what I find to a previously documented monolayer differentiation technique.

i. Models: in vivo vs. in vitro

Typically, the simplification of a system like the CNS is done in a model of sorts. The two main types of models are in vivo and in vitro, or animal models and cell culture models. The benefit of utilizing an animal model is to assess the balance between risk and benefit of new therapies in an organismal system before they are passed to clinical trials (Jucker, 2010). One of the major diseases that would most benefit from stem cell therapy is neurodegenerative disease. Neurodegenerative diseases are chronic or acute diseases characterized by the loss of either a

specific subtype of neuronal cells or a broad swath of neuronal cell types (Lindvall & Kokaia, 2010). Most animals, however, do not often experience the types of neurodevelopmental or neurodegenerative diseases as humans do, even though there may be a highly similar genome and gene expression (Piotrowska, 2012). Therefore, when an animal model is needed to represent a human disease, an approximation has to be reached. Either human genes can be introduced to the system to express particular phenotypes, known as a humanized model, or genes homologous to the ones implicated in humans can be activated or deactivated to simulate the disease's effects (Piotrowska, 2012).

Animal models in general are highly important as they can parallel human diseases and can be modified in a controlled manner by genetic, chemical, or physical manipulation (Lindvall et al., 2004). Animal models have a few major drawbacks. They often do not endogenously experience neurodegenerative diseases like the ones humans do, and they are still complex organisms in their own right (Qiang, Inoue, & Abeliovich, 2014). Because of this, it is not uncommon for breakthroughs in a model organism to not translate to success in clinical trials (Jucker, 2010). It is often difficult to study disorders of the brain in model organisms due to the intricacies of the neural networks found in the central and peripheral nervous systems (Lancaster et al., 2013). Though in vivo models cannot be devalued due to their ability to allow for experimentation on whole organisms, they may not be the most efficient way to isolate systematic or genetic aberrations (CITE THIS PG).

The biggest advantage of an in vitro neural model is that it is as simple a system as possible. A model, when being designed, is often preferred when it can exclude particular aspects of the external elements of the target system (Lancaster et al., 2013). This creates an environment to more closely observe and manipulate the internal elements of the system and find a causal interaction between them (Piotrowska, 2012). They also give the ability to study an isolated cell type

in a controlled environment, ease of genetic manipulation, and investigation of specific cellular mechanisms (Schlachetzki, Saliba, & de Oliveira, 2013). This includes the ability to determine the use of "support cells" like microglia, astrocytes, and non-neuronal neural cells in an isolated system. Typically, one of the goals of in vitro neuronal differentiation is to increase the efficiency of differentiation of a particular cell type or subtype. The utility of other types of cells, like support cells, has more recently been seen as increasingly useful in in vitro support systems. For example, research has been done on the importance of astrocytes in determining the type of cell differentiated by neural precursor cells (NPCs) prior to or post-transplantation and glial cells have important roles in fate determination (Lindvall et al., 2004; Song, Stevens, & Gage, 2002; Wagner et al., 1999). Efficiency will be defined in this study as percentage of neuronal tissue produced in a plate compared to the whole. Embryonic development is a highly complex and difficult to observe phenomenon, therefore having a simple model of central nervous system organogenesis is key to developing a deeper understanding of the human brain (Lancaster et al., 2013).

ii. Cell types for in vitro models

Primary CNS neurons are difficult to successfully obtain from patients with neurologic disorders; therefore multipotent cell-derived neural tissue is required. There are multiple cell types capable of forming in vitro neural models, embryonal carcinoma cells (EC), embryonic stem cells (ES), and induced pluripotent stem cells (iPSC). Each has individual benefits and drawbacks, but they all exhibit the ability to differentiate into another cell type, neural or non-neural, given a chemical cue. The ideal system is one that is as close to the environment in question as possible and is able to consistently differentiate into the same cell type given the same conditions. The most promising way to do that thus far seems to be iPSCs. They can be derived from cells from various tissues from a host of any age, though due to telomere shortening, younger donors' cells typically

fare better (Thomson et al., 1998). This is less of a problem when using mouse-derived cells, as murine telomeres are longer than human telomeres and are therefore less prone to senescence in vitro (Kipling & Cooke, 1990).

The creation of patient-derived pluripotent cells has allowed, for the first time, the opportunity to study a direct disease/patient specific model (Hermann & Storch, 2013; Takahashi & Yamanaka, 2006; Takahashi et al., 2007). By creating an undifferentiated cell line with the genomic makeup and gene expression of the patient, testing could be more safely done to determine how a patient's specific disease would respond to treatment. There is vast genetic variability present in the human population, which reduces the utility of a generic human stem cell model (Qiang et al., 2014). With an increased interest in human genetic variation, the ability to easily compare the impact of rare and common genetic variations in the human genome on disease has a strong allure. This interest has stemmed from genome-wide studies and modern sequencing methods allowing relatively quick and easy access to individual genomes. The ability to create differentiatable cell lines non-invasively from a broad range of human donors would allow for more reliable and efficient model-to-clinic transitions (Qiang et al., 2014). These iPSCs can also be reintroduced as a precursor cell graft in a therapeutic manner with significantly fewer immunosuppressive needs for the graft recipient than could be necessary with transgenic ES or EC precursor cells (B.-Y. Hu et al., 2010; Marchetto, Winner, & Gage, 2010; Schlachetzki et al., 2013).

Thus far, iPSC-derived neuronal subtypes have included spinal motoneurons, dopaminergic neurons, and cortical glutamatergic and γ-aminobutyric acid (GABA)-ergic neurons (Qiang et al., 2014). When in vitro studies compare the temporal gene expression in differentiating EC cells and iPSCs, similar patterns can be seen, however iPSCs are consistently less efficient (B.-Y. Hu et al., 2010; Qiang et al., 2014). They are also prone to having a high degree of variability in the type of

neuronal subtype produced. Because of this, it seems that ECs and ESCs are currently a more effective model of neurogenesis, though iPSCs could prove invaluable when a more efficient preparation technique has been created. Luckily, it is also thought that iPSCs perform like ESCs during differentiation, therefore protocols developed and tested for ESCs would translate to effective protocols for iPSCs (Hermann & Storch, 2013).

A subtype of iPSCs known as induced neurons (iN) has recently been developed (Qiang et al., 2014). They are the result of transdifferentiation of non-neuronal cell types to terminally differentiated neuronal cell types using genetic cues. This transdifferentiation process entirely skips the time-consuming processes of dedifferentiation and redifferentiation to directly become an entirely different phenotype. The biggest impediment to its usefulness is the fact that these cells cannot expand as a population nearly as much as the iPSC lines can (Qiang et al., 2014). Even without the ability to significantly increase in population, the potential for these cells is significant.

Embryonic stem (ES) cells were originally derived in 1981 from the inner cell mass (ICM) of mouse blastocysts (Evans & Kaufman, 1981). Cultures of these cells could form teratocarcinomas when implanted in the extrauterine system of a mouse, they could totally replenish their population from a single cell, and could differentiate into any number of cell types. These pluripotent cells have been traditionally seen as a source for refurbishing any number of tissues and organs (Babuška et al., 2010). As long as they remain undifferentiated, they can almost perpetually self-renew while maintaining their ability to terminally differentiate. This is achieved by a specialized set of transcription factors that maintain the chromatin of the pluripotent cells to prevent telomere degradation (Gaarenstroom & Hill, 2014). Previously it was thought that these cells, in order to maintain their pluripotency, had to be grown in very specialized conditions on feeder cells to maintain pluripotency. They key factors that enabled this unchecked growth were bone morphogenic protein (BMP) activated inhibitor of differentiation protein in conjunction with

the cytokine leukemia inhibitory factor (LIF). This activates the transcription factor STAT3 to activate MAPK cascades and drives self-renewal through inhibition of differentiation (Lanner & Rossant, 2010; Q. L. Ying, Nichols, Chambers, & Smith, 2003; J. Yu & Thomson, 2011).

Interestingly, there seems to be a difference between ES cells derived from human sources and ES cells from mouse sources. Though they are both derived from the inner cell mass (ICM) of the blastocyst and require similar transcription factors, like Nanog Oct4 and Sox2, they require totally different culture conditions to maintain proliferative renewal in vitro. For example, LIF did not appear to enhance proliferation in culture, instead FGF2 seems to play a more prominent role in maintaining pluripotency. It has been proposed that they actually resemble postimplantation state murine cell lines rather than murine ES cells (Lanner & Rossant, 2010).

Embryonal carcinoma (EC) cells have been well established as a valuable method of modeling complex systems in finite in vivo environments (Babuška et al., 2010; Hwei Ling Khor, 2007; Jin et al., 2009; Jones-Villeneuve, McBurney, Rogers, & Kalnins, 1982; Jones-Villeneuve, Rudnicki, Harris, & McBurney, 1983; Lin, Yang, & Chen, 2012; Martin, 1980; Monzo et al., 2012; Rossant & McBurney, 1982; J. Yu & Thomson, 2011) They are characterized by the ability to differentiate into cells in any of the three primary germ layers: endoderm, mesoderm, and ectoderm. These multipotent stem cells can be derived from teratocarcinomas, which are cancerous tumors that contain a mix of undifferentiated cells and a myriad of differentiated tissues (Martin, 1980). The undifferentiated carcinoma cells, when isolated and immortalized, are morphologically and functionally similar to the undifferentiated inner mass of cells in mammalian embryos. They can be terminally differentiated into non-carcinogenic cells, although they retain some abnormal gene expression compared to normal terminally differentiated tissues (Martin, 1980; Rossant & McBurney, 1982; Sharma & Notter, 1988). EC cells are especially useful due to their ability to retain a highly conserved chromosomal constitution that is close to the karyotype of normal cells in

a clonal line to serve as an infinite supply of cells, yet consistently differentiate into a desired cell type (Bain, et al., 1994; Martin, 1980; Rossant & McBurney, 1982).

