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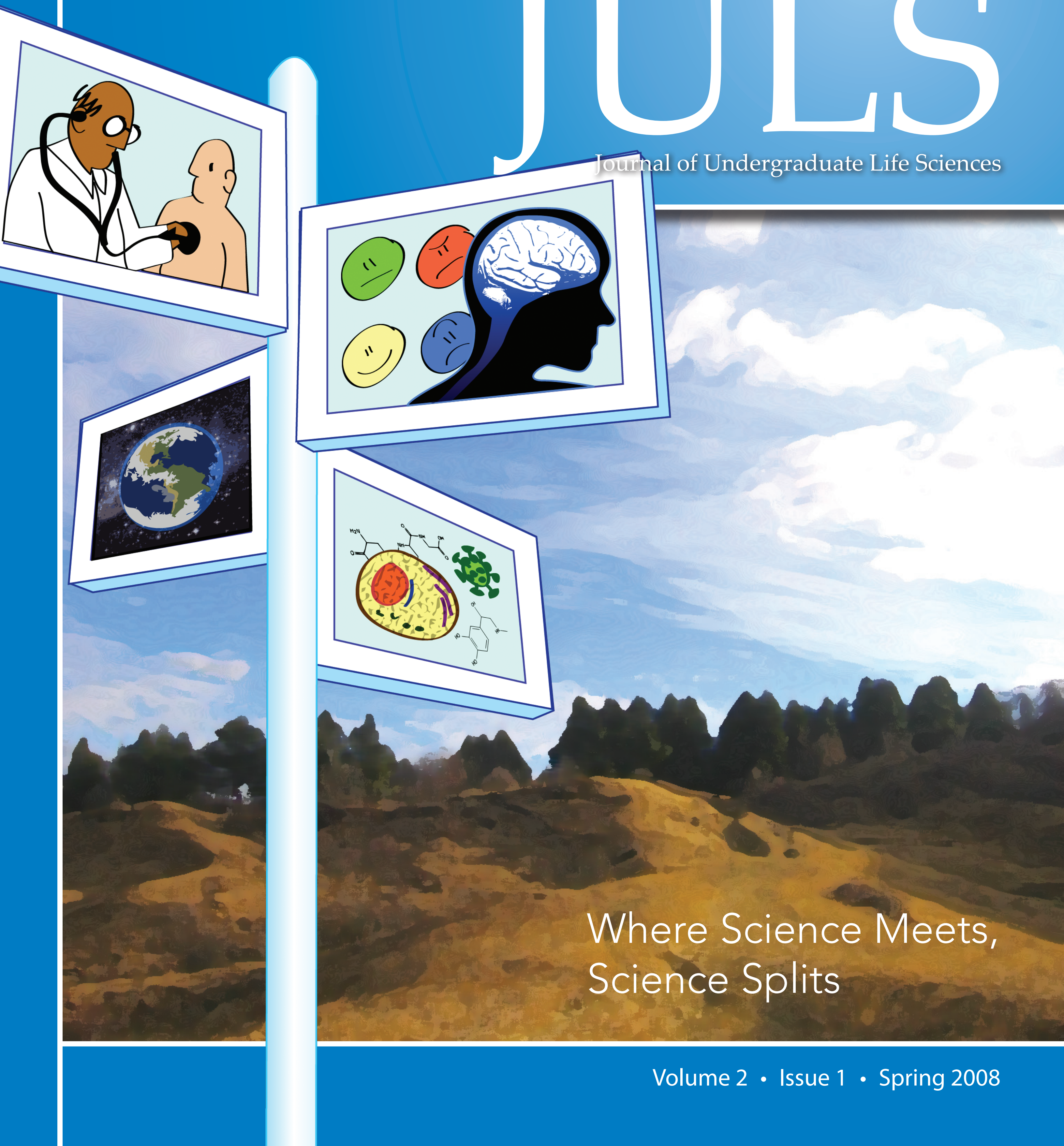
JULS

Journal of Undergraduate Life Sciences



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Volume 2 • Issue 1 • Spring 2008

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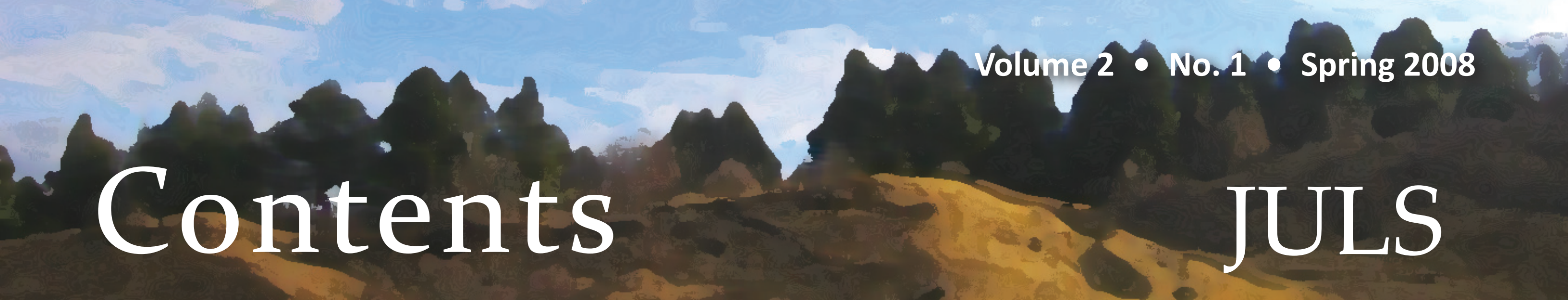

Volume 2 • No. 1 • Spring 2008

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About the Cover

In this issue, a combination of articles on psychology, geological sciences and chemistry attempt to strike a harmonious chord with the life sciences in the hope of finding and utilizing ideas which may otherwise seem to apply only in distinct spheres.

Cover illustration by Mingyu Huang.

JULS

Journal of Undergraduate Life Sciences
University of Toronto

Call for Submissions

The *University of Toronto Journal of Undergraduate Life Sciences (JULS)* is always looking for submissions that showcase the research achievements of undergraduate life science students. We welcome manuscripts in the form of Research Articles or Reviews. Submissions must come from University of Toronto undergraduate students or undergraduate students outside of U of T who have conducted research for at least three months under the supervision of a faculty member at U of T.

Research articles should present original research and address an area of the life sciences. Mini-reviews should focus on a specific scientific topic of interest or related to the research work of the author. Research articles should be between 2,000-3,000 words and mini-reviews between 1,500-2,000 words. All works must not have been previously submitted or published in another undergraduate journal. The deadline for submissions for each issue will appear on the JULS website http://juls.sa.utoronto.ca or http://jps.library.utoronto.ca/index.php/juls.

