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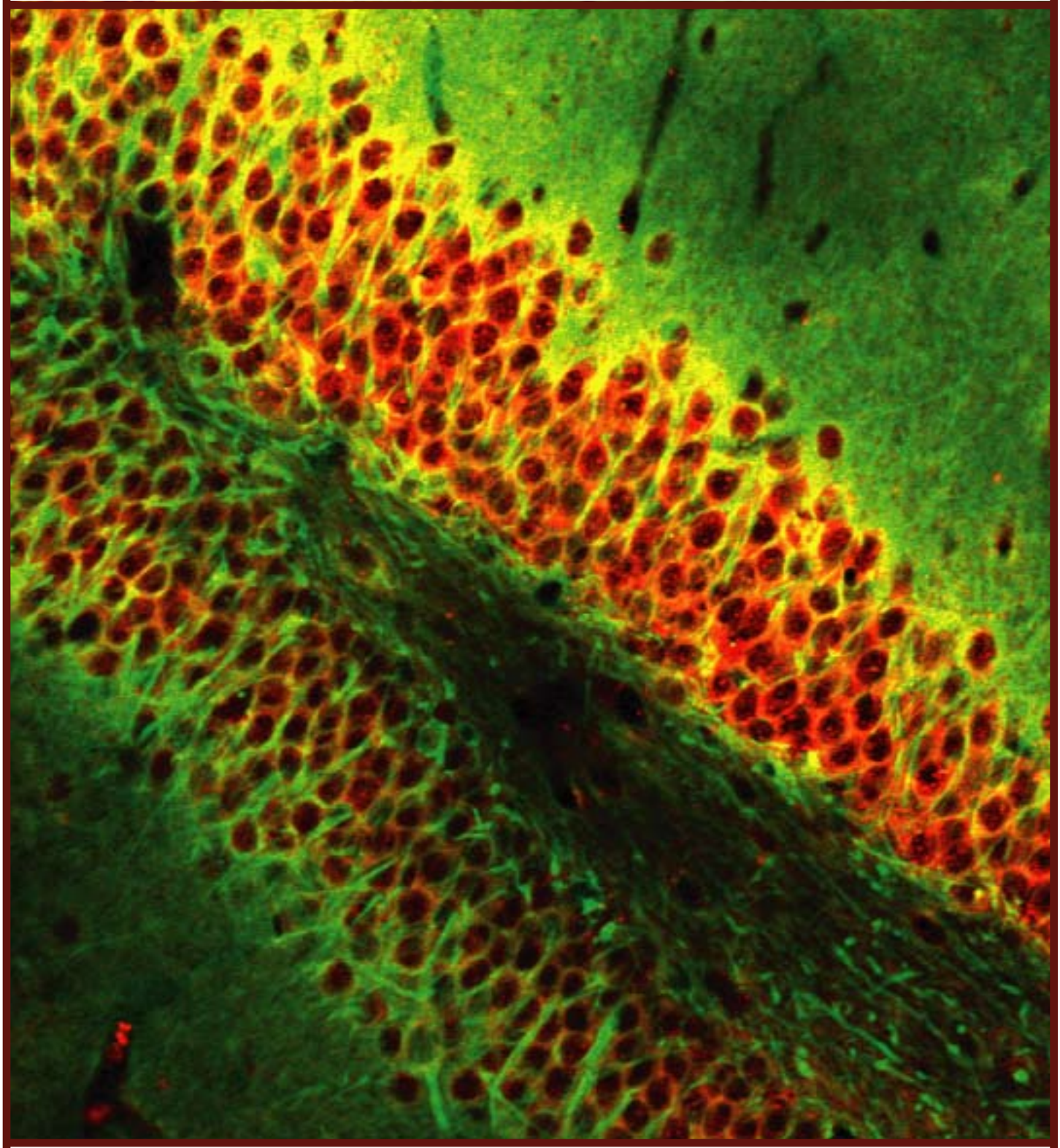
Undergraduate Research Forum



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Green Care and Promoting Environmental Responsibility: Professor DeYoung

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Introduction

Professor Raymond DeYoung is an Assistant Professor in the School of Natural Resources and the Environment at the University of Michigan. He received his Bachelor's degree in Engineering and Master's in Ocean Engineering from the Stevens Institute of Technology in Hoboken, New Jersey. In 1984 he received his PhD in Urban, Technological and Environmental Planning from the University of Michigan.

He is currently teaching courses in the psychology of environmental stewardship, the relationship between behavior and the environment, and the transition to a more localized society. His research interests include environmental planning and conservation behavior. Motivating people to actively practice environmental stewardship is a difficult task. We spoke with Professor De Young about the notion of Green Care, a new approach that aims to promote environmental responsibility from a variety of angles.

What is Green Care?

Green Care is a program that started initially in Europe. What they do in Europe is use agricultural and nature sites, particularly with at-risk people, bring them to nature and have them get directly involved with it. There are farms for people who are depressed or anxious to stay. There are places for mentally or emotionally challenged kids and adults to live. It is being paid for by many of the countries in the European nations-their health insurance covers it. What they have only started doing is the research to back it up. There are lots of programs, and they see lots of positive effects, but they are only starting to figure out what works and what does not, why it works and how they can improve it. One of our colleagues here at UM and a few other people have helped them think about methods, measurements and theories.

But Green Care also is relevant for people who are not at risk for any particular psychological problem. Trying to strengthen everyone's ability to function and be effective in everyday activities is part of what we think of as Green Care. It is easy to imagine that adapting to climate disruption, adopting a simple and local lifestyle in a post-carbon world, and dealing with various social issues will mentally challenge and fatigue people in all countries and in all ages and walks of life. Green self care is a way of trying to strengthen our ability to problem-solve, cooperate and coordinate our responses.

Have corporations integrated Green Care and have there been any incentives for corporations to do so?

I think they are slow to pick up on it. Psychology sometimes gets categorized as a soft, flimsy kind of science. I think it takes someone inside a company "to have seen the light," if you will, to really understand the psychological relationship to nature and to really push it as something in the corporation's self-interest. Left without such a psychological entrepreneur, the corporations have difficulty taking the first step. In contrast, hospitals have been getting involved in this area for a couple of reasons; one is that they themselves realize that patients heal better in the presence of nature. We have considerable research on that. In terms of cancer patients, who are dealing with long term uncertainty, some of the mental fog that has been attributed to chemotherapy is perhaps an attentional fog, a mental fatigue resulting from their struggle to keep their life focused under this prolonged uncertainty. When hospitals realized that nature can help, they became sensitive to how much nature their patients were exposed to, but nature also helps their staff of nurses, doctors, etc. They have begun to talk about having healing gardens in hospitals, having more access to nature through the windows and bringing nature inside hospitals. Here at U of M they have a wonderful meditation garden placed in the midst of the hospital buildings, and they also have the new Cardiovascular Center's (CVC) atrium, the cafeteria that overlooks the Huron River Valley, and a great many walkways with views of nature.

Does it work? There is controversy, a couple of competing theories with one developed here at U of M, and research publications emerging all the time. One recent study done locally (Berman, Jonides & Kaplan (2008) "The cognitive benefits of interacting with nature") was written up in Michigan Today, but while we have a good start on the research, it remains a fascinating question and needs more work.

Nature has begun to be brought into some corporate settings, high tech companies in particular. I think the other part of it is the corporations that allow people to work at home by telecommuting. They have begun to realize that when people are in a more natural home environment and have the flexibility of getting outside, taking their breaks in a backyard or walking their kids to school, they seem to perform better. We cannot be sure that all telecommuters take advantage of nature so this also remains an area needing more research, and we definitely need more people doing this research. Corporations are cautious adopting these notions. This probably seems like an amenity to offer their employees later when they are doing better economically . Right now I doubt that they think of access to nature as an important survival tool, but from our perspective as researchers, if you want effective people, you want a vibrant company. If you want to maintain your competitive advantage, then take care of your employee's mental state; otherwise you're likely to have a bunch of bad decisions being made.

Figure 1: *Cardiovascular Center Atrium (Photo Credit: University of Michigan Health System)*

How do you measure worker satisfaction? Do you use a number scale or do you go through hundreds and hundreds of surveys?

It is a mixture of things. In terms of citizen satisfaction with various aspects of life, there are standardized scales that have been developed. We usually use these standardized scales but adapt them to the client we are working with, the specific population being studied. If we were studying worker satisfaction we might ask a series of questions that are each rated on a scale of, say, one-to-five exploring such things as "Do you find life fulfilling?", "Do you find it enjoyable coming to work?", "Have these been difficult times staying focused on your work?","Do you feel like your personal goals are being achieved?" You take all of those items and subject them to a series of statistical tests called dimensional analysis, which tries to find structure among the answers. The structure is often a series of independent factors that you can use in other parts of the study, perhaps comparing them to worker or workplace characteristics, the amount of nature access individuals have during a typical week, or other social or psychological aspects of their work life.

Another way of doing this sort of study is to look for secondary data: how the company is performing, how a small group of workers is doing within a company, or reports of behavior from teachers, neighbors, or city offices. Some of the studies that are widely cited on the effect of nature on human wellness have explored urban living arrangements. One such series studied low-income housing where access to nature has been shown to reduce violence within families, increase school performance, and increase psychological wellness.

I understand that Green Care can support better lifestyles, encouraging conservation and sustainability. What can the average student do to encourage other students to adopt this Green Care philosophy?

That is a good question for a number of reasons. It touches on a series of related issues. We are a society that has gotten away from norms that support social influence, even social control. It is no longer appropriate for us to strongly influence each other's behavior. The notion of consumer sovereignty that is at the basis of our market-based economic system has morphed into an individual sovereignty at every level of life. We feel entitled to do whatever we want, whenever we want. We are much more likely to reject people telling us what reasonable behavior is. The response is more often, "What right do you have to tell me what to do?" But students are in a situation where they can begin to change that. They can try to encourage others to change their behavior. If students were to buy into this Green Care idea, the idea that attentional fatigue is a problem that affects all of us and has implications on all our well-being, then they might begin to gently nudge each other to spend more time in nature or at least rest their mental mechanism more often.

Green Care, or similar efforts to restore our mental effectiveness, may be essential to creating the kind of communities that we think are valuable and worth living in. A colleague of mine once said that *burned out people can't help restore the planet.* Clearly, it is not enough to just know what you ought to do (i.e., take time in nature to restore your mental abilities). We actually need to act regularly to revitalize our mental capacity. While our society doesn't currently have a norm of time-off for mental restoration, students can change that right here, right now. Environmentally we need to *think local and act local* but to do that we first need to *restore local*.

The Social Characterization of Price: Professor Wherry

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Introduction

Professor Frederick Wherry is an Assistant Professor of Sociology at the University of Michigan. He received his Bachelor's degree in Public Policy Analysis and Creative Writing from the University of North Carolina – Chapel Hill in 1996,his Master's in Public Affairs and PhD in Sociology from Princeton University in 2004. He is currently serving as a faculty associate for the Center for Southeast Asian Studies and faculty fellow at the Yale Center for Cultural Sociology. Professor Wherry's research interests include economic and cultural sociology, using qualitative methods and comparative approaches to study market interactions, consumption, global flows, and production processes.

Professor Wherry has recently published a research article titled "The Social Characterizations of Price: The Fool, the Faithful, the Frivolous, and the Frugal" in Sociological Theory. In the publication, he suggests that prices are not culturally neutral, rather they are set as comparative standards for the purchasers of different products, and he further discusses the various social classifications based on price that differently situated people pay. The "differently situated" refers to the fact that people occupy a different status in society based on their socio-economic status and on their race or ethnicity as well as their gender. Consequently, equal prices may evoke different evaluations of the differently situated people, resulting in the diverse ways that individuals and groups are characterized in market situations.

These characterizations are, as the title of the article indicates, the fool, the faithful, the frivolous, and the frugal. These are based on a person's reaction to price which, to a certain extent, seems to speak of the person's inherent attributes. Wherry assigns the term "calculating" to those who "may accept prices for individual gain or for transcendental ends" and "noncalculating" to those who accept prices due to habituation or yielding to urges. According to his classifications, the fool is noncalculating and makes purchases when one can barely afford it, the frivolous is also noncalculating but has the means to afford it, the frugal is calculating and wishes to save as much as possible, while the faithful is calculating and aims for transcendental ends.

What led you to conduct research on this subject?

I had been reading a lot on ethnographic studies and I noticed that people would often talk about price and it struck me as odd how price suggested people's value and how prices made generalizations about people and society. To be sure, I started to look for other instances where people talked about price and those discussions also included narratives about character and that is what eventually began to emerge. I started digging in the direction of price and its social significance within society. I wanted to make sure that I was not reinventing the wheel and was taking advantage of existing theory in the field.

Did it have anything to do with the current financial crisis?

I actually started drafting the article a couple of years ago and I did my final edits last spring. In 2006 it was already under review, which was before the housing meltdown. After the housing meltdown happened, it struck me that the implications of my findings were dependent on whether homebuyers were characterized as foolish or whether these people were simply victims of larger market forces. Part of what I anticipated in that paper was the notion that we are often not making judgments on whether that was the right price or the right interest rate but it depends, in part, on the judgments made by people in mainstream society about the types of people who would make a decision such as that. So you may have one group that makes a foolish (non-calculating) decision, but relative to the social position he's in, it is not characterized as foolish. Your social position has a lot to do with the way you are being judged.

Do you mean that people of different social classifications may be judged differently even if they spend money the same way?

Yes. Someone spending money on something that is considered to be a very bad investment is going to be judged differently if they are in a higher income, well-educated group than a less educated one. That way, we establish social position using more objective indicators. How they react to prices compared to their mainstream counterparts says a lot about their character in terms of calculating and non-calculating. I even came across in Georg Simmel's *The Philosophy of Money* this notion that the candidates for the head of state should be people who are "rational" with money. A lot of our evaluations of individuals are based on how they deal with money and the prices they pay. Money is considered neutral. It has no race, class, color, ethnicity. Therefore, we use price and money as a neutral arbiter to assess character, but this hides the rather non-neutral basis to which the judgments of price are being put.

How did you determine the four general classifications for consumers?

The classifications came about from an emergent process. I had forty narratives and historical excerpts of people talking

about money. I studied these and used qualitative data analysis to group the words and phrases into clusters according to similar meanings such as rational and irrational calculations. Some of these clusters fell off because there were key words that did not seem to fit anywhere, but I kept adjusting until I had four major groups. I did it this way so that others could check the clusters and bring up new ideas as well as how one might better organize them.

Did you use any quantitative data for your research?

No. All of it was based on qualitative data. I used this program called HypeResearch and it has a function called Hypothesis Tester so once I decided that certain key words would cluster together, I would test the coherence of these clusters across my cases.

I understand that quantitative data might be difficult to obtain with this study. How would quantitative data be helpful?

I think at this stage of the research, qualitative data are more appropriate. For a second phase, what one could do is use some excerpts from actual testimonies and study how people respond to different scenarios of particular purchases and let them describe these transactions. Once we have an idea of the characteristics and reactions of an average person, then we could come up with a set of scenarios and do a widespread testing of that. Gathering qualitative data is good for building and generating testable hypotheses. I hope that there are researchers out there interested in testing this. Knowledge is meant to build, and our conclusions are tentative, but to the extent that my approach is transparent enough so that other researchers can test my theories, I have contributed to the scientific enterprise.

How do you think the mainstream behavior is changing as a result of the economic crisis?

That is a good question and I am always reluctant to make predictions because what I think would be useful to do is to look at discussions people have about making purchases, such as holiday shopping. Behavior may have changed a little as shoppers are more calculating, trying to be more aware of the current crisis regardless of the immediate impact of the crisis on them. This is due to the social reference group to which people want to feel a part of.

Do you have any words of advice or encouragement for undergraduate research?

The best way to learn about research is to go in and get your hands dirty. The beauty of it is to see the pitfalls of doing research and its restrictions because once it arrives in print, it looks pretty straightforward, but often times, research is not. This experience helps you gain more comfort with ambiguity and setbacks. For undergraduate research, the most important thing is to get a feel for the craft of research and learning by doing is always the best. There are many researchers here who are happy to have students come and help. Do it often. Every time you do it you will learn a little.

Of Pathogens and Men: Professor Johannes Foufopoulos

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Introduction

Dr. Johannes Foufopoulos is an Assistant Professor at the University of Michigan. He is currently teaching in the School of Natural Resources and Environment as well as in the Department of Ecology and Evolutionary Biology. He received his Bachelor's degree in Biology from the University of Illinois-Urbana-Champaign and his Master's and PhD in Zoology from the University of Wisconsin-Madison.

His research interests include conservation biology as well as the ecology and evolution of infectious diseases. Along with his research interests in the effects of habitat fragmentation and global climate change on species extinction, he also studies the impact diseases have on wildlife populations and the processes leading to disease emergence.

To better understand the impact of pathogens affecting both wildlife and humans we caught up with Professor Foufopoulos.

Parasites and pathogens differ in their impact on the host. What role does the virulence of a pathogen play in its survival?

What we experience as virulence is basically the rate at which a pathogen/parasite extracts resources from the host and uses them to reproduce or do whatever it needs to do in order to get transmitted. It is a fundamental parasite trait because it determines whether the parasite survives by being transmitted from one host to another. Because multiple pathogen strains generally compete against each other, either inside a single host or within a population of hosts, at first glance it would seem that each parasite should evolve to extract the maximum amount of resources from the host, i.e. evolve the highest possible virulence. The problem is that as virulence rises, the host is likely to die more quickly, meaning that there is a shorter time period available for the pathogen to transmit itself to another host. So, simplifying matters a bit, there are trade-offs between virulence and time available for transmission. Dependent on what this relationship looks like for different transmission modes, there exist different optimal levels of virulence. For example, sexually transmitted pathogens tend to be on average less virulent because if they make their host too sick, he/she will not go out and have sex and as a result the pathogen will not get transmitted.

What appears to have happened in the last few hundred years is that virulence for most human pathogens has decreased. There are several possible explanations for this, including humans just being able to deal better with infection because of improved living conditions. But perhaps the most interesting hypothesis postulates that reduced virulence may be the inadvertent outcome of effective medical treatments. Essentially, each time you choose to go to the doctor and you receive antibiotic treatment, the pathogen population that infected you gets wiped out. When you have different strains that have varying levels of virulence, those patients infected with the most virulent strains are also most likely to go to the doctor, get treated and thus eliminate their infection. So essentially all the truly virulent strains, the ones that really make their host sick, suddenly are at a disadvantage because they are being treated and are not being transmitted anymore. So the only strains that now survive are the ones that do not make you sick enough for you to choose to go to the doctor. Since only the least virulent strains now survive to replicate, the disease as a whole becomes progressively less virulent.

This is one of the interesting unintended evolutionary developments that no one really thought about when they developed drugs. When we first developed antibiotics, the basic idea was that we would be eliminating infection, but what we apparently ended up doing, by applying selective pressure, was to make some pathogens less virulent.

What are humans doing to promote the spread of infectious diseases?

Humans are right now engaged in a broad range of actions that promote disease; perhaps most important among these are particular activities that disrupt the environment, therefore facilitating the emergence of new pathogens. Despite their name these are generally pathogens that are already existent in natural ecosystems. Although sometimes they can be newly evolved pathogens, in general these are organisms that have been around for long periods of time. When we disturb the natural environment we put ourselves in contact with them and suddenly we have an epidemic in humans. If you look at all the new emerging diseases that have hit humanity in the last twenty years or so, almost all of them have been zoonotic. This means that these are pathogens that normally circulate in some kind of animal host population in nature and then were able to switch hosts and are now infecting humans. This has often been the result of our interference with natural ecosystems. AIDS is a good example. HIV, the causative agent of AIDS, is really just "the revenge of the vanquished." HIV entered the human population as the result of the intense hunting of chimpanzees, which are the virus's regular host species. We are currently in the process of cutting down the central African rainforest while at the same time hunting chimps - who are also our closest relatives - to extinction. We are literally eating the species to extinction - there are very few chimps left right now—just a few thousand. They are in precipitous decline and from an ethical perspective, I wonder what that says about

humans— the fact that we are butchering and eating our closest relatives at a time when we have plenty of alternatives. But essentially what happened was that in the process of us eating our way through the chimp population, we contracted one of their pathogens, which was HIV.

Similar things occurred for other zoonotic pathogens. SARS, for example, produced a major outbreak about five years ago when it was introduced into the human population in Chinese wet markets, which are basically wildlife markets where people bring various kinds of live animals for sale. These places are hotbeds for cross-species infection because you have many different species crammed together in tight proximity in truly abhorrent conditions. People just butcher the animals and often eat them right there, under very unsanitary conditions which then promote the transmission of all kinds of pathogens. So it was not surprising that humans were exposed and infected with this new group of viruses called coronaviruses, one of which causes SARS.

Another example is West Nile Virus, which causes bird disease and that one careless traveler apparently brought in from the Middle East. The whole North American epidemic started in New York City in the vicinity of JFK International Airport. A traveler probably brought in an infected bird which then transmitted the virus to the local mosquitoes, and from there the infection spilled over into the resident bird populations. The pathogen then spread rapidly and has now become established over most of North and Central America, has been reported from over hundred species of birds, mammals and reptiles, and is estimated to have caused the death of several million native birds.

Environmental degradation is another major problem. It is a problem in many regions of the planet because it stresses local wildlife and makes it more susceptible to existing parasites. This in turn can have effects on human health as well, because the more prevalent a pathogen is, the higher the probability that it will spill over into the human population. Therefore if we want healthy wildlife and human populations—these two things are intimately interconnected—you need to make sure the ecosystems are in good shape. If we can not find it in our hearts to protect the forests, oceans and grasslands because they are beautiful and we care about them, we have to at least do it because it is in our own narrow health interest. Whenever people do not do that, they end up ultimately undermining their own health and their own wellbeing. You have to think long-term and you have to be prudent.

To summarize, international trade and transport, habitat degradation, and trading of bushmeat are all human activities that promote the emergence of new pathogens. A wealth of data has demonstrated that they are some –but not all- of the main processes that have led to the emergence of new diseases.

In what way does your research examine these issues?

Much of my research currently focuses on two projects. The first project looks at avian malaria in bird populations in Colorado. We are studying this because avian malaria is a very common pathogen in bird populations. It is not a disease that humans can contract, but it is a pathogen that can cause serious conservation problems. It is responsible for the extinction of endemic species in Hawaii after it was first introduced there by Europeans. The native birds in Hawaii had never been exposed to avian malaria and had therefore no resistance to the disease. Humans accidentally brought mosquitoes to Hawaii and later on introduced malaria-infected game birds and avian pets. The mosquitoes transmitted the disease from the exotic, largely resistant birds to the native species which died by the thousands; this led eventually to the extinction of many species. Many of the unique Hawaiian birds became extinct because people were not thinking about the long-term implications of their actions.

We study avian malaria to understand how it affects birds. In Colorado it is a native parasite so it is not an organism that is new or introduced. In general, in nature there is some sort of dynamic balance between parasites and their hosts. However, if the host population is stressed because the environment is being degraded—there is not enough food for example—then it is not going to have, among other things, enough resources to maintain a competent immune system. As a result, the balance can shift in favor of the parasite and you could end up with a serious epidemic. If you truly want to understand diseases in natural ecosystems, you really need to understand the stressors that the hosts face, and this is one of the main topics we are currently investigating. For example, we are examining how food availability, in particular, shapes the balance of the host-parasite interaction.

The other project we are working on is looking at small populations of lizards that live on islands in the Mediterranean Sea. There are a great number of different islands, and because lizards can not swim, they are stuck on these islands. The reason they are found there today is because sea levels a few thousand years ago used to be much lower, and all the islands used to be connected. When sea levels rose, the lizards became isolated on the hilltops, which are now islands, so there exist all these populations that differ in their size due to their period of isolation. Because both of these characteristics, population size and duration of isolation shape the genetics of a population, these populations differ greatly in their levels of genetic diversity. The larger and the younger the island, the less inbred the population is, which has implications for their ability to mount an effective immune response against parasites. In particular, we are looking at the relationship between mites, the worms that live in the gastrointestinal tract and lizard malaria. For example, we examine how lizard malaria changes between different island populations of hosts that have differing levels of genetic diversity.

Why is this interesting? It turns out that one of the main mechanisms by which humans impact natural habitats is through habitat fragmentation. For example, when humans enter a large forest expanse and put a road through it or develop the area, they end up fragmenting the habitat and the wildlife populations living there. Then these fragmented populations start facing all kinds of problems: loss of genetic diversity, edge effects, etc. In addition, they are not able to deal very well with the regular parasite communities that they have always harbored. So one of things we are trying to figure

out in the Mediterranean lizard project is how fragmentation affects the outcome of host-parasite interactions in these organisms.

What can the average college student do to suppress the impact of diseases?

There are two different ways to answer this question. On the immediate, trivial level, it would be to wash your hands and go to the doctor and that type of thing, but that is pretty obvious. I think the issues we are facing are actually much bigger. These are large problems that have to do with what choices we make as a society. In turn, these societal choices are ultimately the result of individual values and choices, and only a society of informed citizens is likely to do the right thing, so being informed and remaining politically and socially engaged is going to be key for any student who cares about these issues. Right now we are entering an age of unprecedented environmental change, and this is going to have important health implications. As far as scientists can tell, environmental changes are going to be massive, and people will be surprised by what will be happening to the planet over the next fifty years. One of these things is going to be shifts in global climate. From a health perspective a warming climate will, among other things, likely allow a number of tropical pathogens to enter the temperate regions of the United States and Europe.

One of the most important things students can do to reduce the impact of infectious diseases in the long term, is to make sure the planet and its climate remain in good shape. For example, it is crucial that we try to limit our activities that accelerate global climate change. We all need to start thinking about our carbon footprint—how our activities generate CO_2 and then change the climate—because this will have implications for our health in terms of infectious diseases. It is going to be a nasty wake-up call if people in the U.S. suddenly start contracting malaria or yellow fever again. Malaria used to occur in Michigan. It was eradicated from the U.S. because the relatively cool climate enhanced the effects of good public health practices. However if the climate warms up enough, we could see the disease return again with a vengeance as some computer models predict is likely.

What does this mean in practical terms? We need to drive less, turn the lights off, fly less, and reduce overall consumption. Two of the most effective practical things students can do is give up eating meat (which has a huge carbon footprint) and choose to have one less child - the planet can simply not support the current human population. More humans translate into more damaged natural environments, a ruined climate, as well as higher probabilities of disease transmission. So these are two simple things that in the long term are going to make a big difference.

The interesting thing is that if you ask people, no one wants to damage the climate, destroy the rainforest or cause species extinction. But the truth is when you go to the mall and you buy that nice little pair of sneakers or that CD you do not really need, you are really contributing to global warming and to species extinction. Those sneakers did not fall from the sky. They were manufactured from oil and leather from somewhere, most likely Latin America or Asia, where it is cheap to produce them. They were made from oil that had to be pumped out of an oilfield, shipped in an accident-prone supertanker across the ocean and processed in a polluting refinery. The leather came from a cow that likely fed on a cleared piece of tropical rainforest. Ultimately, it does not matter what you say but rather what you do - you vote with your wallet. Each time you go and shop at the mall, you promote extraction and destruction of the natural environment. Everything is connected. The key for promoting environmental and human health – and the two are intimately interconnected – is to limit our impacts on the environment.

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September 15, 2009

The Culture of Defensive Medicine: Is Tort Reform Necessary?

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Introduction

This paper examines the culture of defensive medicine that has spread rapidly across the United States in response to an increase in malpractice lawsuits and staggering malpractice insurance premiums in certain high-risk medical and surgical specialties. Due to the presence of this culture, it is important to question whether tort reform (e.g. placing caps on jury awards) is necessary to curb rising malpractice insurance premiums and reduce the implicit costs of defensive medicine. Based on compelling data, placing caps on jury awards for pecuniary and non-pecuniary damages will reduce malpractice premiums, ensure fair compensation for victims of medical negligence, and encourage physicians to practice sound medicine in high-risk specialties by dismantling the culture of defensive medicine prevalent throughout the United States.

