KCl-induced depolarization facilitates neuronal differentiation of P19 embryonic carcinoma cells

Terrence Minkyu Yang[2], Michael D. Uhler[3]
Molecular and Behavioral Neuroscience Institute, University of Michigan, Ann Arbor, Michigan

Introduction

Studies of neuronal stem cells have intensified over the past few decades because of the relevance of these cells to the treatment of neurodegenerative diseases such as Parkinsons or Alzheimers diseases. The hope of better understanding the detailed mechanisms of development of the vertebrate nervous system, and the potential discovery of long-term treatments for neurodegenerative diseases has drawn the interest of many scientists. The recent derivations of pluripotent stem cells from fully differentiated cells suggest that even "personalized" stem cells may be available for gene therapy approaches in the future.[1, 2]

Stem cells are not the only cells known to be pluripotent. Embryonic carcinoma cells, like stem cells, have been known for some time to be another pluripotent type of cell, isolated from murine teratocarcinomas.[3] Teratocarcinomas are malignant tumors consisting of different tissue types. These tumors could develop in early mouse embryos that are transplanted from the uterus to ectopic sites.[4] Cell lines derived from teratocarcinoma have many advantages including their ease of culture and selection.

P19 Embryonic Carcinoma as a model for developmental neurobiology studies

The P19 line of embryonic carcinoma cells was originally derived from a teratocarcinoma formed following the transplantation of a 7.5 day-old embryo into mouse testis.[5] The primary tumor of the teratocarcinoma was used to establish undifferentiated stem cells.[6] P19, a specific line of this embryonic carcinoma has been long known to be remarkably stable, with the requirement that they are maintained in exponential growth.[6] These cells possess a euploid male karyotype,[5] and were found to grow continuously in serum-supplemented media.[6]

Early research with the P19 line aimed to induce differentiation in the cells. Retinoic acid (RA) and DMSO were eventually found to be the two main agents that successfully induced differentiation in P19 cells at non-toxic concentrations.[7] For studies pertaining to neuronal development, RA became the choice inducing reagent.

RA was found to produce neuroectoderm-based cell types: neurons and glia.[3] Even maturation of oligodendrocytes specifically occurs under conditions where normal myelination occurs. Development of oligodendrocytes from RA-treated P19 has only been found in neonatal rat brain.[6, 8] In contrast to RA, DMSO has been found to induce non-neuronal cell types. Mesodermal and endodermal cells, especially cardiac and skeletal muscle have been easily identified in DMSO-induced P19 cells.[5, 9]

Interestingly, RA and DMSO induce differentiation via different cellular targets, perhaps suggestive of RA and DMSO's specific abilities to induce differentiation of different cell types. Mutants have been selected that fail in response to one drug and normally differentiate in response to the other.[9] For example, a P19 cell line designated D3 was incapable of muscle development, but was found to differentiate into neurons and glial cells in response to high concentrations of RA. Within six days of initial RA exposure, up to 85% of the cells expressed markers like tetanus toxin binding sites, which are characteristically found in neuronal cells.[7, 10] Non-neuronal cells placed in the same cultures did not bind tetanus toxin.[11]

Following RA application, the fraction of P19-derived neuronal cells declined with time after six days, due to the neurons being post-mitotic, and with non-neuronal cells continuing to proliferate.[6] Survival of the neuronal cells is seemingly dependent on whether or not non-neuronal cells or growth factors are present.[6, 12]

In vivo studies of P19 embryonic carcinoma cell differentiation

In vivo survival of P19-derived neurons has been studied previously, and P19-derived neurons that are placed into a natural anatomical setting have been observed to survive long-term. When transplanted into the mammalian CNS, P19-derived neurons not only survive, but differentiate into morphologically and electrophysiologically mature neurons.[13] RA pre-treated P19 cells that have been grafted into the striatum of an immunosuppressed mouse (whose striatial neurons have been eliminated with an excitatory neurotoxin) survived at least 13 weeks.[6]

P19-derived neurons have also been shown to have electrophysiological activity. Action potentials were induced in these RA-treated P19 grafts by membrane depolarization, with evidence suggesting the presence of voltage-dependent sodium and potassium channels.[6] In older grafts, both depolarizing and hyperpolarizing events were observed.[14] Synaptic potentials in younger grafts were exclusively depolarizing.[14] These observations suggested P19-derived neurons possess similar neuronal properties to those of neurons in the developing forebrain.