There has been a recent push to transfer from the 2D plate-bound models and to go into 3D organ models. Lancaster et al. (2013) grew a 3D brain-like system in a petri dish deemed cerebral organoids. These models combine the benefits of an in vivo system with the benefit of isolation and simplification not as easily accessible in animal models. Though these systems might add some more complexity to the in vivo/in vitro dichotomy, they have the potential to provide a resource that is highly multifunctional in ways as of yet unseen (Lancaster et al., 2013; Pera et al., 2014). An interesting field that could spawn from this separation would be the integration of the two model systems. A combination of transplantation therapy and 3D models could be used to observe how stem cell derived neural progenitor cells differentiate, migrate, and proliferate. In the fully in vitro system exists a fully manipulatable, observable, and functional model system. Producing a 3D functional model of a brain system and then manipulating it to undergo neurodegeneration is likely to produce some invaluable results.

iii. Transplantation therapy

A method for transferring in vitro methods to use in in vivo systems is to use differentiated stem cells in transplantation therapy. In instances such as Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and stroke, there is a degradation of endogenous neural tissue. Though transplantation therapy has been a proposed treatment method for about 30 years (McConnell, 1988; Vicario-Abejón, Cunningham, & McKay, 1995), it had previously been limited to fetal neural tissue grafted onto the damaged site (Lindvall et al., 2004). Therapy for these neurodegenerative diseases has recently expanded to include transplantation of NPCs in hopes of reinnervating some of the damaged regions to attain symptomatic relief or

regaining functionality (Björklund & Lindvall, 2000; Lindvall et al., 2004; Ransom et al., 2013; Schlachetzki et al., 2013). The benefit of using EC cells above fetal grafts is that EC cells can be manipulated in a controlled manner for improvements such as increased survival, more specific differentiation, and neuronal migration (Lindvall et al., 2004).

Techniques like pluripotent stem cell induction have revealed a surprising amount of plasticity in the adult mammalian brain (Qiang et al., 2014). It is this plasticity, in combination with the NPCs, which is being exploited to form functional linkages after transplantation of neuronal precursors in neurodegeneration cases. A major therapeutic benefit of EC cells is their ability to terminally differentiate to different cells based on their context (Björklund & Lindvall, 2000) in addition to an enormous capacity for self-organizing to form whole tissues (Lancaster et al., 2013). The goal for transplantation therapy is that after transplantation of neural precursors into the damaged site, the environmental cues would provide enough impetus for the formation of the necessary neuronal phenotypes required to reinnervate the affected area. Measured through synapse formation and the establishment of polarity, effective development of neural cells and some reinnervation of damaged tissue has been observed in clinical trial (Björklund & Lindvall, 2000). These two components, synapsing and polarity are also what Finley et al. (1996) defined as critical steps for the benchmark of CNS neuron differentiation.

Induced pluripotent stem cells have been highly regarded as viable transplants, as they are cultured from the patient themselves. The reprogramming of iPSCs currently has not been fully successful at removing the reprogramming cue after the pluripotent state has been reached (Qiang et al., 2014). Even through this, they remain the most promising model as they would maintain the genetic makeup of the donor, but still be able to differentiate as needed. Though the success of this is dependent on the age of the cell donor, the differentiation state of the neuronal precursor cells integrated into the graft site, the cues of the neural environment and the ability of the neurons to

successfully integrate into the preexisting nervous system and be able to self-regulate (Björklund & Lindvall, 2000; Lindvall et al., 2004). Even given this, the undifferentiated EC cells will not necessarily differentiate in a given context if not initially differentiated towards their terminal cell type (Rossant & McBurney, 1982). This uncertainty results in complications such as the formation of teratomas arising from the grafted cells (Hermann & Storch, 2013; Miura et al., 2009). This is being counteracted by inducing terminal neuronal differentiation prior to grafting the precursor cells into the damaged areas (Lindvall et al., 2004; Magnuson et al., 1995; Monzo et al., 2012; Morassutti et al., 1994).

Though there have been some experimental successes in stem cell therapy, the translation between in vitro stem cells and clinical trials has not been entirely successful [OU2]

There is a possibility that adult brains produce new dopaminergic neurons in the substantia nigra (Lindvall et al., 2004), even though the adult brain is limited in its capacity for self-repair (Björklund & Lindvall, 2000). Though the study proposing dopaminergic specific growth was not repeatable in a follow-up study, adult brain plasticity has been an integral part of shaping neuronal transplantation therapy. Neurogenesis in the adult brain is typically limited to the subgranular zone (SGZ) of the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Eriksson et al., 1998; Suh, Deng, & Gage, 2009). The NPCs in the SGZ differentiate into granular neurons and integrate into preexisting neuronal circuitry, while the NPCs in the SVZ will migrate and differentiate into interneurons in the olfactory bulb (Suh et al., 2009). Another approach to transplant therapy is to increase the mobility of the preexisting NPCs and influence them to migrate to the lesioned sites in neurodegenerative diseases (Lindvall et al., 2004).

iv. P19 embryonal carcinoma cell line

P19 cells are a line of murine pluripotent embryonal teratocarcinoma cells that have been

used as a model of early neural tube development (Jin et al., 2009; Jones-Villeneuve et al., 1982, 1983; Monzo et al., 2012; Tang et al., 2002). They were developed initially by Rossant and McBurney in 1982 by implanting an embryo at 7 days of gestation (E7) behind the testes of an adult male mouse (Bain et al., 1994; Rossant & McBurney, 1982; van der Heyden & Defize, 2003) and isolating the carcinoma cells in tissue culture. Teratocarcinomas are an ideal system to create pluripotent stem cells because they can be easily produced by introducing a murine fetus into an extrauterine system of the a mouse and the resulting cell mass can express both an undifferentiated set and terminally differentiated set of cells (Martin, 1980). This allows the undifferentiated cells to be collected and then terminally differentiated in vivo. These cells retain their pluripotentiality, as seen in their ability to differentiate into cardiomyocytes and other mesodermal derivatives when differentiated with DMSO, as well as neural cells when differentiated with all trans-retinoic acid (RA) (Bain et al., 1994; Jones-Villeneuve et al., 1982, 1983; Lin et al., 2012; Monzo et al., 2012; Rossant & McBurney, 1982; van der Heyden & Defize, 2003). The differentiation process of these cells changes the phenotype from a malignant cell type to a nonmalignant one by differentiation (van der Heyden & Defize, 2003). They also retain a relatively normal karyotype, as shown by Bain et al., (1994).

The P19-derived neural cells have been reported to resemble those in the developing central nervous system (CNS) at functional and morphological levels, which makes them an invaluable tool as a model for the developing embryonic brain stem (Hong & Bain, 2012; Monzo et al., 2012; Tang et al., 2002). They are easy to maintain as undifferentiated cells, grow rapidly and can regenerate their population from a few cells. Though they are prone to spontaneous differentiation when overly confluent (Bain et al., 1994; Monzo et al., 2012), they do not display contact inhibition and are anchorage-independent (Kanungo & Chandrasekharappa, 2012). Jin et al., (2009) proposed that they are even more specified as progenitor cells for the caudal nervous system by

nestin expression. Early detection of nestin, rarely found in the developing CNS during neural plate induction, indicates precursors for the subependymal zones of the brain and spinal cord (Mckay, 1997). As the neurodevelopmental processes of the CNS are highly complex and largely inaccessible to experimentation, a simple and effective mouse model would be very incredibly valuable (Finley, Kulkarni, & Huettner, 1996). P19s are susceptible to a number of morphogens and are capable of differentiating into primitive endoderm, mesoderm, and ectoderm, neural cells, and beating cardiomyocytes. They have been consistently seen as an invaluable model for early embryonic development (Kanungo & Chandrasekharappa, 2012). As they resemble ES cells in mouse and human, it is possible that they could be an ideal model for mouse and human embryonic development (Farah et al., 2000).

P19-derived neurons seem to express NMDA and metabotropic glutamate receptors, but not AMPA/kainate receptors (Morley, Macpherson, Whitfield, Harris, & Mcburney, 1995)[BC3] When aggregate-derived neuronal cultures exhibited γ-Aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the CNS, receptors. Khor (2007) reported that they expressed specifically $GABA_A$ receptors. When previously treated with GABA, P19s appeared to express glutamate receptors. Initially, in the developing cultures, GABA had an excitatory action, but when the neurons matured, the effect became inhibitory. Khor theorized that the initial excitatory action by GABA is required to stimulate glutamate expression.

The use of P19s as a source of NPCs for transplantation has been tested by injection into mouse blastocysts (Bain et al., 1994), and by grafting them into the striata of an adult host rat (Morassutti et al., 1994). The resultant chimeras displayed P19-derived cells in a broad range of tissues. When a single P19 cell was injected into a mouse blastocyst, 20% of the resultant masses were chimeric in nature, and P19 cells were found in all three germ layers (Rossant & McBurney, 1982), revealing the extent of the multipotent character of this cell line. However, the chimeras

expressed some morphologic abnormalities and the transplanted cells did not necessarily differentiate without pre-inducing a terminal phenotype (Rossant & McBurney, 1982).

Houdek et al. (2012) tested the ability of P19-derived neuroprogenitor cells to form a graft in a Lurcher olivocerebellar degradation mouse model. This mouse model is commonly used in neurodegeneration studies as it experiences a natural degradation of glutamatergic purkinje cells. This occurs through a heterozygous mutation on the glutamate receptor delta2 in cerebellar purkinje cells, the receptor is altered to become a leaky membrane channel, which leads to constant depolarization of the cells. This causes a loss of other cerebellar cells and inferior olive neurons through lack of signaling. The resulting Lurcher cerebellum is flattened and mostly degenerated. When a neuroprogenitor cell suspension was injected into the cerebellar area, there was a distinct lack of graft growth in the cerebellar degenerated region of the Lurcher brains. Therefore, Houdek et al. indicated that P19 cells are not optimal for this kind of transplantation therapy (Houdek et al., 2012).

v. Pathways implicated in neuronal differentiation with and without RA

From the initial differentiating signal, there have to be several pathways followed to reach the terminal homogenous neural culture. Gerhart (1999) proposed that the five most important pathways in early embryonic development are wingless-type MMTV integration site family (Wnt) signaling, Transforming Growth Factor β (TGF-β) signaling, Hedgehog (Hh) signaling, select receptor tyrosine kinase (RTK) pathways, and Notch signaling. These all act by binding to a surface receptor and enacting change through intracellular signaling that eventually results in transcriptional modification of DNA (Duester, 2013). In the time since, each of the above pathways has been explored and nearly all of them interact with each other in the formation and maintenance of the nervous system. As seen in Figure 1, the pathways can result in nuclear

modification of gene expression. Each pathway has at least a minor regulatory role through mutually interactive feedback loops in the other four as well as its independent action in gene regulation (Pera et al., 2014).