The culture of defensive medicine is defined by the Office of Technology Assessment as the practice of "order[ing] tests, procedures, or visits, or avoid[ing] high risk patients or procedures, primarily (but not necessarily solely) to reduce [doctors'] exposure to malpractice liability."[1] Indeed, as two prominent economists, Kessler and McClellan, argue, "Defensive medicine is a potentially serious social problem: if fear of liability drives health care providers to administer treatments that do not have worthwhile medical benefits, then the current liability system may generate inefficiencies many times greater than the costs of compensating malpractice claimants."[2] Even more troubling, Studdert et al. surveyed 824 physicians on the prevalence of defensive medicine in their practice: 93 percent reported that they sometimes or often engaged in defensive medicine outlined in the survey.[3] Many of the respondents to the survey also reported that they had restricted the scope of their clinical practice because of liability concerns (42 percent) and/or were likely to do so further in the next two years (49 percent).[3] It is critical, therefore, for policymakers and future healthcare professionals to have a sound understanding of the defensive medicine culture (and the benefits of tort reform) to understand how it contributes, along with other factors, to current "expenditures [of U.S. healthcare which are predicted to rise] from $1.6 trillion in 2002 (14.9 percent of gross domestic product) to $3.6 trillion by 2013 (18.4 percent of gross domestic product)."[4]

Basis for Soaring U.S. Healthcare Costs

First, this paper will discuss how the defensive medicine culture has contributed in a small (yet significant) manner to the overwhelming cost of American health care today. Next, this essay will propose and support that a viable though contentious solution to this culture is tort reform. It will also consider opposing arguments that suggest defensive medicine is not a substantial problem, but instead that other problems (diminishing stock market returns, culture of technology, etc.) play a greater role in increasing malpractice premiums and healthcare costs. Then, it will explain why no sweeping, national tort reform measures exist in the United States. Finally, it will conclude by summarizing both sides' arguments and discuss the broader implications of this debate for future policymakers to comprehensively attack the problem of rising healthcare costs in the United States.

The culture of defensive medicine that has taken root in the United States in recent decades has led to escalating health care costs caused by an increase in medical procedures and tests designed to prevent high-cost malpractice lawsuits. This is accurately summarized by Kessler and McClellan, who state, "Many physicians and policymakers have argued that the incentive costs of the malpractice system, due to extra tests and procedures ordered in response to the perceived threat of a medical malpractice claim, may account for…the explosive growth in healthcare costs."[2] Additionally, patient care outcomes may suffer as a result of "the practice of defensive medicine…if liability induces providers either to administer harmful treatments or forego risky but beneficial ones."

Clearly, the practice of ordering extra and unnecessary tests and procedures indirectly increases patients' costs. Indeed, as Studdert et al. argue, "…the prevalence of assurance behavior, coupled with the unit of cost procedures typically ordered (e.g. MRIs), lends weight to arguments that the total cost of defensive medicine is substantial."[3] As patients use a larger quantity of services, insurers must reimburse physicians for more; this drives up health insurance premiums as insurers must make a profit to stay in business. Since medical resources are finite (the supply) while the medical services requested increase exponentially (the demand), the cost of each service must increase accordingly. As such, higher insurance premiums combined with higher costs for individual services contribute to an overall increase in healthcare costs for the country.

Implications for Patient Care

Physicians have a greater incentive to take precautions with certain risky procedures and medically liable patients. However, it is an incentive which has long-term, negative consequences. Regarding defensive medicine, state and national surveys have reported that 16 to 64 percent of physicians across all specialties stopped providing or limited the frequency of providing certain high-risk procedures or they simply avoided certain patients because of the malpractice environment.[5] As more providers run more precautionary

exams and tests to avoid medical liability while limiting the frequency of the high-risk procedures which may improve the patient's quality of life, more patients will suffer from chronic illnesses and may develop more serious problems due to lack of access. Studdert also supports the prevalence of the negative consequences of defensive medicine by noting that large numbers of physicians reported engaging in avoidance behavior. Indeed, some surgeons appeared to limit their practice to "bread-and-butter" cases, no longer performing difficult procedures, while avoiding sicker patients, those with prior complications, and those who had sued before.[3] Jacobson and Rosenquist contest, however, that "the inability to control [the culture of technology]…is likely to put a far greater strain on the nation's health care resources than is the practice of defensive medicine", though they concede that "the existence and extent of defensive medicine comprise an important policy issue that needs to be addressed."[6] Additionally, U.S. government institutions such as the now-defunct Office of Technology Assessment concluded in 1994 that "[defensive medicine only] accounts for approximately 5 to 8 percent of all diagnostic tests."[7] Although the impact and extent of defensive medicine is disputed, most observers agree that it is imperative for policymakers to examine it as one of multiple contributing factors in the national dialogue on preserving the physician-patient relationship and protecting the quality of patient care.

> "It is imperative for policymakers to examine [defensive medicine] as one of multiple contributing factors in the national dialogue on preserving the physician-patient relationship and protecting the quality of patient care."

Indeed, there is evidence that the implicit, mutual trust of the physician-patient relationship is eroding as physicians see patients as adversaries and potential plaintiffs. In fact, "certain types of patients commonly prompt specialist physicians to behave defensively, especially those who are seen as demanding, emotional, or unpredictable…patients with prior complications…noncompliant patients, workers' compensation cases, and obese persons…[This reflects] a level of suspicion that itself is arguably detrimental to quality [and leads to dissatisfaction among physicians]."[3] If unhappy physicians cannot reciprocate the trust placed in them by their patients, they cannot provide quality care; likewise, if the patient cannot trust the physician, he is less likely to adhere to medical advice and more likely to have a poor outcome. Kaiser Permanente researcher Donald Freeborn agrees, stating, "Low levels of job satisfaction among physicians may affect doctor-patient relationships and compromise quality of care."[8] Consequently, it is clear that the culture of defensive medicine in the U.S. has multiple negative consequences: higher healthcare costs through unnecessary tests and procedures, limited access to high-risk procedures or for high-risk patients, lower quality patient care/outcomes, and a more hostile, adversarial physician-patient relationship.

Solution: Tort Reform?

In response to these harmful consequences of defensive medicine, many groups (American Medical Association, American Tort Reform Association, and the U.S. Chamber of Commerce) have proposed tort reform through caps on jury awards to rein in the escalating costs of malpractice insurance and encourage physicians to practice sound medicine. Indeed, many physicians "maintain that escalating, multimillion-dollar awards are driving premium increases and that restricting malpractice payments will lower health care expenditures by reducing the practice of defensive medicine."[9]

To be clear, it is not merely speculation that malpractice insurance premiums are increasing for many physicians in high-risk specialties. According to Emory University public health expert Kenneth Thorpe, "Depending on the specialty and state, the median increase in malpractice premiums ranged from 15 to 30 percent. Rate increases in other states, such as Pennsylvania, ranged from 26 to 73 percent in 2003."[10] Linking malpractice premiums to defensive medicine, several studies have found evidence "supporting…that the scope and extent of defensive medicine is greater in areas with high malpractice claim rates and high premiums." For additional evidence, the Hellinger study noted that "rates of growth in malpractice premiums and claims payments have been slower on average in states that enacted certain caps on damages for pain and suffering—referred to as non-economic damage caps—than in states with more limited reforms… from 2001 through 2002, average premium rates rose approximately 10 [percent] in states with non-economic damage caps of $250,000 compared with approximately 29 [percent] in states with more limited tort reforms."[9] Therefore, setting caps on jury awards has a positive impact on reducing defensive medicine and malpractice premiums.

The Thorpe article "The Medical Malpractice 'Crisis'" supplies additional data to support the passage of caps on jury awards: "premiums in states with a cap on awards were 17.1 percent lower than in states without such caps."[10] To further bolster this argument, the authors from Indiana University, Gronfein and Kinney, discuss the effects of tort reform in Indiana of caps on both economic and non-economic damages. In 1975, Indiana adopted a comprehensive set of malpractice insurance and tort reforms, including a cap on all damages, a state-operated insurance fund for claims over $100,000 and mandated pre-trial medical review before trial. Compared with similar sized, neighboring states Michigan and Ohio, which do not have these reforms, Indiana has enjoyed one of the lowest rates of malpractice insurance in the United States. In fact, Gronfein and Kinney go on to argue that "under Indiana's system, all major parties are better off in the aggregate. Malpractice insurers…[are better off because] claim severity is controlled and therefore more predictable. Providers are better off because they are assured the availability of coverage and comparatively low malpractice insurance costs. Most importantly, most claimants with large claims are better off because they receive more (30 to 40 percent higher than in Michigan or Ohio) for their injuries and their attorneys' fees are capped."[11]

However, there is an important tradeoff to consider with the reduced compensation of elderly and unemployed plaintiffs under a tort system capped for non-economic damages. As Hyman et al. report in the case of Texas, "Payouts…varied across groups; for example aggregate (per claim mean) payouts declined by 38% (19%) for elderly plaintiffs, compared to 22% (10%) for babies…there is a striking gap [as well] between the 53% aggregate reduction payout for unemployed deceased plaintiffs, versus 17% decline for employed deceased plaintiffs."[12] Tort reform through caps on jury awards clearly decreases malpractice premium rates and helps reduce defensive medicine, but proponents must carefully design and implement plans to minimize disparities in fair compensation for victims of medical negligence based on age, employment status, or other factors.

Criticism of Tort Reform

As seen in parts of this article, opponents of tort reform argue that defensive medicine is not the problem (or only contributes negligibly to total health care costs), but rather that insurance companies' declining stock market returns are to blame for the current medical liability crisis. They argue that tort reform through caps on jury awards reduces fair compensation to injured plaintiffs, allows physicians to escape punishment, makes no guarantee that insurance companies will reduce malpractice premiums, and results in unsafe medical practices. They contend that defensive medicine is not a substantial problem contributing to the rising costs of healthcare in the United States.

First, according to many of the studies cited in this paper, both defensive medicine and insurance company losses play a role in the current malpractice crisis. While Thorpe states that "higher investment returns offset the need to raise premiums", indicating that insurance company losses in the stock market may contribute to rising premiums, Jacobson et al. admit that "liability fears have no doubt influenced the climate of medical practice…[though] isolating the use of specific tests or procedures to avoid liability remains a difficult task." Second, tort reform actually increases fair compensation for injured plaintiffs. For instance, Indiana actually pays out "nearly 40 percent [more]…than the mean claim payment in Michigan [and] 33 percent [more] than the mean claim payment in Ohio." Under their reformed civil law system, Indiana physicians are assured "availability of [malpractice] coverage and comparatively low malpractice insurance costs." Third, victims of real medical negligence, as determined by a pre-trial medical review board, can still sue physicians for generous awards commensurate with their injuries. Fourth, as the tort reform in Indiana shows, malpractice premiums dropped relative to the rates in the rest of the country as "claim severity [was] more controlled and predictable." Last, with lower malpractice premiums, physicians would be encouraged to treat high-risk patients and perform high-risk procedures more often; the financial and emotional costs of defensive medicine would decrease as doctors limited tests and procedures to only those that were necessary.[8]

Current Obstacles

Despite the benefits of tort reform and legislation to place caps on jury awards in 28 states, no federal legislation has passed to provide sweeping damage caps in the entire nation due to the efforts of various lobbying forces. According to a 2001 article by Scott Tarry, the American Association of Justice (AAJ) lobby, formerly known as the Association of Trial Lawyers of America, is "one of the nation's most well-connected and biggest spending interest groups."[13] In fact, the Center for Responsive Politics reported recently that the AAJ lobby contributed over $1.4 million to various political campaigns in the 2008 election cycle. In comparison, the American Medical Association lobby has spent a meager $300,000 in campaign contributions thus far.[14]

Unfortunately, politicians respond to campaign contributions through the legislation they push through Congress, which often serves the interests of their richest supporters. As such, the AAJ and others lobby aggressively to block legislation that would threaten hefty fees in malpractice lawsuits (i.e. tort reform through caps on jury awards). Ironically, as mentioned above, plaintiffs would actually obtain larger jury awards ("40 percent more than in Michigan, 33 percent more than in Ohio") with tort reform as seen in Indiana. Trial lawyers would still draw generous fees from their clients' lawsuits, but perhaps not the exorbitant sums they often receive. "During 1990, 1.5 percent of all paid claims exceeded a million dollars. By 2001 the percentage had risen to 8 percent," cites Thorpe, underscoring the rising rate of large jury awards. Additionally, even though juries award large sums to plaintiffs, much of it is swallowed up by "standard contingency fees charged by plaintiffs' attorneys (35 percent of the indemnity payment),"[15] which affirms Studdert's prior convictions. As such, as long as there are lobbyists in Washington, it is clear that legislation will favor the most generous lobbyists; to combat this problem and push tort reform through, like-minded organizations must band together to spend more money explaining its merits to key members of Congress who can then effect change to pass bipartisan, nuanced legislation with the cooperation of opposing parties.

Conclusion

The culture of defensive medicine that has taken root across the United States calls for greater discussion of change in the tort law system among all parties involved (physicians, insurers, lawyers, patients). The presented data from a wide variety of studies and reports suggest that setting caps on jury awards through tort reform will appreciably decrease malpractice premiums, encourage physicians to practice sound medicine, and decrease the number of malpractice lawsuits through the elimination of the pervasive defensive medicine culture. Indeed, as Kessler and McClellan conclude in their study, "We find that malpractice reforms that directly reduce provider liability pressure lead to reductions of 5 to 9 percent in medical expenditures without substantial effects on mortality or medical complications…liability reforms can reduce defensive medical practices."[2] Keeping in mind the Hyman et al. article, wise reformers will take a nuanced approach to tort reform, particularly with setting caps on jury awards, to ensure fair, equitable compensation for all plaintiffs regardless

of age, employment status, or other variable[12].

Nevertheless, the passage of meaningful tort reform similar to that of Indiana will require a significant majority of supporters, especially among those who staunchly oppose any caps on jury awards. While tort reform opponents make their case articulately against caps on jury awards, which they claim would protect physicians and insurers over patients, the data for the benefits of tort reform is more compelling. In particular, explaining the current, stabilized malpractice situation in Indiana (an example of effective tort reform) to the AAJ is a wise move in building a task force across aisles to best serve the economic interests of lawyers, physicians, insurers, and the economic interests of and quality of care for patients. Future policy makers and politicians interested in progress on this clearly divisive issue should focus on generating healthy debate on the various factors contributing to the costliness of American healthcare (defensive medicine, diminishing stock market returns, culture of technology) and negotiating comprehensive, bipartisan legislation "[chiefly] to promote patient safety [and] not merely to provide safe harbors for potential defendants."[5]

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Exploring the treatment of epilepsy through intrahippocampal GABA modulation with an HSV-vector expressing GAD67

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Abstract

This study explores the effect of a herpes-simplex viral (HSV) vector expressing glutamic acid decarboxylase 67 (GAD67) in the rat hippocampus. GAD67 gene transfer increases GABA release in transfected neurons by 3 days and its effect diminishes over 3 weeks. Transgene expression was associated with a decrease in voltage-gated sodium channel α-subunits 1.2 (NαV1.2) and 1.6 (NαV1.6) in the hippocampus. Changes in VGSC levels, along with enhanced GABA neurotransmission, are likely responsible for the prevention of status epilepticus in rats expressing the GAD67 transgene using a pilocarpine-based model of epilepsy. Thus, gene transfer of GAD67 under a latency promoter for prolonged expression may have therapeutic value for patients with intractable epilepsy.

Introduction

Epilepsy is a serious neurological condition characterized by recurrent, unprovoked seizures.[8] An epileptic seizure is a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain.[32] It is among the most common neurological conditions[78], affecting approximately 5 out of every 1000 people in developed countries[96], and it is reported that up to 5% of people will experience seizures at some point in their life.[6] Decades of epilepsy research have identified genetic and acquired abnormalities of synaptic and neuronal network instability, and over forty subtypes of epilepsy are recognized today.[24,27] Thus, epilepsy is not a single disease but a symptom of brain dysfunction: a final common pathway of many different cerebral insults.

Epilepsies may be classified based on etiology as either primary (idiopathic) or secondary (symptomatic). Primary epilepsies are those that are not caused by another known disorder or syndrome and their etiology is presumed to be genetic. Secondary epilepsies, on the other hand, are the result of an insult to the patient's brain, such as a tumor, stroke, infection, or trauma.[42] Types of epilepsy are further divided into partial or generalized based on the clinical presentation; partial seizures remain confined to a particular brain region while generalized seizures occur bilaterally and throughout the forebrain.[27]

Despite the variety of subtypes, all epileptic seizures can be unified as abnormalities in the ongoing electrical activity of the brain, and most, if not all, epileptic events arise from some type of imbalance between excitatory and inhibitory activity within the neuronal circuitry.[71] The interconnectivity of neurons makes them particularly susceptible to seizures through "runaway" excitation. Here, activated neurons stimulate neighboring neurons in a feed-forward loop, and the result is the initiation of synchronous electrical events that are characteristic of seizures.

In the case of partial epilepsy, the region of the brain that is responsible for initiating the excitatory activity is often identifiable by the sensations, known as auras, perceived at the onset of a seizure. Electroencephalography (EEG) aids in the localization of focal epileptogenic abnormalities by detecting aberrant electrical firing through electrodes placed on the scalp, and today, functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) are often utilized for the accurate localization of abnormal brain activity.[24] These tools provide physicians with the insight necessary for diagnosis and treatment of a particular type of epilepsy.

After diagnosis, the first line of treatment utilized in epileptic patients is pharmacotherapy. A range of anti-epileptic drugs (AEDs) are currently in use, but no single drug has proven to be universally effective, and combinations of AEDs are often used in an attempt to control seizures. Most AEDs have similar mechanisms of action, either blocking voltage-gated sodium channels (carbamazepine, phenytoin, and lamotrigine) or enhancing inhibitory neurotransmission (barbiturates, vigabatrin, and tiagabine). Understanding the effects of these drugs requires knowledge of the neuronal voltage-gated sodium channels (VGSCs) and their role in generating and propagating action potentials, as well as a familiarity with GABA synaptic transmission; these topics will be briefly reviewed below.

VGSCs consist of three protein subunits: a pore-forming α-subunit and two auxiliary β-subunits, β1 and β2.[76] There are nine distinct mammalian α-subunits, and specific α-subunits are expressed differentially throughout the central and peripheral nervous system. The embryonic hippocampus predominantly expresses α-subunit 1.3 (NαV1.3), while the adult hippocampus expresses α-subunits 1.1, 1.2. and 1.6 (NαV1.1, NαV1.2, and NαV1.6, respectively).[5,29,36]

These proteins are described as voltage-gated because their sodium ion (Na^+) pore opens and closes in response to changes in cellular membrane voltage. Gating allows for rapid changes in electrical activity to be transferred down the axon, and it is essential for the propagation of the action potential. At rest, VGSCs are closed and do not allow Na^+ to flow through their pores. However, they have threshold voltages that cause them to open and allow for Na^+ flow through their pore region. Once this threshold is reached and the pore opens, the Na^+ that is accumulated on the outside of the cell is allowed to flow into the cell, and the cell becomes depolarized. This depolarization of the membrane allows more VG-

SCs along the axon to reach their threshold voltage and open their pores, allowing the electrical signal to proceed down the axon.

By a similar voltage-gated mechanism, the VGSC pore closes to ion flow once the cell reaches a specific membrane voltage, and this is known as inactivation. The closing of the pore allows the cell to re-establish the baseline ion gradient and to prepare for the firing of another action potential. Thus, the timing of gating events and the membrane voltages at which they occur determine the properties of a particular neuron and are important to consider when analyzing the excitatory/inhibitory events associated with different types of neurons.

Carbamazepine, phenytoin, and lamotrigine are AEDs that alter VGSC gating events in order to decrease neuronal excitability. These drugs bind to sodium channels that have been inactivated and stabilize the inactivate conformation so that the VGSCs remain closed to ion flow. This has the effect of inhibiting action potentials in these neurons. Based on probability, neurons that fire rapid action potentials are more likely to be bound and constitutively inhibited, while neurons that fire action potentials intermittently are less likely to be inhibited. Therefore, in theory, the proper dose of these AEDs should inhibit the aberrant firing of seizure foci while leaving the firing associated with normal processes relatively unaffected.[80]

A second class of drugs that is capable of controlling seizures is effective at the level of the synapse. In the prototypical neuron, depolarizing events in the cell body trigger the action potential to be propagated down the axon and neurotransmitters to be released from the axon terminals into the synaptic cleft. Here the neurotransmitters can bind to receptors and trigger changes in the molecular or electrical environment of the neighboring cells. Barbiturates and benzodiazepines are known to be effective at the level of the synapse and were some of the first drugs utilized for epilepsy treatment. The efficacy of barbiturates and benzodiazepines lies partly within their ability to mimic the principal inhibitory neurotransmitter in the brain, γ-amino butyric acid (GABA).

GABA is produced in nerve terminals from the brain's principal excitatory neurotransmitter, glutamate, and is released into the synaptic cleft by both vesicular and non-vesicular mechanisms.[119] Once present in the synapse, GABA can bind to receptors and inhibit the firing of action potentials in neurons. This is achieved by evoking hyperpolarizing currents in cells. Hyperpolarizing currents shift the membrane potential away from the threshold required to fire an action potential, and thereby inhibit neuronal firing. $GABA_A$ receptors achieve this by allowing chloride ions (Cl^-) to flow into the cell through an ion pore, while $GABA_B$ receptors achieve this by intracellular signaling cascades that open potassium channels and allow K^+ to flow out of the cell.

In recent years, other AEDs have been developed around mechanisms of enhancing GABA-mediated neuronal inhibition. Vigabatrin and tiagabine, for example, are drugs that serve to increase the brain's endogenous levels of GABA. Vigabatrin accomplishes this by inhibiting GABA transaminase[39], the enzyme that degrades GABA in the nerve terminals. Tiagabine, on the other hand, increases GABAergic transmission by inhibiting the plasma membrane's GABA transporter (GAT).[38] GAT typically serves in the reuptake of GABA from the synaptic cleft. Therefore, inhibition of GAT serves to increase the concentration of GABA in the synapse, facilitating GABA binding to receptors and increasing inhibitory neural transmission.

Despite the development of new AEDs, approximately 20-30% of patients with epilepsy remain refractory to drug therapy[54,100], and surgical resection of the seizure focus must be considered for improving seizure control. This method was first explored as a treatment option in the 19th century, and the first successful resection was reported in a patient with temporal lobe epilepsy (TLE) in 1879.[64,24] TLE is an important subtype to consider when discussing surgical treatment options since it is the most common type of focal epilepsy, the most refractory to pharmacotherapy, and the most accessible to surgical therapy.[98,24] Unfortunately, despite extensive improvements in surgical techniques, not every epileptic patient is a good candidate for surgery and not every seizure focus is readily localizable.[7] Furthermore, nearly all patients that undergo surgery still require additional anticonvulsant therapy[117] and suffer from the cognitive and behavioral side effects of AEDs.

If surgery has been rejected, very few treatment options remain. As a result, alternative treatments are being explored using animal-based models of epilepsy, and a variety of techniques have been established that can closely mimic TLE in humans. Most commonly, electrical stimulation, electrical kindling, and excitotoxin (kainic acid or pilocarpine) administration are used for this purpose. Each of these techniques has been proven to mimic the pathophysiologies associated with human TLE, including hippocampal sclerosis and electrophysiological alterations.[83,109,110] In our studies, the pilocarpine model of seizures was used to induce status epilepticus (S.E.) and to explore the possibility of using gene transfer as a novel therapeutic approach to epilepsy.

The following studies were designed to explore the efficacy of a non-replicating herpes-simplex virus (HSV)-based vector in mediating the gene transfer of glutamic acid decarboxylase 67 (GAD67) for the treatment of epilepsy. GAD67 is an enzyme that synthesizes GABA from glutamate. Neurochemical studies suggest that a decrease in γ-aminobutyric acid (GABAergic) inhibition, particularly in the dentate gyrus of the hippocampus, contributes to epilepsy[53], and that there is potential for treatment through modulation of GABA inhibitory neurotransmission.[13]

In our laboratory, the HSV-based vector that encodes GAD67 (QHGAD) has been shown to increase GABA synthesis and release by transduced neurons.[59] The principle objective of our study was to test whether focal injection of QHGAD in the rat hippocampus would decrease abnormal electrical discharges and thus reduce seizure activity in an animal model of epilepsy.

Based on current AEDs' ability for seizure prevention (discussed above), we hypothesized that enhanced GABA neurotransmission would reduce seizure activity in a pilocarpine-based model of epilepsy. In addition to its behavioral

effects, we hypothesized that enhanced GABA release from transfected neurons acting on $GABA_A$ and/or $GABA_B$ receptors in these, or neighboring, neurons would alter neuronal expression of VGSCs. We aimed to explore this effect and to determine the mechanisms by which our vector alters these channels.

Methods

Experimental Animals. Female Sprague Dawley adult rats (210-230 g) were used. They were housed with a fixed 12 hr light/dark cycle and *ad libitum* access to food and water. Housing conditions and experimental procedures were approved by the University of Michigan Committee on Use and Care of Animals.

Vector Construction. The HSV-based vector had four immediate early genes deleted, (ICP4, ICP22, ICP27, and ICP47) to block viral gene expression and abort the HSV lytic cycle. The human GAD67 gene is under the control of the human cytomegalovirus immediate early promoter (HCMV IEp) at the UL41 locus in QHGAD. A green fluorescent protein (GFP) reporter gene is also under control of HCMV IEp at the UL54 locus. The control vector, QOZHG, is defective in the same four IE genes and contains the GFP reporter gene, but the GAD67 gene is substituted with the *Escherichia coli lacZ* reporter gene under control of the herpes promoter infected cell polypeptide 0 (ICP0), at the UL41 locus. Vector stocks were produced and stored at −80 °C until thawed for use. The QHGAD and QOZHG vectors were purified to a titer of 5 x 10^6 pfu/μL. Vector Constructs are shown in **figure 1**.

Stereotactic injections. Two vectors were used in these experiments: Control vector, QOZHG, and experimental vector, QHGAD. Animals were injected with the appropriate vector bilaterally into the dentate gyri (nose bar, 0 mm; lateral, +/-2.8 mm from Bregma; anterior/posterior, -3.8 mm from Bregma; ventral, -3.5 below the dura) using a 10 μL Hamilton syringe. 3 μL of the vector was injected at a rate of 0.33 μL/min and all animals were fully anesthesized throughout surgery (4% chlorohydrate, 1 mL/kg, intraperitoneally (i.p.)). Damage to the dentate gyrus was avoided to our best ability, and no behavioral differences were seen in these animals post -stereotactic injection. For seizure experiments, control vector and QHGAD vector were aliquoted and labeled numerically by an outside source. The numbered vectors were injected, and behavioral testing was performed before the identity of the vector was known, eliminating researcher bias. Pilocarpine was administered three days post-stereotactic injection.

Pilocarpine administration. As **figure 2** illustrates, each rat was pre-treated with scopolamine (1 mg/kg, i.p.) before pilocarpine administration (400 mg/kg, i.p.). The animals were closely monitored and any seizure behavior was recorded for 90 minutes. At 90 minutes, each animal, regardless of seizure severity, was given a dose of diazepam (5 mg/kg, i.p.) regardless of seizure severity. The animals were then given a dose of 4% chlorohydrate (1 mL/kg, i.p.) to ensure full anesthesia before sacrificing the animals.

Behavioral Measurements. After pilocarpine injection, animals were observed for 90 minutes for seizure-specific behaviors. The latency to mouth/facial movements and head bobbing, defined as stage 1 and stage 2 seizures, respectively, was recorded.[87] In strongly affected animals, these behaviors were followed by recurrent myoclonic convulsions, rearing, falling, and S.E.[109] S.E. was defined as persistent seizure activity that was uninterrupted by voluntary or coordinated movement. The occurrence of S.E. was recorded in all animals, and the latency to S.E. was noted when applicable.

Cell Culture. Hippocampal neurons from Sprague Dawley rat pups on post-natal day 1 were obtained, dissociated, and plated over poly-lysine coated 24-well plates. Hippocampal neurons were cultured in defined Neurobasal media supplemented with B27, Glutamax I, and Albumax II. After 3 weeks in culture, cells were incubated with the appropriate viral vector (MOI 3) for 2 hours before the media was removed and fresh media was added. The cells were left to express the vector for 48 hours, at which point approximately 95% of cells expressed the reporter gene, GFP, as observed using fluorescent microscopy.