Furthermore, P19 neurons have shown early responsiveness to basic neurotransmission. Initial studies of RA-treated P19 revealed the presence of the neurotransmitters acetylcholine and catecholamine as well as the enzymes tyrosine hydroxylase, dopamine beta-hydroxylase and phenyletha-

nolamine N-methyltransferase.[15] Low levels of histofluorescence for endogenous catecholamines were found in these neurons.[15] Further research has revealed that about 60% of these neurons contain GABA, 20% somatostatin, 20% neuropeptide Y.[6, 16] Tyrosine hydroxylase, DOPA decarboxylase, serotonin, calcitonin gene-related peptide, galanin and substance P have also been found in these cells, but at low levels.[16]

Brain slices of grafted RA-treated P19 cells have revealed neurotransmission in vivo. Brains implanted with RA-treated P19 cells, within two weeks, had neurons that had developed responsiveness to the excitatory neurotransmitter glutamate and the inhibitory neurotransmitters gamma-aminobutyric acid (GABA) and glycine. Pharmacological studies suggested the presence GABAA, NMDA and non-NMDA glutamate receptors.[14]

In vitro studies of RA-induced P19 cells

In vitro models of P19-derived neuronal activity were more recently discovered. The inhibitory neurotransmitter GABA was detected in RA-stimulated P19 cells in culture, co-localizing with glutamate in some neuron processes.[17] These neurons, when depolarized, responded by releasing both GABA and glutamate and the same neurons have been identified to express the glutamate receptor subunits GluR1, GluR4 and NMDAR1.[17] Thus, P19-derived neurons were found to mature in culture and form electrically active neural networks involving glutamate and glutamate receptors. The research implications of a P19-derived neuron in vitro model were significant because use of the cell line would allow large amounts of homogenous material for studies of neurogenesis and synaptogenesis.

P19 cells have also been a model of choice due to the ease of genetically manipulating the cells, making them very useful for investigating molecular mechanisms concerning neuronal differentiation.[6] With RA having been the model drug used to study P19 neuronal differentiation and properties for many years, recent studies have revealed that with RA induction, increased expression of neurogenic basic helix-loop-helix (bHLH) transcription factors expression has been observed.[18] Basic helix-loop-helix transcription factors are proteins with a structural motif characterized by two alpha helices connected by a loop. These bHLH proteins are dimeric, with each helix containing basic amino acid residues that facilitate DNA binding.

MASH1 as a transcriptional activator of neuronal differentiation

A particular bHLH transcription factor of interest in recent years has been MASH1 (also called ASCl1). MASH1 is a mammalian homologue of the Drosophila neural determination gene achate-scute complex-like 1. Research suggests that murine P19 cells are a good model system to study this gene.[19] Studies have also shown that MASH1 is expressed in distinct subsets or areas of cells that give rise to only neurons.[20] This conclusion was made based on studies on the mammalian homologue of BarH (MBH1). When expression was analyzed MBH1 revealed a complementary pattern to MASH1 expression in the developing nervous system. Forced expression of MBH1 down-regulated MASH1. This suggested MBH1's role as a potential regular of mammalian bHLH genes, and reinforced MASH1's important role in 00……neuronal differentiation.[20]

It was recently discovered in the Turner laboratory at the University of Michigan that overexpression of MASH1 alone could induce neuronal differentiation. Prior to these studies, MASH1 was known to be required for neuronal differentiation in RA-induced P19 cells. Studies in 1993 revealed that differentiation of P19 cells into neuronal cell types by exposure to retinoic acid resulted in the induction of MASH1 RNA expression.[21] Dr. Turner's laboratory a few years later demonstrated that RA was not necessary for differentiation when used with MASH1. Transient transfection of MASH1 into P19 cells fully resulted in neuronal differentiation,[18] suggesting MASH1's sufficiency as a neuronal activator. Many neuronal proteins seen expressed in P19 cells upon application of RA have been found in MASH1-transfected P19 cells.[18]

Mouse knockouts of MASH1 have revealed its critical role as a regulatory gene in neuronal development and differentiation. Mice that have a single knockout revealed weak but significant neurogenesis,[22] whereas a full knockout of MASH1 had massive neuron depletion.[22] Cells that were normally destined to become neurons formed glial cells, indicative of MASH1's regulatory role in neuronal fate determination.[22] Mice that have been bred with a null allele of the gene die at birth due to breathing and feeding defects.[23] In regards to the breathing defect, previous studies have demonstrated loss of pulmonary neuroendocrine epithelium in MASH1 full knockout mice.[24] MASH1-null mice completely lack neuronal olfactory epithelium (which could describe the feeding defect).[23] Perhaps the most important effect of the null mutant is that the sympathetic, parasympathetic, and enteric ganglia are severely affected in these animals.[23] The massive lack of neuronal generation with a MASH1 null mutant has revealed its necessity to induce neuronal differentiation (and retinal differentiation[25]).

Electrophysiological properties have also been observed in neurons transfected with MASH1.[16] With MASH1 acting as the sole transcriptional activator in differentiating embryonic cells into neuronal cells, MASH1 has been suggested to be sufficient in conferring a neuronal fate on uncommitted mammalian cells.[18] Without the need of RA and cell aggregation to induce neuronal differentiation, the generation of neurons via bHLH transfection has opened up new avenues in studying molecular neurogenesis.