Contact Us

JULS is always looking for contributions from writers, artists, designers, and editors. Please contact JULS at juls@utoronto.ca if you are interested in joining the JULS team, or have any questions regarding any matter pertaining to the journal, or visit our websites: http://juls.sa.utoronto.ca or http://jps.library.utoronto.ca/index.php/juls.

Acknowledgements

We thank Mingyu Huang for illustrating our brilliant cover graphic this year.

Staff List

Letters From the Editors

Dear friends and colleagues,

We are very pleased to present the second issue of the University of Toronto Journal of Undergraduate Life Sciences (JULS). Owing to the thousands of hours volunteered by staff members and contributors throughout the past year, this publication of the journal contains an expanded scope and content in more than 80 pages. In *Our Current State* section, graduate students discuss their experiences and perspectives in a candid manner, and Professor Miles Johnston, a leading investigator at the Sunnybrook Health Sciences Centre, provides an update on the often-neglected field of lymphatic research. Along with undergraduate research articles in the areas of hematopoietic stem cells, chemical conformational analyses and cognitive flexibility, Tudor et al. and Modi et al. present two *Letters* to introduce an appreciation of the methods of investigation in the earth sciences and, we hope, to promote multidisciplinary thinking in all fields of the life sciences. As our theme for 2008, we believe that all streams of investigation which follow systemic inquiry and attempt to unify the acquired evidences merit consideration from an invariable pedestal.

The undergraduate life sciences community at our university is placed in a very unique position. Just in the past year, investigators at U of T and University Health Network (UHN) made significant contributions to domains such as the metabolic theory of cancer [1], neurodegenerative diseases [2], health informatics [3] and lentiviral vaccination against tumours [4], to name a few. Undergraduate students are actively involved in many of these laboratories, and their skills and creativity are essential for maintaining productivity and innovation in these fields at our university. JULS hopes to become the primary choice for publication of research conducted at the undergraduate level, and we have no doubt that our future members will do their utmost in continuing to improve the path of the journal in the years to come.

Most sincerely,
Sepehr Ehsani

1. Pan, J.G. and T.W. Mak, *Metabolic targeting as an anticancer strategy: dawn of a new era?* Sci STKE, 2007. **2007**(381): p. pe14.
2. Rakhit, R., et al., *An immunological epitope selective for pathological monomer-misfolded SOD1 in ALS.* Nat Med, 2007. **13**(6): p. 754-9.
3. Ehsani, S., et al., *Creation of a retrospective searchable neuropathologic database from print archives at Toronto's University Health Network.* Lab Invest, 2008. **88**(1): p. 89-93.
4. Mossoba, M.E., et al., *Tumor protection following vaccination with low doses of lentivirally transduced DCs expressing the self-antigen erbB2.* Mol Ther, 2008. **16**(3): p. 607-17.

The student authors featured in this edition of JULS all began their work with what is arguably the most fundamental element of scientific research: asking a question. This question served as the basis of the prolonged efforts that ultimately led to the publication sitting before you. Over the course of their investigations, the authors added to our body of scientific knowledge. This idea becomes more profound when we come to realize that seconds before their Eureka moments, nobody had the answers to the questions they were asking.

So we gather that asking questions is important. What do we do about it? Ask more questions. As Plato once said, “The unexamined life is not worth living.” But where do we find questions? Look around you. When I was having trouble finding a topic for my fourth-year project, my supervisor advised me to take note of something that bugged me about the way things were done, convert it into a question, and find a new solution. But what if your questions have already been answered? Reframe them. The reviews contained in the following pages are testament to the notion that despite knowing so much about a variety of fields, there are still questions that warrant further study.

One of our aims with JULS is to encourage the flow of questions from early on in the undergraduate experience. Sitting in lecture halls, we are we are often provided the answers to questions that others once posed. JULS provides a forum for students to ask their own questions. It is no coincidence that our advertisements encourage students to “leave their mark in science.” Not all of these authors will go on to become researchers or pursue careers in academia. Yet, they took the time to ask a question that interested them and answered it, thereby broadening their own knowledge, and ours in the process.

The next time you walk along King’s College Circle, point something out and think of a question you might be interested in knowing the answer to. It doesn’t have to be scientific. If you get a minute, log on to the library mainframe and find out if anyone has answered the question. If no one has, you may just have yourself your very own research project (and an article in next year’s JULS). If it does have an answer, reframe the question. Or take another walk.

All the best,
Abbas Panju

Lymphatic Research

Prof. Miles G. Johnston

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Research on the lymphatic circulation at the international and national levels is a good news, bad news story. Let's start with the bad news first.

BAD NEWS

Interest in lymphatic research

As you are no doubt aware, scientists publish their research findings in journals and the number of articles in any given field provides an index of interest in a particular area. The news on the lymphatic front is not good. I did a quick literature search for all articles published on blood vessels. My computer search yielded 381,408 papers. When I enquired about papers on lymphatics my search yielded only 3,334 studies (less than 1% of the studies on blood vessels). Similarly, if we were to compare investigations on cancer (1.3 million) to lymphedema (~5,000), we can see clearly that there is not a great deal of interest in research on diseases of the lymphatic circulation.

Identification of clinical expertise in lymphedema

With most diseases, the pathway from initial diagnosis to identification of the appropriate medical specialist is clear. However, with lymphedema this is not always the case. Many health care professionals are unfamiliar with experts in this area. Indeed, in my experience, I have learned that some physicians investigate treatment options through the internet. This is not meant as a criticism of the physicians who work very hard to find appropriate treatments for their patients. Rather, this indicates the scarcity of lymphedema knowledge provided to members of the medical profession during their training.

Lack of funding agencies dedicated to lymphatic research

The lack of funding for research in Canada continues to be a problem. Additionally, there are no agencies that provide money for research in lymphedema specifically; although this situation is changing in the USA as the National Institutes of Health (NIH) has a few special programs in which they have requested researchers in the lymphatic area to submit proposals (see below).

Perception of lymphedema by young academics and physicians

It continues to be very difficult to entice young researchers into the lymphatic field. We need to develop scholarships and fellowships that are specifically targeted to research on the lymphatic system in order to attract new blood into this discipline.

GOOD NEWS

New initiative in the United States

I was invited recently to participate in a meeting in the United States that was jointly sponsored by the Lymphatic Research Foundation and the NIH. This meeting was organized as a 'think tank' session. Lymphatic experts were collected together from around the world to discuss issues related to lymphatic research and to define the most important areas for future investigation. All participants found the exercise to be informative. There is hope that this will be the first of many such meetings. In addition, the Lymphatic Research Foundation has developed new funding opportunities. Hopefully, as financial resources in this area increase, scientists will be able to apply for grants to support lymphatic research, and fellowship and scholarship awards to new clinical and basic science investigators will be available.