Western Blot Analysis. Animals were perfused transcardially with 100 mL 0.9% NaCl and the brain was collected and frozen on dry ice immediately. The hippocampus was then dissected from the surrounding cortex and the tissue was homogenized in sample buffer (2% SDS, 1% glycerol, 0.5% Tris Buffer) containing phosphatase inhibitors (Sigma, p2850 & p5726) and a protease inhibitor (Sigma, P8340). Care was taken to avoid the precipitation of SDS. The homogenate was then sonicated and subsequently centrifuged at 14,000 rpm for 5 min before the supernatant was collected. High-speed fractionation studies were performed on noted samples, which included a spin at 80,000 rpm for 60 minutes. Bio-RAD Dc protein assay was performed to determine all protein concentrations and 50 μg aliquots were prepared for

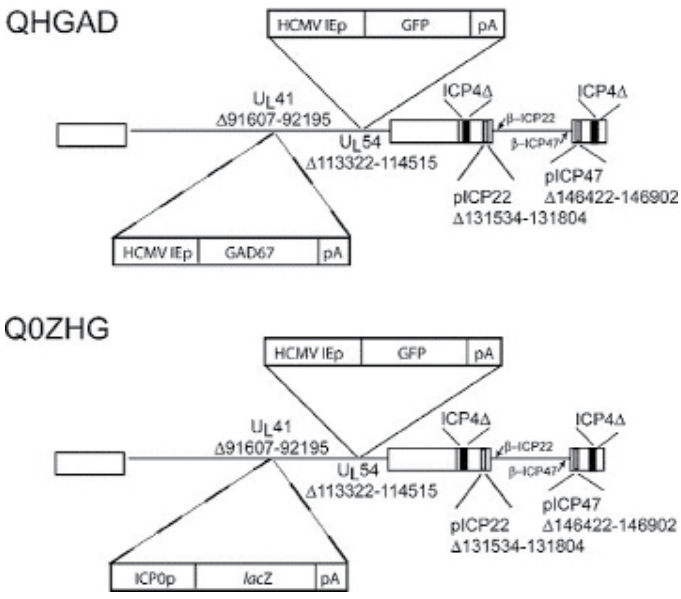


Figure 1: *A schematic representation of the vector constructs.*

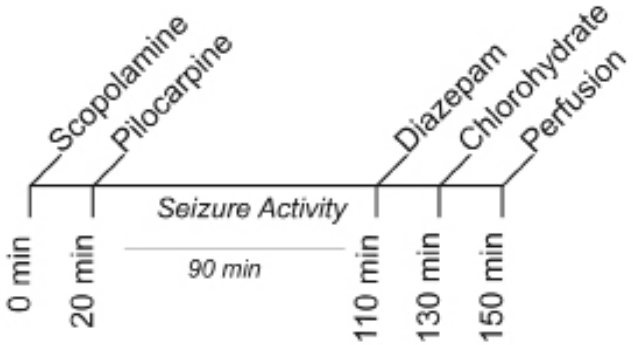


Figure 2: *A schematic representation of the seizure induction. protocol.*

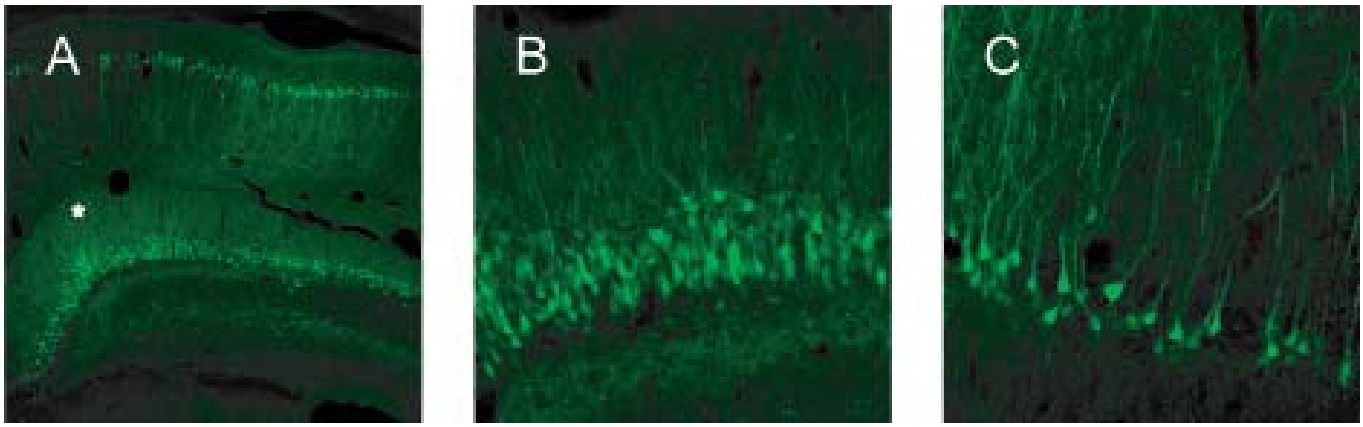


Figure 3: *GFP expression in the rat hippocampus upon QHGAD inoculation. 3 days post-injection, GFP is present throughout the hippocampus, and the injection did not seem to cause any serious tissue disruption (a). Granule cells of the dentate gyral superior blade display the strongest expression (b), and significant expression is also seen in the CA1-CA3 regions (CA1, c). The injection site is indicated with an asterisk(*).*

analysis. All samples were run on 4-15% or 7.5% tris-HCl gels from Bio-RAD. Primary antibodies were obtained from Chemicon, Temecula, CA (GAD67, 1:1000; NαV1.2, 1:200; NαV1.6, 1:200; β2, 1:300) and Sigma-Aldrich, St. Louis, MO (β-actin, 1:2000)), and species-specific secondary antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA (1:2000). All antibodies were diluted in 5% milk solution prepared in 1x PBS. Exposure of antibody binding was performed with chemiluminescent HRP antibody detection reagent from Denville Scientific (E-2500). Densitometry analysis was performed using Quantity One software. Experiments using cell lysates were also prepared according to the methods listed above.

Immunohistochemistry. Animals were perfused transcardially with 150 mL 0.9% NaCl and subsequently with 200 mL 4% paraformaldehyde (PFA) buffered at a pH of 7.4. The brains were then dissected and post-fixed for 24 hours in PFA and dehydrated for 48 hours in a 30% sucrose solution. Forty μm thick sections were taken using a sliding microtome, and slices were kept in antifreeze solution (sucrose, ethylene glycol, phosphate buffer) at -20°C until use. Antibodies used include those listed above, as well as c-fos (1:500, Santa Cruz, SC-7202). Fluorescent, species-specific, secondary antibodies (1:2,000, Alexa Fluor) were used in immunofluorescent assays, and DAB stains were performed using reagents from Vector Laboratories, Inc.

Immunocytochemistry. Cells were directly fixed in 10% formalin solution, blocked (5% NGS, 1% BSA, 0.01% 1000x sodium azide, 0.2% Triton), and probed with the appropriate antibodies diluted in solution (5% NGS, 1% BSA, 0.01% 1000x sodium azide). Cells were then probed with fluorescent, species-specific, secondary antibodies (1:2000, Alexa Fluor)), and cells were mounted using Fluoromount-G (Electron Microscopy Sciences, 17984-25).

Imaging. Confocal microscopy was performed using the Zeiss 510 Meta Microscope from the Microscopy and Image Analysis Core Facility in the University of Michigan Biomedical Science Research Building.

Statistical Analysis. Statistical significance was obtained for the seizure animals using a Fisher Exact Test.[32] For Western blot analysis, one-tailed Student t-tests were applied to densitometry ratios when the number of samples per group was sufficient.

Results

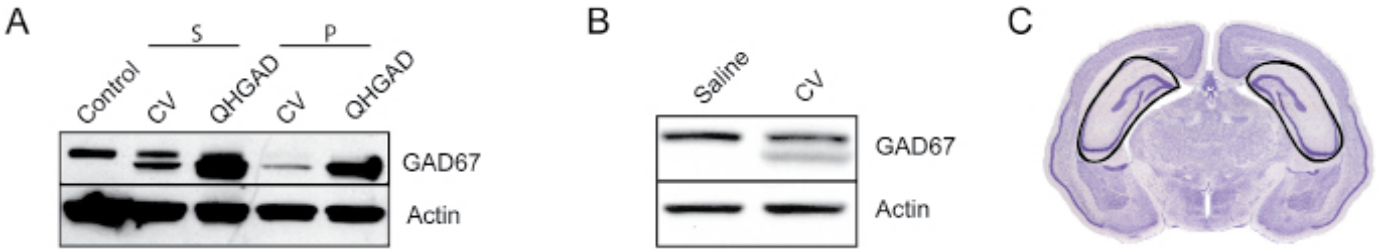
GFP and GAD67 transgene expression in rat hippocampus following HSV-vector delivery

To determine the extent of neuronal infectivity and transgene expression in rat hippocampus after HSV-vector delivery to the dentate gyrus, a series of histological and biochemical studies were performed.

Injection of the HSV-vector into the rat hippocampus was adjusted to give the maximum amount of expression in the dentate gyrus region and to minimize the damage to surrounding tissue. Ultimately, 3 μL of the vector injected at a rate of 0.33 μL/minute in a region dorsal to the dentate gyral superior blade was determined to be ideal; these settings were utilized for all subsequent experiments.

The extent of vector-mediated transgene expression was visualized using a green fluorescent protein (GFP) reporter gene present in QHGAD and QOZHG vector control (**Figure 3**). Injection of the vector resulted in widespread expression in the granule cells of the dentate gyrus (3b), as well as the pyramidal cells of the hippocampal CA1-CA3 regions (3c) 3 days post-injection. The expression in the dentate gyrus was specific to the granule cell layer and did not show notable expression in the neurons of the dentate hilus. GFP is observed in neuronal cell bodies, as well as neurites, indicating widespread transgene expression intraneuronally. These results were maintained at the 1 week time point, but were largely diminished by 2 weeks and were absent by 3 weeks.

The level of GAD67 transgene expression was measured by Western blot of protein homogenates from animals injected in the dentate gyrus with QHGAD, control vector (CV), or saline. Results showed that QHGAD treated hippocampi expressed very high levels of GAD67 protein at 3 days post-injection as compared to sham animals and control vector injected animals (**Figure 4**). The electrophoretic mobility of the GAD67 transgene protein appears slightly faster than the



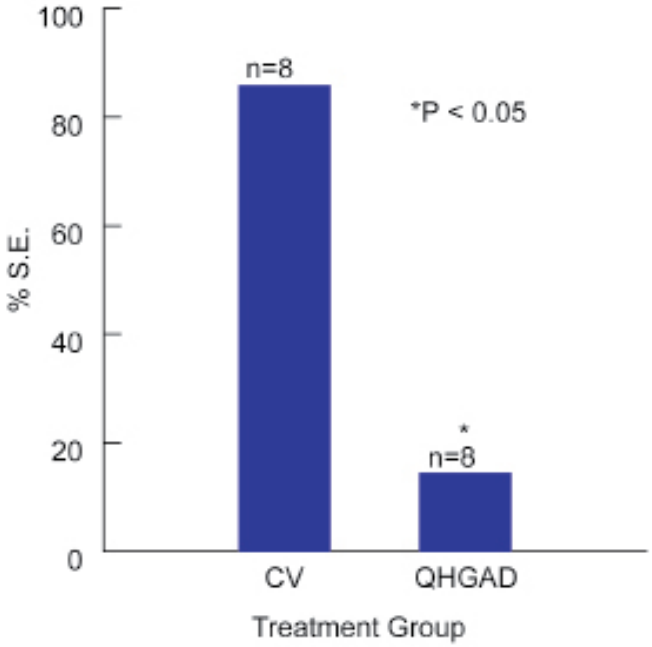


Figure 4 (Top): *GAD67 transgene protein expression from rat hippocampus. A shows the Western blot analysis of hippocampal protein three days post-injection and the strong expression of GAD67 with the QHGAD vector. CV is control vector, S is the supernatant fraction and P is the pellet fraction of homogenates. B illustrates that the lower band detected with the GAD67 antibody is not due to trauma (saline injection), but is present with injection of control vector. C depicts the regions of the hippocampi that were isolated from rat brain for Western blot analysis.* **Figure 5 (Left)***: QHGAD inoculated animals are resistant to status epilepticus (S.E.) upon pilocarpine administration. Six out of eight control vector inoculated animals reached S.E. in this seizure assay while only one out of eight QHGAD inoculated animals reached S.E. when given the same dose of pilocarpine. Statistical analysis was performed using a Fisher Exact Test and the difference observed is significant, $p < 0.05$.*

endogenous band seen in sham animals. Animals injected with saline alone showed the same band as sham animals, suggesting that the change in mobility was not due to trauma. We are unclear about the exact nature of this slight increase in electrophoretic mobility of the GAD67 transgene protein, but it is possibly due to differences in glycosylation levels or phosphorylation state. In the high speed pellet fraction of these samples (100,000g), a single molecular weight band was observed instead of the doublet, indicating a possible membrane associated fraction of GAD67. Again, protein expression was robustly increased in the QHGAD sample.

These results showed that the direct delivery of a non-replicating HSV-vector to a focal brain regions leads to a high efficacy of neuronal infection and robust levels of transgene expression. Also, the time course observed was characteristic of what we have previously seen using the HCMV promoter. Thus, these vectors were determined suitable for further studies in epilepsy.

Over-expression of the GAD67 transgene provides resistance to pilocarpine-induced status epilepticus

Using the behavioral analysis described above (see Methods), seizure activity characterized by mouth/facial movements, head bobbing, convulsions, and status epilepticus was recorded. All animals were observed for 90 minutes following the administration of pilocarpine, and latencies to different seizure activities were recorded. Preliminary experiments were performed to determine an optimal dose of pilocarpine for inducing status epilepticus (S.E.) in vector injected animals, and it was concluded that a dose of 400 mg/kg, preceded by scopolamine (1 mg/kg), was the maximum dose that could be administered without risking mortality. This dose was capable of producing S.E. in 75% of QOZHG-injected animals, which is similar to the percentage reported by others for sham animals.[53]

Three days prior to pilocarpine administration, all animals were injected with 3 µL of the appropriate vector containing $5x10^6$ pfu/µL. These animals were injected bilaterally in the dentate gyri, as described previously in studies of GAD67 transgene expression.

Treatment with QHGAD was capable of reducing the percentage of animals to reach S.E. in this model of seizures, and the results were statistically significant ($p < 0.05$) (**Figure 5**). Six out of eight animals injected with control vector experienced S.E. while only one out of eight animals injected with QHGAD experienced S.E. The latencies to onset of stage 1/2 seizure activity and S.E. were highly variable between groups and results were not statistically significant.

In addition, we examined brain sections from these animals for c-fos expression with immunocytochemistry. Analysis of c-fos expression in the hippocampus can be correlated with seizure activity, as reported by others.[21,43,22] Animals that did not reach S.E., independent of treatment group, show low basal levels of c-fos expression in the hippocampus, and QOZHG injected animals that reached S.E. showed a strong up-regulation of c-fos in dentate granule cells and CA1-CA3 pyramidal cells (**Figure 6**). Interestingly, the single animal injected with QHGAD that reached S.E. did not show c-fos up-regulation in the dentate granule cells but did show some level of c-fos im-

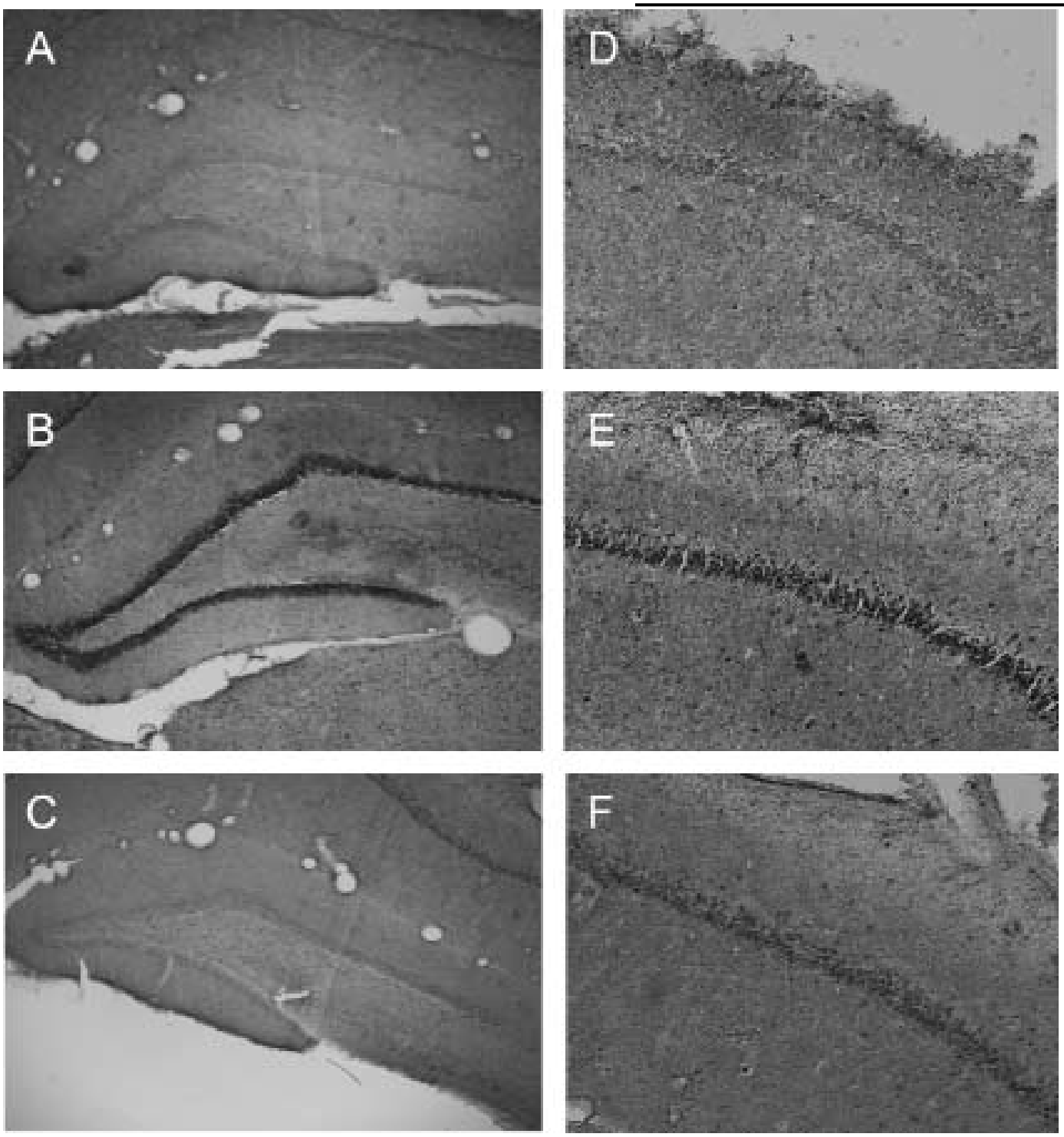


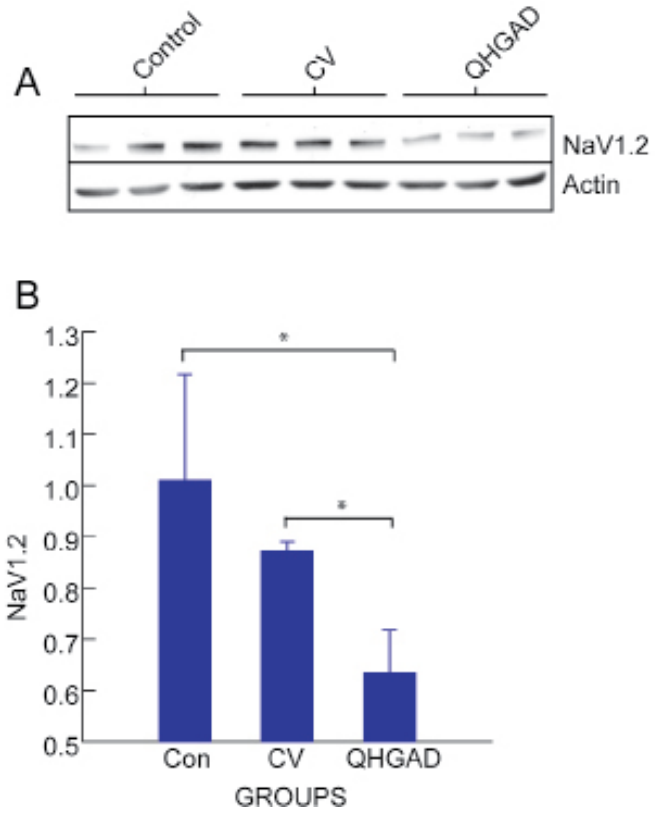
Figure 6: *Expression of c-fos in the dentate gyrus (a,b,c) and CA2 regions (d,e,f) after pilocapine administration. a and d are sections from a QHGAD injected brain that did not reach S.E. b and e are sections from a control vector injected brain that reached S.E. Strong expression of c-fos is seen in the dentate gyrus and CA2 region. c and f are sections from a QHGAD vector injected animal that reached S.E. The dentate gyrus of this animal resembles the non-S.E. dentate gyrus (a) with low c-fos expression, but the CA2 region of this brain shows expression of c-fos similar to that in the S.E. brain (e). The CA2 region is depicted as a representation of levels in CA1-3 regions.*

munostaining in the hippocampal CA1-3 regions as compared to animals with no seizures, indicating that another pathway may have been responsible for initiating S.E.

The results from these experiments showed that prevention of clinical seizures may be obtained though HSV-mediated GAD67 transgene delivery to the seizure focus.

Long term enhanced synthesis and release of GABA from over-expression of GAD67 down-regulates VGSCs in treated hippocampus

Changes in VGSC expression and properties have been



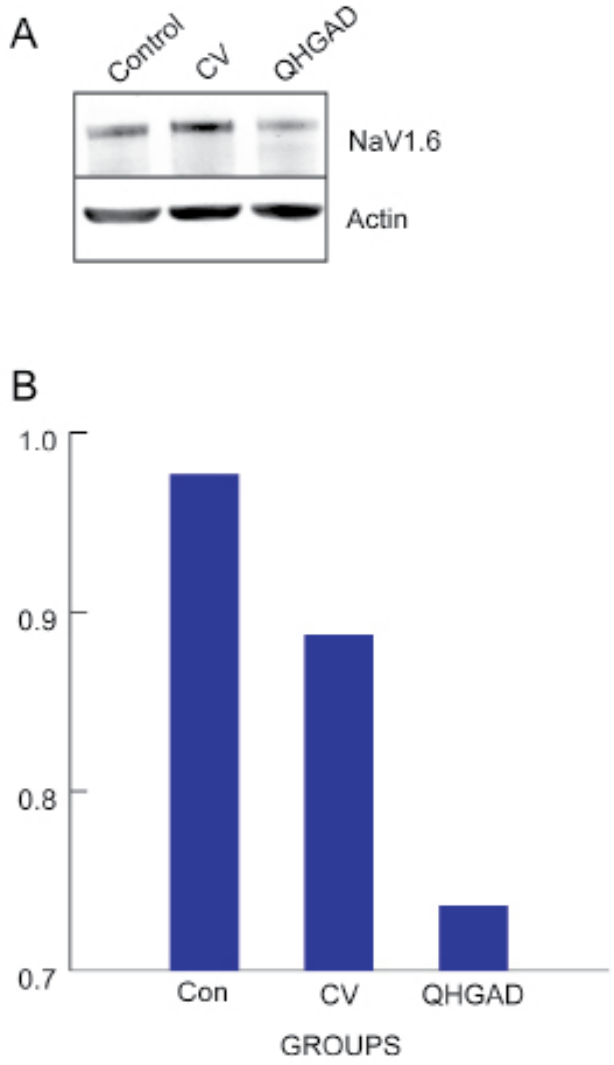


Figure 7 (Top): *NαV1.2 is down regulated in QHGAD injected animals. Western blot analysis of hippocampal tissue shows decreased levels of NαV1.2 at 3 days post-injection (A). A ratio of the mean intensity of the NαV bands to control (β-actin) is depicted in B. Bars represent standard error of the mean. Differences between the groups indicated were statistically significant, p<0.05, using a one-tailed Student's t-test.* **Figure 8 (Right)***: NαV1.6 is down regulated in QHGAD injected animals. Western blot analysis of hippocampal tissue shows decreased levels of NαV1.6 at 3 days post-injection (A). A ratio of the mean intensity of the NαV bands to control (β-actin) is depicted in B. The blot shown is representative of several trials showing NαV1.6 down regulation.*

described in different animal models of epilepsy, and it has been reported that these changes can play a critical role in the development of seizures.[1, 23, 51, 61, 116] For this reason, we hypothesized that the enhanced release of GABA resulting from QHGAD inoculation may affect the levels of the voltage-gated sodium channels. We examined the presence of NαV1.1, 1.2, and 1.6, since these are the subunits present within the adult hippocampus. Protein levels were examined 3 days after vector injection in animals that were not treated with pilocarpine.

We observed a strong decrease in the presence of NαV1.2 and NαV1.6 in response to QHGAD compared to QOZHG and control brains (**Figure 7, Figure 8**). We then examined the time dependence of this down-regulation and concluded that NαV1.2 and NαV1.6 were down-regulated to the greatest degree when the GAD67 expression was the highest. By Western blotting, we show that these α-subunit protein levels are greatly diminished at one week but are increasing at two and three weeks when GAD67 transgene expression is diminishing (**Figure 9, Figure 10**). NαV1.1 was unable to be examined with this method due to antibody limitations. Immunofluorescent staining of α-subunit proteins was also attempted, but the resolution of this method was not sufficient to display these changes in protein levels.

We examined the levels of β2 protein in the hippocampus since the β-subunits of VGSCs are important for the insertion and maintenance of α-subunits in the plasma membrane, but we did not observe significant change upon administration of QHGAD (**Figure 11**). These findings suggest that the regulation of the NαV1.2 and 1.6 protein levels by GAD67 transgene expression may be independent from the regulation of the β2-subunits in these cells.

The occurrence of VGSC α-subunit down-regulation upon QHGAD inoculation signifies that QHGAD prevention of S.E. may not have been strictly due to enhanced GABA release, but may have been partly due to a decrease in the VGSC α-subunits that are crucial for action potential propagation.

VGSC α-subunits are localized in hippocampal neurons

In order to examine if the regulation of VGSC α-subunits was localized in neurons or glial cells of the hippocampus,

immunocytochemistry was performed. NαV1.1, 1.2, and 1.6 α-subunits were all seemingly localized to neurons, as visualized by the co-localization of VGSC proteins with the neuronal marker MAP2 (**Figure 12**). The co-localization we detected was specific to granular layer neurons of the dentate gyrus and pyramidal neurons of the CA1-CA3 regions, and strong expression of NαV1.1 and 1.2 was observed in neuronal plasma membranes. NαV1.6 was detected more uniformly throughout the cell body, but this effect may be due antibody quality.

These findings suggest that the down-regulation in VGSC α-subunits observed upon QHGAD injection in the hippocampus is specific to neurons, not glial cells, and therefore may have a direct effect on action potential propagation in these cells.

Absence of QHGAD regulation of VGSC α-subunits in vitro

Experiments using hippocampal neurons in culture were performed in an attempt to further characterize the cellular mechanisms involved in the down-regulation of VGSC α-subunits in response to QHGAD.

Hippocampal neurons were obtained from neonatal rat pups on post-natal day 1 and cultured for 3 weeks. VGSC α-subunits were examined in this cell culture by immunocytochemistry, as they were examined *in vivo*. Co-localization experiments were performed with antibodies to NαV1.1, 1.2, 1.6, and the neuronal marker MAP2. These experiments showed dominant VGSC α-subunit expression in neurons, rather than astrocytes (**Figure 13**).

It should be mentioned that low levels of VGSC staining were observed in occasional astrocytes *in vitro* when double labeled with GFAP, but this effect was not uniform, and the levels were substantially lower than those detected in neurons.

Upon transfection with QHGAD, cultured hippocampal neurons displayed pronounced GFP expression and displayed a large increase in GAD67 protein observed with Western blotting (**Figure 14**). However, in preliminary experiments these neurons did not show the regulation of VGSCs observed *in vivo*.

These experiments lead us to believe that the VGSC α-subunit regulation that we observe *in vivo* is occurring in neurons, but the effect is somehow specific to adult hippocampal neurons or is dependent on the intact neuronal circuitry present in an *in vivo* model.