The major benefit of MASH1-induced neuronal differentiation in mouse P19 is its ability to produce neurons void of glial cells, which are present when induced with RA. In studying specifically neurogenesis and/or molecular properties of neurons, MASH1 is especially helpful. The absence of glial-specific proteins in MASH1-transfected P19 cells[18] ensures the sole presence of developing neurons in vitro.

MASH1 transfection studies in embryonic stem cells

MASH1 has in recent years become a popular and powerful transcription factor for embryonic stem cell research. MASH1-transfected embryonic stem cells (ES) have been

shown to express high levels of Nestin mRNA from RT-PCR studies.[26] Nestin is a type VI interfilament protein that is expressed in nerve cells. Presence of Nestin typically implicates the radial growth of an axon. These MASH1-transfected ES cells also began to differentiate much earlier than ES cells treated with retinoic acid, and differentiated into neurons at high efficiency.[26] Also, MASH1 has recently been shown to promote autonomic neurogenesis from neural crest stem cells.[27] The protein is expressed in the mouse ventral region, specifying GABAergic neurons.[28]

MASH1 as a neurogenic bHLH gene product, binds and forms a heterodimer with a bHLH-activating factor E47, and this complex activates gene expression by binding to the E box consesus sequence (CANNTG).[28] It is known that proteins of the regulatory bHLH gene family Hes inhibit neuronal differentiation. Hes1 is a major bHLH repressor gene regulated by Notch that represses MASH1 activity by binding directly to the promoter sequence.[28] The Notch pathway as a whole inhibits neuronal differentiation, maintaining the neural stem cell state. Recently, it was discovered that a unique Hes protein called Hes6 supports MASH1 activity by inhibiting Hes1 activity.[29, 30] The absence of Hes1 consequently results in Notch's failure to inhibit differentiation. Hes6 promotes neurogenesis via MASH1 in a positive feedback loop.[30]

Therapeutic approaches using MASH1 are being developed. In spinal cord injury (SCI), research has found that Nogo Receptor (NgR) plays a critical role for axon growth inhibition in vitro, and inhibition of axonal regeneration and plastic responses post-SCI. MASH1-transfected ES cells have recently been shown to produce spinal motoneuron precursors not expressing NgR via immunoblotting.[31] SCI model mice that were transplanted with MASH1-transfected ES cells received significantly higher scores on motor tests than control SCI mice. By four weeks post-transplantation, the scores indicated that the MASH1-ES transplanted mice had coordination between their hind and fore limbs.[31] The MASH1-ES SCI mice also revealed electrophysiological tests results that nearly reached the level of a sham-operated normal mouse. These motorneuron precursors not only survived in vivo, but also inhibited scarring and gliogenesis at the SCI site. Nestin is a neural stem/immature neural cell marker, and its expression disappeared 4 weeks post-transplantation, suggesting that the graft placed into these mice matured in vivo.

Amidst this exciting research with MASH1 inducing neuronal differentiation, it is particularly interesting then to note that there has yet to be a study concerned with the potential effects that depolarization, a trademark of nervous system functionality, may have on neuronal differentiation. Specifically, there does not seem to be any recent literature concerned with the effects of potassium on MASH1 or any bHLH transcription factors in either embryonic carcinoma or stem cell models. The possible role of depolarization in MASH1-induced neuronal differentiation has not been fully explored. Although no neuronal relationship has been established, an outward-rectifying sensitive potassium current in P19 cells that differentiate into cardiomyocytes has already been recently identified.[32] This paper tests the hypothesis that depolarization increases neuronal differentiation of MASH1-induced P19 cells in culture. Using P19 cells transiently transfected with a P19 expression vector, the studies described here characterized the differentiation of P19 cells in the presence and absence of elevated potassium chloride, which depolarizes neurons by opening voltage sensitive potassium channels. In addition, we also determined whether elevation of intracellular calcium had any effect on the differentiation of the P19 cells using the calcium ionphore, ionomycin.

Methods

P19 Cell Line Growth/Passage. The complete growth media solution (αMEM (Gibco)) contained 10% of a 3:1 calf serum: fetal calf serum mixture and penicillin-streptomycin. P19 cells were continuously passaged in 100 x 20mm tissue culture plates containing 10mL complete αMEM, and maintained in a 37°C incubator at 5% CO2. Cells were passed before confluency using trypsin and diluted 1:10 before plating every two days.

Transfection. Plates of P19 cells at 40-50% confluency were transfected with a mixture reagent of: 1.5mL αMEM, 81μL Mirus Bio Trans-IT®-LT1 lipid transfecting reagent, and 27μg of total DNA containing a mixture of US2MASH1 (18μg), US2GFP (6μg), and US2puro (3μg). Transfection efficiency was determined by scoring green GFP fluorescence on a Nikon *X70 fluorescence microscope.