New scientific developments

There are several new discoveries that give one reason to believe that new developments in the treatment of lymphedema are on the horizon. These include the discovery of defective genes that appear to contribute to certain forms of hereditary lymphedema. These are important discoveries since they raise the possibility that scientists will eventually be able to stimulate the growth of new lymphatic vessels to enhance lymph transport in limbs that are affected with lymphedema. Of course, the attainment of this goal will require many more years of study. Nonetheless, lymphedema sufferers should take heart that we now have important clues that will no doubt lead to innovative therapeutic approaches in the future.

Memoirs of a Grad Student

Derek Ng

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Two-and-a-half years ago, I packed my bags and moved to Toronto to pursue a Ph.D. in biochemistry. Being excited was an understatement—I had just spent the entire summer after my undergrad surveying people over the phone at a call centre (which involved convincing them that I wasn't a telemarketer and then reading out loud the same questionnaire at least 100 times per day). I was in a new city, at a different university, and anxious for my own set of pipetmans. I would be lying if I said that I wasn't also slightly nervous. Several questions kept intruding into my thoughts: (1) will I get along with my new supervisor/coworkers; (2) will I like my research project; and (3) will my set of pipetmans even be calibrated?

The Search

Before coming to Toronto for grad school, I was given the opportunity by the Department of Biochemistry to interview several potential supervisors (which I chose based on my interest in their field of research and their recent publication activity). This was a critical opportunity, as it would dictate which lab I would commit the next five years of my life to. As such, I prepared for each interview with a clipboard full of questions, a blue ballpoint pen to record the answers, and several breath mints.

I had spent my undergrad summers and fourth year in different research labs, so I had an inkling of the type of work environment that I would do well in: (1) a medium sized lab with a good mix of graduate students, post-docs, technicians, and research associates; (2) minimal micro-management from the supervisor; (3) weekly lab meetings with the opportunity to present my research at least every few months; and (4) a collaborative rather than competitive lab atmosphere between colleagues. Evaluating how each potential supervisor met these criteria was important for me, since I couldn't imagine coming to work every day for five years in a lab where I hated the environment—even if my research project was the best on the planet.

The supervisors who stood out the most among the pack were the ones that allowed me to speak with their grad students without me even having to ask them. This was perhaps the most valuable part of the interview, since the grad students often had insider information on the management style of the supervisor (and, on occasion, other supervisors that I was seeing).

By the end of my two days in Toronto, I was thoroughly exhausted from the constant interviewing and was happy to board the plane going back home. I pretty much had a good idea whose lab would be a perfect match for me—I only hoped they thought the same.

The Grad Years

For me, being in grad school feels different than being in undergrad.

As an undergrad, assignment deadlines, midterms, finals, and last-minute cramming sessions defined my life, whereas in grad school, it was more like experimental incubation times, lab-meeting presentations, hot-off-the-press science papers, and pizza seminars. By no means is grad school easy. It requires a great deal of self-motivation (especially for those with supervisors that allow them to make their own goals), critical thinking (because the experiments and ideas presented in peer-reviewed papers are not always correct), and patience (for times when experiments or equipment fail for no apparent reason). It feels like being in limbo between a "real job" and being in school.

Looking back at when I came to Toronto for grad school, I realize that there was no real need for me to be nervous, because the lab that I ended up joining was the right fit for me—not only do I get along with my supervisor and colleagues, I am enthusiastic about my research project and even have my own set of newly calibrated pipetmans. This perfect match had to do mostly with the interviews that I went through—although I can't deny that a little bit of luck might have been thrown into the mix.

Life Through the Eyes of a Graduate Student

Yoav Farkash

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Another school year is drawing to a close and once again, you find yourself busily preparing for your upcoming exams and lab reports. You may also be thinking about graduate studies and wondering if it is right for you, just as I was three years ago. The following is a reflection on my experience as a graduate student. It is not a guide to choosing the right school or a roadmap through the application process, but rather a discussion on what my life has been like as a graduate student. I will talk about the importance of my supervisor and lab mates, my day-to-day challenges, my lifestyle and my perspective on what I gained by pursuing a graduate-level degree. In the following paragraphs you will find some of the behind-the-scenes information—things that no tour or fancy brochure will tell you.

I am currently in my second year of my Master of Science (M.Sc.) degree with the Department of Biochemistry. In the life sciences, most of our work is basic research—meaning that we spend most of the time in the lab, designing and carrying out experiments in order to further the body of scientific knowledge. My lab mates and supervisor are among the most important people in my academic life. They are there for me on a daily basis, much like your coworkers will be when you have a long-term job, to listen to me vent when I need it, to help me when I struggle, and most importantly, to simply hang out.

One of the most critical choices you will make is in deciding who your supervisor will be. There are different styles of supervision, and finding the right supervisor for you will greatly impact your progression as a student. On one extreme you have the “hands off” style, where you and your supervisor decide on a project (the problem you are studying) together and from there, you are mostly on your own. You meet on a weekly or biweekly basis to discuss progress and get input, but there is minimal direction. This style of supervision will usually help develop your independence, critical thinking skills and experiment-designing abilities; it also tends to cause your project to progress more slowly as your error rate is high.

The other extreme is a supervisor who will clearly lay out the direction of the project and assist you in the design of your experiments on a weekly basis. This does not mean that you have no freedom at all, but more often than not, you will end up carrying out what your supervisor designs. Your experimental results will progress faster, though it takes you longer to develop your independence. Most supervisors will be somewhere in between these two extremes. Matching your character to your supervisor’s will largely determine your satisfaction with your project.

Another key consideration is what will be expected from you on a daily basis. Some supervisors will expect students to be in the lab from 9 AM to 5 PM, while others will not prescribe a set timeframe so long as you are generating results in a timely manner. Ultimately, your choice of lab, considering who your supervisor and lab mates will be, and how your daily timetable will break down will not only affect your academic progress, but also your mood and life in general. Since you commit yourself to graduate school from two (M.Sc.) up to seven or even eight years (Ph.D.), choosing your lab is not something you want to do haphazardly.