Lupo et al. (2013) proposed that homogeneous cell populations could produce heterogeneous neural populations through several different ways; Notch-dependent inhibition of lateral patterning, variations in FGF signaling as its signal degrades in the culture media, differential exposure to extracellular signals as cells change shape and confluence, or any combination of the above (Lupo et al., 2013). Ransom et al. (2013) present the more specific role of RA in interactions with the RTK stimulated MapK/Erk pathway by binding to a number of co-regulator complexes in the nucleus to influence transcription (Ransom et al., 2013). In Xenopus models, it has been suggested that FGF and Wnt signaling are required for proper formation of the AP axis through induced posteriorization. This action, however, requires the presence of RA. Though FGF and Wnt signaling is enough to suppress anterior gene induction, RA is required to promote posteriorization. Kudoh et al. present that RA is necessary and sufficient for the promotion of several posteriorizing signals, whether or not other pathways like FGF or Wnt are involved (Kudoh, Wilson, & Dawid, 2002). Murashov et al. (2004) succeeded in inducing dorsal/spinal interneurons from ES cells with the application of Shh, RA, and specific TGF-β and Wnt signaling molecules. It was successful due to the timing of addition for each of the signaling molecules, reinforcing the spatiotemporal influences of neurogenic cues (Murashov et al., 2004).[BC4] These intricate feedback loops suggest a yet-undiscovered complex interaction of Wnt, FGF, and RA signaling during regional specification in the CNS (Pera et al., 2014).

a. Notch Signaling

Notch signaling is highly conserved and a major player in the neurodifferentiation of the CNS (Briscoe & Ericson, 2001). This pathway is dependent on cell-cell interactions of single-pass transmembrane proteins. The Notch ligands are Delta and Serrate (also known as Jagged) and they have an extracellular region that interacts with the single pass, transmembrane Notch receptor heterodimer on an adjacent cell (Louvi & Artavanis-Tsakonas, 2006). The binding itself is regulated by extracellular, post-translational events. When the ligand binds, a proteolytic system results in the cleavage of the cytoplasmic region of the transmembrane receptor, which then translocates to the nucleus. Once in the nucleus, the translocated cytoplasmic region of the receptor will act as an activator and recruit the components needed for a complex containing the DNA binding protein suppressor of hairless (Su(H)) and the nuclear protein mastermind (MAM). Su(H), however, is the major effector in drosophila, where Notch signaling is better characterized. Its

ortholog in vertebrates is the CBF1 protein (K. Yoon & Gaiano, 2005), which appears to perform in a highly similar manner. MAM, true to its name, coordinates the assembly of the Notch intracellular domain (NICD) that translocates to the nucleus and the converted CBF1 repressor complex. When converted, the NICD-CBF1 complex becomes an antagonistic activator and upregulates the activity of bHLH transcriptional repressors and target mammalian genes Hes1 (hairy and enhancer of split-1), Hes5 (hairy and enhancer of split-5), Hesr1 (hairy and enhancer of split related-1), and Hesr2 (hairy and enhancer of split related-2). These bHLH proteins inhibit the early neuronal gene expression of markers like Mash1 and various neurogenins (K. Yoon & Gaiano, 2005). Though as many as 140 genes have been implicated in association with Notch signaling, the intricacies of the pathway are still not widely understood(Louvi & Artavanis-Tsakonas, 2006; K. Yoon & Gaiano, 2005).[BC5]

This main Delta/Jagged-Notch-CBF1-Hes/Hesr pathway is integral to the developing nervous system. Though Notch signaling seems to play a role in neuronal maturation, its main role appears to be in glial fate specification. The presence of Notch target Hes proteins is highly correlated with lack of neuronal and oligodendrocytes differentiation juxtaposed with promoted glial cell growth (Louvi & Artavanis-Tsakonas, 2006). The most common effect of Notch signaling appears to be promoting the outgrowth and differentiation of radial glia. This is done through asymmetric division resulting in one neurally determined cell and one basally determined one (Z. Dong et al., 2012; Duester, 2008; Louvi & Artavanis-Tsakonas, 2006). Their development is mediated by several factors, such as Numb and Neuralized (Neur). Neur, along with Mind Bomb (MIB), acts in the signaling cell to promote fate determination in the signal-receiving cell (K.-J. Yoon et al., 2008). It seems as if the major role of embryonic Notch signaling is permissive, not instructive. However, the effect induced by Notch signaling is not retained once the active signal is removed and is totally reversible. The role of Notch in boundary establishment is conserved across

vertebrates and nonvertebrates, but the type of boundary differs. In vertebrates, it is most active in the formation of segments in the developing somites. Once the CNS has more fully developed, the role of Notch is less well understood (Louvi & Artavanis-Tsakonas, 2006).

b. Receptor tyrosine kinase signaling

Receptor tyrosine kinases (RTKs) are a family of factors with a signature type of receptor that is capable of performing like an enzyme (Lanner & Rossant, 2010). They all possess and induce a cascade from a generally dimeric transmembrane receptor with a cytoplasmic kinase specific for tyrosine residues (Lemmon & Schlessinger, 2010). When a subunit of the receptor binds to a ligand, the receptor subunits dimerize in a manner specific to the ligand bound. They do this by cross-phosphorylation, where each RTK dimer phosphorylates multiple tyrosines on the other subunit. There are four specific transduction pathways within the RTK family; the Ras/extracellular signal-regulated kinase (Erk) small G-protein pathway that leads to the activation of one or more of the three protein kinase pathways (MAPKs); the phosphoinositide 3-kinase (PI3K) pathway; the phosphoinositide phospholipase C (PLCγ) pathway; and the Janus kinase/signal transducer and activator of transcription (Jak/Stat) pathway (Gerhart, 1999; Lanner & Rossant, 2010).

Receptor-ligand specificity derives from the extracellular components of the receptor monomers (Lemmon & Schlessinger, 2010). All RTKs share a similar structure otherwise; a single α-helix transmembrane portion and a cytoplasmic region that consists of either one or two tyrosine kinase subunits. The ligands that activate these kinase cascades include the subfamilies of epidermal growth factors (EGFs), fibroblast growth factors (FGFs), and platelet derived growth factors (PGFs). Though their names are the archaic result of the source of their initial derivation, these factors are ubiquitous through most systems. Vertebrates have 22 FGF ligands and five FGF

receptors (FGFR), which are used widely in development, early mesoderm maintenance, notochord development, and a number of other essential functions (Gerhart, 1999; Lanner & Rossant, 2010).

The most active RTK pathways involved in early neurodevelopment are the FGF subfamily pathways. In the extracellular component of the FGFR monomer, there are three immunoglobulin-like (Ig) subunits. The FGF ligand is a monomeric ligand that requires the accessory molecule heparan sulfate proteoglycan (HSPG) to bind and induce activation. Each subunit of the dimerized receptor binds to a FGF ligand and HSPG/heparin, where they induce direct receptor subunit contact through the middle Ig-like subunit (domain 2) (Lemmon & Schlessinger, 2010).The presence or absence of HSPGs in the extracellular space act as a bioregulator of FGF signaling (Lanner & Rossant, 2010). The monomer also has the ability to autophosphorylate by stabilizing the inactive extracellular domain and preventing ligands from binding. This is counteracted by cross-phosphorylation by another monomer to form the activated FGFR dimer. This release of autoinhibition is a key event that triggers the activation of the RTK FGF pathway (Lemmon & Schlessinger, 2010).

c. Wnt signaling

Wnt genes encode a family of secretory glycoproteins that activate G protein-coupled receptor (GPCR) signaling pathways. Though mainly expressed in the CNS during embryogenesis of vertebrates, Wnt genes are well conserved throughout vertebrates and nonvertebrates (Zhang, Yang, Yang, & Zhang, 2011). The three different pathways involving Wnt signaling are the canonical Wnt/β-catenin cascade, the non-canonical planar cell polarity (PCP) pathway, and the Wnt/Ca^{2+} pathway (Zhang et al., 2011). It is thought that canonical Wnt/β-catenin signaling may promote neural differentiation while non-canonical Wnt signaling may inhibit it (Inestrosa & Varela-Nallar, 2013). This is corroborated by the direct relationship β-catenin has with neuronal

differentiation (Hong & Bain, 2012; Zhang et al., 2011). The canonical Wnt/β-catenin signaling pathway is widely recognized to be an integral part of the neurodevelopmental and proliferative process (L. Chen, 2013; Zhang et al., 2011). It depends on halting the degradation of β-catenin to allow it to influence the Wnt target proteins. These proteins include cMyc, cyclin D1, Axin2, and CamkIV (calcium/calmodulin dependent protein kinase type IV) (L. Chen, 2013; Hong & Bain, 2012).

Inhibiting the phosphorylation of β-catenin is done by Wnt binding to the Frizzled (Fz) seven-transmembrane spanning receptor that belongs to the GPCR family (Inestrosa & Varela-Nallar, 2013). This family contains seven-transmembrane proteins that activate signal transduction pathways (Wettschureck & Offermanns, 2005). The G-protein receptor is a heterotrimer, consisting of α, β, and γ subunits. Generally, an inactive G-protein receptor has a GDP (guanidine diphosphate) bound and is activated when a specific ligand binds to the receptor. The α subunit binds the ligand and the GTP, while the β, and γ subunits form an undissociable complex. When the ligand binds the α subunit, the GDP falls away and a GTP is bound. Both the complex and the α subunit, once GTP has been hydrolyzed, can freely modulate the activity of a number of cascades. These include ion channel regulation, mediation of isoforms of adenylyl cyclase and phospholipase C, and regulation of phosphoinositide-3-kinase isoforms. These interactions can continue until the GTP is hydrolyzed into a GDP and P_i. After the P_i is released, the α, β, and γ subunits will bind back together and the cycle can begin again (Wettschureck & Offermanns, 2005).