Puromycin Selection/Cell Dilution. Transfected cells were diluted 1:10 for KCl experiments and 1:30 for ionomycin experiments to avoid cells from overcrowding and layering in the 24-well plate. Between 24-48 hours after transfection, the P19 plates were detached with trypsin, and re-suspended into 10mL αMEM. The 10mL cell mixture was then transferred into a 50mL tube containing 28mL αMEM and mixed via pipetting. 24mL of that mixture was removed, and the remaining 14mL mixture received 24mL αMEM and mixed to make a 1:10 P19 mixture. This process was repeated once more to make a 1:30 P19 cell dilution. 24mL of 1:10 P19 and 1:30 P19 were separately transferred to its own 50mL tube containing 24mL αMEM and 12μL puromycin (desired a 5μg/μL concentration). Tubes were mixed via pipetting. 2mL of the 48mL mixture was then placed into each well of a laminin-coated 24-well culture plate. Cells were exposed to the puromycin for 12 hours in the 37°C incubator, then removed and replaced with 1mL αMEM per well.

24-Well Culture Plate Setup. KCl and ionomycin treatments were performed on the 1:10/1:30 transfected P19 cells at 48, 72, 96, and 120 hours since transfection. Each row (time point) had two control wells, and four experimental wells (i.e. 20mM and 56mM KCl each had two wells per row).

KCl Addition. The 20mM (10μL) and 56mM (28μL) KCl-αMEM solutions (KCL stock is 2 mg/ml) were administered at 48, 72, 96, and 120 hours since transfection. Media and KCl-αMEM were replaced every 24 hours. It is important to note that "96" KCl wells received KCl-αMEM every 24 hours, totaling 4 days' worth of KCl exposure. The "72" treated cells received 3 days' worth, "48" 2 day's worth, and "24" 1 day's worth.

Ionomycin Addition. For the ionomycin treatment, 0.8μL ionomycin (1mg/mL) in 400 αMEM mimicked a 1:500 one-time ionomycin treatment. 4.0μL ionomycin in 400 αMEM mimicked a 1:100 one-time treatment. Experimental wells were exposed to ionomycin for one hour in the 37°C incubator, and then removed. Concentration and exposure time was chosen to avoid conditions that would promote apoptosis (i.e. 1mM ionomycin exposure for four hours). All wells received 1mL αMEM prior to overnight growth in the incubator.
Culture Plate Fixing/Staining. Cell plates were fixed in 4% formaldehyde solution (diluted from 37% formaldehyde with sterile 1X PBS) at 144 hours after transfection, and were subsequently stained with 1:5000 αTUJ1 (primary antibody), and 1:2000 Alexa Fluor 546 (secondary antibody) at room temperature. Fixed samples were placed in a 20°C room when not being stained or having pictures taken.
Photomicrography. All pictures of experiments were taken under the fluorescent microscope using bright field, green light (GFP expression) and red light (TUJ1 expression). Random sampling of GFP-expressing cells was taken at every time point and variable concentration/control, followed by its corresponding TUJ1 picture. KCl: One experiment counting 90-cell samples ("KCl90") was counted and pictured for the KCl experiments.Ionomycin: One experiment entitled "Ca30 (Fig. 2)" counting 30-cell samples per time and concentration was counted and provided.
Counting. Cell counting of the pictures was manually performed using images for GFP and TUJ1 expression. For all experiments excluding the JKCl series, five or six GFP cells were included in each picture and then compared to its corresponding TUJ1 picture. If there were more than five/six GFP cells observed in one picture, five of them were chosen at random prior to comparison to the TUJ1 picture.
Statistical Analysis. Statistical significance was measured by Microsoft Excel® two-sample t-tests assuming unequal variances because sample numbers differed for various conditions. Significance was determined if the two-tailed $p < 0.05$. In the graphs, one and two asterisks were marked to compare between control and KCl concentrations for each time point. Asterisks greater than two in number were used to denote significance between different time points of the same KCl concentration.

Results

Time-dependent KCl-induced depolarization increases MASH1-dependent differentiation

P19 mouse embryo cells were transfected with MASH1 from the US2 plasmid instead of RA, since the formation of glial cells would complicate the counting process (described in Methods). The cell cultures were diluted to 1:10 or 1:30 to minimize cell layering and overlapping (which would complicate the picture taking and cell counting processes). Cells were cultured in a 24-well plate to observe progression of depolarization-induced differentiation over the span of 96 hours. All neurons in culture were identified immunohistochemically by TUJ1, a monoclonal antibody that stains against the neuronal class III β-tubulin.