Discussion

The high prevalence of drug-resistant epilepsy requires patients to undergo surgical resection of the seizure focus

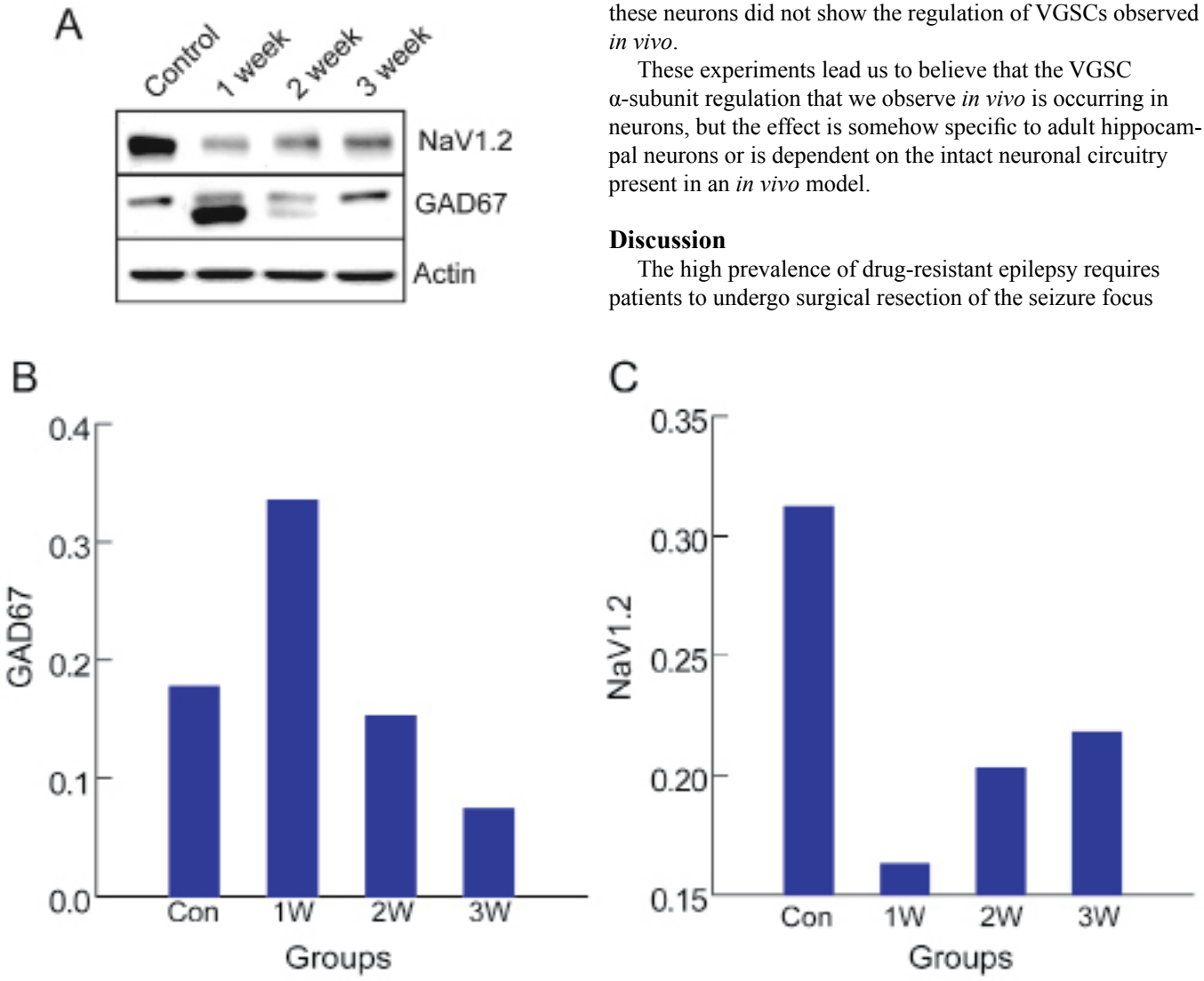


Figure 9: *Response of NαV1.2 to GAD67 transgene expression over a 3-week timecourse. NαV1.2 protein expression is inversely related to GAD67 transgene expression over this time period. B and C illustrate the ratio of the GAD67 band mean intensity or VGSC band mean intensity to control (β-actin), respectively.*

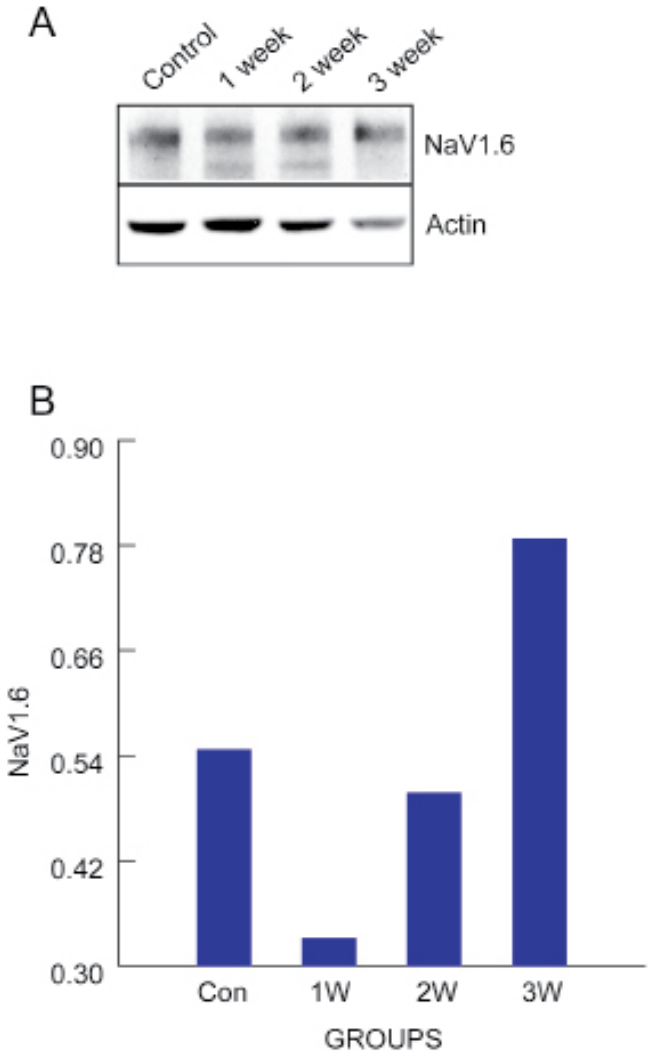


Figure 10: *Response of NαV1.6 over a three week timecourse. The NαV1.6 protein level is regulated in a similar manner to NαV1.2. It is decreased at 1 week, but increases in expression in week 2 and 3 (A). B is a ratio of the mean intensity of the VGSC bands to control (β-actin).*

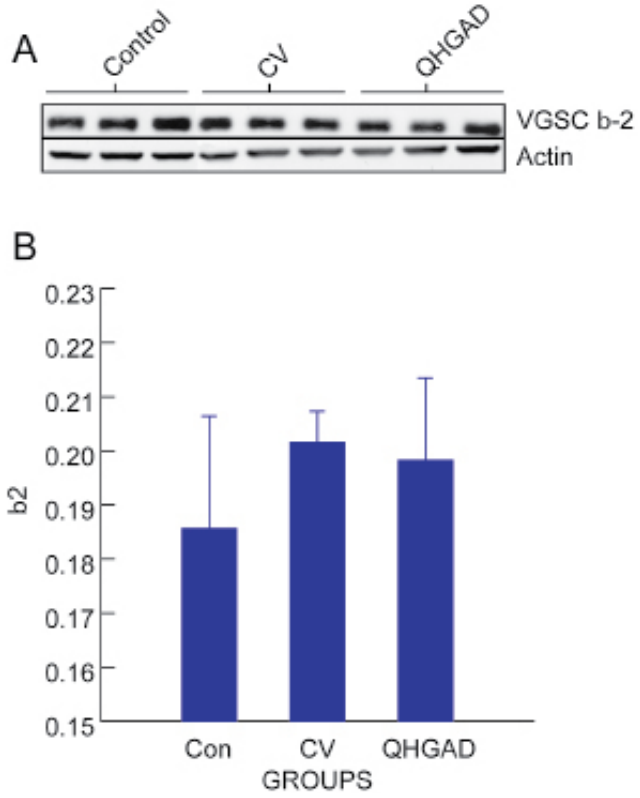


Figure 11: *VGSC β2-subunit protein does not show any alteration between control, control vector, and QHGAD after 3 days of expression, as is seen in the NαV1.2 and NαV1.6 levels. Western blot of the protein is shown in A. B shows a ratio of the mean intensity of the β2 bands to control (β-actin) bands. Bars represent standard error of the mean. Differences are not statistically significant.*

often as a last treatment option. Unfortunately, not all patients are eligible for surgery based on their medical history and/or the location of their seizure focus. The development of other treatment options for these patients is necessary, and our studies suggest that GAD67 gene transfer to the seizure focus may provide a therapeutic option for epilepsy management.

Progress in the development of non-replicating viral vectors has greatly advanced their potential use in clinical gene therapy, and HSV-based vectors have gained a great deal of attention due to its their many desirable characteristics. HSV's ability to establish a latent state in the nucleus of non-dividing cells, such as neurons, as a non-integrated episomal element makes it particularly useful. This mechanism allows the viral genome to remain latent in the host cell without disrupting the host genome.[74] Furthermore, HSV's genome can be manipulated to decrease its toxicity and to carry relatively large genes for transfer to host cells.[31] For these reasons, HSV-based vectors have been explored as clinical treatment options, and they have been shown to be well tolerated and safe when delivered to a variety of tissues, including the brain.[40, 68, 79, 85, 89] The QHGAD vector was previously shown to be effective in the transduction of dorsal root ganglion neurons and was able to greatly increase the release of GABA from these cells *in vitro* and *in vivo*.[59] In our studies, QHGAD was capable of transfecting hippocampal neurons (**Figure 3**) and expressing GAD67 protein in this tissue (**Figure 4**).

Endogenous properties of GAD67 make it particularly favorable for epilepsy treatment. The two isoforms, GAD65 and GAD67, are each encoded by a single, separate gene, and they are distinct in their intracellular distributions in neurons.[11] In general, GAD65 is prevalent in axon terminals and synaptic vesicles, whereas GAD67 is located in cell bodies and uniformly distributed throughout the neuron.[56] The levels of GABA synthesized by these enzymes differ since GAD65 is bound to its cofactor, pyridoxal 5'-phosphate (PLP), less often than GAD67, making GAD67 more constitutively active in neurons.[3] Thus the transfer of the GAD67 gene rather than the GAD65 gene allows for the bypass of this regulation and the constitutive production of GABA in transfected neurons.

Sequestering of neurotransmitters in synaptic vesicles provides another level of control for neurons. Shifts in membrane potential and subsequent increases in intracellular

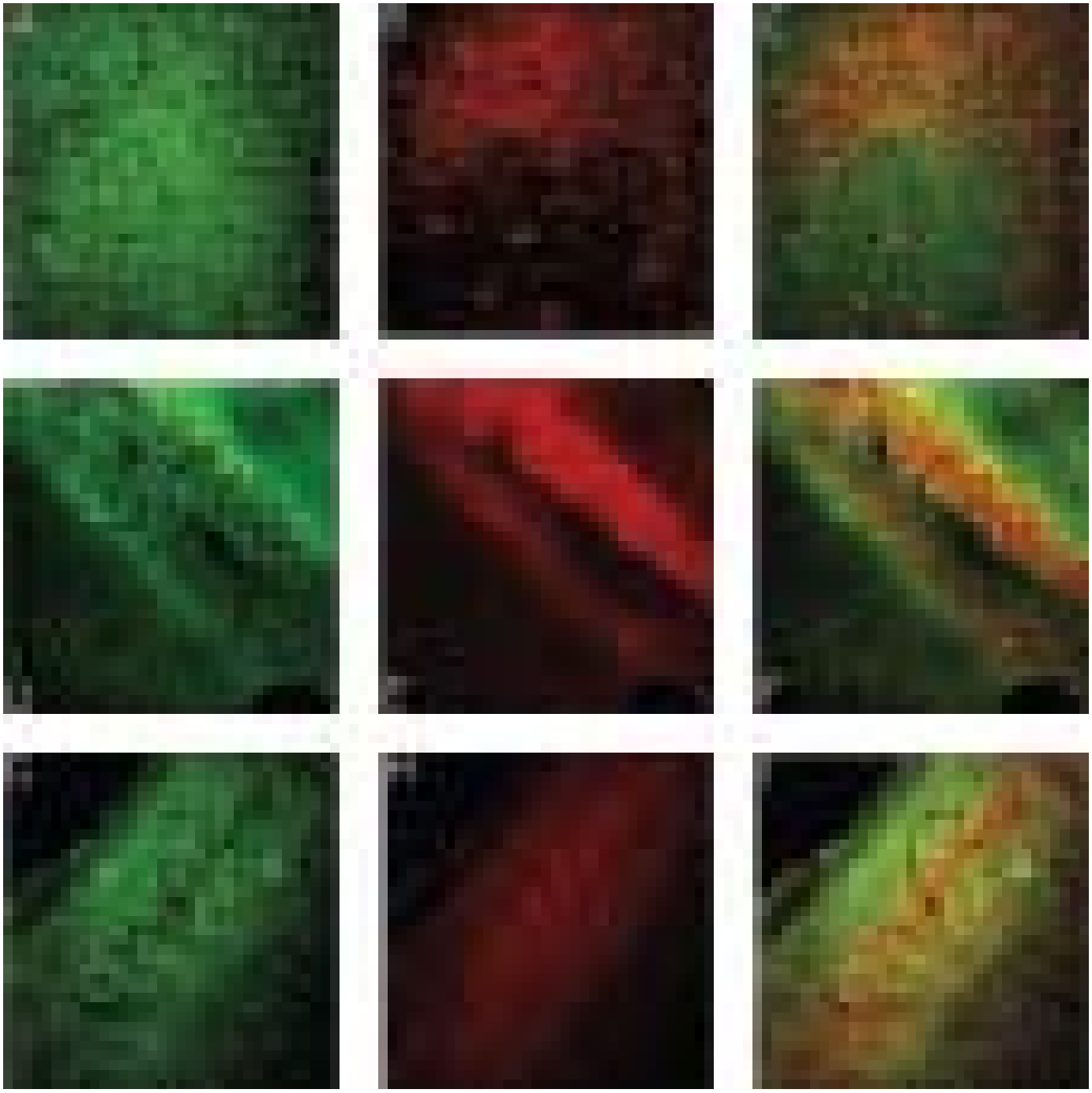


Figure 12: *Tissue sections show the possible co-localization of NαV1.1 (A,B,C), 1.2 (D,E,F), and 1.6 (G,H,I). A, C, and F are hippocampal neurons stained with MAP2, and images B, E, and H are VGSC α-subunits stained with the antibody corresponding to the specific subtype. C, F, and I are overlays of these images displaying co-localization.*

calcium ions are able to control the trafficking of synaptic vesicles to the plasma membrane, where they release their contents into the synaptic cleft. Typically GABA is released at the synapse, binds to GABA receptors, and causes hyperpolarization. Interestingly, it has been reported that a decreased quantal release of GABA from synaptic vesicles may contribute to epileptogenic activity in experimental models of temporal lobe epilepsy.[44]

In addition to synaptic release, the reversal of the GABA transporter, GAT-1, has been observed as a non-vesicular mechanism of GABA release. GAT-1 undergoes the coupled translocation of Na^+, Cl^-, and GABA in a ratio of 2:1:1[63], and the transporter can be reversed by increased intracellular concentrations of Na^+ and GABA (119, 120, 121). QHGAD has been reported to release GABA in this fashion in transfected DRG neurons, and thus it has the potential to release GABA constitutively, independent of membrane potential and synaptic vesicle activity.[59]

Animal models of seizures and epilepsy allow for the mechanisms, as well as the treatments, of epilepsy to be exten-

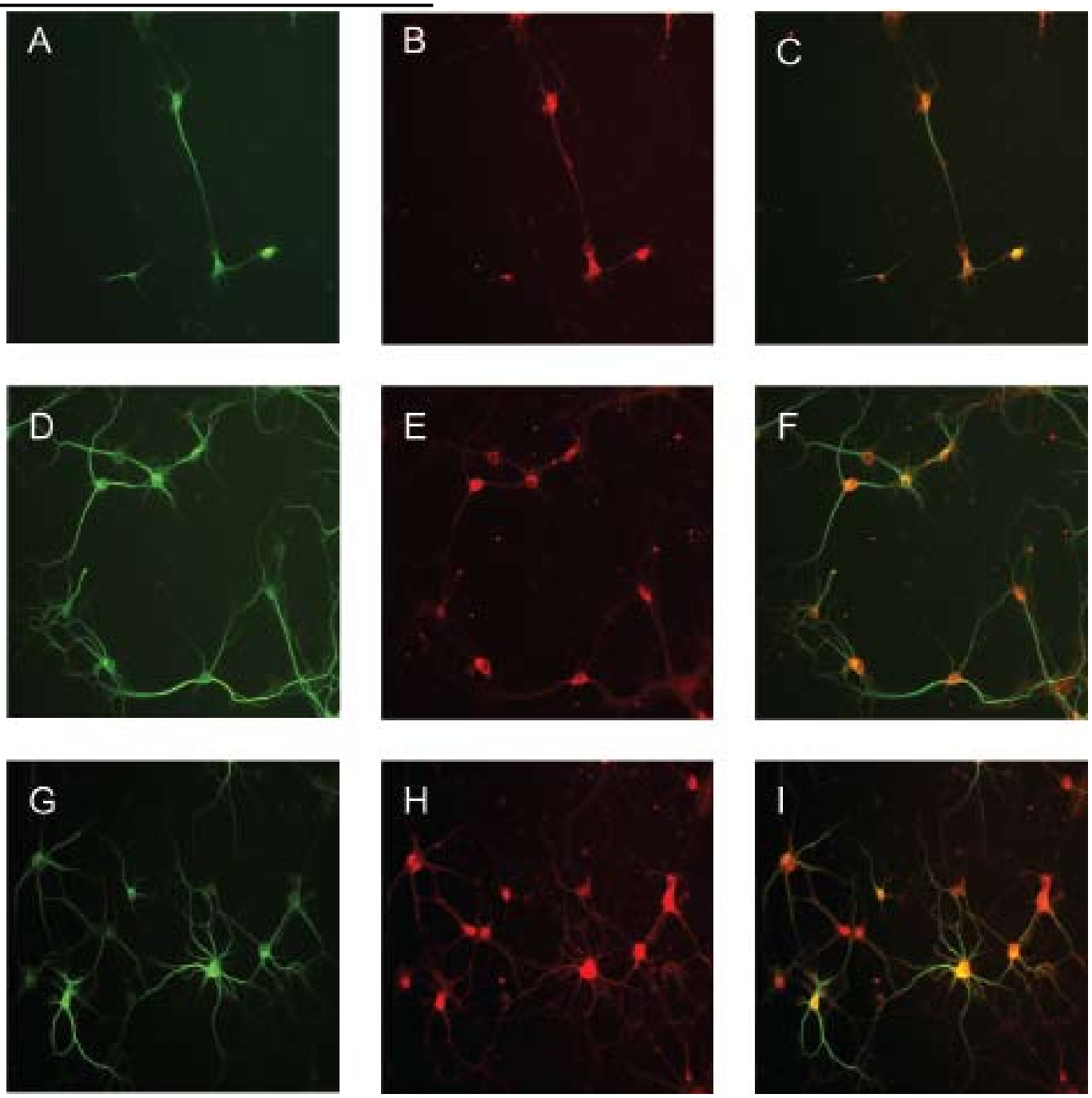


Figure 12: *Immunofluorescent staining of hippocampal neurons in vitro shows the strong co-localization of a neuronal marker (MAP2) with NαV1.1 (a,b,c), 1.2 (d,e,f), and 1.6 (g,h,i). A, C, and Fare hippocampal neurons stained with MAP2, and images B, E, and H are VGSC α-subunits stained with the antibody corresponding to the specific subtype. C, F, and I are overlays of these images displaying co-localization.*

sively researched. In the 1980s, the excitotoxin pilocarpine was determined to be valuable for designing new therapeutic approaches to epilepsy.[109] Pilocarpine was observed to provoke seizure-characteristic behaviors, and to produce long-term effects of epilepsy and seizure related brain damage.[83] Today, the pilocarpine model of epilepsy allows us to conduct two types of pharmacological studies: acute studies concerning our treatment's efficacy against status epilepticus; and chronic studies concerning our treatment's potential for prevention of recurrent seizures.

To date, we have only completed the first round of studies using QHGAD, but the efficacy of the vector is encouraging. We showed that QHGAD inoculation in the rat dentate gyrus was able to prevent status epilepticus in 87.5% of animals injected with pilocarpine, while only 25% with control vector inoculation were protected from S.E. upon pilocarpine administration. Results from the QOZHG group are consistent with other reports from sham animals[53], and inter-animal variability likely accounts for the percentage of these animals that did not reach S.E.[58]

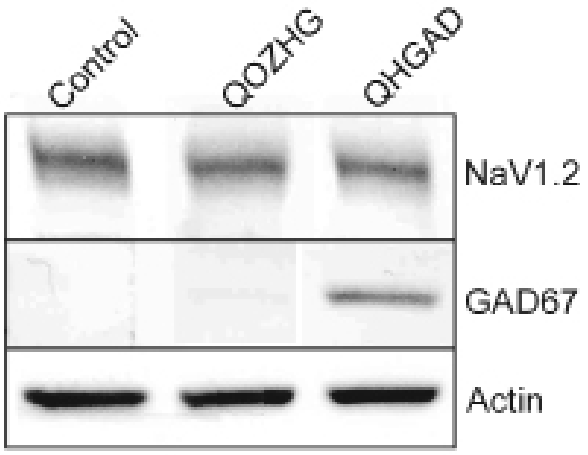


Figure 14: *Western blotting detects a strong increase in GAD67 protein upon QHGAD infection in vitro, but a decrease in NαV1.2 and/or 1.6 is not detected. NαV1.2 is shown here. The blot displayed is representative of several trials.*

It has been previously determined that seizure activity results in rapid and transient c-fos up-regulation in dentate granule cells and CA1-CA3 cells, reaching maximum levels within 30 minutes of seizure termination.[20,21,22] We used this marker as a confirmation of seizure activity in QOZHG and QHGAD injected animals in an attempt to support behavioral findings. We found that the seizures experienced by control and QOZHG inoculated animals were consistent with an up-regulation of c-fos in these regions. Furthermore, QHGAD injected animals that did not exhibit S.E. did not show an up-regulation of c-fos.

Interestingly, the single QHGAD inoculated animal that reached S.E. did not follow this trend. This animal did not show the strong up-regulation in dentate granule cells, but did show some degree of up-regulation in CA1-3 regions (**Figure 6**). We are unable to fully explain this novel finding, but we hypothesize that the high expression of GABA in the dentate granule cells and their strong inhibition remained intact, and status epilepticus was reached through another pathway in the brain. The perforant pathway connecting the entorhinal cortex (EC) to the CA1-3 regions is a possible candidate. The EC is believed to be a seizure origin for many patients with TLE[38], and it has been reported that neurons leaving the EC can stimulate the CA1-3 regions without stimulating the dentate gyrus[26]. Based on *in vitro* studies showing the contribution of the CA regions to seizure initiation and maintenance, we believe that this mechanism was the possible pathway for generalized seizure induction[2].

Many alterations in brain protein composition have been observed in animal models of epilepsy as well as human epileptic hippocampus, and compensatory mechanisms of VGSC regulation are noted to occur in sodium channels' α-subunits, as well as their β-subunits.[23,111,116] We hypothesized that the continuous release of GABA from QHGAD transfected neurons would alter the electrical environment and result in an alteration of VGSCs. We examined NαV1.1, 1.2, and 1.6 because of their marked presence in adult hippocampus[29], and the β2-subunit because of its association with these channels and its reported alterations with seizure activity.[17,76]

Analysis after 3 days of QHGAD expression revealed a strong down-regulation of NαV1.2 and 1.6 proteins (**Figure 7, Figure 8**); the decrease was statistically significant for NαV1.2, and experiments are currently underway to increase the sample size and to perform statistical analysis on NαV1.6 bands. We also observed that this down-regulation correlated with the level of vector-mediated GAD67 expression, as shown in the 3 week time course (**Figure 9, Figure 10**).

The continuous release of GABA having an effect on VGSC α-subunit protein levels is a novel finding that has not been reported by other groups. In order to gain insight to this effect, we designed experiments to determine what cell types were expressing these VGSCs and what mechanisms were responsible for their down-regulation.

Since it has been reported that hippocampal astrocytes, like neurons, exhibit VGSC electrophysiological properties[10,103], we utilized immunofluorescent staining to determine the cellular localization of NαV1.1, 1.2, and 1.6 in rat hippocampus. Immunostaining of tissue slices suggests that the proteins being detected are neuronal in origin, but due to the limitations of light microscopy, we cannot rule out that a portion of the proteins detected are in astrocytes in close association with labeled neurons. These experiments were repeated *in vitro*, and a confirmation of the prevalent neuronal localization was observed (**Figure 11**), with the exception of occasional astrocytes staining for low levels of these α-subunits.

The down-regulation of VGSCs is associated with several neurological diseases and insults, such as epilepsy and ischemia, yet little is known about the mechanisms of down-regulation. It has been proposed that neuronal VGSCs may be the targets of the ubiquitin-protein ligases of the Nedd4 family[33,95], but we have not ruled out decreased transcription of VGSC genes as a mechanism of the down-regulation resulting from QHGAD. Experiments looking at VGSC α and β-subunit mRNA levels by reverse transcription-PCR are currently underway.

In an attempt to explore the mechanism of VGSC α-subunit down-regulation, we utilized the dissociated hippocampal neurons from rat neonates and transfected these cells with QHGAD. We aimed to determine if blocking $GABA_A$ and/or $GABA_B$ receptors would abolish the observed down-regulation. However, the down-regulation of α-subunits was not observed in this cell culture as it was observed *in vivo*, despite strong GAD67 expression (**Figure 14**). We hypothesize that this may be due to several factors including the lack of an intact neuronal network (as is present *in vivo*) or the molecular differences between neonatal and adult hippocampal neurons.

It has been reported that early in development, GABA serves as an excitatory neurotransmitter in the brain due to the decreased expression of the potassium chloride cotransporter, KCC2.[57,92] We hypothesize that this reversed effect of GABA in neonatal cells may be responsible for eliminating the mechanism of voltage-gated sodium channel regulation.

Another possible explanation for this phenomenon lies within the relative abundance of NαV1.1, 1.2, 1.3, and 1.6 in the hippocampus. Levels of these proteins are reported to change between neonatal and adult life, with 1.3 being the

predominant VGSC α-subunit in neonatal hippocampi.[5,29] We hypothesize that NαV1.3 levels are high in our hippocampal neuron culture and that these proteins, rather than NαV1.2 and 1.6, may undergo down-regulation after QHGAD infection. Experiments to test the levels of NαV1.3 have yet to be conducted, but we plan to complete these experiments in the near future.

Also, experiments using an organotypic slice preparation of adult rat brains are being planned to determine if an intact neuronal network is necessary for the regulation of VGSC α-subunits and to eventually explore the electrophysiological properties of this type of VGSC regulation.

In addition to exploring VGSC α-subunit regulation, future experiments will need to assess the ability of QHGAD to be an effective treatment for chronic epilepsy. It may prove that QHGAD does not provide the long-term protection that is needed due to the short time span of GAD67 expression, and in this case, a vector should be constructed with a longer acting promoter in place of the HCMV promoter. Studies using the HSV-latency promoter LAP2 for long term expression have been previously published and show transgene expression for up to one year.[18,91] Chronic studies will allow us to determine whether GAD67 transgene expression is capable of preventing recurrent seizures over months or years. The results of this type of study will provide better insight to the clinical capabilities of gene therapy in the setting of anticonvulsant-resistant epilepsy.

It has been extensively observed that the cellular environment of the cortex and the hippocampus can give rise to seizure activity by a variety of mechanisms. These include alterations in VGSCs[23], "burst-generating" cell activity[71], shifts in EGABA due to K^+/Cl^- cotransporter regulations[86], and synchronization of cell firing due to ephaptic interactions[47]. Despite the variation, all of these epileptic mechanisms could be conducive to improvement by enhanced GABAergic activity, and GAD67 gene transfer provides a reasonable method of achieving this enhancement.