In the case of Fz, Wnt proteins bind to the Fz receptor and the Wnt-Fz complex binds to the co-receptor low density lipoprotein receptor protein 5/6 (LRP5/6) (Zhang et al., 2011). This initiates signaling which is transduced through Dishevelled to inhibit GSK3β activity. GSK3β normally phosphorylates β-catenin leading to decay of β-catenin. Inhibition of GSK3β leads to stabilization

of β-catenin (Ulloa & Martí, 2010). Stabilized β-catenin accumulates in the cytoplasm and translocates to the nucleus. There, it complexes with the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors. This ultimately influences the target genes and upregulates neuronal differentiation cues (L. Chen, 2013; Ulloa & Martí, 2010; Zhang et al., 2011).

There are several members of the Wnt gene family, three of the most thoroughly documented are Wnt1, Wnt3a, and Wnt5a (Castelo-Branco et al., 2003; L. Chen, 2013). Wnt1 has been most correlated with in vitro neuronal differentiation. Its role in animal models is still not entirely elucidated, though it seems integral in the neurodevelopmental process. When Wnt1 expression was disrupted, there was a loss of midbrain and cerebellar structures in embryo (Tang et al., 2002). Tang et al. proposed that Wnt1 signaling activation via N-cadherin overexpression can be used to differentiate P19 cells into neural cells without the use of RA (Tang et al., 2002). This could have been a result of either N-cadherin's interactions with β-catenin in the cytoplasm or through fibroblast growth factor receptors (FGFRs) (Gao et al., 2001; Wang et al., 2006). They observed increased proliferation of orphan nuclear receptor-related factor 1 (Nurr1), upregulation of cyclins D1 and D3, and downregulation of p27 and p57. Nurr1 is a receptor that is expressed in dopaminergic progenitors in the midbrain, cyclin D1 and D3 are indirect cell cycle regulators of the G1/S transition, and p27 and p57 are inhibitors of cell cycle progression (Ye, Shimamura, Rubenstein, Hynes, & Rosenthal, 1998). All of these would be necessary to maintain a state of pluripotency in a cell line and prevent differentiation by maintenance of the mitotic cycle.

d. Hedgehog signaling

The sonic hedgehog (Shh) pathway is mainly involved in vivo in specification of neuronal fate along the ventral neural tube (Briscoe & Ericson, 2001). The protein itself is initially secreted

by the notochord where its presence induces production in the cells at the ventral midline of the neural tube, the floor plate cells (Ulloa & Martí, 2010). It acts through two transmembrane proteins, Patched (Ptc) and Smoothened (Smo). Ptc has an inhibitory effect on Smo when Shh is absent in the system. When Shh binds Ptc, Smo initiates intracellular signaling.

Interestingly, the ventral depth at which Shh is acting will moderate the effect it has. The progressively more ventral regions require a progressively larger concentration of Shh for induction. Due to this, Shh signaling is treated as a gradient, decreasing as Wnt signaling begins to predominate on the ventral side of the neural tube [BC6] (Briscoe & Ericson, 2001). Shh can also induce the differentiation of five distinct neural subtypes in the neural tube (Briscoe & Ericson, 2001; Ulloa & Martí, 2010). This broad effect is the result of a group of homeodomain (HD) proteins divided into two categories based on their function. Class I proteins are repressed at a particular level of Shh expression, and their most ventral expression indicates the edge of the progenitor cell domain. Class II proteins are classified by their dependence on Shh signaling for how they are expressed and conversely, their most dorsally expressed regions indicate the progenitor cell domain. These classes can have paired proteins that share boundaries and respond to changes in their expression in an equal and opposite manner (Briscoe & Ericson, 2001). These two classes of HD proteins, in conjunction with the Gli family of basic Helix-Loop-Helix (bHLH) transcription factors, are responsible for the establishment of the five most ventral neural subtypes. The gradient expression of Shh is thought to induce a gradient of intracellular Gli activity, which would be responsible for the morphogenic properties of Shh (Ulloa & Martí, 2010). [BC7]

e. TGF-β signaling

TGF-β signaling can act as an inhibitor for neuronal cell differentiation (Tropepe et al., 2001). Although how specific contexts affect TGF-β signaling is still largely unknown, the

environment that the signaling takes place in appears to play more of a role than the cytokines in the pathway in determining the effect of the signal. This results in a huge variety of effects resulting from TGF-β signaling, even manifesting in dichotomous gene expression by the same pathway in two different cell types (Massagué, 2012). Ligands in the TGF-β superfamily are separated into two subfamilies on the basis of sequence similarity (Massagué, 2012). They play an initial role in embryonic development by regulation of pluripotency genes and genes for differentiation or germ layer specification (Gaarenstroom & Hill, 2014). The subfamilies are the TGF-β/Activin subfamily, and the BMP subfamily, which both act through the SMAD transduction pathway (Massagué, 2012; Xu et al., 2008). SMAD proteins are homologs to the drosophila gene mothers against decapentaplegic (MAD).

These ligands bind to oligomeric receptors that activate the SMAD protein family and induce transcriptional regulation of cell function and growth. The structure of the receptor is that of a heteromer composed of alternating type I and type II components whose structure is largely dependent on the bound ligand. Both types contain an extracellular ligand binding domain, a single transmembrane domain and a cytoplasmic serine/threonine kinase (Massagué, 2012). Ligands will bind to the extracellular region of the type II component, which binds and activates the type I component by phosphorylation (Moustakas, Souchelnytskyi, & Heldin, 2001). When the type I component is phosphorylated, it goes on to phosphorylate the SMAD protein that corresponds to the subfamily of ligand bound. When the receptor complex is bound by a TGF-β, Nodal, or Activin ligand, the R-SMADs subsequently activated are SMAD2 and SMAD3, while BMP and DGF binding leads to activation of SMAD1, SMAD5, and SMAD8.This activated SMAD protein will them form an oligomeric complex with SMAD4 (Gaarenstroom & Hill, 2014; Massagué, 2012; Moustakas et al., 2001; Shi & Massagué, 2003).

SMAD proteins are a family of highly-conserved signal transducers (Moustakas et al.,

2001). The different variants of SMAD proteins include receptor-activated SMADs (R-SMADs), which complex with the common SMAD (Co-SMAD), SMAD4. The remaining SMAD proteins are SMAD6 and SMAD7, or the inhibitory SMADs (I-SMAD. These I-SMADs do this by competing with R-SMADs for receptors and when bound, marking them for degradation by the proteasome through addition of ubiquitin to the receptor (Moustakas et al., 2001). The unifying traits of SMAD proteins are the two conserved regions in the N- and C-terminals, termed mad homology 1 (MH1) and 2 (MH2) respectively, with a linker region between them. MH1 contains a hairpin structure that binds to DNA, while MH2 contains a series of hydrophobic regions that allow for interactions with cytoplasmic and nuclear proteins (Massagué, 2012). This common structure allows for formation of a C-terminus sequence, Ser-X-Ser-Ser, the necessary structure to bind to the Co-SMAD. There can be any amino acid in place for the X portion (Massagué, 2012).

Once activated by phosphorylation, the type I component of the receptor will bind and phosphorylate the appropriate R-SMAD, which will then form a complex with SMAD4. This oligomeric complex will then travel to the nucleus (Gaarenstroom & Hill, 2014; Moustakas et al., 2001). This pathway is highly involved in the regulation of cell proliferation, differentiation, and apoptosis and is active at both the embryonic stage and fully adult stage of development. Typically it has a negative effect on cell growth, inhibiting tumorigenesis (Shi & Massagué, 2003). Its effect is carried out by post-transcriptional modification of DNA in combination with cofactors and is mediated by master transcription factors like Oct4, Sox2, and Nanog in ESCs (Gaarenstroom & Hill, 2014; Moustakas et al., 2001). Transcription factors (TF) bind promoter regions of DNA and can both up- and downregulate gene expression through direct, or unbound to DNA in indirect ways (Gaarenstroom & Hill, 2014). These factors

f. Pathway interactions

Part of the efficacy of these pathways in neuronal differentiation is the ability to interact with the other pathways, as previously mentioned. Some of the more prominent looped interactions in neurogenesis involve Wnt, FGF, and RA (Pera et al., 2014). Recently, there has been work done to elucidate the role of RA in differentiation. Duester (2013) reviews the use of RA in embryonic development and maintenance of adult systems, and presents the interaction of RA and Hh signaling in the development of motoneurons in the spinal cord and hindbrain. Beyond that inductive signal, P19 derived neural cells appear to be a heterogeneous population of neuronal and non-neuronal neural cell types. In early embryonic development, RA interacts with several pathways in the initial patterning of the two main axes of the nervous system (Kudoh et al., 2002). The dorsoventral (DV) and the anteroposterior (AP) axes are initialized following neural induction and the regionalization of the neuroectoderm. The patterning of these axes is reliant on pathways to determine the positioning and terminal cell fate. In human embryonic stem cells (hESCs), RA plays a caudalizing role in the posteriorization of cells in the AP axis alongside Wnt, Hh, and FGF signaling (Lupo et al., 2013). Only recently has more been elucidated about the specific mechanisms of how retinoic acid induced neural stem cell development is initialized in very early embryos (Pera et al., 2014; Shimozono et al., 2013; Tropepe et al., 2001).

The interactions of Wnt and Shh are most well documented in the dorso-ventral formation and patterning of the neural tube in early vertebrate development. As Wnt signaling decreases and Shh signaling increases, the likelihood of inducing a more ventral neuronal subtype increases. The reverse, increased Wnt and decreased Shh, increases the likelihood of a more dorsal identity precursor formation. Wnt1 and Wnt3a are the most readily found emanating from the most dorsal portion of the neural tube and induce dorsal identities while inhibiting ventral ones. Since Wnt1/Wnt3a act specifically through the canonical pathway, it is most likely that the canonical pathway is most active in competing with the Shh pathway (Ulloa & Martí, 2010). The canonical

pathway is at least partially dependent on and is likely to directly control Gli3's inhibitory activity of Shh signaling. Gli3 is a transcription factor expressed endogenously in a gradient and has a significant inhibitory effect on Shh signaling (Alvarez-Medina et al., 2008).