KCl was initially administered at 48 hours after transfection and was replenished every 24 hours 4 times (i.e. 48, 72, 96, 120 hours since transfection). It is important to note that with this setup, the "96" KCl wells were exposed to 20mM/56mM KCl the longest (96 hours' worth of KCl exposure), and "24" hour KCl wells received the shortest (24 hours' worth of KCl exposure).

Preliminary experiments presented a problem in determining statistical significance, which was attributed to the disparity in the number of cells counted per picture at different KCl concentrations. Instead of counting a maximum of 5 cells per picture to provide consistency in cell count for all pictures, some pictures were counted as high as 10 cells and others as low as 2. The 0mM cells were the most difficult of the three concentrations to find and many more picture sets were taken compared to the 20mM and 56mM concentrations.

Having an uneven distribution of cell counts per field appeared to have an effect on the two-sample t-test assuming unequal variances. Many p-values from the two-sample t-test were either 0.05 or close to 0.05, but could not be considered significant. It was strange to see that a large percentage difference in cell count (i.e. Supplement – JKCl Fig. 3: 96H 0mM and 56mM) would not be statistically significant. It was not unusual to find that if one or two more cells in that specific concentration and time point were TUJ1 positive, the effect would have been considered significant.

Thus, "KCl90" was analyzed in a different manner. Based on observations from preliminary experiments, an evenly distributed number of cells per picture between varying KCl concentrations produced p-values much closer to 0.05 or at 0.05. Hence for the KCl90 data set, five or six GFP-expressing cells were counted per picture set, regardless of how many cells were present in a picture. By keeping the number of picture sets consistent for all concentrations, statistical significance was more accurately determined. The decision to increase the number of cells counted per time per concentration was done to ensure an adequate sample size for statistical analysis.

The KCl90 experiment (**Figure 1**) confirmed KCl's facilitative role in neuronal differentiation in a time-dependent manner. While MASH1 consistently induced neuronal differentiation in 89-90% of the P19 cells, the remaining 10-11% did not differentiate. After 48 hours of exposure to 20mM KCl, there was a significant decrease in non-TUJ1 expressing P19-GFP cells. Percentage of TUJ1-negative neurons decreased about 6.5% with 20mM KCl exposure and 7.7% with 56mM KCl. After 72 hours of 20mM KCl exposure, about 7.7% of the original 10-11% of undifferentiated P19 had differentiated into neurons. With 96 hours of treatment, almost all of the P19 cells were identified as neurons. Statistical significance was also measured between the "96" and "24" hour-long treatment of 20mM KCl, and the "96" or "72" treatment compared to the "24" hour treatment with the 56mM KCl, further implicating KCl's time-dependent facilitative role.

Although not quantified, presence of neurons and axonal length was more pronounced in 20mM and 56mM KCl-induced P19 than the 0mM P19 in a time-dependent manner (**Figure 2**).

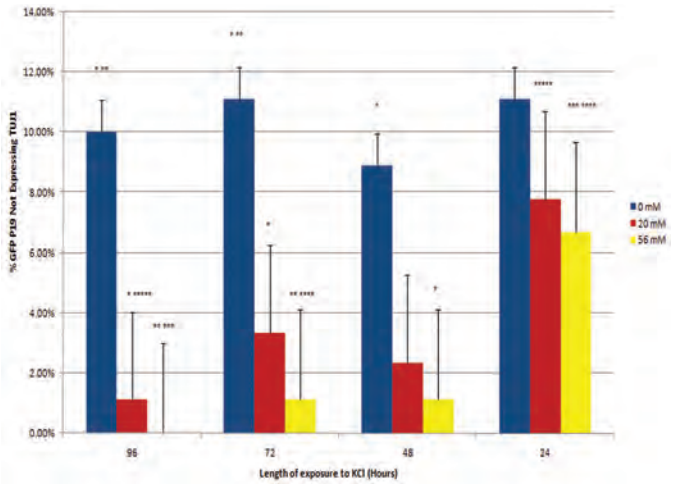

Figure 1: *Exp "KCl90" - GFP-Expressing P19 not expressing TUJ1 (90 Cells/Time/[M] KCl)*
P19 cells were transfected and treated with 0, 20, or 56mM KCl as described in Materials and Methods. GFP fluorescence and TUJ1 staining were determined by fluorescence microscopy. Statistical significance measured at "96" (0mM and 20mM, 0mM and 56 mM), "72" (0mM and 20mM, 0mM and 56mM), and "48" (0mM and 56mM). 20mM KCl treatment produced significant results in P19-GFP cells after 72 hours of exposure to KCl. 56mM KCl treatment produced significant results after 48 hours of exposure. Significance was also measured between different time points of 20mM KCl treatment ("96" to "24"), and 56mM KCl treatment ("96" to "24", "72" to "24").