People often ask about the kinds of challenges a graduate student can expect. Personally, my two biggest challenges are self-motivation and time management. As a graduate student you do not have a precise curriculum, specific material to cover for an exam, or a fixed date at which the course is over (for better or worse!). You are investigating a topic, and for the most part, investigating something that is as yet unknown. It is up to you to find the most up-to-date information; no one will tell you what books or articles to read. You will also need to familiarize yourself with experimental techniques. The senior students will teach you the basics, but you will have to eventually master them on your own through trial and error. Your supervisor will help you design experiments to answer your questions, but it is up to you to carry them out diligently, interpret the results, and determine where to go from there. There is no manual, no guidebook, and no limit to the wealth of knowledge you can acquire. All of this requires a substantial amount of self-motivation. Experiments rarely work on your first, third or even tenth try for that matter. You must not despair, just keep trying! Experimental research is a slow process where progress is measured on a scale of weeks and months, not hours and days. Even if you enjoy the process, it is sometimes extremely hard to push through, which is why self-motivation is, in my opinion, the number one challenge graduate researchers face.

A second challenging aspect of graduate student life is time management. My supervisor does not expect me to be in the lab at specific hours. I can come and go as I please, and whether I carry out my experiments from 9 AM to 5 PM or from 6 PM to 1 AM is a matter of personal preference. I can come in on the weekends and skip weekdays whenever I feel like it. My work schedule needs to be balanced with my personal one—hobbies, workouts, errands and social life. This is often quite the challenge! In fact, I quite often find myself either spending too little or too much time doing work. It is typical to see students working extremely long hours and weekends when a project is advancing well, as they are excited about their discoveries. This kind of enthusiasm is a good thing, but like everything in life, it can be harmful when taken to an extreme. At the other end of the spectrum is the possibility of losing interest in your project or simply not putting enough effort into it while spending too much time on your hobbies. While being in control of your time is certainly one of the attractive aspects of graduate work, at the same time, it is one of the hardest to master.

As far as lifestyle is concerned, you will find that graduate students are a diverse group. For the most part, all of us have our own microcosms outside the lab. From the extremely dedicated-work-only type of people, to the party animals, the sports buffs and everything in between—graduate students do not judge each other by their hobbies and life choices. I find my peers to be welcoming and open-minded. This diversity leads to wonderful opportunities for trying out new activities, learning new topics, and exchanging ideas with interesting people. I find my lab mates to be a wonderful source of knowledge about topics I would not otherwise be exposed to. They are among the most interesting people I know, and are one of the reasons I enjoy coming to the lab.

What can you expect to achieve from pursuing graduate studies? First of all, you will gain specialized knowledge in your field of study. Understandably, this is viewed by some as the main reason for attending graduate school. Depending on the career path you eventually choose for yourself, this knowledge may prove invaluable in the work environment. However, I find this to be an overly narrow view and in fact, your options are not as limited as you may initially think. For the most part, students consider the career paths available to them to be limited to academia, the biotechnology and pharmaceutical industries, or perhaps future professional studies at medical school or law school. Such a view only places value on the specific technical knowledge that you gain at graduate school. In reality, employers will value not only your expertise, but also your independence, research ability, motivation and all of the other transferable skills you gain. I look at my graduate experience as an opportunity for self-improvement, rather than a linear path that solely leads to obtaining specific knowledge.

To conclude, I am extremely happy with my choice to pursue a graduate degree. It is by no means an easy path and frustration is more frequent than success. However, this is only true when success is defined as progression of your project. If you look at the years spent in graduate school as time spent improving your personal skills while making friends and experiencing new things, you will invariably find it a fulfilling and rewarding experience.

Challenges and Difficulties: A Reflection on the Graduate Student Experience

Simon Lee

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Email: mr.lee@utoronto.ca.

My recent experience as a graduate student at the University of Toronto represents a chapter in my life that I will never forget. Although there were many challenges and difficulties along the way, my journey through graduate school was definitely a positive one. As I look back to those years and reflect on them, I realize that I was very fortunate to have met so many great people along the way. The program itself was very enjoyable, but it was truly the people around me that made the biggest difference. At the end of it all, I found myself equipped with a great deal of knowledge, a valuable skill set and, of course, my new degree – but unexpectedly, it seemed that the most important things that I'd be leaving with were the friendships that I had developed over the years. Without a doubt, it has been the people around me who I will miss the most. They have made my experience such a memorable one, and they have all enriched my life in one way or another. When I first started the graduate program, this was not something that I had anticipated, but I am very grateful that it happened. To me, the interpersonal relationships that I had developed are the things that I will always value and cherish. It was the people around me who had helped me throughout the years, and without their support I wouldn't have been able to adjust to my program so smoothly.

From the very beginning, I could see that life as a graduate student would be remarkably different from my undergraduate years. As a candidate for a Master of Science degree, my experience was centered on laboratory experiments and cutting-edge research. Of course, much of this was intimidating to me as it all seemed overwhelming in the early stages. Though undergraduate education provided me with the basic foundation of knowledge required for my work, it was evident that there was much more I needed to know and that the learning environment would be considerably different. There was a steep learning curve involved, but the transition from lecture-based learning to a hands-on practical approach was very welcome. During undergraduate courses, much time was spent absorbing information that, at the time, seemed inconsequential, but it was very refreshing to see how the same principles were applied to my work. In addition, the thinking process required for graduate school is noticeably different from the way we've been trained in the undergraduate years. Memorization and studying were replaced with analysis and practical application. Importantly, much of the training was from peers and others who did similar things. It was nice to work alongside people who were able to teach you on a personal basis, and share their experience with you. It was all very different, but at the same time, it was exciting to be able to switch away from the in-class learning to which I had always been exposed. Though there was still much reading involved – can't seem to ever get away from that! – it was certainly not the same textbook-style approach that I had been accustomed to; in particular, the onus was on the individual to seek out the relevant literature. There was a great emphasis on self-learning and independent study, which added an important dimension to our thought process.

As I look back on my experience in graduate school, I can see how I have personally developed over the years. I feel like I have matured a great deal over a short span, and that I am mentally stronger than before. The whole experience has changed my life, and I'm so glad that it was largely due to the amazing and talented people whom I had the pleasure of working alongside. In time, even if my knowledge fades, I hope that these friendships will last forever.

A Day in the Life of a Graduate Student

Simon Lee

7:30am	Wake up and fight rush hour traffic getting to school.
9:34am	Stumble into lab.
9:36am	Plug in laptop and check e-mails. Chat with lab mates.
10:01am	Meet with supervisor (if necessary). (It's usually necessary.)
10:16am	Check e-mail.
10:30am	Set up my experiments in the lab. Find out if any equipment or supplies are missing. If so, track them down!
11:07am	Check e-mail.
11:20am	Perform experiments. While they are running, complete some data analysis, statistical work, and miscellaneous number crunching.
12:30pm	Check e-mail.
12:48pm	Pull up journal articles to add to the stack sitting on my desk that my supervisor (and postdocs) have been consistently adding to. Read, read more, read more, read more...
1:50pm	Check e-mail.
2:10pm	Grab lunch or a snack (caffeine!)
2:44pm	Talk to people in the halls of the Medical Sciences building.
3:15pm	Browse a bit online. Catch up on YouTube videos. Check stock quotes. Check e-mail.
4:00pm	Take a power-nap if necessary. (It's usually necessary.)
4:25pm	Back to work until it's done! Check up on experiments and record data. Set up experiments to run overnight. Go through more data and crunch more numbers.
6:25pm	Check e-mail.
6:37pm	Usually drained and head home!