Wnt1 has the ability to form a loop with LIM homeobox transcription factor 1, alpha (Lmx1) to assist in establishing the identity of dopaminergic neuronal precursors through antagonism of the Hedgehog (Hh) signaling pathway (L. Chen, 2013). Wnt signaling has been demonstrated to induce the generation of dorsal subtypes of neurons (Ulloa & Martí, 2010). This action is in competition with the Hh pathway's ventral influence. The transforming growth factor β (TGF-β) family of signaling pathways interacts with and can be inhibited by targets of the Wnt signaling pathway. Some aspects of the TGF-β signaling pathway can regulated by Wnt-activated Ca^{2+} calmodulin-dependent protein kinase II (CamKII) (Wicks et al., 2000). SMAD2 is a substrate for CamKII and it has been shown to induce phosphorylation of SMAD2, SMAD4, and slightly induce SMAD3 in vivo. It was also seen to block nuclear accumulation of SMAD2 and could induce oligomerization of SMAD2 and SMAD4 without TGF-β receptor activation. This actually prevented the action of the TGF-β pathway.

BMPs are a type of TGF-β, but work on different receptors that also utilize SMADs for second messenger/effector function but lead to different biological responses. Initially, BMPs were known for their inhibition of differentiation. In conjugation with LIF, their activation of the inhibitor of differentiation protein (Id) was used to retain pluripotency in feeder-layer grown stem cells (Q. L. Ying et al., 2003). Total inhibition of BMP4 by SMAD4 knockout models by Tropepe et al. (2001) resulted in a 3-4-fold increase in neurally differentiated colonies in vitro. Though this suggests that TGF-β signaling is not integral in neuronal differentiation, BMP proteins have been shown to alter the response of neural progenitor cells to Shh in vitro compared to an unaltered response under the same conditions. This signaling results in a dorsal shift in the phenotypic

identity of the cell types generated for both precursors and neuronal subtypes. Additionally, when Follistatin binds to BMPs and blocks their signaling, neural cells become sensitized to Shh signaling (Briscoe & Ericson, 2001).

BMP7 has been shown to counteract glutamate overexposure inhibited dendrite development and enhance dendrite output from cortical and hippocampal neurons in vitro (Esquenazi et al., 2002). Though BMP is expressed endogenously in P19s, media containing 10% serum can prevent differentiation even when endogenous BMP is suppressed using SMAD6 (Tropepe et al., 2001; Wang et al., 2006; Q. L. Ying et al., 2003; Q.-L. Ying et al., 2003). Therefore, it is logical to predict that when there is little serum present in media, there is a higher likelihood that cells will differentiate into neural lineages when neural morphogens are present. This theory is supported by a number of sources and reduction of FBS has been used as a technique to induce neuronal cell lineages (Y. Hu et al., 2013; Wang et al., 2006; Q. L. Ying et al., 2003). P19s, like other NPC producing cell lines, have shown to be highly susceptible to environmental changes; a chemical cue results in a transient phenotypic change that recedes when there is another dominating chemical cue (Wang et al., 2006; Q. L. Ying et al., 2003).

An inhibitor for BMP is the ligand Noggin. Noggin acts alongside chordin by binding in the extracellular space to bone morphogenic proteins (BMPs), potent inhibitors of neural differentiation. It is in the class of ligand binding traps, which act by blocking the ligand's binding regions rendering it unable to access the membrane receptors. Noggin forms a complex with BMP7 specifically (Shi & Massagué, 2003). Though glutamate is the primary excitatory neurotransmitter, it has additional roles in dendritic development. This includes regulation of outgrowth, branching, and spine formation. N-methyl-D-aspartate (NMDA) receptors are ionotropic glutamate receptors and have been found to mediate excess glutamate-induced reduction in dendrite growth in young neurons (Esquenazi et al., 2002). These receptors are sensitive to changes in extracellular Mg^{2+}

concentration as Mg^{2+} is an antagonist of the NMDA receptor in a voltage-dependent manner (Zeevalk & Nicklas, 1992). A common neuronal growth media includes B27 supplement to replace the presence of serum. However, B27 supplemented NBA contains 0.8 mM Mg^{2+}, which blockades the dendritic growth regulating properties of NMDA (Hwei Ling Khor, 2007; Zeevalk & Nicklas, 1992).

FGF can act through phosphorylating the linker regions of SMAD1, inhibiting the BMP nuclear translocation that prevents differentiation (Wang et al., 2006). The traditional LIF-dependent pluripotency model has been challenged by the reported role of FGF4 autocrine signaling. FGF4 was required to allow the cell to exit from the state of proliferation into a differentiated fate (Lanner & Rossant, 2010). FGF's involvement in coordination of gene expression during neuronal differentiation appears to occur by way of the Ras/Erk (also known as Mek/Erk or Mapk/Erk) signaling pathway in P19s (Wang et al., 2006). It doesn't activate PI3K or PLCγ signaling during neuronal differentiation (Lanner & Rossant, 2010). Though murine cell lines exhibit differential dependency on FGF signaling, P19 cells seem to be dependent on it for neuronal differentiation (Lanner & Rossant, 2010; Wang et al., 2006).

In conjunction with FGF4, Wang et al. found that FGF8 was necessary for the initial differentiation of P19 neuronal cultures. Its initial upregulated expression leads to the upregulation and maintenance of the FGF4 signal while the FGF8 signal decreases. This was true when differentiation was done using aggregation and embryoid bodies (EB), but when Wang et al. attempted differentiation in monolayer, they saw no initial surge of FGF8 and no significant differentiation. However, when FGF8 was overexpressed in P19s neural growth occurred, suggesting a dependence on FGF signaling for differentiation (Wang et al., 2006). It has also been reported that FGF4 activation of the Ras/Erk pathway is required for neuronal differentiation. The relationship of FGF8 and RA is that of a cyclic inhibition and promotion cycle. As described above,

it plays both an inhibitory and a promotional role in RA signaling alongside Wnt signaling. FGF8 antagonism of RA along the border of their gradient expressions is a significant component of the initial transition away from the non-neural fate determined by FGF8 (Duester, 2013). This stimulates differentiation of neural cells as RA simultaneously inhibits FGF8 signaling (Zhao & Duester, 2009).

vi. Retinoic acid differentiation

Retinoic acid is a retinol (vitamin A) derivative that is heavily involved in chordate embryoid development (Allenby et al., 1993; Duester, 2008, 2013). RA is derived from retinol through oxidation by alcohol dehydrogenases to retinaldehyde and then converted by retinaldehyde dehydrogenases to the most commonly used all trans isoform, referred to herein as RA (Y. Chen & Reese, 2011). Essential in the regulation of over 500 protein coding genes plus more potential non-coding RNAs, RA is involved in the symmetrical development of somites, in patterning the hindbrain, and in specification of motoneuron fate in the CNS, forelimb, cardiac tissue, as well as a number of other developmental processes (Y. Chen & Reese, 2011; Duester, 2008).

RA is unlike many major signaling pathways in that it interacts directly with DNA. It is a small lipophilic molecule that acts by binding to a heterodimer of nuclear retinoic acid receptors (RARα, RARβ, RARγ) and retinoid x receptors (RXR) and affecting DNA transcription. In the nucleus, cellular RA binding protein-II (CRABP-II) is a protein that assists RA mobility. This protein transports RA to RARs to then undergo its transcription modifying purpose (S. Yu et al., 2012). Even though RA binds directly to RAR, the heterodimer is required to bind to and modify DNA. It regulates transcription by entering the nucleus and acting as a ligand for the RAR/RXR heterodimer. This complex then binds target genes at a specific sequence deemed the RA response element (RARE). However, the RXR component cannot bind trans RA; it can only bind 9-cis-RA

directly, which suggests that RXR's primary function may be to act as a scaffold protein. RA is found endogenously in chordates at a concentration of anywhere from 1 nM to 100 nM (Duester, 2013) and can induce a neural terminally differentiated state in vitro at concentrations above 100 nM (Cai & Grabel, 2007; Duester, 2008; Schneider et al., 2009).

There are several pathways and cascades that have been implicated in assisting P19 neuronal differentiation, with or without RA. The general mechanisms for retinoic acid-mediated signaling are fairly well established (Duester, 2008, 2013), but there is still ambiguity determining the specific neuronal phenotypes produced by RA signaling in various cell lines. RA exists at highly variable levels in various tissues and acts by paracrine signaling endogenously. Depending on the cell type exposed to RA, there could be a forced neural differentiation from cells that are not neurally determined (Duester, 2013). The most common theory appears that P19s are CNS-specific (Bain et al., 1994; Hwei Ling Khor, 2007; Jones-Villeneuve et al., 1982, 1983; Monzo et al., 2012; Rossant & McBurney, 1982).

Initially, in P19s, Jones-Villeneuve et al. reported that RA induced differentiation does not inhibit the production of non-neural cell types. Rather, it promotes the differentiation of neural subtypes like neurons and glia (Jones-Villeneuve et al., 1982). More recently, the default state of undifferentiated cells has been suggested to be a neurally differentiated state. The main inhibitor of reaching this state is BMP signaling as cells become more specified into endo-, ecto-, or mesoderm (Pera et al., 2014). Given this, RA appears to act in a permissive or instructive manner depending on its spatiotemporal expression (Duester, 2013). It is not required for neuronal differentiation and is not even present in embryonic development until E7.5, well after induction of the forebrain and midbrain neuroectoderm. By E8.5, RA seems to be crucial in development of the hindbrain through its target family, the 3'-Hox genes (Duester, 2008, 2013; Sirbu et al., 2005). These Hox genes play an essential role in the development of the rhombomere. Even in adults, RA is important for

maintenance of essential systems like development and maintenance of alveoli, regulation of adipogenesis, and obesity (Y. Chen & Reese, 2011).

RA-induced neuronal populations have been reported to induce the production of inhibitory neurotransmitter gamma-aminobutyric acid (GABA), but whether it does this through indirect or direct regulation is not currently known (Duester, 2013). Some of the direct targets of RA signaling through the RAR/RXR heterodimer are Wnt-1, Hoxa1, Hoxb2, and sox6. Indirect targets include Mash1, Ngn1, neuroD, N-Cadherin, and Pbx (S. Yu et al., 2012). Initially, it was proposed that RA differentiates P19 cells into neural cells with high acetylcholinesterase activity (Jones-Villeneuve et al., 1982). Notch signaling is involved in asymmetric division of progenitor cells. Asymmetric division is when a cell divides and one retains mitotic ability and the other terminally differentiates. There has been some thought that RA is a neurogenic factor involved in this transfer from purely mitotic to differentiated, but mouse ES cells can be terminally differentiated into cortical and forebrain neurons without the additions of RA (Duester, 2013).