Ionomycin treatment of P19 cells does not increase neuronal differentiation

Calcium is a major second messenger that changes its concentration during depolarization. Calcium influx into the presynaptic terminal is critical to depolarization and release of neurotransmitter release to the postsynaptic terminal. Investigation of the effects of calcium-induced depolarization also needs to be observed. P19 cultures were set up identically to the KCl experiment prior to experimentation with a different set of experiments complementary to the initial set. Instead of manipulating the cultures with KCl, ionomycin which is a pore-inducing ionophore that allows only calcium ions to seep through the neuronal cell membrane, was used. The calcium influx into the cell is sufficient in inducing sodium influx, depolarization, and neurotransmitter exocytosis. This experiment served as a means of comparison to ensure the importance of voltage-gated potassium channel activity in depolarization-induced differentiation in the P19 cells.

Diluted concentrations of 1:100 (0.01mM) and 1:500 (0.002 mM) from 1mM ionomycin were distributed to the appropriate time well for sixty minutes into the P19 cells and incubated at 37°C. Ionomycin concentrations and exposure time were chosen specifically to ensure pore opening of the P19 cells, but to avoid apoptosis (i.e. 1mM exposure for 1 hour). 30 cell-samples per time per concentration were selected for analysis, with even cell count per picture set.

Ionomycin experiments had a lower percentage of GFP-positive P19 cells throughout the course of each experiment. The exact reason for lower GFP-expression was not determined. However, it was observed that after administering ionomycin to the P19 cells, many of these cells detached from the lamini-coated plate. This was observed particularly at the 0.002mM ionomycin concentration. Hence, a smaller population of cells per time per concentration was available for analysis.

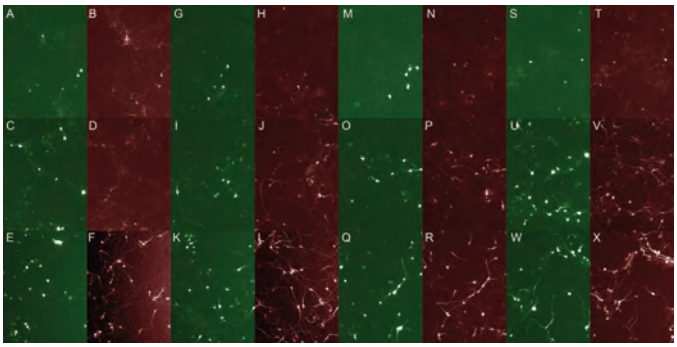

Figure 2: *KCl90 Picture Compilation*
P19 cells were transfected and treated with 0, 20, or 56 mM KCl as described in Materials and Methods. GFP fluorescence and TUJ1 staining were determined by fluorescence microscropy.Each row corresponds to KCl concentrations (Top-Down: 0, 20, 56 mM), and each green-red column set corresponds to time (Left-Right: 24, 48, 72, 96 hours). Pronounced GFP (green)/TUJ1(red) correlation can be readily observed especially in 20mM "72 (O-P)" and "96 (U-V)" hours, and 56mM "48 (K-J)", "72 (Q-R)", and "96 (W-X)" hour-long treatments.

Treatment of P19 cells with the ionophore produced no significant increase or decrease in differentiation in Ca30. The percentage of GFP-expressing P19 cells exposed to ionomycin that did not express TUJ1 was fairly consistent, with small but not statistically significant decreases. Interestingly, more TUJ1 P19 cells were discovered with respect to the control when exposed to 0.002 mM ionomycin at the "72" hour wells. The mechanism responsible for this observation was not determined.

Discussion

Our findings suggest that KCl-induced depolarization may enhance differentiation of P19 cells transiently expressing MASH1, although improvements to the experimental protocol

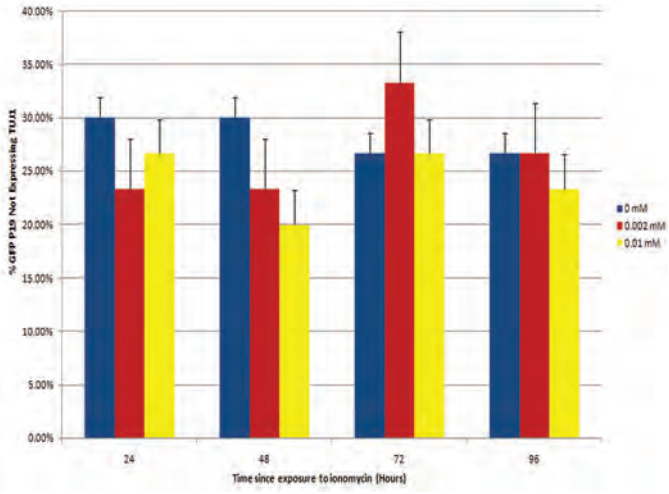

Figure 3: *Exp "Ca30" -GFP-Expressing P19 not expressing TUJ1 (30 Cells/Time/[M] ionomycin)*
P19 cells were transfected and treated with 0, 0.002, or 0.01 mM ionomycin as described in Materials and Methods. GFP fluorescence and TUJ1 staining was determined by fluorescence microscopy. Statistical significance was not observed at any time point treated with 0.002mM or 0.01mM ionomycin for one hour.