PHOTO BY JAMES WILSHER

The Blueprints of Your Career: Planning to Prevent the Procrastination Pitfall

Mustafa Kamani

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Now that I am about to graduate, what do I do with my life-science degree? All life-science students face this question at some point in their undergraduate careers. Throughout these years, students regularly talk amongst friends about their bad procrastination habits. What they don't realize is that, arguably, the most evident example of procrastination is in dealing with the question of their future. Students have at least four years to reflect on their post-baccalaureate course of action; yet, more often than not, this thought process kicks into gear in their final year of study.

In first year, students go through a transition phase from high school to post-secondary education, and briefly think about their future in selecting a program of study. The following two years, students adjust to the specialized courses that constitute their programs, and are more concerned with reaching the end of their studies than the beginning of their careers. Come fourth year, however, people find themselves saying, "What am I going to do next year?"

For those wishing to stay within the confines of the scientific environment, the most obvious postgraduate option is research. Depending on circumstances or personal goals, research via graduate studies may not be the best option. The biotechnology and pharmaceutical industries are always in search of laboratory technicians. However, though some are available to new graduates with bachelor's degrees, the majority of these positions require previous lab experience and often a postgraduate education. One of the advantages of working in the biotechnology sector is the flexibility of transferring to departments other than research and development, such as pharmaceutical sales, medical writing, quality control, finance, and business analysis. With experience, one can upgrade to project managerial positions. Research can also be undertaken in government laboratories, such as through Environment Canada and Health Canada. The pay scale is noticeably different for researchers in the biotechnology, academic, and government settings, with the first being the most financially rewarding. It is important to note that salary should not be the only occupational facet to take consider. The academic research setting is likely to be more flexible in terms of working hours, and if teaching at the post-secondary level is one of your interests, this route is the way to go.

Apart from research, typical science-related careers are medicine, dentistry and pharmacy. Many students decide early in their academic careers that they want to pursue one of these professional programs. While it is great to have this ambition, a back-up plan must also be drafted. It is only too clear that a very small percentage of medical and dental school applicants actually gain admission into these highly competitive programs. So, what happens if you don't get in?

Other scientific careers include, but are not limited to, forensic scientist, elementary or secondary school teacher, medical technician, nutritionist, and physiotherapist. Some of these will require postgraduate education. It is important to realize that a life science degree does not limit graduates to science-based careers. For those intent on a career in business, one option is to work for a couple of years and then apply to Master of Business Administration (MBA) programs. Some schools now offer MBA programs that cater specifically to science students. Some other non-science options are consulting, banking, marketing and law.

In completing the University of Toronto's biochemistry undergraduate program, I, like many of my friends, addressed the question about my future most intently in my final year. I would deliberate over this issue on a regular basis and decided to evaluate the skills that my program enabled me to develop. As a research-intensive program, we were given the opportunity to acquire various laboratory skills and dexterity with lab techniques, but at the same time, a great deal of emphasis was placed on critical thinking and problem-solving. The research and laboratory components of the program also stressed the importance of both oral and written communication. Proficiency with these skills is essential to becoming successful regardless of the line of work. Although I always had the desire to make contributions to the world of medical research, I felt a sense of comfort knowing that I had developed skill-sets that were not only transferable, but also necessary, in other disciplines.

As it is often said, it really is never too early to start planning for your future. Evaluating your future, however, is a continual process. It does not end when you have decided to pursue the postgraduate route, or when you enter the workforce. It may seem easy to advise, but you'll find yourself capable of avoiding, or at least, minimizing, the possibility of making hasty or unsatisfactory decisions, and the associated woes of this process. So, while you may work frantically to finish projects for a year-long course at the last possible moment, try not to delay working on the assignment that will have much greater influence: the blueprints to your future career.

JULS

Research Article

Silencing the polarity protein Scribble in murine hematopoietic progenitor cells

Ann Marie Macara, Kelly Pike, and Anthony J. Pawson

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Abstract

It has been shown that the polarity protein Scribble is required for T-cell migration, which itself is important for lymphocyte development. To study its effects on B-cell development and migration, Scribble gene expression was silenced using a self-inactivating retroviral expression vector that produces a small hairpin RNA in murine hematopoietic progenitor cells (HPCs). Infected HPCs were then allowed to differentiate into B lymphocytes on an OP9 bone marrow stromal cell line. Silencing of Scribble expression in HPCs did not inhibit commitment to the B-cell lineage. However, these B lineage cells failed to survive in culture over a period of three weeks. The loss of B lymphocytes may indicate a role for the protein Scribble in B-cell proliferation, survival, and cell death.

Keywords: B-cell development; hairpin RNA; hematopoietic progenitor cell; retroviral expression vector; Scribble

Introduction

The immune system provides active defense against pathogens within an organism. B- and T-cells are the key players in the detection and termination of these foreign invaders. While B-cells are primarily involved in producing antibodies that detect antigens [1], T-cells have a wide range of functions, including macrophage recruitment and attack of infected cells [1].

Lymphocyte progenitors are derived from bone marrow stem cells. These stem cells commit to either the B- or T-cell lineages within the bone marrow [2]. T-cell-committed HPCs migrate to the thymus, where the stromal environment induces T-cell differentiation. On the other hand, B-cell progenitors stay in the bone marrow, where a different local environment helps stimulate B-cell differentiation [2].

As the B- and T-cells migrate within their respective generative tissues, the cells extend in the direction of cell movement, developing protrusions at the front and a uropod at the rear [3]. Uropods are important in various aspects of lymphocyte polarity, such as cell-cell interactions and migration. As defined, polarity is the asymmetric distribution of molecules to different regions of the cell, and is an essential property of most cells. In general, polarity proteins regulate polarity by recruiting other proteins and molecules to various sections within the cell, interacting with the cytoskeleton to change lymphocyte shape or function, and influencing the activity of other polarity complexes [3] . The polarity protein Scribble is found in the uropod of migrating T-cells [3]. Ludford-Menting and colleagues showed that silencing Scribble gene expression in murine T-cells prevented cell polarization and migration, illustrating Scribble's essential role in T-cell motility [1, 2]. It is not known, however, if Scribble is also important in B-cell migration.