Shimozono et al. (2013) allowed for visualization of the gradient expression of RA in zebrafish embryos through specialized fluorescent markers. They observed that RA is expressed most strongly in the mid trunk, where retinaldehyde-2 (Raldh2) expression is greatest, and the RA signal tapered off at both the anterior and posterior ends, where retinoic acid (RA) 4-hydroxylase (cyp26) is most strongly expressed (Shimozono et al., 2013). This lead to further elucidation of the RA mediated AP axis patterning and neuronal induction. Diffusion of RA through the anterior trunk mesoderm peaks at the boundary between the hindbrain and spinal cord (Pera et al., 2014). In an excellent review, Pera et al. (2014) go into depth of the pathway interactions involved in early neural development. The end result they present is that the main actors in RA signal modulation are Wnt signaling pathways and FGF signaling pathways. These two signals contribute to a maintained pool of naïve axial stem cells in the caudal epiblast. These cells are influenced by

FGF8 and various Wnts to arrive at their terminally differentiated state. FGF signal represses the all-trans RA producing Raldh2 expression in the paraxial mesoderm while it simultaneously promotes the RA degrading Cyp26 in the caudal epiblast. This signaling protects the tail end of the developing spinal cord. FGF8, while partially directing the RA gradient, also stimulates the expression of Wnt8. Wnt8, in turn maintains Raldh2 expression. This increases the amount of RA in the system, which in turn inhibits FGF8 and Wnt8 and promotes the differentiation of neural cell types and somite development (Pera et al., 2014).

There has been recent work elucidating a non-canonical RA signaling pathway involving the peroxisome proliferator-activated receptor β/δ (PPAR β/δ) (Schug et al., 2007). The cognate lipid binding protein in this pathway is the fatty acid-binding protein type 5 (FABP5), which plays the role of CRABP-II. This pathway can outcompete the canonical RAR pathway through an imbalance in the ratio of lipid binding proteins CRABP-II and FABP5. While both of these pathways have been suggested to be involved in neuronal differentiation, Yu et al. (2012) propose that the RAR/CRABP-II role is to initiate early differentiation. PPAR β/δ and FABP5 are proposed to instruct neuronal precursors and immature neurons to develop into mature neuronal cells. The transition from the RAR/CRABP-II pathway to the PPAR β/δ/FABP5 pathway is triggered by a change in the ratio of the lipid binding proteins in the nucleus. When the ratio between CRABP-II/FABP5 is in favor of CRABP-II, the RA pathway predominates and vice versa (S. Yu et al., 2012).

vii. Prior differentiation methods

The ability of P19 EC cells to differentiate into neurons was first documented by Jones-Villeneuve et al. (1982) and since then, their methods have been slightly modified but consistently used by most literature (Hamada-Kanazawa et al., 2004; Jin et al., 2009;

Jones-Villeneuve et al., 1982, 1983; Sharma & Notter, 1988; Tang et al., 2002). Undifferentiated cells are grown in MEMα containing 10% FBS and pen/strep. They wash with Ca^{2+} and Mg^{2+} free PBS containing 0.25% trypsin in 1mM EDTA to detach the cells from the plate prior to plating at a density of 10^5 cells/ml in bacteriological grade petri dishes to induce spontaneous aggregation. The aggregates were then treated with 10^{-6} M RA and plated on cell culture dishes 2-3 days after aggregation. Some methods use different buffers and dissociation enzymes (Finley et al., 1996), some provided different cues for differentiation (Lin et al., 2012; Ostrakhovitch, Byers, O'Neil, & Semenikhin, 2012; Tang et al., 2002), while others used N2 serum free (DMEM/F12) media supplemented with 5 µg/ml insulin, 50 µg/ml human transferrin, 20 nM progesterone, 60 µM putresine, and 30 nM sodium selenite, and1 µg/ml fibronectin (Pacherník et al., 2002; Tang et al., 2002).[BC8]

The idea behind the aggregation was to use the natural tendency for EC cells to differentiate in monolayer at very high concentrations and be able to manipulate it as needed to produce the desired differentiation effect (Jones-Villeneuve et al., 1982, 1983; Pacherník et al., 2002). However, this does not specify for homogenous differentiation to homogenous neural differentiation, merely promotes induction of neural cells of many different subtypes (Jones-Villeneuve et al., 1982). This aggregation method was used as the primary differentiation method for P19s for decades (Bain et al., 1994; Monzo et al., 2012). RA treatment of P19 EC cells grown in monolayer rather than aggregates had been previously thought to only stimulate the formation of endodermal and mesodermal derivatives (Wang et al., 2006) and have neuronal cells only in very small quantities or to be wholly impossible due to the assumed three-dimensional structure of the resulting neural network (Bain et al., 1994; Hwei Ling Khor, 2007; Jones-Villeneuve et al., 1982; Tang et al., 2002).

Boudjelal et al. (1997) were able to differentiate P19s in monolayer using the

overexpression of Stra13 (stimulated by retinoic acid 13) in conjunction with RA, though they did not report any success with neural differentiation without the overexpression of stra13. Dong et al. (2012) overexpressed FoxA1 in P19 cells to induce differentiation. These FoxA1 overexpressing cells had characteristics of neural stem cells without RA and were able to differentiate much more rapidly compared to wt cells upon addition of RA (D. Dong et al., 2012). Hwei Ling Khor (2007) reported that unmodified P19-derived neurons were unable to differentiate in monolayer and it has been repeatedly reported that only fibroblast-like cells were able to be derived from monolayer neural differentiation (Jones-Villeneuve et al., 1982, 1983; Sharma & Notter, 1988). Pacherník et al. (2002), 20 years after Jones-Villeneuve et al.'s initial findings, delved into monolayer neural differentiation with RA using unmodified mouse ES cell lines D3, C3H, and F1. Though they did not use P19s, work done by Monzo et al. (2012) used P19s and differentiated in a monolayer through a highly efficient set of methods with significant longevity. The benefit of a monolayer differentiation as opposed to the previously used aggregation technique is that the neurons develop a more two-dimensional network when matured. They are also easier to visualize during the neurogenesis period, which gives insight into the growth patterns inherent in the induced system (Q. L. Ying et al., 2003). Otherwise, they retain some of the aggregate tendency, which prevents as effective electrophysiological analysis of an isolated set of neurons. This two-dimensional structure also simplifies the interactions between the neurons, allowing for a more efficient differentiation process (Monzo et al., 2012). Chen & Reese (2011) state that they experimented on monolayer cultures, but did not maintain neural cultures.

Monzo et al. (2012) published a monolayer differentiation method, which details a multistep process using two different medias and multiple rounds of additives on matrigel plates to reach their fairly spectacular results. Initially, the undifferentiated cells are passaged in MEMα containing 10% FBS with 100mM penicillin, 0.1 M streptomycin, and 2 mM L-glutamine. For

differentiation, they were washed and split using trypsin EDTA, spun down, and resuspended in MEMα with 2.5% FBS and differentiated using 10^{-7} M RA at a density of $6x10^4$ cells/cm^2. For post differentiation treatment, the cells were split using trypsin-EDTA and resuspended in MEMα with 2.5% FBS, washed again and resuspended in supplement-free neurobasal A medium (NBA), before being plated at a concentration of $9x10^4$ cells/cm^2 in NBA with 1x N2 supplement and 2mM GlutaMAX on matrigel plates with a 1:45 dilution of matrigel to NBA. A 20 day media regiment followed; for the first five days after the secondary plating, they were maintained in the NBA with N2 and GlutaMAX; the sixth to the tenth day, they were changed to NBA containing 2mM GlutaMAX, 1x B27 supplement, 8μM Cytosine β-D-arabinofuranoside (AraC; Sigma), 8nM 2' Deoxycytidine (2dCTD); finally, they were changed to NBA containing 2mM GlutaMAX and 1x B27 supplement. They report that this regiment resulted in neuronal networks that were maintained for up to four weeks without being overgrown by undifferentiated or glial cells.

viii. Non-RA differentiation of P19s

Though RA has been used consistently to induce differentiation to neural cell types, there are numerous methods that differentiate P19s to neuronal cell types without the use of RA. Tang et al. (2002) used overexpression of N-cadherin and subsequently, Wnt-1 to induce neuronal differentiation through the Wnt-1 pathway. This produced cells that expressed neurofilaments and microtubule associated protein 2 (MAP2), but no glial fibrillar acidic protein (GFAP). This resulted in upregulation of basic helix-loop-helix genes such as Mash1 and Ngn1 (Tang et al., 2002). Hamada-Kanazawa et al. constitutively overexpressed sox6, which directed neural differentiation and aggregation without the use of RA. Wang et al. (2006) were able to accomplish the same through overexpression of FGF8 in the absence of RA.

There have been documented correlations between overexpression of neural bHLH proteins

and neural induction in P19s. Using transient transfection of naïve P19s with neural bHLH proteins like MASH1, ngn1, neuroD2, etc. stable neuronal differentiation was observed. This effect, however, seems to be limited in its efficacy to P19 cells, as transient transfection did not produce lasting significant neural growth otherwise. This suggests that there is an interaction present in P19s that is promoting this differentiation, or that there is one absent that is allowing it to proceed (Farah et al., 2000).

ix. Genetic indicators of differentiation

Neural progenitor cell (NPC) indicators

Mammalian sex determining (Sry)-related HMG box (Sox) genes serve a broad set of functions in neurodevelopment (Hamada-Kanazawa et al., 2004). They are characterized by the presence of a DNA binding domain in the high mobility group (HMG) box. Several of the family members are expressed in only the CNS, including Sox1, Sox2, Sox3, Sox6, Sox9, and Sox11. Sox2 and Sox3 are expressed mainly in the earlier developmental period of the CNS and Sox2 is often used as a genetic marker for neural progenitor cells as it is downregulated once differentiation has begun (Pevny & Nicolis, 2010). Sox3 is also a regulator of embryonic development and helps determine cell fate, it is mainly expressed in the developing CNS. Sox2 is a transcription factor that is involved in stem cell maintenance in the CNS. Its expression is initially restricted to neural plate cells (Wilson, Graziano, Harland, Jessell, & Edlund, 2000) and is most prevalent in neuronal precursors and undifferentiated cells, but its expression fades as neuronal cells mature (Cavallaro et al., 2008). However, in sox2 deficient cultures, there was an observed lack of markers for mature neurons (Pevny & Nicolis, 2010).