may have increased the statistical significance of the results obtained. For instance, there is concern that adding such high levels of KCl to the extracellular media could have undesirable effects on osmotic responses of the cells. The addition of 56mM NaCl solution to untransfected cells could serve as an important control in this respect. Theoretically, the NaCl would have produced the same osmotic effects as the 56mM KCl, but would have not had an effect on the voltage-gated potassium channels. This control was not tested. . With respect to the staining, we could have also measured Ca2+ activity via staining with Fura2 instead of TUJ1. This technique also was not tested on the KCl-affected P19 cells.

Nonetheless, the KCl90 experiment results suggest that membrane-bound voltage-gated potassium channels on P19 cells facilitate MASH1-dependent neuronal differentiation. The ionomycin experiment results suggest that calcium influx into the cell is insufficient to facilitate depolarization, and that a molecular mechanism downstream of the voltage-gated potassium channel may be responsible for the activity seen in the KCl experiments. However, further experiments are required to truly demonstrate that both KCl and ionomycin resulted in changes in intracellular concentration of potassium for the treated P19 cells. If there is another channel downstream that the voltage-gated potassium channel works through, identification of the channel and its relationship to the voltage-gated potassium channels is critical to better understanding depolarization-induced differentiation.

Membrane depolarization has been long known to activate voltage-gated sodium and calcium channels. Neuronal physiological processes from action potentials (voltage-gated sodium) to synaptic transmission, local signaling and processing, and gene expression (voltage-gated calcium) are dependent upon these channels.

In regards to voltage-gated calcium channels, they are critical for calcium influx entry at the axon terminal for proper exocytosis of neurotransmitters into the synaptic cleft via the SNARE complex.[34] The Cav2.1 channel has been shown to be the predominant pathway in rapid release of neurotransmitters such as glutamate, acetylcholine, and GABA.[34] This channel has been coimmunoprecipitated with CamKII, a ubiquitously expressed protein kinase and one of the most important calcium receptors in neurons. The kinase has been found to bind to the C-terminal domain of the Cav2.1 channel and to enhance its activity by slow inactivation and shifting the voltage dependence of inactivation positively.[34] Regulation in situ in skeletal and cardiac muscle cells has been attributed to Cav1.1 and Cav1.2 respectively.[34]

With the understanding that voltage-gated calcium channels are critical to neuron function, it is important in further researching potential candidate voltage-gated channels that act downstream of the membrane-bound voltage-gated potassium channel.

Three candidate genes currently may serve to mediate the effect of KCl in P19 cells. The mRNA expression of Cacna1h, a T-type calcium channel in P19 cells, has been shown to increase nine-fold within twenty-four hours since MASH1 transfection, and ten-fold six days after transfection. T-type calcium channels are low-voltage-activated channels that control neuron excitability and responsiveness under physiological conditions near resting states, and are widely expressed in various types of neurons.[35] These channels have been suggested to be potential therapeutic targets in the nervous system.[35]

The two other candidates are genes that upregulate potassium channels, Kcnkl3 and Kcnc4. These genes have also shown elevated mRNA expression in P19 cells once transfected with MASH1. Within twenty-four hours of transfection, expression of Kcnkl3 has been shown to reach seventeen-fold. Elevated mRNA expression of these three genes has been observed in microarray analysis at Dr. Uhler's laboratory.

Since the mRNA expression of these genes magnify greatly within only a span of twenty-four hours, it raises the question as to whether the potassium effect on P19 cells is actually time-dependent, or needs to be expressed as a specific time point. It would be imperative to also run the same KCl experiment, but make the exposure of KCl equal between all the time points. Observations then must be made to see whether or not the length of axonal processes between the "96/72/48" and the "24" fields replicate the results observed in the KCl90 experiment.

Any, all, or none of these three genes could be involved in P19 signal processing downstream of the KCl-induced depolarization. Verification of the gene's activity downstream of depolarization could be determined by blocking or silencing the channel. Pre-treatment of conotoxin, a neurotoxic peptide that specifically blocks T-type calcium channels, onto P19 cells followed by KCl treatment would determine Cacna1h's role downstream of voltage-gated potassium channels. Gene silencing of each candidate via RNAi silencing technique (short-hair pin constructs ordered from Open BioSystems®) followed by KCl treatment is another method that could be used to evaluate each gene's requirement for depolarization-induced differentiation.