As a B-cell matures, it expresses various extracellular membrane protein markers. The CD45R isoform B220 is one of the first indicators that a lymphocyte has committed to the B-cell lineage [4]. Immature B-cells only contain the surface membrane protein immunoglobulin M (IgM), whereas the mature B-cell contains both surface membrane proteins IgM and IgD. IgM and IgD are B-cell antigen receptors (BCRs), which give B-cells antigen specificity. Additionally, the IgD receptor acts as a signal to release the mature B-cell into the circulation [5].

T-cell differentiation undergoes a three-stage process of lymphopoiesis within the thymus [2, 6]. First, the double-negative lymphocyte progenitor (CD4-/CD8-) enters the thymus at the corticomedullary junction. Then, the pro-T-cell migrates to the outer cortex in the thymus, where it becomes a double-positive immature T cell (CD4+/CD8+). The T-cell fully matures into a single positive T-cell (either CD4+ or CD8+) in the medulla.

A line of bone marrow cells (OP9 cells) was used to study lymphopoiesis *in vitro*. It has been shown that OP9 cells support B-cell differentiation and not T-cell differentiation [2], suggesting that some element of the bone marrow environment is specifically-conducive to B-cell differentiation. Alternatively, some factor that normally facilitates T-cell differentiation in the thymus may simply be lacking in the bone marrow. Notch-ligand signaling has been shown to play a very important role as a cell fate regulator [2]. Studies have indicated that inducing OP9 cells to express the Notch-ligand delta-like-1 protein (DL1) causes bone marrow cells to lose the ability to differentiate into B-cells, but enables them to differentiate into T cells [2]. Schmidtt and colleagues showed that normal OP9 cells fail to express DL1, whereas DL1 is endogenously expressed in the thymic stromal cells [6]. When the authors experimentally induced the OP9 cells to express DL1, it was found that OP9-DL1 cells began to support T-cell differentiation while suppressing B-cell lymphopoiesis. Therefore, we used both the OP9 cells and the OP9-DL1 cell lines in order to examine both B- and T-cell lymphopoiesis, respectively.

We aimed to build on previous work examining the functional role of the polarity protein Scribble in lymphopoiesis. In particular, we aimed to determine whether Scribble is required for B-cell differentiation. To do so, we silenced the Scribble gene using RNA interference (RNAi) retroviral vectors to infect the embryonic murine liver cells, and noted expression rates.

Materials and Methods

RNA interference

A retroviral vector-based RNA interference system was used to silence the endogenous Scribble gene of liver hematopoietic progenitor cells (HPCs). The vector is called RNAi-Ready pSIREN-RetroQ-ZsGreen (pSZG) and expresses short hairpin RNAs (shRNAs) [7]. The pSZG vector expresses the green fluorescent protein (GFP) ZsGreen, which is detected by flow cytometry in pSZG-expressing cells. Synthetic oligonucleotides encoding the target sequences were annealed and ligated into the pSZG vector. Two shRNAs against Scribble were used in these experiments (Scribble1, Scribble2), while luciferase was employed as a negative control. The retroviral vectors were transfected into retroviral packaging cell lines (such as the Phoenix or PlatE cell lines), to produce infectious viruses encoding the shRNAs. The supernatant from these cells, containing the virus particles, was then added onto the target cells (HPCs).

Ligation of RNAi-Ready pSIREN-RetroQ-ZsGreen (pSZG) retroviral vector

The pSZG was digested with Eco*R1* and Bam*H1* (Roche). Then 50-100ng of the targeted gene's luciferase shRNA, Scribble 1 shRNA and Scribble 2 shRNA were ligated into the linearized pSZG retrviral vector (Clontech Laboratories, Inc.).

Generation of Retroviral Vector and Infection of BI-141 T cells.

The Phoenix or PlatE retrovirus producer cells were passaged on *Day 0*. The cells were transfected on *Day 1*. The ligated pSZG retroviral vector (10 µg of DNA) was diluted into 750 µl of Opti-MEM media (Invitrogen). 25 µl of polyethylenimine (PEI) was added to the diluted DNA, and was then incubated at room temperature for approximately five minutes. The DNA suspension buffer was then added to the Phoenix or PlatE cell lines. The media was changed on *Day 2*. On *Day 3*, the BI-141 cells were harvested, washed, and resuspended to 0.25×10^6 cells/mL, then aliquoted to 0.5×10^6 cells/well in a six-well plate. The transfected Phoenix or PlatE cell supernatant was then harvested to collect the pSZG retrovirus. The viral supernatant was added (2 mL/well) to the BI-141 cells. Next, 2 µl per well of polybrene (10 µg/mL) was added, and the plate was centrifuged at 2200 rpm for 1.5 hours at 30 ^{o}C to ensure good contact between the virus and the cell surface.

OP9 cell media

The medium was made from 450 mL of aMEM (Gibco 12561) with 10% FBS, 5 mL HEPES (Gibco 15630-080, 100×, 1M), 5 mL sodium pyruvate (Gibco 11360-070, 100×, 100 mM), 5 mL Pen/Strep (Gibco 15140-122, 100×, 10000 U/ml pen, 10000 µg/mL strep), 5 mL Glutamax (Gibco 3505-061, 100×, 200 mM) and 0.5 mL 2-mercaptoethanol (1000×, 55 mM).

Washed/No-wash HPCs post-infection and 10ug/mL or 20ug/ul of polybrene

One set of HPCs was set aside as the no-wash group, while the other set of HPCs were washed once with OP9 media after infection of the pSZG retrovirus. The groups of washed and unwashed HPCs were further divided into groups whereby the viral media was given either 10ug/mL of polybrene or 20ug/mL of polybrene, along with the addition of appropriate cytokines.

Anti-HSA Killing

5mL of fetal liver cells were harvested from suspension, and 4mL of J11d (anti-HSA) supernatant and 1mL of the rabbit complement were added. The cell suspension was then incubated for 30 minutes at 37 ^{o}C. After the incubation period, 10mL of Lympholyte-M (Cedarlane Laboratories Ltd) was added. The cell suspension was then spun down at 2200rpm for 20 minutes at room temperature. The Lympholyte-M solution separated the layers of cells. The HPC cells were harvested at the interface between the Lympholyte-M solution and the cell suspension solution.

Differentiation of hematopoietic progenitor cells (HPCs) on OP9 cells

Day 0: The fetal murine livers were harvested (day 14.5) and homogenized to generate cell suspensions. The cell suspension was then filtered through a 70µm filter. An anti-HSA killing protocol (as described above) was used to isolate the HPCs. The HPCs were seeded at 1.5×10^5 cells per 80% confluent plate of OP9 in media containing 5µg/mL cytokines Flt3-Ligand (R and D 308-FK) and IL-7 (R and D 407-ML) to assist HPC proliferation.