Thus, the development of novel treatments for epilepsy is ongoing, and the results from our experiments, as well as other studies in gene therapy, show promise toward management of intractable epilepsies.[49,72,91,121] As previously shown, HSV-vectors can provide a safe and effective means for transgene delivery, and hold great potential for the future of gene therapy. The findings presented above are encouraging for the possible use for GAD67 transgene expression as a treatment for epilepsy, but many critical experiments remain to be pursued.

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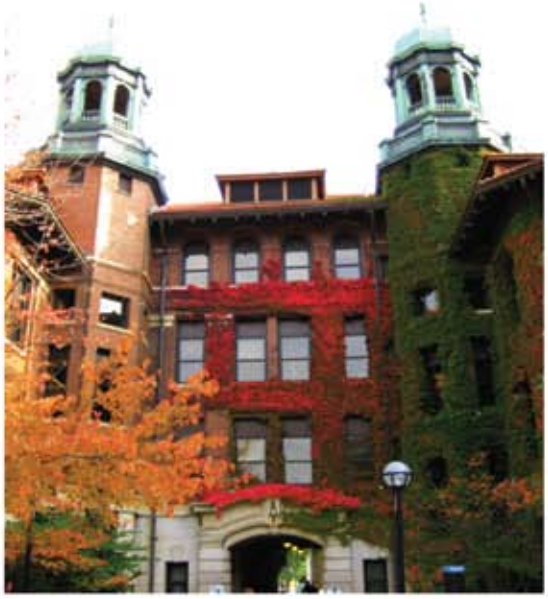

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Modern, Local Doulas: Birth Advocates and Activists in Ann Arbor, Michigan

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Abstract

Labor and delivery support has occurred at least since the beginning of documented human history. But the circumstances under which women labor and give birth have markedly changed in many cultures. In order to document one such change, I researched the modern profession of birth support via interviews with six birth and/or postpartum doulas[1]. A doula is an "experienced professional who provides continuous physical, emotional, and informational support to the mother before, during, and after birth, or who provides emotional and practical support during the postpartum period. They do not perform clinical tasks."[1] It is important to note that there is no mention of the term "medical support" in the definition which will become significant later when I evaluate the relationship between nurses and doulas. This research found doulas to be extremely qualified communicators and excellent labor advocates, earning rave reviews from new parents. Unfortunately, though doulas enjoy their work, they struggle with what they see as unneeded medical intervention on a regular basis.

Introduction

A doula is not only a birth companion, but a friend. According to the organization DONA (Doulas of North America) International, a doula's primary job is to mother the mother, providing psychological support during childbirth. Doulas also give tips and encouragement to the mother's partner (without displacing the partner), in order to help provide the best possible birth experience. Some doulas, known as postpartum doulas, also work with the family after birth, to ease the transition into a new or growing family.[1] A doula is not a medical worker; it is not her job to deliver the baby, although it could happen by accident, so "doula" is not synonymous with lay midwife or nurse-midwife. Another major difference between doulas and lay midwives is location. Lay midwives typically do not work in hospitals, and even though some doulas may attend many home births in their careers, none of the doulas I interviewed were exclusive to non-hospital births while one only had the opportunity to work with hospital births. Many are certified as birth and/or postpartum doulas or "labor assistants," not as nurses, unlike many midwives. Furthermore, doulas are not trained to assess and react to emergencies as certified professional midwives (CMs and CPMs) are. A doula can have up to five main "roles" in the profession, which include labor support skills (including skills that keep the partner involved, such as massage), guidance and encouragement, covering gaps in the care of the mother, team-building, and communication improvement among all those involved.[5] All six doulas I interviewed summed up what the research suggested as "my job is to do whatever I can do to support the woman and keep lines of communication open and continuous."

There are several reasons why women would hire a doula. DONA International stresses that birth and the immediate postpartum period are socially and emotionally complicated, and individuals experienced in mothering new mothers can be very helpful during this time to provide reassurance that the birth is proceeding normally.[1] One doula said "it's so sad to see someone get an epidural; [She] is doing so well, so in rhythm with herself, unaware of her pain, and then a seemingly compassionate nurse or doctor says, "You're tired do you want the epidural now?" which may make the mother think something is not normal, and the natural flow is totally disturbed." Doulas also have the opportunity to get an understanding of the mother's personality at a deeper level than medical professionals, who are often distracted. This deeper knowledge of the mother's personality means she has someone with her who can help her sort out several complicated issues. For example, a doctor may be glossing over medical information at a fast pace and be unavailable to answer questions. A doula can either explain what certain words mean or encourage the mother to make the doctor slow down to give the mother clear explanations. Communication between hospital staff, who can provide labor tips to the other, can be inhibited if the personalities of the mother and staff do not align. A doula can act as the bridge between the staff and the mother in such situations.

Doulas are critical for keeping the lines of communication open during labor and delivery. Unfortunately, doulas can be caught in a tough middle ground between "natural" and "medicalized" birth, especially with the increasing dependence on caesarean sections births in the medical arena. "Natural" and "medicalized" are variously defined and understood; in this paragraph, I am referring to the interviewees' perceptions of these terms. The middle ground between "natural" and "medical" births is unpleasant and difficult for many doulas. Many interviewees perceived this middle ground as unpleasant because their own personal philosophy suggests that birth is, most often, a safe and natural event requiring little or no medical intervention, versus the perceived conventional view that birth is a high risk event requiring expensive, rapid, and occasionally uncomfortable procedures in order to be "cured." Pushing a natural event towards a biomechanical event removes important social bonds and turns what can be a personally and socially fulfilling occasion into an intense doc-

1 All names used are pseudonyms

tor's visit. However, doulas continue their work because they know doula support makes a difference to individual families. Doula care is a grassroots movement towards acknowledging the more human side of childbirth, even in a sterile environment such as a hospital. My research goal was to learn doulas' perspectives on medicine and its role in childbirth and also how their own lives have been affected (i.e., personal relationships and "worldviews"). I also wanted to know the prevalence of medical interventions when a doula is present and when one is not. In addition, I wanted to learn the role of nurses and doctors in hospital births, especially when there is tension between doulas and nurses.

Methods

In the winter of 2007, I conducted research on the profession of doula care as well as the medicalization of childbirth. I consulted pediatric, obstetric, and nursing journals to find data on the prevalence of medical procedures in doula-supported births and non-doula supported births. In addition to journals, I also consulted books that provided information on the role of the medical community in childbirth. The study was limited to doulas that practice or place their business cards in Ann Arbor. Many of the doulas were identified by business cards made available at locally owned businesses, such as Center for the Childbearing Year and People's Food Co-op. I interviewed six doulas in the vicinity of Ann Arbor, Michigan about their profession and found them to be a diverse group of women who have unique and lasting places in the lives of the women and families who hire them. I contacted two through their business cards, two were referred to me by friends, and two contacted me through a sign I placed at Canter for the Childbearing Year (CCBY). Each doula signed an informed consent letter promising confidentiality of names and permission to not answer any uncomfortable question. This study underwent a preliminary screening with the IRB and was exempted from further review.

Results

Medical Journals and Doulas

All of the doulas I spoke with believed they play beneficial role in birth. Medical research supports this anecdotal evidence. At least two of my interviewees know about this medical literature, which they could cite, should they ever encounter a skeptic. In one article, the authors note that when a doula is consistently present, labor is 25% shorter, 60% fewer epidurals are used, forceps deliveries are 40% less frequent, and c-section rates decline by 50%. Postpartum effects are also significant. Women who had doulas report more self confidence, while suffering from less depression than women who did not have a doula. Fifty percent reported more breastfeeding. Doula-supported women also appear to have more affectionate interactions with their babies in tests done two months after birth.[2]

After reading such articles which document the benefits of doula support, it may be difficult to understand why someone would not want a doula. Yet, there is alternate research suggesting there are not many differences between women who had doulas and those who did not, according to reports on hospital-based doulas in HMO-managed hospitals. However, this report of 214 births (149 births with doulas) also shows decreased use of epidurals (by 11.7%) and reports of "better" birth experiences among women supported by doulas. However, it appears as though the rates of caesarean delivery (1% difference), forceps delivery (9.6% difference), postpartum depression, breastfeeding (2.7% difference), and self-esteem were not significantly different among doula-supported women.[3]

Both of these studies can be problematic. The first paper claiming numerous benefits did not cite how many women were studied. The authors of the paper showing fewer benefits admitted that the study was centered around white, well-educated women, while other studies are more diverse in their sample populations. Using white, well educated women is problematic because this type of group is much more likely to attend childbirth classes and be better prepared, making them less fearful of the process.[3] After all, not every woman who goes into a hospital to give birth is well-prepared, and this study did not acknowledge the effect of doula support for women who are not prepared for birth. Also, the research stating doulas as being only marginally beneficial was published in an obstetrics and gynecology journal, which may mean there is a professional gap between doctors and doulas. Doctors may not understand what doulas do, or may feel threatened by the presence of someone who may seem "unskilled" compared to a physician. This would be a conflict of "authoritative knowledge," or whose opinion carries the most weight.[4] Doctors are essentially in charge of a hospital birth, but women listen to their doulas and communicate well with them, so a doctor may feel like his/her authority is being challenged by someone with fewer skills. The research that supported the benefits of doulas, however, was published in a pediatrics journal. Pediatricians rarely deliver babies in the United States, so they may be more open-minded towards doulas since they only hear birth stories from new parents instead of actually being at a birth to scrutinize and critique a doula, or be critiqued by a doula.

Are Doulas Paraprofessionals?

In spite of excellent reviews by new parents, doulas are struggling to become a respected childbirth paraprofession. A paraprofessional can be defined as a person with fewer credentials or less training who works along with a professional. Naturally, there is debate about what a paraprofessional actually is. Some doctors see nurses as paraprofessionals, while people outside of the medical profession may see a nurse (especially one with many years of experience) as a highly trained specialist, rivaling a doctor in knowledge and quality of care. Since it is difficult to determine the professional status of a nurse, it may be even harder to say if a doula is a paraprofessional since doulas are not only rare but also have many different levels of experience and training. Doulas themselves are primarily well-educated (fewer than 7% with a high school diploma or less), white (93.8%), married women with children (87.8%), with a significant number planning careers in midwifery (27.2%).[6] On the surface, doulas might seem like a group that would be welcome as paraprofession-

als because they are primarily white and well-educated, which makes them members of a reasonably privileged strata of society. However, this is not the case, even though there is no evidence that doulas are harmful.[2] Only one in three doulas claimed that the work is financially rewarding, and many said they were not respected by clinicians. Several said they struggled to balance their own family with their career. Many major obstacles in the profession concern pay. Doulas are not covered by insurance, and few get any "third party" payback in spite of the presence of a doula reducing the need for interventionist procedures, saving insurance companies money. However, involving insurance companies might create limitations on what doulas can and cannot do, making a doula nothing more than a cog in an enormous medical machine. The idea of a doula as a childbirth professional is still relatively new and more study is needed.[6] Another question also arises: does a doula really want to be associated with the medical field at all, either as a professional or paraprofessional? Many doulas are doulas partially because they believe it is helpful to have a non-medical person present at a birth.

Doulas and Nurses: Cooperation or Competition?

If a doula is indeed a childbirth paraprofessional (or a profession), could it be that the doula actually has a complementary role with a nurse during hospital deliveries? A nurse is responsible for the woman's physical safety, while a doula helps make a woman *feel* safe and in charge emotionally. Nurses can tell the doctors what a woman wants, while a doula can help the woman tell the nurse what is desired, as sometimes hospital professionals do not interpret the wishes of patients very well. A nurse may indeed be very helpful and caring, but has other responsibilities to attend to, while a doula is present the entire time and can keep everyone updated and well-informed, without their job being complicated by other patients or paperwork.[7] Although the combination of nurse and doula may sound complementary overall, it is not perfect. The largest conflict may be "turf" issues.[7] A nurse may feel the doula is trying to take over, while a doula may see a nurse as pushy and invasive to the woman in labor. My interviewees said both nurses and doctors are too forceful and do many unnecessary procedures, such as very frequent vaginal exams and monitoring that disturbs the rhythm of labor. This may lead to a medical practitioner suggesting a c-section due to "failure to progress." One interviewee described hospital staff as "mean." Another interviewee said there is no reason for medical intervention in a delivery with no emergency medical complications[3], and if she voiced this opinion in the presence of a nurse, that could be taken as a challenge to the nurse's authority.

Another potential point of tension between nurses and doulas is the c-section rate. The profession of the modern doula began to grow in the 1980s, partially out of the growing concern over the climbing c-section rate in that decade. Women who wanted to avoid cesarean deliveries were encouraged to hire labor advocates. Although these early doulas were sometimes individually militant in their anti-c-section views, no professional doula organization has a premise of being anti-c-section.[5] Nurses and doctors, however, may not know professional doula organizations do not advocate a "no c-section policy," and if one doula has a history of militancy for avoiding c-sections, that doula may be labeled as a "troublemaker" at a particular hospital obstetric unit. Nurses are also, by definition, medical workers. At some level, they have accepted a mechanized model of childbirth simply by choosing that profession, while for a doula, that is not necessarily true, since they are not working in the medical profession.

Doulas Speak on their Profession

In my interviews, I asked doulas about medical aspects of childbirth and questions about their evolution as doulas up to the present time. I wanted to know how these women became interested in doula care, what their training was like, their favorite and least favorite parts of the work, and how travel, other careers, and their own families have affected their relationships with their clients or otherwise contributed to their career. I also asked about how their own personal feelings play into the work, and how seeing so many births may have changed their outlook on life, professional roles, and society.

I interviewed six women, all birth doulas (and three who are also postpartum doulas), all American-born and between the ages of 20 and 40. Despite these similarities, I still found each of them very unique. All of them became interested in different ways. The first interview I conducted was with a young woman (Wendy) who became a doula because she realized her need to be in a job where she can practice empathy and care about others. She discovered this after a difficult relationship, and affirmed it by speaking with her precocious sister.[8.1a] In my second interview, the doula (Julie) had been very dissatisfied with the medical team at her daughter's birth ten years earlier and decided if she could help just one woman have a good birth, that was a positive thing to do. She had also worked as a licensed practical nurse in oncology, and decided she would be a better fit in profession that deals with keeping normally healthy people healthy as opposed to the constant illness present in oncology.[8.2] The third doula's (Lisa) sister is a home birth midwife. She attended the birth of her sister's daughter, and the sister said she had a "good touch" and "good hands."[4] This was also during a difficult time in Lisa's employment, so the switch to being a doula was very well-timed[5].[8.3] Yet another doula (Kate) felt empowered by a natural delivery with one of her children and wanted to share her knowledge.[8.4] One of the youngest doulas I interviewed (Shelly) was inspired by Lisa Kane Low's "Perspec-

2 It would indeed be difficult for a doula to be physically harmful since they do not medically intervene. Probably the worst that could happen with a doula would be a serious personality clash between the mother and doula during the delivery that ends in a number of people becoming upset.

3 This doula actually said "natural and medical do not belong in the same sentence." However, she did acknowledge that medical emergencies do happen, and some hospital procedures are warranted. Another doula mentioned that to someone who has not studied childbirth academically and is very comfortable with a doctor, is simply blissfully ignorant of how good a birth could be.

4 Doulas often do massage and help the laboring woman with other relaxation techniques. In this case, "good hands" and a "good touch" are quite literal..

5 At the time, Lisa had an unsatisfying, unfulfilling job, and the birth of her sister's child came right in the midst of this career transition.

tives of Women's Health" class at the University of Michigan and other women's studies classes relating to women's empowerment in many aspects of life, including careers, schooling, sexuality, and community roles. The picture was completed during a class called "Women in the Community," which was taught by an individual who is active at Center for the Childbearing Year, where many local doulas train.[8.5] In my final interview, another young doula (Mia), who was applying to nursing school and wants to be a nurse midwife, said she had seen a television show where someone had a doula, and it looked like a good connection to nursing. When her sister trained as a doula, she decided to do the same.[8.6]

> "A doula is a person of communication, which is a health benefit in and of itself."

Training

None of the interviewees had been doulas more than approximately thirteen years, which seems appropriate because doula care in this country became more common in the 1980s.[5] Since the profession of the modern doula is still relatively young, it is reasonable to think there would be many similarities in the styles of training, even among doulas who had trained at different times, and this research found this to be true. Mia, Wendy, and Shelly all trained at the workshops at Center for the Childbearing Year (CCBY) in Ann Arbor. They were also the youngest interviewees - all in their early twenties. They described the training as a very warm and welcoming experience where they were able to network and become very close to the other women in their class. They felt everyone wanted to be there, unlike many college classes. More than once, it was stated that the training was intense, as much material was covered over just three days. In addition, emotions ran high, mainly because of birth films and mothers in the class sharing their birth stories. At least two of the doulas said a weekend long training session may not seem like much but could be nerve-wracking. Since being a doula is about emotionally supporting someone, sometimes the doula just has to get out there and do it. The "trial by fire" period is also what makes the Doulas Care program so helpful. Doulas Care is a volunteer doula program run out of CCBY, and it helps place new doulas with five successive clients as a volunteer, in order to learn and gain skills before they go on to further certification and/or advertising themselves independently in the community. Naturally, without that volunteer experience, it could be more difficult to find the first client when the new doula has never actually seen a live birth. Kate also attended the weekend workshop at CCBY, but she was already a childbirth educator. Her childbirth educator training included an intensive workshop, reading several books, observations of birth care and breastfeeding support, and a test. She entered Doulas Care as well, which was quite comfortable for her since she had prior experience with teen mothers and other women who may have limited means of support.[8.4] Lisa also trained at CCBY, though she said most of her initial training actually came from her sister, a home birth midwife who helped her into the field as a postpartum doula.[8.3] Julie was trained in the local area via an ALACE (Association of Labor Assistants and Childbirth Educators) weekend workshop that sounded very similar to the training at CCBY, with reading materials, informative speakers, and demonstration of support techniques such as massage.[8.2] The big difference seemed to be with what happened after the training. Doulas Care is a typical follow-up for CCBY trainees, where volunteer doulas attend five births and then go on independently and/or with additional training to be DONA certified. DONA certification is a commitment to attend a sixteen hour workshop that is DONA-approved, read five books from a required reading list provided by DONA, read and understand a certification packet, take an "Introduction to Childbearing" class with the workshop, attend, document, and receive good professional reviews from three births, write an essay on the value of labor support, and understand and sign the Code of Ethics. Currently, there are 5,500 certified DONA members doing both birth and postpartum doula care[1]. [6] ALACE expects its birth attendants to attend eight to ten births for certification and to keep up the work after the eight to ten births. For certification, ALACE also has a reading list and requires a self-evaluation of six births, a written test, three performance evaluations by someone who observed the doula, as well as auditing a series of childbirth classes.[9]

Their Work

Overall, the doulas enjoyed their training, so it is important to investigate the reasons for their sustained interest in their profession. Although I received many answers, they all fell into the category of "healthy, happy mom who says all the difficulty was worth it and a happy baby." More specifically, popular responses for sustained interest included observing the first five seconds of a baby's life, aiding the mother in the release of long-term tension after the building intensity of pregnancy, and helping nervous women learn that everything possible will be done to make the birth go well. Other favorites were seeing and feeling that being a doula makes a difference in this major life event, the amazing wonder and privilege of watching a birth, the diversity of women and births, the myriad of surprises, the sense of community, and a spiritual connection.

Like any other job, there are also drawbacks. Wendy says sometimes she thinks epidurals are "weird" because they interrupt the natural flow of labor. The doula also can not leave, so it is easy to get lightheaded and exhausted while simultaneously not letting the laboring mother see the doula's discomfort. In other words, the main support person has the second hardest job.[8.1a] Also, being on call is stressful, since everything has to be dropped to attend the birth, which can be challenging with family and school responsibilities. Lisa says

6 I asked an Ann Arbor midwife how many doulas she thought were in the area. She estimated that Doulas Care has trained over 300 doulas, most of whom live in Southeastern Michigan. It is possible Ann Arbor alone has 50 or 100 doulas; but it is hard to tell because they are at varying levels of certification, and some may no longer be active.

any unnecessary medical procedure is very frustrating because a woman may not even realize how hard she is working and how tired she is until someone offers an epidural and says "you're tired, want this?" Dysfunctional families and unkind hospital staff are also problematic; they are upsetting to both mother and doula, and interrupt the natural flow of labor[7].

A recurring theme in my interviews was that many doulas believe most medical procedures can and should be avoided, as a woman's body is usually capable of delivering a baby without drugs or surgery. I noticed a lot of frustration aimed not at laboring women themselves who choose drugs or surgery, but at medical personnel who insist the procedures are safe and painless, when, in fact, many take away pain only temporarily and induce much longer recovery periods. It is also important to keep a civil tongue with families as well as unkind obstetric teams. A doula may end up feeling like a hostage negotiator, as Julie said. She also said there is a razor thin line a doula must walk with everyone involved; a doula can not irritate anyone. Not only may an irritating doula upset the family, word of an irritating doula will spread around the hospital, and when the doula goes back, the doctors and nurses are already cynical and untrustworthy of that particular doula.[8.2] Overall, my interviewees overwhelmingly saw their role in medicine as a support person to the person going through some procedure or process, which makes sense, since a doula is not even allowed to take someone's temperature.[7] Most said they like to stay away from being associated with medicine and want to have as much a role as possible with the family and keep the association with medicine and medical procedures as minimal as possible, without losing any strings of communication.

Several of my interviewees have traveled to many parts of the world, such as Australia, France, Jamaica, Costa Rica, Guatemala, Mexico, and Italy. None have attended births in other countries. However, a recurring theme in their memories of travel was that experiencing all these different cultures promotes respect for all people in general, which subtly or not-so-subtly translated into increased respect for their own clients here in the States. One doula said her travels allowed her to see how a society functions, so when a person gains respect for a culture, respect is gained in all or most aspects of that society, up to and including birth. I suspect travel around the world allows someone to speculate about how American culture evolved, and notice things about American culture that fade into the background when it is "lived" every day in the USA. Nuances of American culture appear during birth, and a doula that has traveled may have more respect for how that nuance grew and changed.

Another possible change I thought being a doula might bring is a change in outlook on life or the world, or in relationships. All my interviewees said they enjoy interacting with people they probably would not otherwise meet, and have expanded their circles of friends and acquaintances greatly. All appreciate the experience of being involved in such an intimate aspect of someone's life. Some changes and experiences are very profound. Kate recalls a birth she attended with a single mother where she was in the hospital bed along with the mother vocalizing with her during this very intense labor. When the baby was born, Kate held the baby, an honor usually reserved for the father. She said she felt baptized as she cried right along with the new mother. Another profound realization is that things are not always what they seem to be. Sometimes, as Kate told me, family members may seem "mean" and unsupportive when they are really just scared.[8.4] Shelly said seeing people for who they really are in a very intense situation is hard to describe but nonetheless "enlightening."[8] Similarly, Mia loves pregnant women and has gained immense respect for them, their strength, their commitment, and what they go through. Lisa described that her travels abroad helped her to realize that the way birth is treated affects a whole culture and the way people parent. Birth starts an entire chain of relationships, for better or for worse. Lisa was also more frustrated at biomedical culture, but being a doula is also about being diplomatic and being present for emotional support.[8.3] Julie also concurred, saying if she had never been a doula, she never would have seen the fundamental problem starting at birth, how biomedical culture does not respect women and their beliefs. She said biomedical culture affects how people parent, while supportive birth services get parenting started on the right foot. Wendy has some interesting philosophical connections to her experiences with birth. Birth is powerful and moving, according to her and many others. (More than one time, the word "miraculous" came up, independently, in several interviews). It is also full of symbolism and spirituality. For example, Wendy said it is spiritually "disarming" and amazing to see how powerful women are.[9] Difficulties in life can be akin to labor and delivery.[8.1b/c] The emotional and philosophical connection may not be very obvious, but it is still there. She said life is like labor: you may have support people along the way, but it is ultimately your experience. Kate said birth is the most sacred event in someone's life, more so than any man-made institution. With regards to symbolism, cultures all over the world show evidence of reverence for birth and the power of women in their writings and art.[10]

Doulas and the Role of Medicine in Childbirth

The core of American 20th and 21st century childbirth research is studying the role the medical field plays during birth, even if a delivery is at home attended by midwives. Everyone in this country is somehow influenced by American biomedicine. In many traditional cultures across the world, birth still takes place in a dimly lit, enclosed place, often with several female attendants, while birth in the United States typically takes place in a sterile hospital room. Robbie Davis-Floyd describes this as the technocratic model of birth. The technocratic model of birth views the body as a machine, and pregnancy and birth a mechanical failure of the machine, needing to be fixed by doctors and medical procedures. A technocratic model is also a way of legitimizing patriarchy. Birth is a female event, but men and other authorities (who may, in fact,

vii "Dysfunctional family" has a broad definition. This can mean relatives visibly arguing with each other, or worse, with the mother, families that are so loud and obnoxious that the mother gets upset during labor, and women who are depressed over relationships.

8 "Enlightening" was her main description of birth in this section of the interview, she told me there really are not better words.

9 She also mentioned a strong sense of reverence for birth and the women who give birth.

be female, but have selected jobs where men are in charge, i.e., nurses being supervised by male doctors or departmental heads) can cut into this female event in many ways. By perpetuating stereotypes that birth is inherently dangerous and a disease able to be fixed only by the medical system, birth falls into the hands of men and medicine. Wherever birth falls under a technocratic model, the expectant woman no longer has control.[11] Bridgette Jordan would describe the conflict between medical personnel, doulas, mothers, and traditional birth practices in other societies as a competition over who has authoritative knowledge. The person with authoritative knowledge is the person with the final say in what happens and whose opinion carries the most weight.[4] A doula's role and level of authoritative knowledge, especially, can be unclear in a hospital birth. The uncertainty of the role can be fueled by something as simple as the attire of a doula. Hospital employees usually wear uniforms that vary depending on what hospital they are working at, and in all the hospitals I have been to, all staff have a visible identification card with the title of their profession. Women giving birth in the hospital wear hospital gowns, which removes their individual identities normally provided by personal clothing and denotes them as a patient.[11] Doulas wear their usual clothes at births. There is nothing showing professional status and nothing showing patient status, so it may be hard to tell where a doula fits into the "team" of people participating in a birth.

American biomedicine and its rituals and practices have come to symbolize modernization and progress all over the world, while eschewing other ethno-obstetric systems (many of which leave authoritative knowledge to a mother or midwife) as primitive and inadequate.[12] Spreading biomedicine also spreads the patriarchy Davis-Floyd describes. Indigenous knowledge and knowledge passed through the generations by women in this country is not necessarily all bad.[4] For example, if some hands can easily catch the baby, there is nothing considerably dangerous about giving birth while squatting or standing, which is common in many traditional cultures. A squatting or standing birth is not as convenient for doctors, so they endorse the lithotomy position, or lying on the back.[4]

In a global society, American medicine in general has certainly spread, with some positive effects. For example, rates of polio have fallen dramatically all over the world since the introduction and spread of an effective vaccine. Not all effects of American medicine have been positive. In some countries, the technocratic model of birth has been introduced, but medical facilities are not up to par with American hospitals. A c-section in this country is reasonably safe, but a c-section in a resource-poor country can be very dangerous, because the hospitals may not be able to afford to clean their facilities or properly sterilize surgical equipment.[13] Naturally, it would not be surprising to hear women in these countries saying "I am not going to the hospital." They may seem non-compliant and uneducated to "modern" Westerners at first glance (and a birth at home may also be hazardous); but if women in these situations are likely to die anyway, it is reasonable to let them die at home surrounded by their friends or family than alone on an un-sterilized operating table in a poorly-equipped hospital. Also, a woman in a developing nation may forgo a trip to the hospital for fear that she may make a long journey and not be waited on at all. An example of this is a woman who gave birth in a Nigerian waiting room even after she paid and attended prenatal checkups.[14] In addition to the possible hazards of a delivery room or inattentive doctors, women in traditional cultures who give birth under an American methodology also lose a valuable piece of their cultural history,[13] which can have a devastating effect. The loss of cultural history is beautifully illustrated in *The Spirit Catches You and You Fall Down*, in which a number of psychologists in southern California suggested that "role loss" as a result of a quick and treacherous escape from Southeast Asia, shaky settlement in the United States, and the lack of worthwhile jobs was a major cause of severe depression and other mental illnesses among Hmong refugees.[15] The loss of female support systems in any country is a disempowering event for women. Birth is a ritual and social event, because it does not just have to do with our bodies. It has to do with what is going on in our minds. Extensive and intrusive vaginal exams, fetal monitoring, and other medical procedures interrupt or entirely discredit the sociability and ritual practice of birth because it breaks the female solidarity and bonding that is so common in traditional communities and was present in America's past.[10,13] The doula extends a touch of this traditional community of women as much as possible in the hospital, and also in the event of a home birth where the pregnant woman has more freedom. Doulas and midwives clearly see birth as a natural process that should not be interrupted, therefore rejecting the technocratic model.