Another avenue of experimental pursuit is observing the importance of KCl in forming and maintaining functional synapses. Previous research has shown that PC12 (primary bovine chromaffin) cells depolarized with elevated K+ levels releases human growth hormone in similar proportions to endogenous catecholamine release.[36] With potassium having been shown to mimic synaptic depolarization,[36] and with the understanding that activation of calcium-sensitive potassium channels occurs at the synaptic level, it is pertinent to investigate the effect of KCl in P19-derived synaptic formation, functionality, and maintenance. This study has only observed the effect of KCl exposure on P19-derived neurons at maximum for four days, and the cells were stained with an antibody that detects neuron cytoskeletal proteins. Long-term effects of KCl on P19 cells (15-30 days) would better suggest its role(s) at the synaptic level.

Studies of P19 embryonic carcinoma cells have significantly advanced our understanding of neuronal development. Much research has yet to be done to better understand the molecular mechanisms underlying depolarization-facilitated neuronal cell differentiation. While the results of the current studies are not conclusive, the finding supports our original hypothesis that depolarization of P19 cells would enhance neuronal differentiation. Additional experiments in the future

to include other markers of neuronal differentiation such as synaptic proteins and electrophysiological measurements of synaptic transmission will be required to conclusively test our hypothesis. However, our findings do provide additional justification for more rigorously testing the role of depolarization in P19 neuronal differentiation. With this relationship established, new therapeutic strategies for neurodegenerative disorders can be discovered and developed.

Acknowledgements

The author primarily thanks Dr. Michael Uhler for sponsoring and mentoring the author, the project and its progress. The author also thanks Holly Huang, Ginger Kubish, and Tanya Morocco-Redmond for their help in teaching the author proper laboratory techniques vital to the success of this project. Finally, the author thanks Dr. Richard I. Hume, who co-sponsored the project and Dr. Haoxing Xu in agreeing to evaluate the thesis.

References

1. Takahashi K., et al., Cell. 2007. **131**(5), 861
2. Okita K., et al., Nature. 2007. **448**(7151),313
3. Jones-Villeneuve, E.M., et al., J Cell Bio, 1982. **94**(2),735
4. Stevens, L.C. Dev. Biol., 1970. **21**(3),364
5. McBurney, M.W., Rogers, B.J., Dev. Biol., 1982. **89**(2),503
6. McBurney, M.W., Dev. Biol, 1993. **37**(1),135
7. Jones-Villeneuve, E.M., et al., Mol. Cell. Bio. 1983. **3**(12),2271
8. Staines, W.A., et al., Neuroscience. 1996. **71**(3),845
9. Edwards, M.K.S. and McBurney, M.W., Mol. Cell. Biol. **3**(12),2280 (1983)
10. McBurney, M.W., et al., J. Neurosci. **8**(3),1063 (1988)
11. Mirsky, R., et al., Brain Res. **148**(1),251 (1978)
12. Schubert, D., et al., Nature **344**(6269),868 (1990)
13. Morassutti, D.J., et al., Neuroscience **58**(4),753 (1994)
14. Magnuson, D.S., et al., Brain Res Dev Brain Res. **90**(1-2-),141(1995)
15. Sharma, S., and Notter, M.F.D. Dev. Bio. **125**(2),246 (1988)
16. Staines, W.A., et al., Neuroscience **58**(4),735 (1994)
17. MacPherson, P.A., et al., Neuroscience **80**(2),487 (1997)
18. Farah, M.H., et al., Development **127**(4),693 (2000)
19. Johnson, J.E. et al., Development **114**(1),75(1992)
20. Saito, T., et al., Dev. Biol. **199**(2),216(1998)
21. Franco del Amo, F., et al., Biochim. Biophys. Acta. **1171**(3),323 (1993)
22. Tomika, K., et al., EMBO J. **19**(20),5460 (2000)
23. Guillemot, F., et al., Cell **75**(3),463 (1993)
24. Ito, T., et al., Development **127**(18),3913 (2000)
25. Tomika, K., et al., Genes Cells **1**(8),765 (1996)
26. Kanda, S., et al., Int J Dev Neurosci. **22**(3),149 (2004)
27. Lo, L., et al., Development **129**(7),1553(2002)
28. Kageyama, R., et al., Exp Cell Res. **306**(2),343(2005)
29. Bae, S., et al., Development **127**(13),2933 (2000)
30. Koyano-Nakagawa, N., et al., Development **127**(19),4203 (2000)
31. Hamada, N., et a., Neurobiol Dis. **22**(3),509 (2006)
32. Sotkis, H.V., et al., Fiziol Zh. **52**(1),49(2006)
33. Garcia, AG., et. al., Physiol Rev. **86**(4),1093(2006)
34. Catterall W.A., et al., J Recept Signal Transduct Res. **26**(56),577 (2006)
35. Shin H.S., et al., Curr Opin Pharmacol. **8**(1),33 (2008)
36. Wick, PF., et al., J Biol Chem. **268**(15):,10983 (1993)