Day 3: The OP9 co-cultures were passaged by filtering the harvested cells through a 40um filter to select for HPCs. The progenitors were then pelleted and resuspended in OP9 media containing the appropriate cytokines, and then added onto an 80% confluent plate of OP9 or OP9-DL1. The co-cultures were then passaged every 2 days.

Antibodies

Several antibodies were used, including anti-HSA-FITC (BD Biosciences), anti-HSA-PE (BD Biosciences), and anti-CD19-PE (BD Biosciences). Flow cytometry data were analyzed using the CellQuest software package (BD Biosciences).

Statistical Analysis

The independent two-sample t-test was used to determine statistical significance of the transfection and infection rates at the p=0.05 significance level.

Results

Determination of the most efficient packaging cell line

To determine which retroviral packaging cell line was most efficient at viral production, retroviruses were produced with either PlatE or Phoenix cells and infection efficiency was verified through infection of the BI-141 T cell line. Analysis of GFP expression showed that the transfection rates of Phoenix cells and PlatE cells were comparable (Fig. 1).

The infection rates of the Phoenix and PlatE BI-141 T cells were measured 24 hours post-infection (Fig. 2). The infection rate of the PlatE-produced viruses was significantly larger than that of the Phoenix-produced viruses ($p \leq 0.05$), suggesting that the PlatE cells produce the virus and infect other cells more efficiently than the Phoenix cell line.

Optimization of infection protocol of fetal liver progenitor cells

The effect of washing the HPCs after infection with a pSZG virus (either from the PlatE or Phoenix cell lines) was investigated by comparing washing with two different concentrations (10μg/mL and 20μg/mL) of polybrene. Both the washed PlatE- and Phoenix-generated pSZG-infected HPCs had significantly lower infection rates and fewer cells than the unwashed HPCs ($p \leq 0.05$). Data are shown in Figure 3.

Diminished Scribble expression in fetal liver progenitor cells

PlatE cells were transfected with pSZG-luciferase shRNA, pSZG-Scribble1 shRNA, and pSZG-Scribble 2 shRNA. Transfection rates were measured using flow cytometry 72 hours post-transfection. The pSZG-luciferase transfection rate was 72%, the pSZG-Scribble1 transfection rate was 100%, and the pSZG-Scribble 2 transfection rate was 78%. Two different infection conditions were setup. In the first infection condition, the HPCs were infected once every 24 hours over a 48-hour period. After 48 hours post-infection, enough cells were not present to check for GFP expression. In the second infection condition, the HPCs were stimulated using the cytokines FMS-like tyrosine kinase 3 ligand (Flt-3L) and interleukin (IL)-7 for 24 hours to increase the likelihood of infection during HPC division. HPC growth was stimulated for 24 hours, after which they were infected with the viral supernatant from the PlatE cells. Infection rates of the

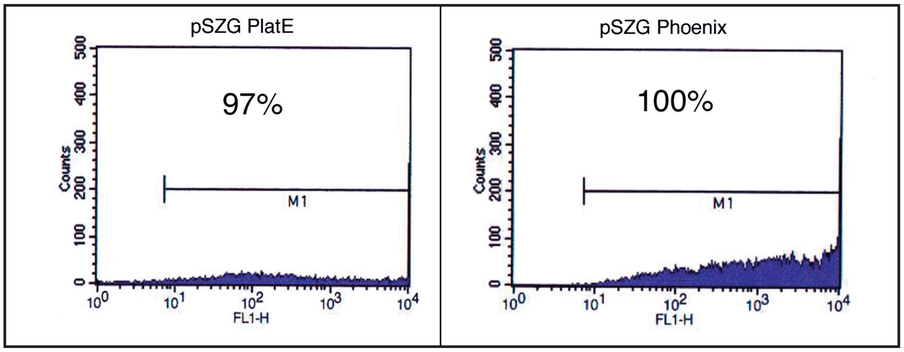

Figure 1 (above). PlatE and Pheonix cell transfection rates. The amount of GFP expression was measured using the green spectrum FL1-H of the flow cytometer. The difference in transfection rates of PlatE (left) and Phoenix (right) packaging cell lines was not statistically-significant at p=0.05.

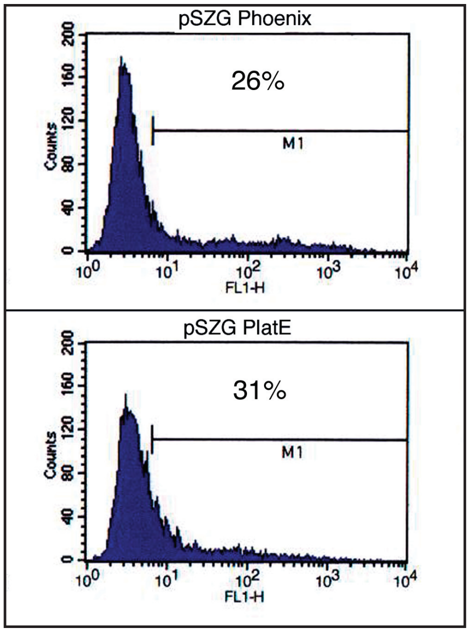

Figure 2 (above). pSZG Pheonix and PlatE cell infection rates. The amount of GFP expression was measured using the green spectrum FL1-H of the flow cytometer. The infection rate of pSZG Phoenix cells (26%; top) was significantly smaller than of pSZG PlatE cells (31%; bottom) ($p \leq 0.05$). The naïve control sample appeared to be contaminated, and was omitted from analysis.

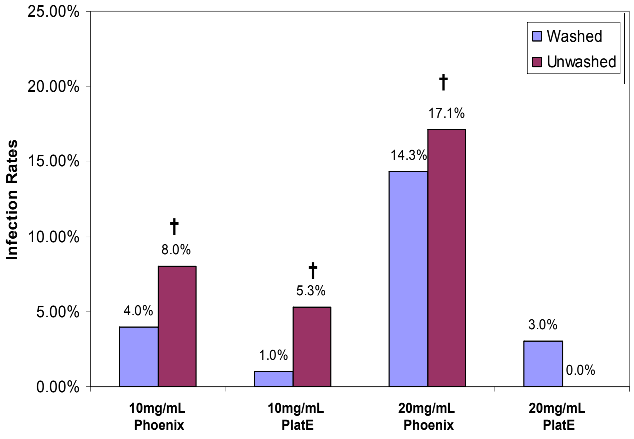

Figure 3 (left). Effects of washing HPCs with polybrene on infection rates. Both the washed PlatE- and Phoenix-generated pSZG-infected HPCs had lower infection rates and fewer cells than the unwashed HPCs. Data for the unwashed PlatE cells (at 20μg/mL) is unavailable due to sample contamination. Statistical significance ($p \leq 0.05$) is denoted by (†).