In spite of the technocratic-natural birth split, there is a balancing point where a woman can care about herself and also be reasonably sure her child is safe. In fact, part of a doula's job is reminding the expectant mother to actually communicate her physical concerns with her doctor or midwife, who is trained to recognize and intervene with truly dangerous situations. A doula is a person of communication, which is a health benefit in and of itself. Adequate communication saves lives.

Home birth is actually safer than it may initially sound. Julia Allison, an English midwife who did research between 1948 and 1972, found that 52% of women who delivered at home with a midwife and no doctor would actually be classified as "high risk" today, and thus would not fulfill the criteria for English midwifery services. The perinatal death rate for the home births was 0.3%. The perinatal death rate for the hospital group was 7.5%. If the high risk women were eliminated from the study, homebirth would likely look even safer.[13] In 1988 in Arizona, the low-risk, home-birth neonatal death rate was 1.1 out of 1000, compared to a 1989 country-wide survey of hospital birth (i.e., varying levels of risk), where the neonatal death rate was 10 per 1000 births.[11] Overall, the doulas I spoke with thought home birth was a very viable option for healthy women. Interestingly, it was the doulas who were thirty and older (two had children, one does not) who were more enthusiastic about home birth. Younger doulas did not seem to be as likely to promote home birth, although two hinted they would like to try a home birth when and if they have children.

During my observation at meetings at a local midwifery

center, a number of women mentioned they turned to home birth because they were appalled at the treatment they received by doctors in the hospital. However, family influences and fear can turn a woman who disliked her first hospital birth back to the home for subsequent children. One trend Americans seem to like is a "homey experience in the hospital," which can include a nicely furnished, private hospital room and nurse midwives present. However, one of my interviewees said even though the "homey" facility in the hospital may be nicer than a typical delivery room, there really is no place like home. In fact, she wishes she could videotape a hospital birth and a home birth to show her clients the stark differences before they make a decision where to give birth. She also said being a doula is a rare glimpse into both worlds of home and hospital delivery.[8.3] Other childbirth professionals do not have this opportunity because they either work for a hospital or an agency that caters to home birth. None of my interviewees ever left a client or refused her case because the client ended up in hospital or chose to go there in the first place. A doula has fewer boundaries as to her clientele and is exposed to a variety of locations as well as interacting with a broad client base. There really may be no place like home, and home is a safe option for low-risk women. However, if a woman has a terrible hospital experience in a standard delivery room, anything may be better, even if it is a nicer part of the same hospital. It is still blissful ignorance, though, and some doulas are more passionate than others about illustrating the differences between hospital and home. Interestingly, it was the younger doulas who seemed to be less concerned about the split between home, hospital, and the "homey" part of the hospital. Another current trend in American obstetrics, between traditional midwife and doctor, is a certified nurse midwife. I have heard people casually refer to certified nurse midwives as "medwives," while certified nurse midwives have their "turf" issues with both doulas and lay midwives who apparently try to "take over." An explanation was presented to me stating, "Lay midwives think nurse midwives sold out, while nurse midwives think lay midwives are irresponsible." Legally speaking, a lay midwife might be prosecuted for practicing medicine without a license, depending on which state the birth occurs in.[4] A lay midwife may defend herself by saying "yes, I am practicing without a medical license but birth is not a medical procedure." In fact, no doula I interviewed said she was exclusively anti-medical, just against unnecessary procedures. The problem is, according to the doulas, doctors do make women feel as though their babies are in constant danger. Even the slightest mention of risk can encourage a woman to put all of her trust in the hospital and little or none in herself.

There is no doubt that American birth is much more of a medical procedure than it was earlier periods, such as before the early 1800s, before medicine as a profession was fully legitimized. At the beginning of obstetrics as a legitimate medical profession, there was little social outcry. Doctors gained popularity among the urban middle class, and women of that social stratum accepted doctors' claims that they were better than midwives, and the practice of midwifery declined among this large social group. Some people thought men delivering babies were socially and morally inappropriate, but there were no known protests.[16] Still, how much medicalization is too much? For example, in the video "Giving Birth, Four Portraits" Margaret Mead asks "who determines what a natural birth is?"[17] A person could say drug-free hospital delivery is natural compared to repeated c-sections, while someone who gave birth outdoors might think a drug-free hospital delivery is too tightly controlled by the medical profession. This, again, is a debate about authoritative knowledge in childbirth. In many traditional societies, the mother or midwife (or both) has all the authoritative knowledge, while in the US it is the doctor or nurse midwife. On the rare occasion when someone delivers with a lay midwife in this country, authoritative knowledge is more balanced.

The Future of Doulas

None of my interviewees seemed disinterested or dissatisfied with their work. Rather, many described it as incredibly emotionally rewarding. With a high level of emotional fulfillment, it is clear that the medical profession will not push doulas away easily, as doulas are here for families, not doctors. Doulas maintain their critical link in a good birth by encouraging an open line of communication and a physically and emotionally supportive presence that garners respect from parents.[2] Even though doulas will not go away without a fight (should they ever be asked to stop practicing), I had to wonder about how the doula profession may alter with the changing legal and medical systems. Kate said the essential role of emotional support will not change, but doulas will have to adapt to more medical procedures and continue to help make women more comfortable in the midst of medicalization. Mostly, she thinks the doula's role may become more in depth as communication intensifies. Lisa said things are always changing, but she is unsure of how that would affect a doula's role. However, she is sure the magnitude and number of medical procedures will increase, although she has met many activists who are working to change that. Kate believes birth will become more polarized, although the amount and magnitude of medical procedures may (or may not) stagnate. She says the medical model is very powerful, yet people in the natural birth movement are very passionate and tenacious.[8.4]

America is a very litigious society, and doulas may be held legally accountable for what they do. Lisa believes the best a doula can do is not make decisions for her client, in order to limit responsibility; if one doula gets sued, the effect may be catastrophic. Kate is not sure if legal accountability will increase, although she certainly hopes not. Mia thinks there may be a small possibility that birth doulas may one day be out of work because of high c-section rates, although she remains hopeful that at least single mothers, who often have the least amount of support, will continue to use doula services regardless of the circumstances of the birth. Wendy thinks there is little chance the doula's role will change or that doulas will get sued. She believes if lawsuits were possible, doula prices would go up, and pregnant women will not want to pay extra expenses. In other words, doulas would find themselves out of work if prices changed dramatically. Legal accountability and changing roles also fit in with what Jordan describes as

cosmopolitical obstetrics. Cosmopolitical obstetrics encompasses all that is trendy, popular, and thought of as modern and progressive about birth in a given society.[12] Lawsuits are part of cosmopolitical views on the legal system in this country. It is legitimized in this society to sue a restaurant for hot coffee if the restaurant does not post warnings about the food temperature. If a doula's role changes to include hands-on medical care, suing a doula could become a legitimized trend. Lisa said lawsuits do tend to snowball, but as long as the doula's role is kept where it philosophically wants to be, as an emotional support person and not a medical worker, legal action may be limited. Doulas are a diverse group of women well-adapted to changing circumstances, and I am confident they will keep up with their learning and teaching to make the best birth experiences possible, regardless of what doctors and hospitals say. There is no substitute for a person with empathy and people skills.

Conclusion

Doulas are a unique group of emotionally supportive women who are not medical workers and want to keep their status that way, much preferring to be associated with the families they help. They persevere through difficult hospital births, often complicated by tension with medical professionals such as nurses and doctors, who can make a doula feel left out. Doulas tend not to see themselves anywhere in the medical hierarchy because they do not work for medical facilities. Increasing c-section rates partially spurred the growth of doulas and medicalized childbirth made the doula critical. However, this same trend may alienate them from the hospital setting.

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Study of Cell Orientation Alignment in Response to Cyclic Mechanical Stresses

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ABSTRACT

A number of studies have been performed on the subject of 'Cell Orientation Alignment' caused by mechanical stresses applied to sample substrates. Cells in most solid tissues (muscles, tendons, skin) have a characteristic alignment. How does this develop? Current research indicates that when cells on a two-dimensional substrate are subject to stress, either by stretching the substrate, by subjecting them to a well-aligned flow, or by other means, they reorient and align themselves in preferred directions.

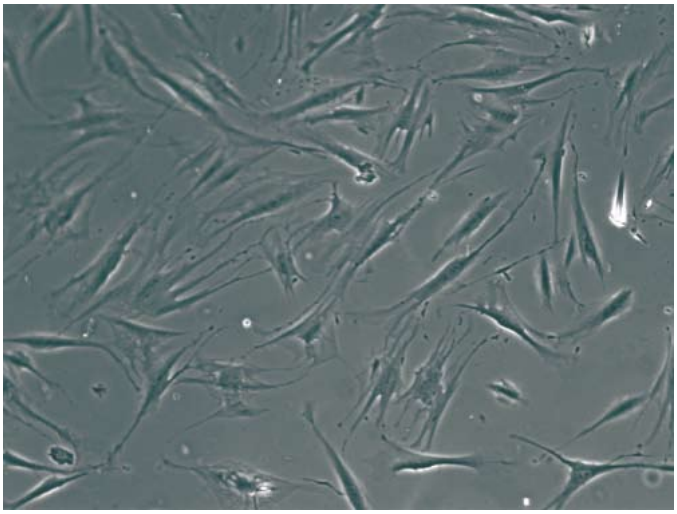

Figure 1: *Cells before Stretching*

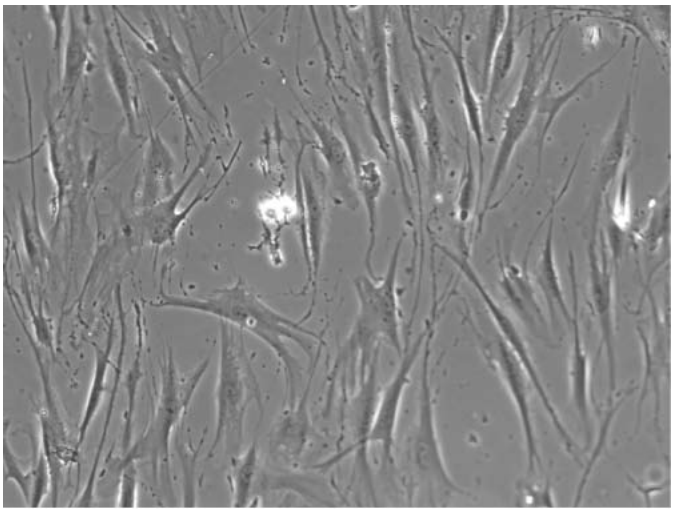

Figure 2: *Cells after Stretching*

(Images courtesy of Simon Jungbauer and Ralf Kemkemer, Max Planck Institut fuer Metallforschung, Stuttgart)

An understanding of these responses of cells to mechanical stimuli will have implications for mechanical inhibition of cancer cells and promotion of stem cell activity. If the behavior of cells can be formulated mathematically it would make it would aid in detecting cancerous cells, making it easier for medical specialists in eliminating the disease in its initial stages before it spreads around the entire body. It is well established that biological cells (i.e.: Fibroblasts and Rat Embryonic Fibroblasts (REF)) tend to align away from the stretching direction.[1] Most observations suggest that these cells tend to align in the direction of least substrate stress or in other words, they tend to align perpendicular to the direction of applied stress. The exact mechanism by which this process takes place has not been investigated thoroughly. The objective of this research is to perform experimental measurements on sample cells and to use mathematical modeling to help quantitatively describe a cell's morphological response to stretching. Specifically, the goal of this research project is to determine the relationship of cell orientation relative to the horizontal with respect to time. This was done by generating an exponential relation of cell orientation as a function of time and validating it by fitting this mathematical model to actual experimental data.

OVERVIEW

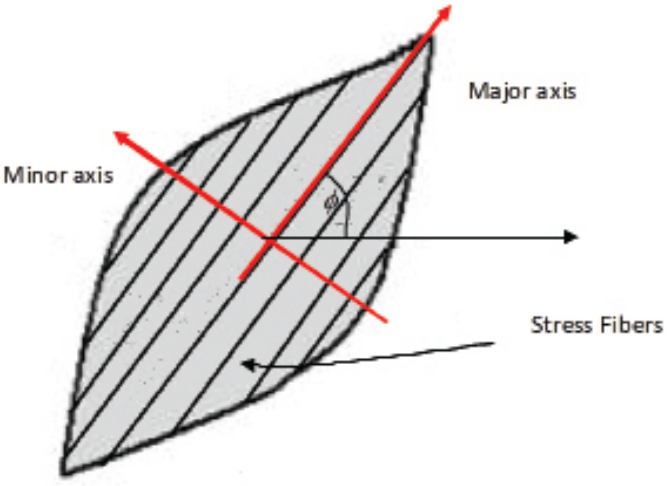

Figure 3: *A schematic model of a biological cell indicating the major & minor axes and the orientation of cell.*[1]

A simple schematic model of a cell is shown in figure 3. A typical 2D cell model is made up of a chain of polymers called stress fibers arranged parallel to each other lying along the major axis of the cell. Orthogonal to the major axis is the minor axis. These axes are useful in defining the orientation of a cell. In figure 1, ϕ corresponds to the cell orientation with respect to the horizontal.

To study the cell orientation of such a model we derived

a mathematical expression that represents the orientation of a cell under cyclic stretching. It is based on the idea of net polymerization of stress fibers.

The strain energy ϕ at an arbitrary orientation cos(ϕ) in a cell sample is given by equation 1:

$$U = U_0(\cos^2\phi)^2 = \frac{1}{2}EAl\varepsilon^2(\cos^2\phi)^2 \quad \text{(Eq\#1)}$$

$$U(m) = U_0(\cos^2\phi)^2 = U_0(\frac{\cos 2\phi + 1}{2})^2 = U_0(\frac{m+1}{2})^2 \quad \text{(Eq\#2)}$$

Where E is Young's modulus, A is the area; l is length, U_0 is the initial strain energy and ϕ is the strain amplitude.

The net polymerization rate = $k^F(U_0 - U(m)) - k^R$ (Eq#3)

In the above expression k^F and k^R are the Forward and Reverse reaction rates.

Equations 1-3 are the primary equations which aided us to construct a concise mathematical formula describing the cell orientation. After performing numerous intermediate operations the final expression reduces down to:

$$cos\emptyset(t) = Z_o[\frac{1 + r\,e^{\left(\frac{-2Z_f t}{\tau}\right)}}{1 - r\,e^{\left(\frac{-2Z_f t}{\tau}\right)}}] \quad \text{(Eq\#4)}$$

$$r - \frac{Z_o - Z_f}{Z_o + Z_f} \quad \text{(Eq\#5)}$$

Equation #4 is the exponential fitting function which describes the orientation of substrate cells as a function of time. Z_o is the initial fit value and Z_f is the final fit value of the exponential curve, t is time and τ is the time constant. r is simply a ratio of the difference of the fit values to the sum of fit values. Time constant is used to indicate how rapidly an exponential function decays. Equation #4 is the most important equation in our research study. Most of the raw data collected from the experiment was finally utilized to create a fitting function for cell orientation over a period of time.

Experimental Setup

Under a separate study of cellular activities in response to cyclic mechanical stresses, tendon fibroblasts were attached to Silicone dishes with grooved surfaces and were bi-axially stretched in parallel and normal directions. The fibroblasts would then align with the microgrooves before any cyclic stresses were applied to the Silicone substrate.[2] Such experiments were performed under different temperatures and cyclic frequencies.

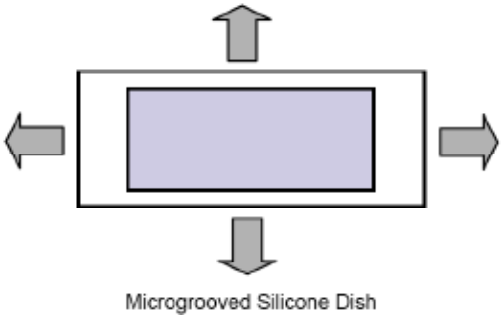

Figure 4 *illustrates a schematic model of a silicone dish (Cell sample inserted) undergoing cyclic stretching in vertical and horizontal direction.*[2]

Cells were cultured in custom designed microgrooved silicone dishes under controlled temperatures. Three temperatures chosen for such experiments were 17°C, 29°C, and 37°C. Custom built apparatus were used to cyclically stretch the substrate sample under different frequencies ranging from 1-20Hz at the three desired temperatures. Temperatures lower and higher than room temperature (~ 25°C) were chosen to determine if the cell sample behaved uniformly and normally. The silicone dish was placed at the center of the apparatus to maintain uniform stretching of cells both in the horizontal and vertical direction in 2D space (refer to figure 4). The loaded specimen would then be cyclically stretched for about 500 minutes under constant temperature and frequency. Numerical data corresponding to the cells' 2-D coordinates and orientation were collected in intervals of 50 seconds. (*All image processing was done by our collaborators at Max Planck Institut fuer Metallforschung in Stuttgart Germany producing raw data for post processing).*

The recorded data consisted of the following quantities:

Quantity	Description
Pic	Designates different time sets when cell data is recorded
X center	x position of mass center of cell
Y center	Y position of mass center of cell
Minor	axis orthogonal to stress fibers
Major	axis parallel to stress fibers
Perimeter	Perimeter of individual cell sample
Phi	Cell orientation with respect to horizontal in 2D plane

Table 1 *lists all the physical quantities recorded from lab experiments.*

Since the cells are not labeled, the initial task was to sort the data and group in to each single cell. The above listed quantities along with computer codes were used to sort data for specific cells. From there on we investigated the cell orientation in further detail.

Data Analysis

Most of the data processing was done using MATLAB. Most experimental data corresponded to temperatures of 17°C, 29°C & 37°C for different frequencies ranging between 1-20 Hz. The four main type of file used for analysis were: Data input files, sorting file, and two files used for data fitting. Further details are provided below.

Input Data File: input_FL/REF_##C_##Hz.m

After the recorded data was collected in a spreadsheet, it was imported into a Matlab file which included quantities like perimeter, major & minor axes, x & y coordinates, pic number, cos(2ϕ) values, nmax and ntimcut. nmax is the number of data entries and ntimcut is the number of time sets recorded during the experiment. Data was collected into groups of different temperatures and frequency; for instance, 'FL17C_1Hz' indicates Fibroblast Cells at 17 degree Celsius operating under a driving frequency of 1Hz. The same classification was used for Rat Endothelial Fibroblast Cells (REF).

Original Sort File: sort_angle_092508_arsalan.m

The original sorting file was written by Professor Krishna Garikipati and used to produce a complete preliminary analysis of the data files. From the MATLAB data files, the variables are first separated and stored in individual arrays. The

number of cell data for each time step is also noted and stored in the array ncell. Using the time step ntmin with the least number of cells, the data is then sorted according to individual cells. This is done by identifying the x and y coordinates of a particular cell at time step t, and matching them to the closest cell at time step t – 1 and t + 1. This matching process propagates until a cell is fully matched up from the start to the end of the experiment. This is done for all the cells available at time ntmin. The final data array is stored in xs (x-coordinate), ys (y-coordinate), and ϕ's (orientation angle) with individual cell data down the rows, and time steps increasing across the columns.[3]

A check is performed to ensure that the coordinates of individual cells do not exceed a certain limit value. Cells that fail this condition are flagged.

The final data of cells is arranged in three orientation groups: $0^\circ \le \phi \le 30^\circ, 30^\circ \le \phi \le 60^\circ, 60^\circ \le \phi \le 90^\circ$

The cell orientation is then averaged by dividing the orientation by the number of cells (ncell) in that group. If a specific orientation group does not contain any cells, then the analysis associated with that group is ignored. Once all the sorting has been done, the mathematical fitting function described above is implemented in the code. The user is prompted to enter three unknown quantities: 'tau, xib, xi0' for each group set followed by a least square calculation. Further details on how the three quantities were obtained are explained in following subsections. (*All image processing was done by our collaborators at Max Planck Institut fuer Metallforschung in Stuttgart Germany producing raw data for post processing).*

Fitting function #1: recfun1.m

This is a short piece of code that reads in time and experimental data. Using this data it computes the exponential function for the specific orientation group. This function is called in the second fitting function called *recfit1.m* explained below.

Fitting function #2: recfit1.m

This MATLAB file is used for computing the exponential fitting curve and determining the least square value associated between the fit curve and experimental data. Least square value indicates the accuracy of the fit. The closer the value is to 0, the stronger the fit. Boundary conditions are applied to determine tau, xib and xi0. Tau is the time constant of the fit curve, xi0 is the starting value and xib is the final value of the exponential curve. This piece of code uses the MATLAB command "lsqnonlin". 'lsqnonlin' solves nonlinear least-squares problems, including nonlinear data-fitting problems.

Computation Method

To obtain optimal values of xib, xi0 and tau, a number of specific operations are performed. Starting with the input data file, we run this file, which creates an array of all the physical quantities listed in this report followed by compiling *sort_angle_092508_arsalan.m*. We then collect the time and cosphi1, 2, 3 arrays and import them into recfit1.m. cosphi1,2 & 3 correspond to cell orientation groups of $0^\circ \le \phi \le 30^\circ, 30^\circ \le \phi \le 60^\circ, 60^\circ \le \phi \le 90^\circ$ respectively**.** Performing this iteration helps us in determining optimal values for xib, xi0, tau and least square values for each orientation group.

Computational Results

After performing multiple experiments and data analysis, optimal values were obtained. These results are presented in the figures below:

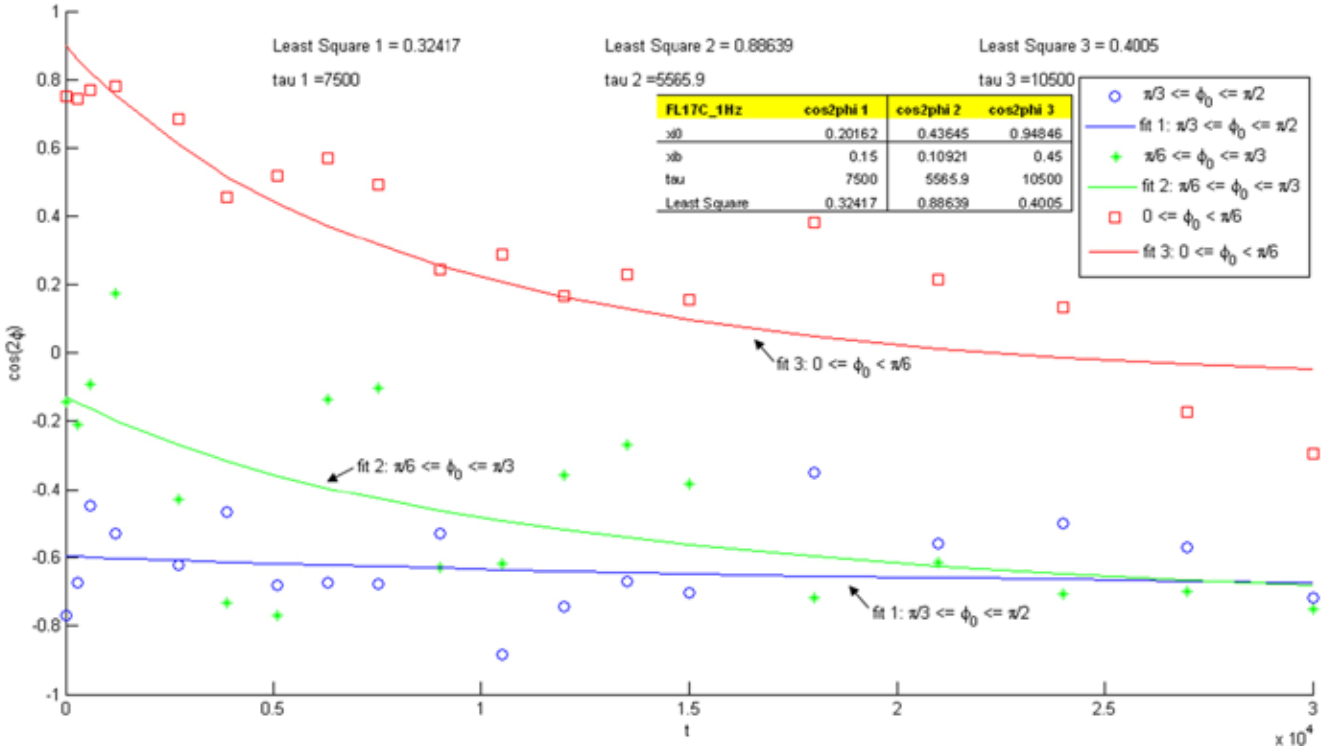

Figure 5 *Data and Curve fit plot for FL 17C 1Hz*

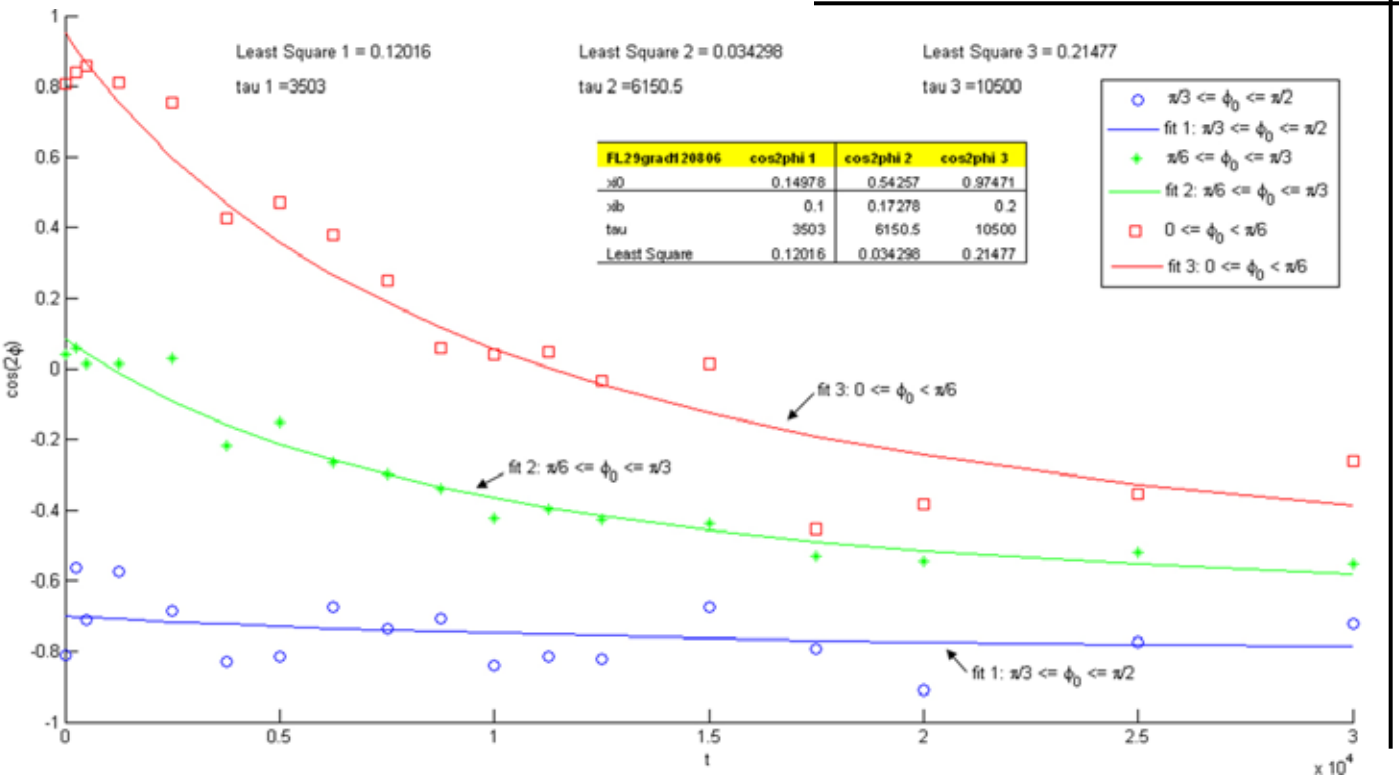

Figure 6 *Data and Curve fit plot for FL 29C grad120806*

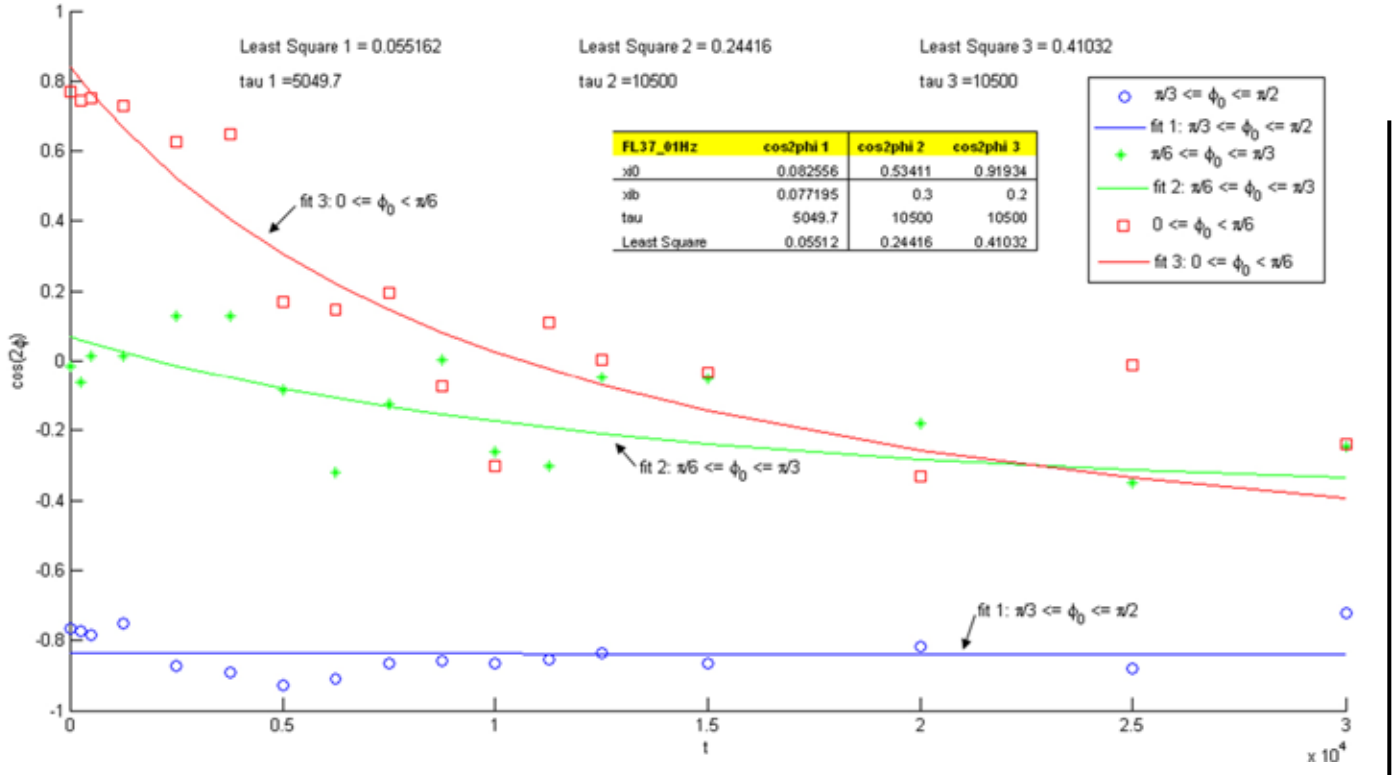

Figure 7 *Data and Curve fit plot for FL 37C 1Hz*

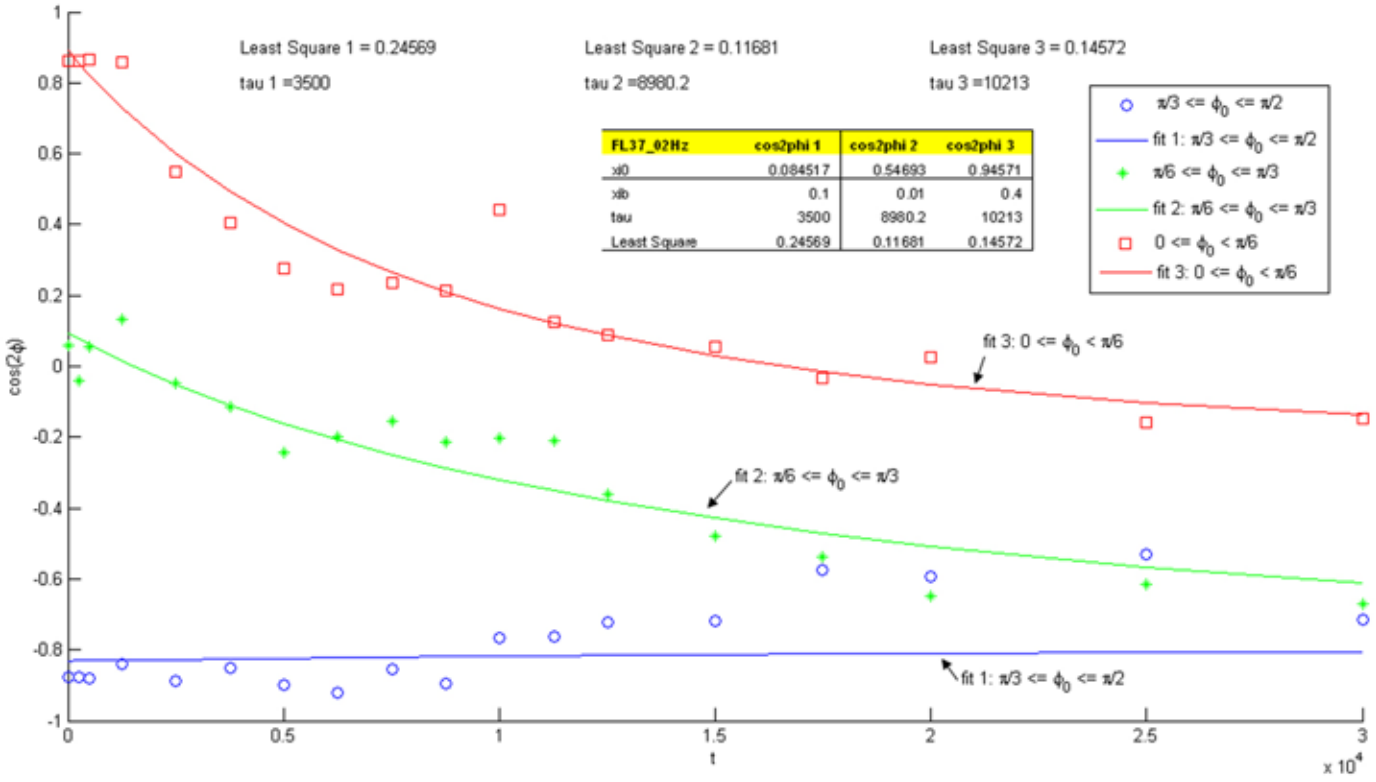

Figure 8 *Data and Curve fit plot for FL 37C 2Hz*

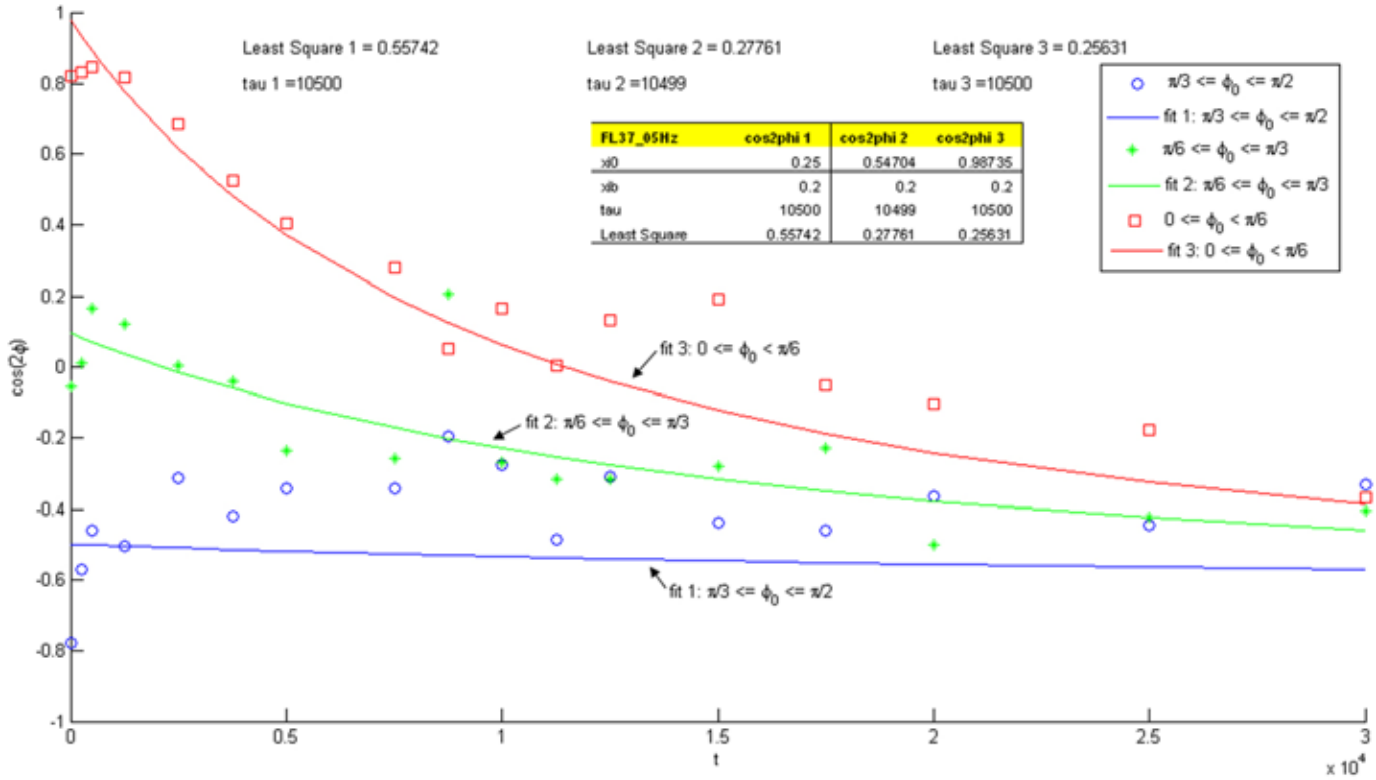

Figure 9 *Data and Curve fit plot for FL 37C 5Hz*

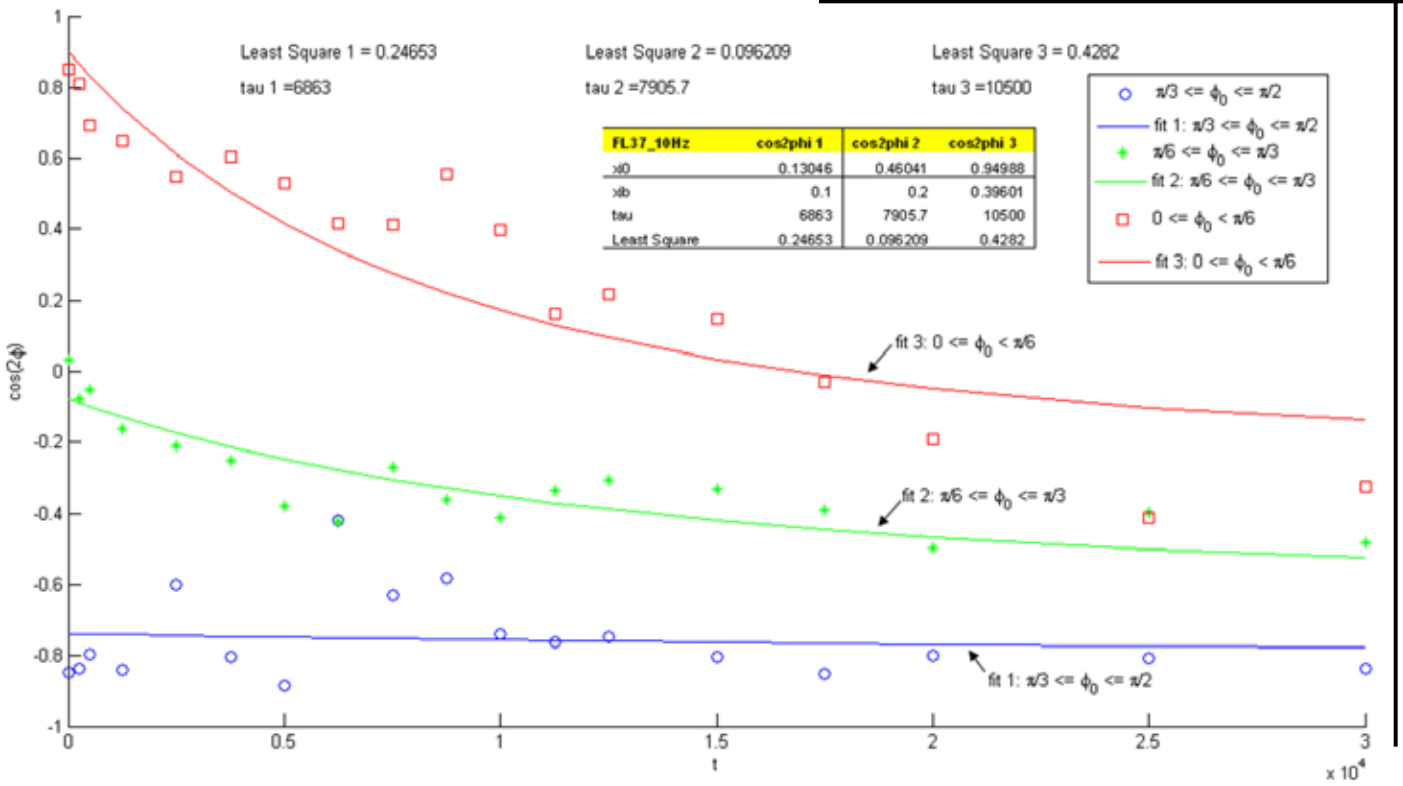

Figure 10 *Data and Curve fit plot for FL 37C 10Hz*

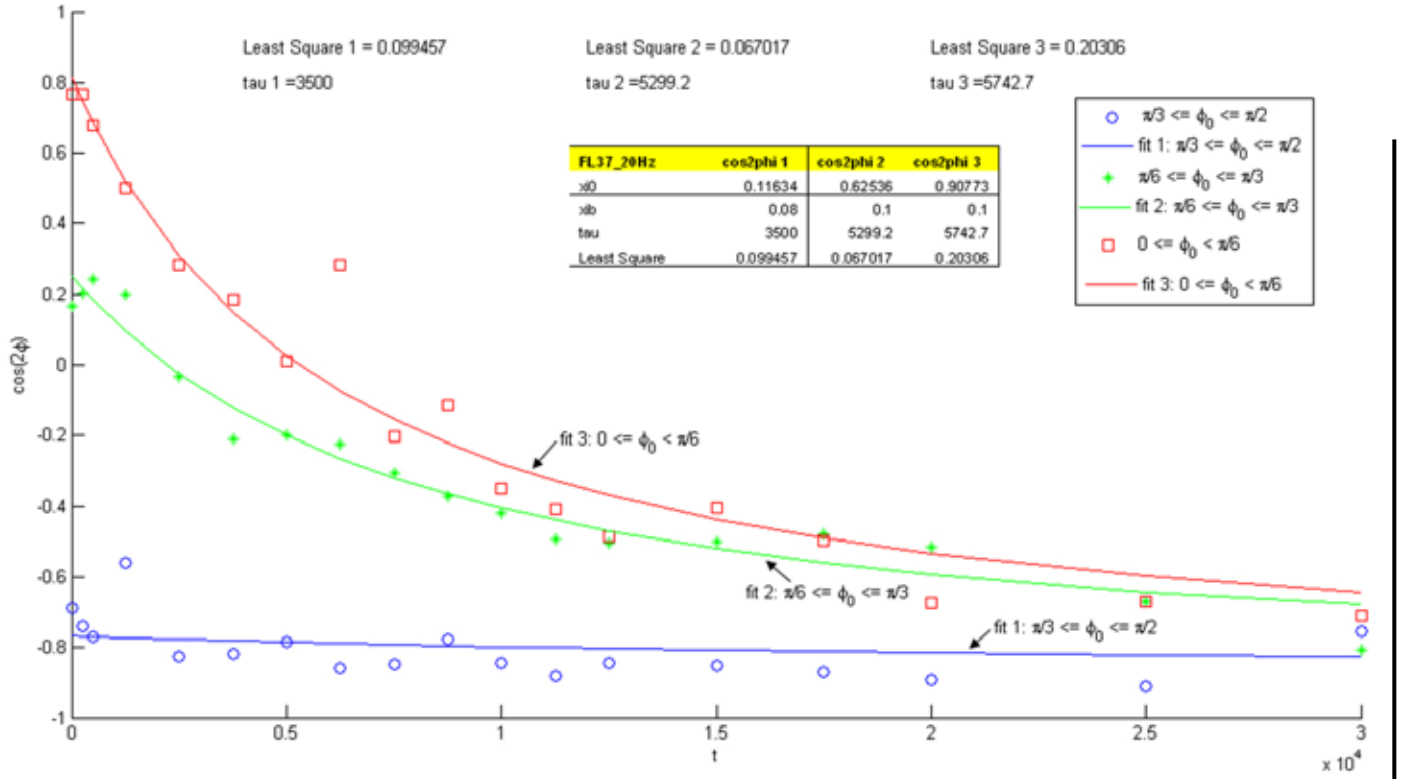

Figure 11 *Data and Curve fit plot for FL 37C 20Hz*

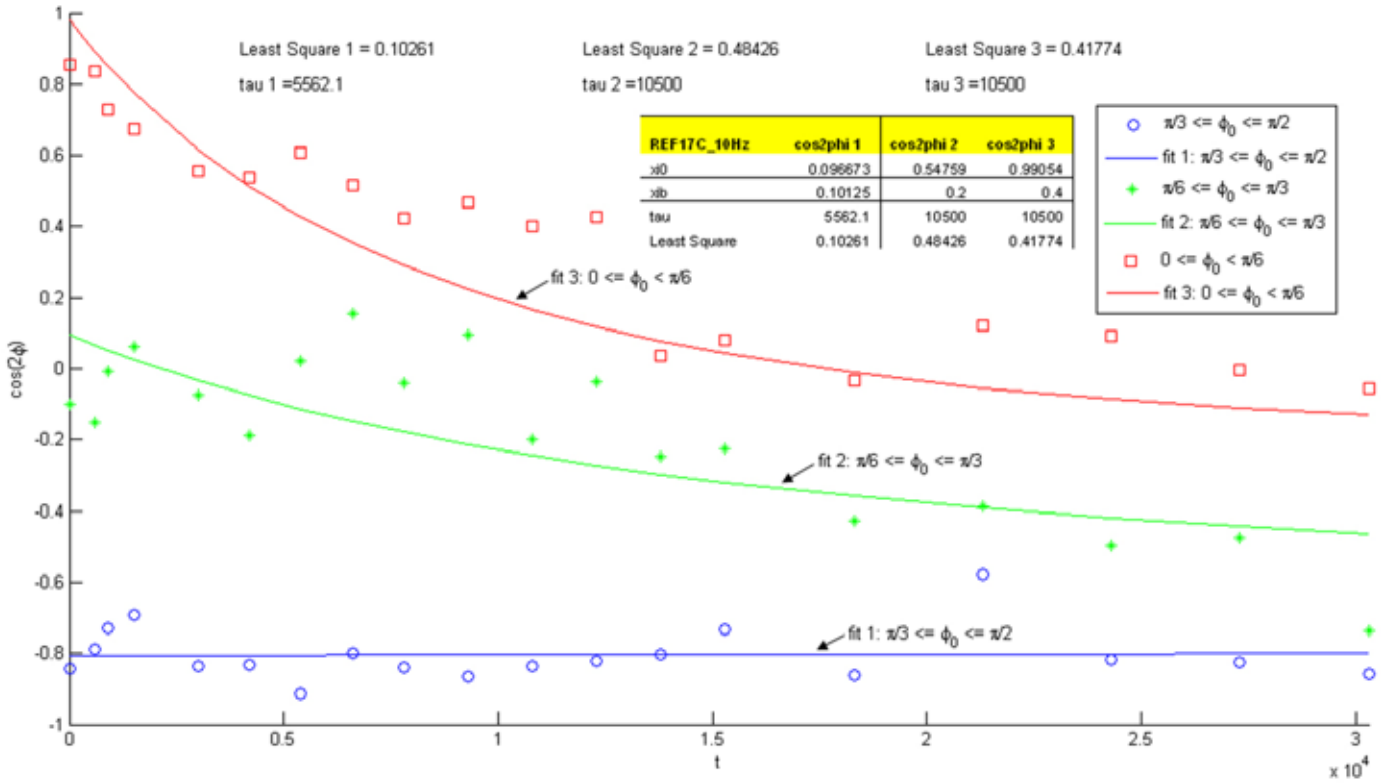

Figure 12 *Data and Curve fit plot for REF 17C 10Hz*

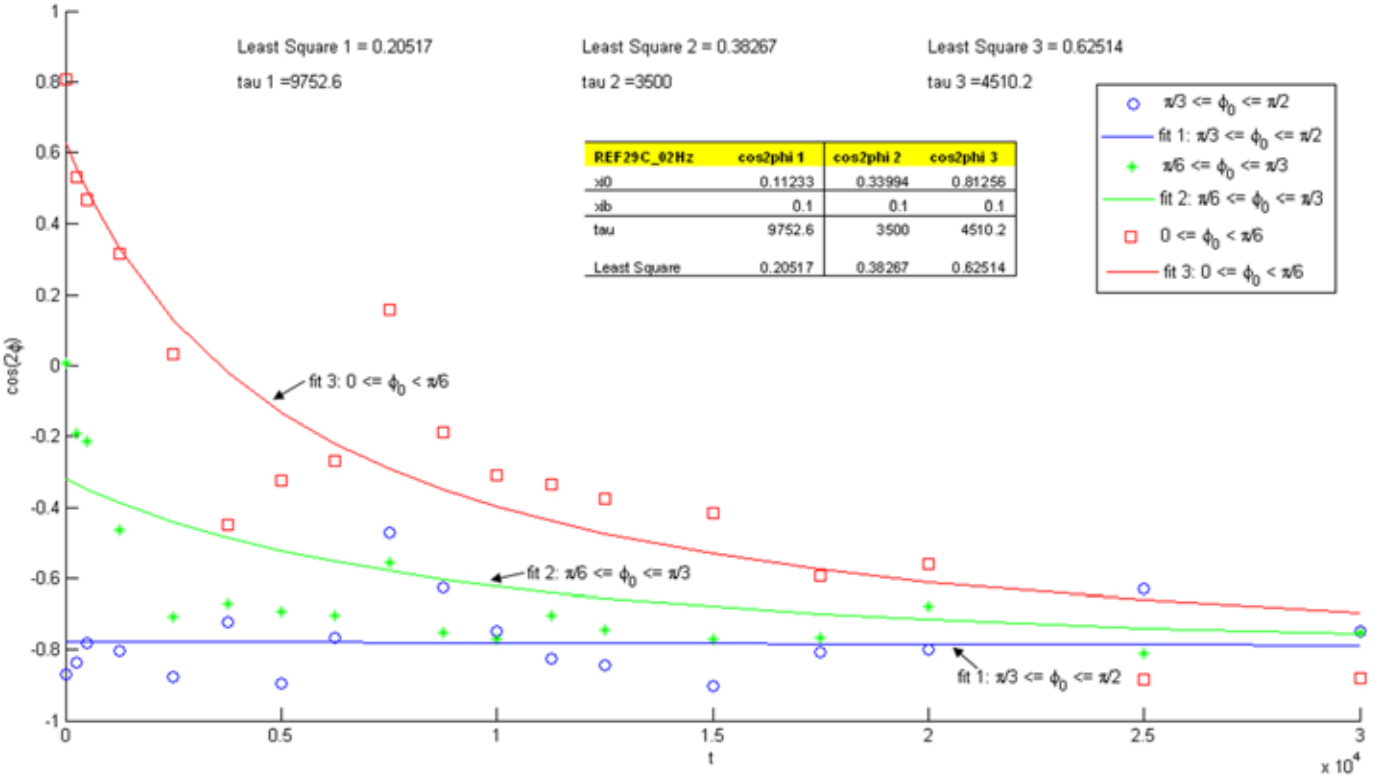

Figure 13 *Data and Curve fit plot for REF 29C 02Hz*

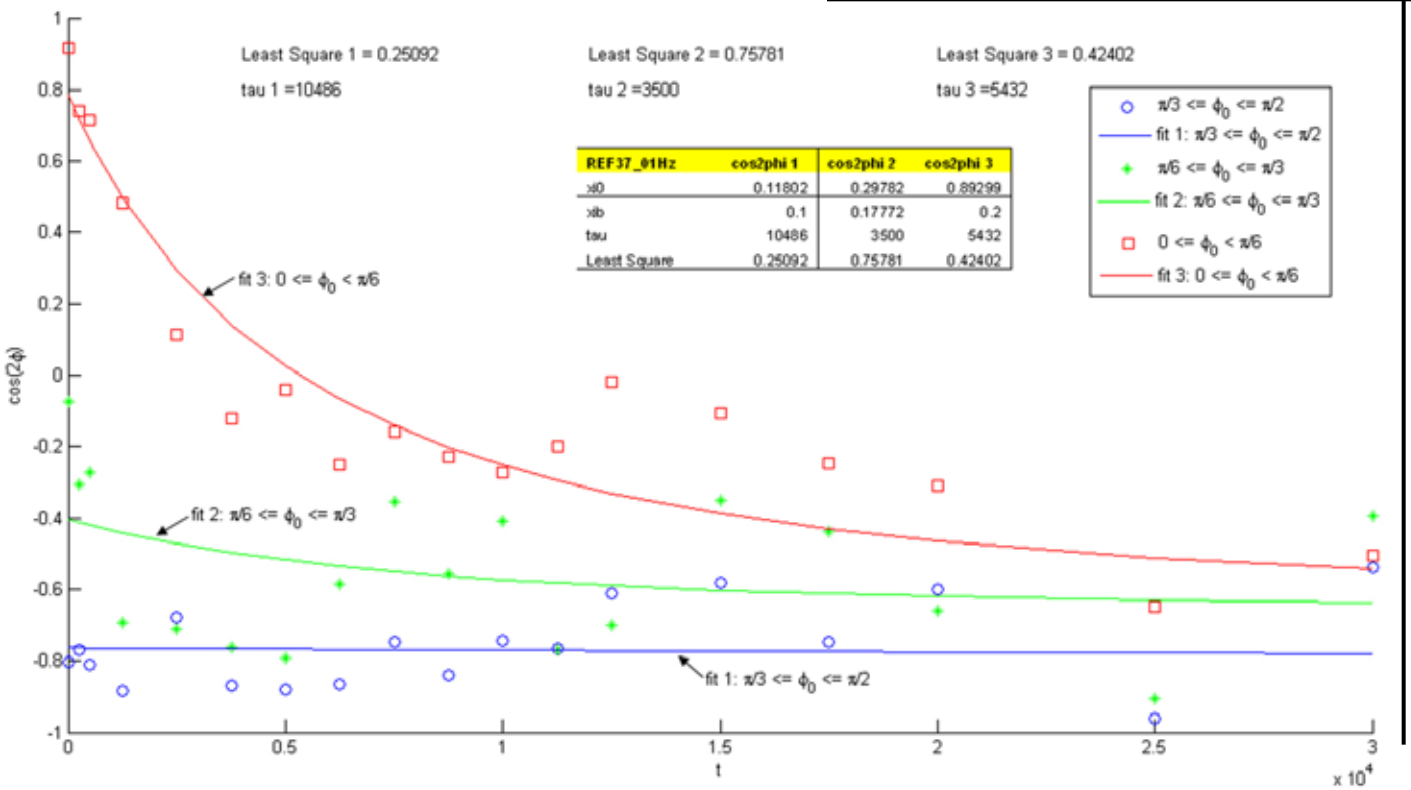

Figure 14 *Data and Curve fit plot for REF 37C 01Hz*

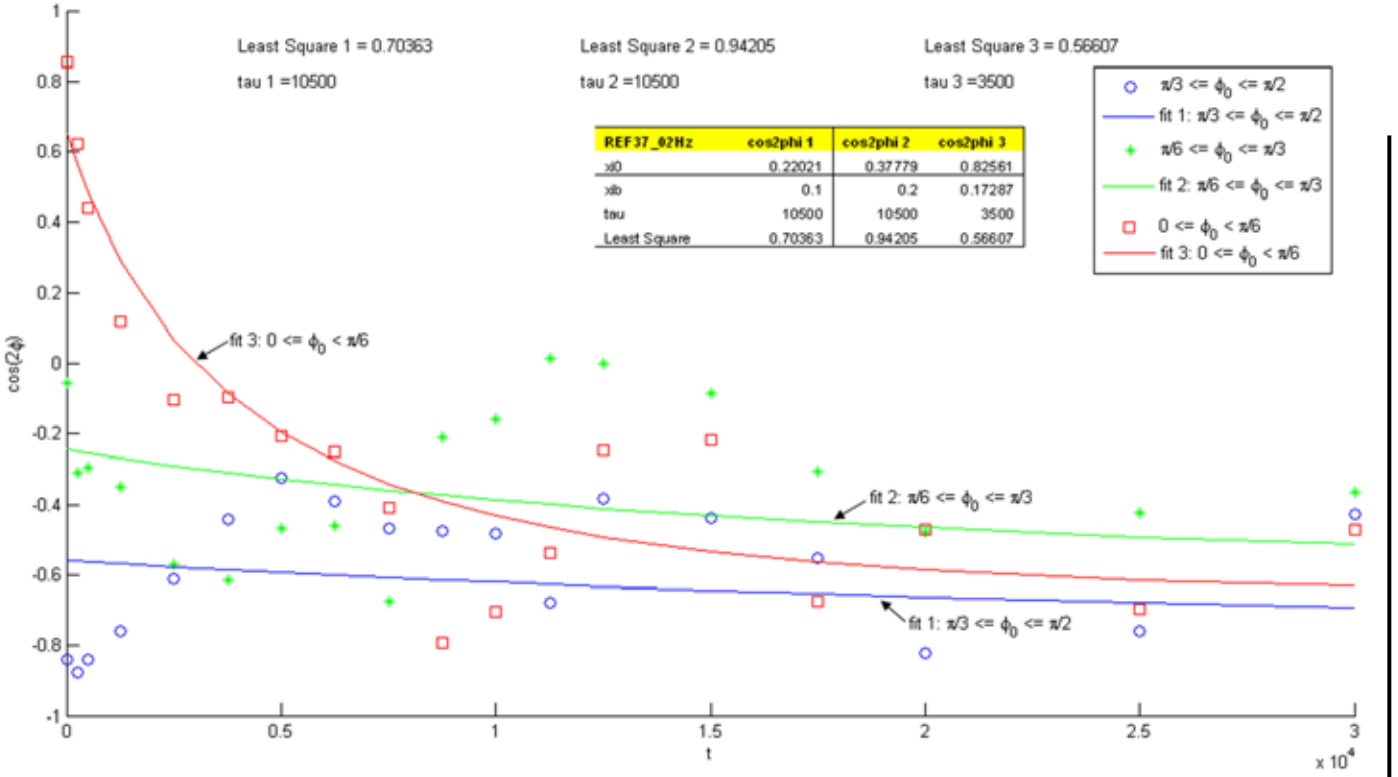

Figure 15 *Data and Curve fit plot for REF 37C 02Hz*

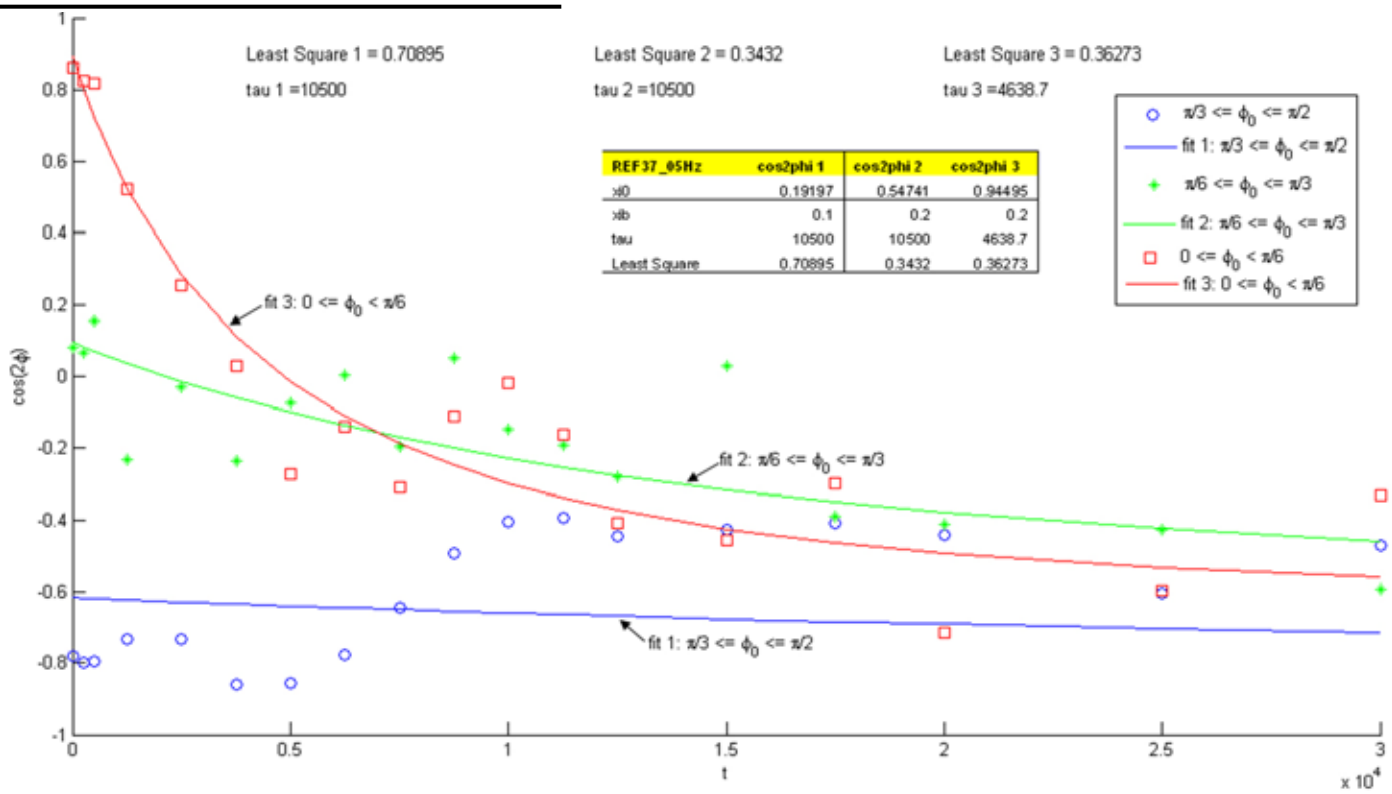

Figure 16 *Data and Curve fit plot for REF 37C 05Hz*

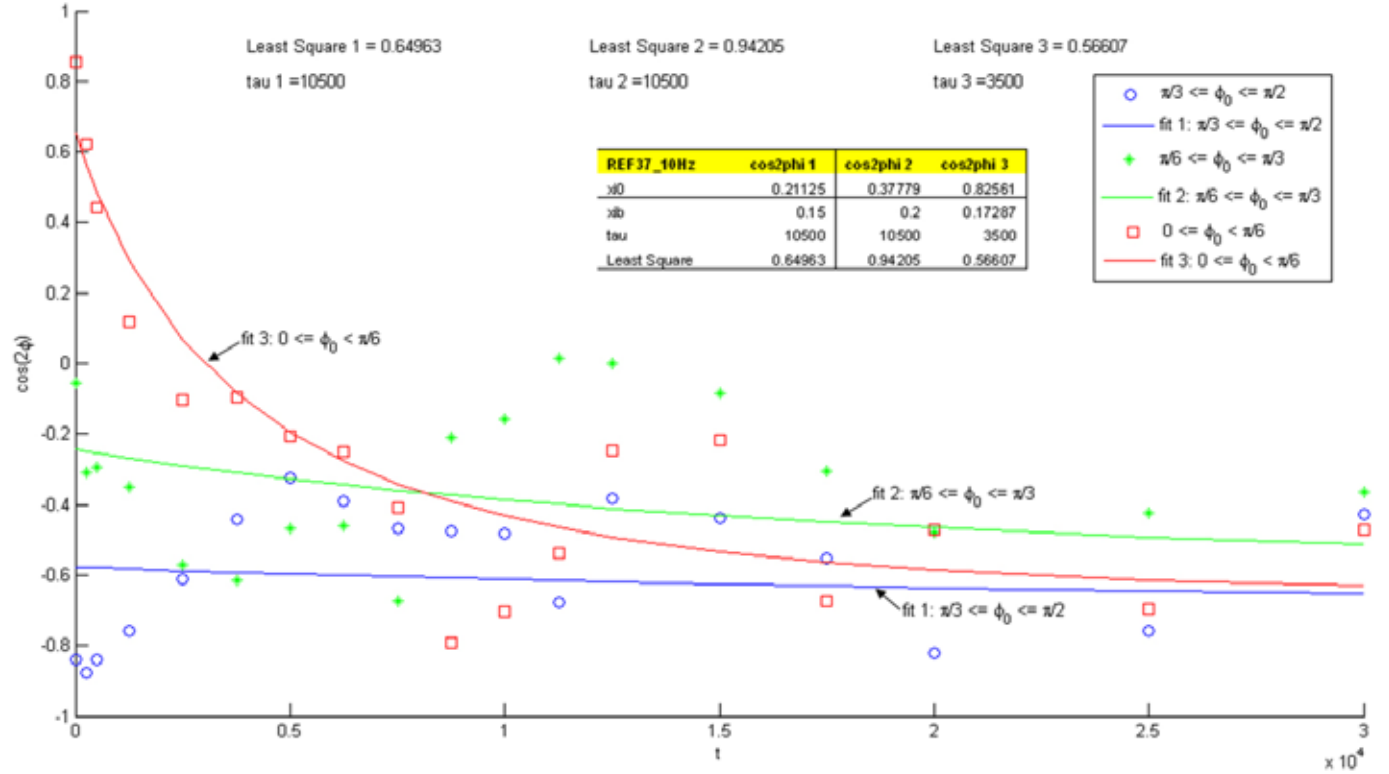

Figure 17 *Data and Curve fit plot for REF 37C 10Hz*

Figures 5-17 illustrate best-fit curves and the least square values for different conditions. These results show that the mathematically generated exponential curves are a good fit to the experimental data. In all cases the least square value is close to zero, indicating a strong correlation between the exponential curve and the actual test data. For different temperature and strain rate the cell orientation obeys the exponential relation with time. This suggests that our mathematical model for cell orientation is reasonably accurate. Thus we have successfully quantified the cellular orientation with respect to time. We observe that in most cases all three orientations in each experiment converge to a common value, indicating that over a long period of time all cells tend to align in the same orientation.

CONCLUSION

The data analysis of rates of cell-reorientation is able to provide us with useful information about the mechanism that the cell adopts to achieve the observed results. Why is this important? Restate that cells tend to align in same orientation and what this means for any applications. Revisit the idea of inhibition of cancer and promotion of stem cells. The reader need to know why this research is relevant. By characterizing their stress response what has the scientific community gained? These experimental data can be further refined in order to provide results that are close to the actual behavior of the cells and to eliminate many errors associated with our results. The experiments produced very useful results. Different temperatures and frequencies were applied to validate our hypothesis. A strong correlation is present between the experimental data and the mathematical model. In order to further excel in cell research, repeated experiments should be performed under the same conditions in order to make further comparisons. In this way discrepancies between data sets of similar conditions can be easily determined. If such discrepancies exist, this research could be further expanded to re-derive a more accurate model. More physical quantities should be included to make our model more accurate and minimize errors. A better algorithm should be constructed to collect and process raw data, speeding up the computational process.

This research was primarily performed to quantify biological studies in order to prepare accurate mathematical models for future research studies in the field of cell mechanism. A list of MATLAB codes have been provided in Appendix A which have been useful for calculating experimental data.

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2. H. C. Wang, G. Yang, Z. Li, W. Shen, "Fibroblast responses to cyclic mechanical stretching depends on cell orientation to the stretching direction", Journal of Biomechanics 37, 2004
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Appendix A: MATLAB Codes

sort_angle_092508_arsalan.m

```
%Sort data on cell positions and orientations over a range of time steps.
%First sort by time.

%time step
ntmstp = 0;
% nmax is the number of data entries in the input file
for i = 1:nmax
   if (pic(i,1) == 100000)
%time step increment
      ntmstp = ntmstp + 1;
%allots different times for different pic values. for instance
%for pic=6:(6-1)*50=250 sec
t(ntmstp) = pic(i+1,1)*1.0;  %(pic(i+1,1))*1.0;
      if (ntmstp > 1)
%measures how many cells are between successive "00's" (a counter:ncell)
         ncell(ntmstp-1) = j ;
      end
      j = 0;
   else
%when pic is not == 0 then input data values are assigned to new set of
%arrays xst,yst,phist,etc.
      j = j+1;
      xst(j,ntmstp)   = x(i,1);
      yst(j,ntmstp)   = y(i,1);
      phist(j,ntmstp) = phi(i,1);
      minorst(j, ntmstp) = minor(i,1);
      majorst(j, ntmstp) = major(i,1);
      perist(j,ntmstp) = peri(i,1);
   end
end
%repetition of ncell above
ncell(ntmstp) = j;
%Find time with minimum number of cells. ntmin is the time number with
minimum number of cells
ntmin = 1;
for i = 2:ntmstp
%simple for loop that checks for the time set that has the minimum number
of cells
%using 'ncell' and 't'
  if (ncell(i) < ncell(ntmin))
%in this for loop 'ntmin' is the array number with the minimum number of
cells.
%'tmin' being the time of the minimum cell group
     ntmin = i;
     tmin = t(ntmin);
  end
end
%Read data from this time into final sorted array
for j = 1:ncell(ntmin)
   xs(j,ntmin) = xst(j,ntmin);
   ys(j,ntmin) = yst(j,ntmin);
   phis(j,ntmin) = phist(j,ntmin);
   minors(j,ntmin) = minorst(j,ntmin);
   majors(j,ntmin) = majorst(j,ntmin);
   peris(j,ntmin) = perist(j,ntmin);
end
%Propagate the sort to times i < ntmin. Always compare data from successive
%times. The algorithm below ensures that ncell in a sorted
%array = ncell(ntmin).
for i = 1:ntmin-1
   k = ntmin-i;
   for j = 1:ncell(ntmin)
      dmin = sqrt((xs(j,k+1)-xst(1,k))^2 + (ys(j,k+1)-yst(1,k))^2);
            xs(j,k) = xst(1,k);
            ys(j,k) = yst(1,k);
            phis(j,k) = phist(1,k);
      for l = 2:ncell(k)
         dchk = sqrt((xs(j,k+1)-xst(l,k))^2 + (ys(j,k+1)-yst(l,k))^2);
               if (dchk < dmin)
            dmin = dchk;
                     xs(j,k) = xst(l,k);
                     ys(j,k) = yst(l,k);
                  phis(j,k) = phist(l,k);
```

```
            end
        end
            dminn(j,i) = dmin;
    end
end
%Propagate the sort to times i > ntmin. Always compare data from successive
%times. The algorithm below ensures that ncell in a sorted
%array = ncell(ntmin).
for i = ntmin+1:ntmstp
    for j = 1:ncell(ntmin)
        dmin = sqrt((xs(j,i-1)-xst(1,i))^2 + (ys(j,i-1)-yst(1,i))^2);
            xs(j,i) = xst(1,i);
            ys(j,i) = yst(1,i);
            phis(j,i) = phist(1,i);
        for l = 2:ncell(i)
            dchk = sqrt((xs(j,i-1)-xst(l,i))^2 + (ys(j,i-1)-yst(l,i))^2);
                if (dchk < dmin)
            dmin = dchk;
                    xs(j,i) = xst(l,i);
                    ys(j,i) = yst(l,i);
                phis(j,i) = phist(l,i);
                end
        end
            dminn(j,i) = dmin;
    end
end
%Run check on maximum changes in centers of mass
for j = 1:ncell(ntmin)
    if (max(dminn(j,1:ntimcut)) > 100.0)
        nflag(j) = 1;
    else
        nflag(j) = 0;
    end
end