Sox6 has a more significant role later on in embryonic development, though it has been

more closely associated with maintaining normal muscle function. However, overexpressing Sox6 has been found to induce MAP2 expression and EB aggregation in the absence of RA. Several genes have been implicated in this process, including Mash-1, N-cadherin, MAP2, and nestin. Interestingly, in Sox6 upregulated P19s, there was an increased expression E-cadherin, whose expression precedes N-Cadherin's expression, compared to wt P19s. However, the expression of N-cadherin in the Sox6 upregulated cells was approximately half that of the wt P19 cells differentiated with RA. Sox6 is necessary for normal development of the CNS, its expression is expressed in initial nervous systems in embryo (Hamada-Kanazawa et al., 2004).

Neuronal indicators

β-III tubulin is predominately a neural microtubule indicator (Qiang et al., 2014; Wang et al., 2006). Calb1 (calbindin) is a vitamin D-dependent calcium-binding protein that is found mainly in neuroendocrine cells in the cerebellum while the highly similar protein Calb2 (calretinin) is involved in signaling and modulation of neuronal excitability. N-cadherin is a calcium dependent cell-cell adhesion glycoprotein. In some CNS synapses, it acts to anchor the pre and postsynaptic terminals (Tang et al., 2002). NeuN/Fox3 is a neuronal nuclear antigen that is commonly used as a neuronal indicator (Eriksson et al., 1998; Monzo et al., 2012; Pevny & Nicolis, 2010). Shh is a marker of the ventral neural tube, AP limb axis, and the ventral somites (Duester, 2013) Stra13 is an indicator of the uptake of RA, has also been implicated in kinetochore structure maintenance. It has been proposed to promote neuronal differentiation over a mesodermal cell fate (Boudjelal et al., 1997). ChAT (choline O-acetyltransferase) is a characteristic of cholinergic neurons (Jones-Villeneuve et al., 1982, 1983). EGFR is a cell surface protein and its activation leads to cell proliferation. Its overexpression could induce a transition from neuronal fate to glial fate (Mckay, 1997). It's expression in neurons is not yet fully understood, but it has been found in adult cortical,

cerebellar, and hippocampal cells (H. Chen, Liu, & Neufeld, 2007)[BC9]

Synaptic indicators

Cacna1a is a voltage gated Ca^{2+} channel that is part of the presynaptic control of neurotransmitter vesicle release. Scn2a is a voltage-gated Na^{+} channel that is responsible for generation and propagation of action potentials in neurons and muscles. Syp (synaptophysin) is a membrane protein present in synaptic vesicles and Stx1a (syntaxin 1a) encodes a protein thought to be involved in presynaptic vesicle docking.

IV. Methods

i. Cell culture and differentiation

The protocol developed for this study is referred to hereafter as the MEM protocol. The naïve P19 cells were grown in MEMα (HyClone) containing 5% FBS, L-glutamine, and pen/strep and passaged every 2-3 days or at 75-85% confluence on 35mm uncoated cell culture dishes using 0.05% trypsin EDTA. Initially, to differentiate, the cells were split 1:15 into uncoated plates in MEMα containing 1% FBS (Y. Hu et al., 2013), L-glutamine, and pen/strep, with 1μM RA added to the plate and would morphologically resemble neural cells within the first four days. After 48 hours, RA was no longer added and the cells would terminally differentiate into neuronal cells before being overrun with undifferentiated and epithelial-like cells after approximately 12-15 days.

The MEM protocol was run in parallel to the one described in Monzo et al. (2012). Their protocol will be referred to as the Monzo protocol and is described previously. The only exception to the Monzo protocol is that the cells were split 1:15 at 70-80% confluence in parallel to the MEM protocol. The cells were processed with Trizol reagent or fixed and stained at days 1, 5, 10, 15, and

20 post initial differentiation with RA.

ii. Immunohistochemistry

Cells were fixed using 4% paraformaldehyde diluted in PBS (phosphate buffered saline) for half an hour, then permeabalized with 0.15% Triton X-100 for 5 minutes, washed 2x with PBS-T (PBS containing 0.1% tween 20), and blocked with 5% BSA in PBS-T for twenty minutes before adding the primary antibody (mouse αTub, GenScript 1:1000; rabbit βIII-tubulin, GenScript 1:500, mouse GFAP 488 conjugate, Cell Signaling tech 1:300) diluted in 1% BSA in PBS-T overnight at 4°C. The secondary antibody was diluted in PBS-T and centrifuged for 10 min at 10,000 x g at 4°C as the cells were washed 3x with PBS-T. The cells were exposed for 30 minutes at room temperature to the secondary antibodies (Alexa Fluor 488 goat anti-mouse 1:1000; Alexa Fluor 555 goat anti-rabbit 1:500, Life Technologies) before being washed 3x and mounted using DAPI fluoromount-G (Southern Biotech).

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iii. PCR

mRNA was extracted using the phenol chloroform method with Trizol reagent (Ambion) (Y. Chen & Reese, 2011; Jin et al., 2009; Tang et al., 2002), and reverse transcription of purified RNA was performed using the superscript II kit (Invitrogen, #11904-018). Gene expression was analyzed by polymerase chain reaction (Applied Biosystems, GeneAmp PCR system 2700) with TUBB which was also used as a normalization factor. There were a total of 27 primer pairs used, shown in Table 1. Primers were ordered from Operon.

V. Results

i. Morphological effects of differentiation

The α-tubulin (αTub) antibody revealed a complex network of neuronal-like cells, whose identity was supported by βIII-tubulin staining. The cells grown in both the Monzo and MEM protocols developed similar temporal expression of morphological features as shown in Figure 2 with one major divergence. This occurred at day10, where the Monzo cells clearly displayed a different neuronal morphology than the MEM cells. Lancaster et al. (2013) recently attested to the ability of pluripotent cells to self-organize into structures, and aggregation has been previously thought to be the only way to induce neuronal phenotype. One of the more common structures in both the MEM and Monzo cells was a globular cluster of cells that stained positively for αTub and β-III tubulin as in the abnormally exaggerated structure in Figure 3. There would frequently be neural projections radiating out from the spheroid structure interacting with others of the same in an undefined manner as seen in Figure 4.

Simultaneously and spontaneously, cells grown in the MEM protocol would not adhere to the uncoated plates and would instead form aggregates akin to embryoid bodies floating in the media. This would occur in cells split from the same source and plated in the same exact conditions as other pates of cells that would stick to the plate and differentiate. They were fed by centrifugation and washed of dead cells each time. These aggregates would, after a period of approximately 7 days, spontaneously adhere to the plate without trypsinization without obvious cue. The data is not included herein.

FIG 2

FIG 3

There were large, flat, undifferentiated cells prominently distributed throughout that have been previously documented in protocols using aggregate EB formation as well (Q.-L. Ying et al., 2003). Interestingly, many of the flat cells stained positively for tubb3. Large domed clumps of cells were a prominent feature of differentiated cells; they were typically surrounded by neuronal projections, confirmed by tubb3 staining, and typically contained neuronal features in the mass. Mitosis was observed by DAPI staining in non-neuronal phenotypic cells, but not in cells that exhibited neuronal phenotypes (Bain et al., 1994). Cursory GFAP staining of MEM protocol cells revealed that only a small number of GFAP positive cells were present even in the presence of large flattened cells. Most of the non-neuronal flattened cells stained for αTub and βIII-tubulin but not GFAP.

FIGURE 4

FIGURE 5

The ability of P19s to differentiate into neuronal phenotypes has been long documented with specific traits (Bain et al., 1994; Jones-Villeneuve et al., 1982, 1983; Monzo et al., 2012; Rossant & McBurney, 1982). One of the most distinct is the presence of long striated projections coming from small circular to ovular nuclei. These striations are clearly visible when stained with αTub, but βIII-tubulin does not appear to retain these striations. The neuronal cells consistently had smaller and more compact nuclei than the flattened cells while still being distinct from the GFAP positive

fibroblast-like cells and their distinct elongated nucleus[BC10]. The neuronal cells appeared to have dendritic projections, though their presence was not confirmed by gene expression in this study. Another trait of the neuronal cells was that they were not witnessed to undergo mitosis.

-functional ionotropic glutamate receptors of both the NMDA and AMPA/kainite types are present – markers of CNS, not expressed in most of the commonly used neuronal cell liens (only other cell line known to express these is Ntera2, also RA-inducible) (Younkin et al., 1993)[BC11]

ii. Gene expression pattern of P19-derived neural cells

Several markers were used to gauge presence of mitotic and pluripotent EC cells after differentiation. Oct4 (Pou5f1) is a key player in embryonic stem cell pluripotency (Hamada-Kanazawa et al., 2004) and Pcna is a cofactor of DNA polymerase delta and is involved in DNA replication. The presence of Oct4 was strongest in the naïve P19s and faded almost entirely by day 10 in both protocols, consistent with previously published data (Q.-L. Ying et al., 2003). The levels of Pcna consistently decreased in the Monzo protocol cells and remained essentially the same in the MEM protocol cells. This indicates that while the pluripotency of the cells was essentially eliminated, the MEM protocol cells retained their proliferative ability throughout while the Monzo cells lost that trait over time.

FIGURE 6

Sox2 expression had opposing trends in the MEM and Monzo protocols. In the MEM cells, the expression increased and then decreased, while in the Monzo cells, the expression decreased and then began increasing again. Sox6 had a consistently higher expression in the MEM cells than

the Monzo cells, with its expression peaking around day 15 in the MEM cells and around day 1 in the Monzo cells.