%Obtain cosines
for i = 1:ntmstp
    cosphi1(i) = 0.0;
    cosphi2(i) = 0.0;
    cosphi3(i) = 0.0;
end
%Group cells by initial orientation
ncell1 = 0;
ncell2 = 0;
ncell3 = 0;
for j = 1:ncell(ntmin)
    if (phis(j,1) >= 2*pi/3)  %%%% al angles changed from degrees to radians
        ncell1 = ncell1 + 1;
    elseif ((phis(j,1) < 2*pi/3) & (phis(j,1) >= 2*pi/6))
        ncell2 = ncell2 + 1;
    else
        ncell3 = ncell3 + 1;
    end
end
for j = 1:ncell(ntmin)
    for i = 1:ntmstp
        cosphi(j,i) = cos(phis(j,i)*1.0); %%%%%%%%%%%%%changed from
*pi/180 to *pi/90
        if (phis(j,1) >= 2*pi/3)
            cosphi1(i) = cosphi1(i) + cosphi(j,i);
        elseif ((phis(j,1) < 2*pi/3) & (phis(j,1) >= 2*pi/6))
                cosphi2(i) = cosphi2(i) + cosphi(j,i);
            else
                cosphi3(i) = cosphi3(i) + cosphi(j,i);
        end
    end
end
cosphi1 = (1.0/ncell1)*cosphi1;
cosphi2 = (1.0/ncell2)*cosphi2;
cosphi3 = (1.0/ncell3)*cosphi3;
%Fitting for cosphi1........................................
tau = input('Tau for cells above 60 deg: ');
xib= input ('xib for cells above 60 deg: ');
xi0 = input('xi0 for cells above 60 deg: ');

ratio_1=(xi0 - xib)/(xi0 + xib);
for i = 1:18
    fit_1(i) = xib*(1 + ratio_1*exp(-2*xib*t(i)/tau))/(1 - ratio_1*exp(-
2*xib*t(i)/tau));
    %fit_1(i)=2*((fit_1(i)).^2) - 1;  %%%%%%%%EXTRA LINE ADDED
FOR TEST
    fit_1(i) = 2*(fit_1(i)) -1;    %%%%%%%%%%%%%%% NEW LINE
ADDED
end
%least square cosphi1
lsquare_1 = 0;
for i = 1:18
    lsquare_1 = lsquare_1 + (cosphi1(i) - fit_1(i))^2;
end
ls_1 = ['Least Square 1 = ' num2str(lsquare_1)];
tau1 = ['tau 1 =' num2str(tau)];
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

%Fitting for cosphi2........................................
tau = input('Tau for cells between 30 and 60 deg: ');
xib= input ('xib for cells between 30 and 60 deg: ');
xi0 = input('xi0 for cells between 30 and 60 deg: ');
ratio_2=(xi0 - xib)/(xi0 + xib);
for i = 1:18
    fit_2(i) = xib*(1 + ratio_2*exp(-2*xib*t(i)/tau))/(1 - ratio_2*exp(-
2*xib*t(i)/tau));
    %fit_2(i)=2*((fit_2(i)).^2) - 1;  %%%%%%%%EXTRA LINE ADDED
FOR TEST
    fit_2(i) = 2*(fit_2(i)) -1;    %%%%%%%%%%%%%%% NEW LINE
ADDED
end
%least square cosphi2
lsquare_2 = 0;
for i = 1:18
    lsquare_2 = lsquare_2 + (cosphi2(i) - fit_2(i))^2;
end
ls_2 = ['Least Square 2 = ' num2str(lsquare_2)];
tau2 = ['tau 2 =' num2str(tau)];
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

%Fitting for cosphi3........................................
tau = input('Tau for cells between 0 and 30 deg: ');
xib= input ('xib for cells between 0 and 30 deg: ');
xi0 = input('xi0 for cells between 0 and 30 deg: ');
ratio_3=(xi0 - xib)/(xi0 + xib);
for i = 1:18
    fit_3(i) = xib*(1 + ratio_3*exp(-2*xib*t(i)/tau))/(1 - ratio_3*exp(-
2*xib*t(i)/tau));
    %fit_3(i)=2*((fit_3(i)).^2) - 1;  %%%%%%%%EXTRA LINE ADDED
FOR TEST
    fit_3(i) = 2*(fit_3(i)) -1;    %%%%%%%%%%%%%%% NEW LINE
ADDED
end
%least square cosphi3
lsquare_3 = 0;
for i = 1:18
    lsquare_3 = lsquare_3 + (cosphi3(i) - fit_3(i))^2;
end
ls_3 = ['Least Square 3 = ' num2str(lsquare_3)];
tau3 = ['tau 3 =' num2str(tau)];
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
figure(2);
xlabel('t');
ylabel('cos(2\phi)');
xlim([0,t(18)]);
ylim([-1.0 1.0]); %CHANGE LIMIT FROM -1<Y<1 TO -2<Y<2
hold on;
```

```
plot(t(1:18),cosphi1(1:18),'bo',t(1:18),cosphi2(1:18),'g*',t(1:18),cosphi3(1:1
8),'rs',t(1:18),fit_1(1:18),'-b',t(1:18),fit_2(1:18),'-g',t(1:18),fit_3(1:18),'-r');
legend('\pi/3 <= \phi_0 <= \pi/2','\pi/6 <= \phi_0 <= \pi/3','0 <= \phi_0 < \
pi/6','fit 1: \pi/3 <= \phi_0 <= \pi/2','fit 2: \pi/6 <= \phi_0 <= \pi/3','fit 3: 0
<= \phi_0 < \pi/6');
text(0.4e4, 0.9, ls_1);
text(0.4e4, 0.8, tau1);
text(1.2e4, 0.9, ls_2);
text(1.2e4, 0.8, tau2);
text(2.0e4, 0.9, ls_3);
text(2.0e4, 0.8, tau3);
```

recfun1.m

```
function y=recfun1(b)
global data;

t=data(:,1);
Rexp=data(:,2);
%b(1)=xi0    b(2)=xib     b(3)=tau
Rcal=b(2)*(1 + ((b(1)-b(2))./(b(1)+b(2)))*exp(-2*b(2)*t/b(3)))./(1
- ((b(1)-b(2))./(b(1)+b(2)))*exp(-2*b(2)*t/b(3)));    % the calculated
value from the model
Rcal_2theta=2*(Rcal) - 1;
%y=sum((Rcal-Rexp).^2);
y=Rcal_2theta-Rexp;
% the sum of the square of the difference between calculated value
and experimental value
```

recfit1.m

```
global data;
data=[
0           0.8613
250         0.8616
500         0.8661
1250        0.8565
2500        0.5498
3750        0.4057
5000        0.2778
6250        0.2189
7500        0.235
8750        0.2149
10000       0.4424
11250       0.1236
12500       0.0889
15000       0.0549
17500       -0.033
20000       0.0246
25000       -0.1591
30000       -0.1475];                % experimental data FOR "COS
2PHI"
t=data(:,1);
Rexp=data(:,2);
plot(t,Rexp,'ro');                   % plot the experimental data
hold on
b0=[0.875 0.17 5500];   %b0=[0.6 0.001 5000];                    %
start values for the parameters
options=optimset('MaxFunEvals',100000000);   %Max number of
function evaluations
options=optimset('MaxIter',10000000);     %Max number of
function evaluations
lb = [0.75 0.1 3500];                %Lower bound on b
ub = [1.00 0.4 10500];               %Upper bound on b
b=lsqnonlin('recfun1',b0,lb,ub,options)       % run the lsqnonlin with
start value b0, returned parameter values stored in b
Rcal=(b(2)/1)*(1 + ((b(1)-b(2))./(b(1)+b(2)))*exp(-2*b(2)*t/b(3)))./
(1 - ((b(1)-b(2))./(b(1)+b(2)))*exp(-2*b(2)*t/b(3)));     % calculate
```

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

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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 phenylethanolamine 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 pow-

erful 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

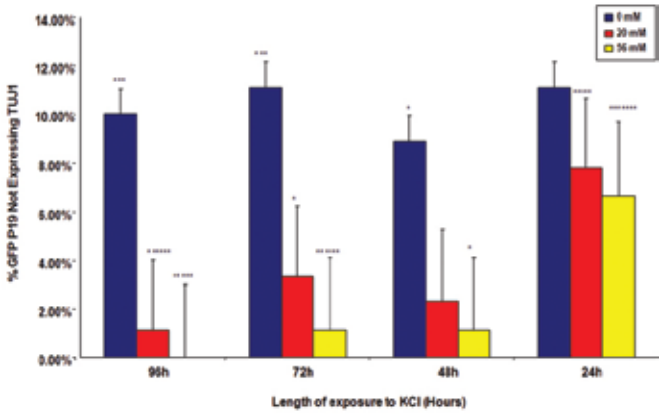

Figure 1: *Exp "KCl90" - GFP-Expressing P19 not expressing TUJ1 (90 Cells/Time/[M] KCl*
P19 cells were transfected and treated with 0, 20, or 56mM KCl as described in Materials and Methods. GFP fluorescence and TUJ1 staining were determined by fluorescence microscopy. Statistical significance measured 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").

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 (Fig. 2).

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).

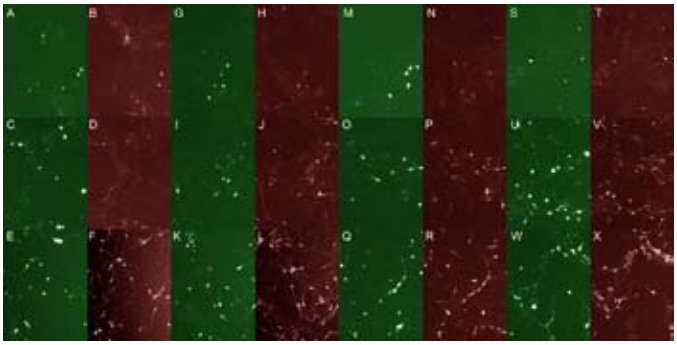

Figure 2: *KCl90 Picture Compilation*
P19 cells were transfected and treated with 0, 20, or 56 mM KCl as described in Materials and Methods. GFP fluorescence and TUJ1 staining were determined by fluorescence microscopy.Each row corresponds to KCl concentrations (Top-Down: 0, 20, 56 mM), and each green-red 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.

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.

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.

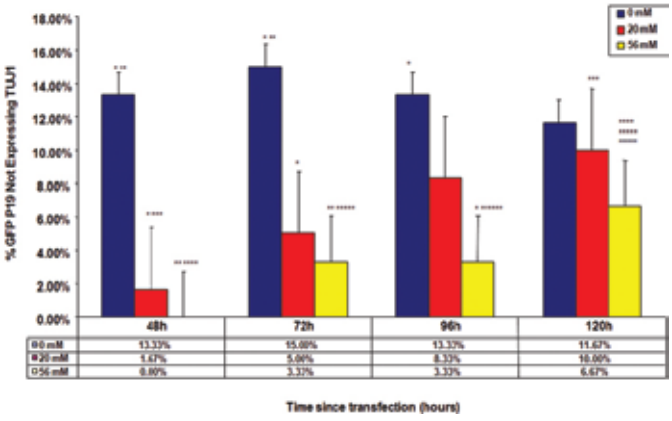

Figure 3: *Ca30*
P19 cells were transfected and treated with 0, 0.002, or 0.01 mM ionomycin as described in Materials and Methods. GFP fluorescence and TUJ1 staining was determined by fluorescence microscopy. Statistical significance was not observed at any time point treated with 0.002mM or 0.01mM ionomycin for one hour.

Discussion

Our findings suggest that KCl-induced depolarization may enhance differentiation of P19 cells transiently expressing MASH1, although improvements to the experimental protocol 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 facilitateMASH1-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.

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