Nestin is expressed immediately after differentiation around E7.5 in neural progenitor cells (NPC), fades rapidly as neural cells mature past E10.5, and is basically nonexistent post neurogenesis (Jin et al., 2009; Tropepe et al., 2001). As such, it is an important molecular marker for neural initiation. It is a CNS specific enhancer marking NPCs, predominately in the neuroepithelial and radial glia of the neural tube. It is a type of intermediate filament and its expression is followed by the production of neurofilaments and GFAP (Bain et al., 1994). Once the expression of nestin initially fades, it can be only slightly detected in the dentate gyrus of the hippocampus and subependymal zones of the brain and spinal cord (Jin et al., 2009). Nestin regulation and purpose is as of yet unknown, but its interactions with TGFβ1 in proximal tubules of the kidney suggest that it could be involved in the migration of dedifferentiated cells to promote regeneration (Wen et al., 2012). Its expression had an increase before its signal decreased in both the Monzo and MEM protocols, but the timing was staggered. The Monzo protocol cells peaked by day 5 and then decreased to below the level of the control cells, but the MEM protocol cells had a peak around day 10 that retained a higher signal strength by day 20.

FIGURE 7

The observed neuronal phenotype was observed and the gene expressions observed seem to corroborate their identity. The expressions of β-III tubulin, Calb1, Calb2, EGFR, NeuN, N-Cadherin, and Shh were consistently upregulated and their expression reduced as the neuronal morphology appeared and declined. The expression of Stra13 was unusual and though it peaked in the MEM cells as the neuronal phenotype was at its most obvious, there was a consistently high

expression even in the absence of RA. It has been reported that cells that simultaneously express sox2 and β-III tubulin, as is evident in both the MEM and Monzo protocols, indicate a neuroectodermal identity (Lupo et al., 2013)[BC12].

The TGF-β ligand is a member of the TGF-β superfamily that appears to be required to induce expression of Shh expression (Briscoe & Ericson, 2001) and is involved in a number of different processes depending on the cellular context (Massagué, 2012). Its expression did not directly precede the expression of the neuronal phenotype, but followed a similar expressive trend. As discussed previously, Wnt1 and TGF-β are highly involved in the proper development of neuronal cells. In the MEM cells, Wnt1 seemed to be mostly highly upregulated around day 5 and had consistently higher expression than the Monzo cells. Wnt1's expression seemed to preface the upregulation of Shh on day 10 and TGF-β on day 15 and appeared to have a higher expression in the MEM protocol cells.

FIGURE 8

There were some contrasting results in synaptic gene expression. Several genes were analyzed, Cacna1a Scn2a, Syp, Snca, and Stx1a. While the Monzo cells exhibited higher levels of Scn2a than the MEM cells, the opposite was true for Stx1a. Snca is α-synuclein, a component of amyloid precursor. It's involved in presynaptic signaling and seemed to be more upregulated in the MEM cells for a brief period of time than the Monzo cells expressed. Both protocols expressed Syp in a similar pattern, though there were some discrepancies in timing and strength. While the MEM cells exhibited an increase in Cacna1a expression and then the signal faded, the Monzo cells only exhibited increased Cacna1a expression consistently across the trial.

FIGURE 9

Peripheral myelin protein 22 is a common marker of peripheral myelinated axons. It should not be present in a strictly CNS neural population. It is fairly evident that this is not true for both the MEM and the Monzo cells. What is interesting is the pattern of temporal expression. The peak neuronal morphology appeared at day 10 and the majority of the neuronal genes were most upregulated from day 10 to day 15. The expression of pmp22 appears to be precisely the opposite. Its expression is most upregulated on day 1, 24 hours after induction and on day 15 and 20 for the MEM cells. The Monzo cells had more gene repression between days 5 and 15, but the expression of pmp22 reappears on day 20. This could indicate an initial step of neuronal differentiation of the CNS that passes through expression of peripheral nervous system markers.

FIGURE 10

VI. Discussion

Preliminary results suggest a distinct difference in gene expression and growth patterns between monolayer neurons grown in two different ways. The cells grown in the Monzo protocol appeared to be consistently more confluent with neuronal cells, but also appeared to lose neuronal character as rapidly as the MEM protocol cells did. There were many similarities between the two protocols. Although, as previously mentioned, concentration of FBS has been documented to influence induced neuronal differentiation (Y. Hu et al., 2013), there did not appear to be an outstanding difference between the use of 5% and 10% in the naïve undifferentiated culture dishes. The decrease in FBS, from 5% to 1% and from 10% to 2.5% initially, reduced FBS to

approximately 1/5 of the original dosage. In the MEM and Monzo cells, there appeared to be cell death correlated with this abrupt change, but there was no direct analysis of this transition.

Development of NPCs appeared to begin the more obvious differences between the two protocols. The expression of Sox6 and Nestin are consistently higher in the MEM cells than the Monzo cells. The lingering expression of Nestin combined with the continued expression of Pcna could indicate that the MEM cells retained their NPC character for a longer period than the Monzo cells. The interaction of sustained proliferation and neuronal marker upregulation in the MEM cells is a possible indicator of Notch signaling activity. The expression of Sox2 seems to invert between the MEM and Monzo cells. It is at its highest from day 5-15 in the MEM cells and in the control and day1 of the Monzo cells before it fades and then demonstrates an increasing trend at day 20.

Typically, when NPCs retain expression over a long course of time, there is a lack of mature neuronal expression. However, between the two protocols there appears to be a fairly consistent trend of upregulated neuronal character. There was an earlier and more prominent spike in β-III tubulin for the Monzo cells around day 5 than the MEM cells achieved in the documented periods. Given that EGFR can be construed as a marker for more mature neuronal structures (H. Chen et al., 2007), it is likely that the Monzo cells developed a more mature set of neuronal cells. However, with the loss in calcium binding protein activity, there is an indicated loss of synaptic signaling. This is supported by the synaptic gene expressions. The MEM cells appeared to have a higher expression of synaptic activity consistently, which is curious given the consistent decrease in neuronal markers.

There are several ways to build on this preliminary data. One of the most significant would be to repeat the experiment and confirm or reject the trends observed so far. In developing the MEM protocol, several iterations that could prove interesting were tested in a cursory manner.

These included addition of a mitotic inhibitor such as AraC/2dCTD in conjunction and AraC independently as well as use of insulin, transferrin, and selenium (ITS). Since neuronal cells are terminally differentiated and are not prone to mitosis in the neuronal state, this should solely inhibit glial and undifferentiated cell growth. However, the growth of the neuronal network is also negatively affected by the introduction of AraC. Monzo et al. countered this by the addition of 2'-deoxycytidine (2dCTD) (Hwei Ling Khor, 2007; Monzo et al., 2012).Cursory experimentation into these methods revealed that use of ITS supplement in MEMα on uncoated cell culture dishes resulted in almost total cell death. While AraC did not appear to improve cell growth on its own, the combination of AraC/2dCTD appeared to postpone the overgrowth of non-neuronal cells. Additionally, use of Matrigel coating seemed to improve aspects of cell culture growth.

A gradient of FBS concentration was tested initially to determine the optimal condition for neuronal growth while decreasing the concentration of non-neuronal cells in culture. At any concentration higher than 1%, the cells would become overrun with non-neuronal cells much faster while simultaneously expressing fewer cells of neuronal phenotype. An interesting difference would be to see if reducing the percentage of FBS in solution too quickly would have any effect on differentiation. NPCs are highly susceptible to stress and stress induction appears to be a viable way to differentiate or undifferentiate cells. RA concentration, when tested in a gradient from 0.1μM to 5μM appeared to induce the most obvious neuronal expression at a range from 0.5μM to 1μM. This was not confirmed by genetic expression, but by qualitative analysis. How trypsinization affects NPCs is not documented, however, as it is a source of stress, there is a possibility that there is a genetic modification as a result.

A way to improve upon this method would be to confirm the benefits of these conditions by genetic and protein expression. The protein expression levels of these cells remained untested, given the interplay between FGF8, RA, and Wnt8, observing the varying concentrations of these

proteins in a temporal manner would improve the strength of any proposed trends.

An interesting future development would be to use caged RA in the context of optogenetic controlled ligation to incorporate into chimeric in vivo studies of P19 NPC transplantation (Neveu et al., 2008).

VII. Conclusion

The complexity of the CNS demands a model system to study. In the last 30 years, many different variants have been employed to differentiate cells into neuronal cultures. P19 mouse teratocarcinoma cells have been considered an ideal model system (Farah et al., 2000) and their differentiation protocol has been extensively modified since their initial differentiation by Jones-Villeneuve et al. (1982). While there have been several misconceptions of the ability of these cells to differentiate in monolayer, there are several successful protocols differentiating P19s and other stem cells into neural cells in monolayer (Boudjelal et al., 1997; Monzo et al., 2012; Pachernik et al., 2002; Tang et al., 2002; Wang et al., 2006). The efficiency of differentiation and type of cell produced appears to vary based on chemical input, which could be further manipulated to induce a single cell line to express multiple different neuronal subtypes. When comparing the cells grown in the MEM and Monzo protocols, it becomes apparent that while the two protocols produced neuronal differentiation, there is the possibility of a distinct difference between the gene expression patterns.

The hypothesis in question was determining an observable difference in the neuronal population produced by differentiation following two different protocols. Using a novel protocol and the one developed by Monzo et al. (2012), we observed the differentiation of cells over a period of

20 days. In initial immunofluorescent staining and gene expression analysis, a distinct difference between the morphological features and gene expressions of the MEM and Monzo cells is evident. Morphology appears to be highly similar between the cells grown in the two differentiation methods for most of the observed period. The gene expression is where the biggest differences lie. The MEM cells retained a proliferative state consistently across the trial period [BC13] while the Monzo cells lost expression over time. Both protocols resulted in neural progenitor cells, and had similar expressions of neuronal markers. The synaptic markers demonstrated that there is increased presynaptic activity in the MEM cells compared to the Monzo cells. I found that there is likely to be a notable difference between cells of the same type and induced with the same chemical cue when they are differentiated using distinct protocols.

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[BC1]Notch????

[OU2]Probably just get rid of this sentence or are you planning on starting a new

paragraph? I forget if this is something Megan asked for.

[BC3]EXPAND ON THIS WHEN THE OTHER PRIMERS ARRIVE?

[BC4]Necessary?

[BC5]True in 2006, still true?

[BC6]Is this the definition of the decapentaplegic morphogen gradient?

[BC7]Gli3 inhibition?

[BC8]Rewrite this less weirdly

[BC9]Make this less sucky

[BC10]Figure (GFAP-flat)

[BC11]This can't be confirmed until I do the GRM/GRIN stuff

[BC12]Cck, ChAT, GRM1, GRIN1 not yet here (making a separate figure for them because they're all neurotransmitter related)

[BC13]Notch????