HPCs were measured 24 hours post-infection (Fig. 4).

The HPCs were also checked for GFP expression and infection rate at four, eight and 15 days (Fig. 5). The data indicate that GFP expression dropped for the pSZG-Scribble1 and pSZG-Scribble2 over the 15-day period.

Discussion

We have optimized a system for the delivery of shRNA into fetal liver HPCs, and have used this system to suppress the expression of a Scribble polarity protein. To do so, we silenced the Scribble gene using RNA interference (RNAi) retroviral vectors to infect the embryonic murine liver cells. After infection, it is believe that the RNA from the retrovirus undergoes reverse transcription and integrates into the cell. The infected HPCs now express the short hairpin RNA (shRNA) [8, 9]. The shRNA is then cleaved into small interfering RNAs (siRNAs) by the Dicer enzyme [8]. The siRNAs form a complex called RNA-induced silencing complex (RISC). The RISC unwinds the siRNA and removes one of the strands. The siRNA strand that is left in the RISC complex then binds to the complementary sequence on the endogenous Scribble mRNA. The protein Slicer cleaves the mRNA, allowing the cell to recognize and destroy it. The continuous destruction of the endogenous targeted mRNA silences the gene. These infected liver hematopoietic progenitor cells were then allowed to differentiate on an OP9 background.

We have shown that the PlatE cells produce the virus more efficiently than the Phoenix cell line (Fig. 2), and observed that a decrease in Scribble gene expression reduced the number of cells in the B-cell lineage (Fig. 5). The pSZG-luciferase control group was compared to the experimental pSZG vectors, Scribble1 and Scribble2. Scribble was not silenced in the control group, and B-cells showed normal survival and development. The pSZG experimental vectors, when compared to the pSZG-luciferase control, showed a decrease in B-cell survival. The pSZG-Scribble1 showed a significantly greater decrease in cell survival over time than the pSZG-Scribble2 vector ($p \leq 0.05$).

As expected, the transfection rates for the three pSZG vectors (luciferase, Scribble1, and Scribble2) varied. The infection rate of the vectors carries error due to fact that the number of active viruses was not counted before infection. For this same reason, the infection rates between

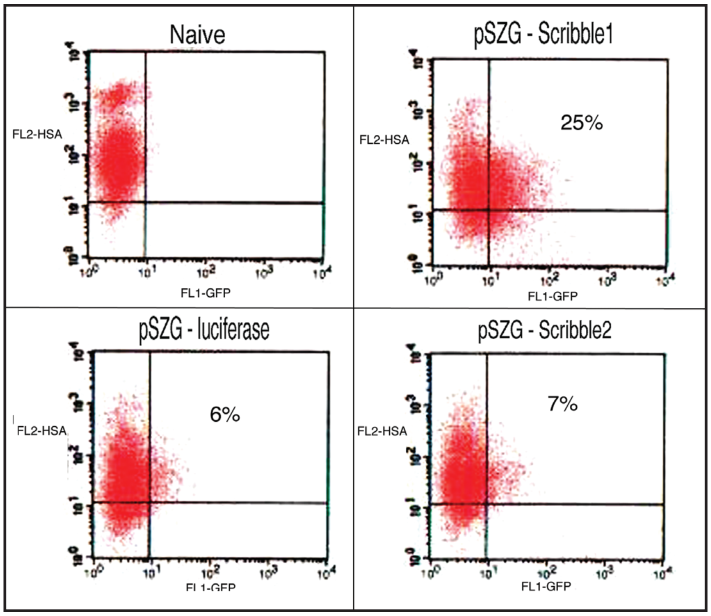

Figure 4. HPC infection rate 24 hours post-infection. In infection condition-2, GFP expression was checked 24 hours after retroviral infection and 48 hours after stimulation by cytokines. The naïve control HPCs (top left) were gated at a 1% false infection rate. The pSZG-luciferase infected cells (bottom left) had an infection rate of 6%, the pSZG-Scribble1 infected cells (top right) had an infection rate of 25% and the pSZG-Scribble2 (bottom right) infected cells had an infection rate of 7%. The infection rate of pSZG-Scribble1 was approximately four-fold higher than of pSZG-luciferase, and the difference was statistically-significant ($p \leq 0.05$). This suggests that a greater number of active viruses contained the pSZG-Scribble1. A significant improvement infection rate was not observed in pSZG-Scribble2 over pSZG-luciferase ($p \geq 0.05$).

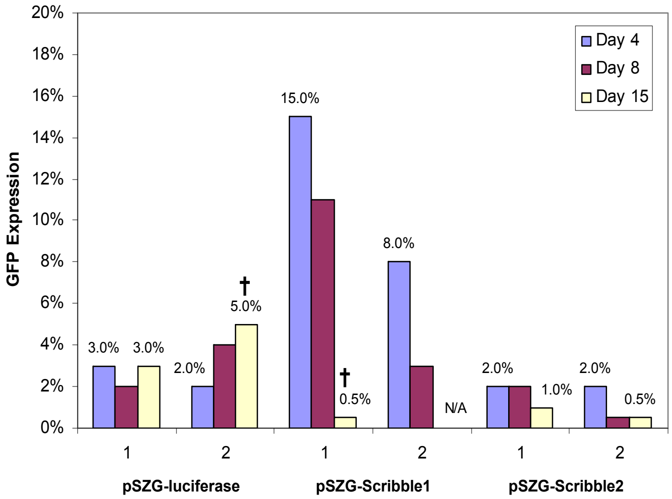

Figure 5. GFP expression of HPCs over 15 days. GFP expression using both protocols (1 and 2) are shown for the various cell lines. HPCs began to differentiate on the eighth day. Data were unavailable for the second Scribble1 treatment at day 15 due to contamination of the sample. Statistically-significant ($p \leq 0.05$) differences between day four and day 15 are denoted by (†). The 30-fold reduction in GFP expression in infection condition one of pSZG-Scribble1 suggests that the loss of Scribble negatively-affects B-cell survival.

the fourth and eighth days of the OP9 co-culture also varied for the control pSZG-luciferase virus in both infection conditions (Fig. 5). The variation of infection rates leads to a level of standard error when comparing the control pSZG-luciferase to the two pSZG-Scribble rates of infection.

Depletion of Scribble is known to prevent lymphocyte migration. Because migration is important in B-cell differentiation, we expected Scribble silencing to have an effect on commitment to the B-cell lineage. However, this effect was not observed. It is possible that Scribble is important in B-cell survival and that our depletion of Scribble resulted in programmed cell death. Our experiments indicate that in a culture of OP9 cells, under conditions that provide an amiable environment for the differentiation of B-cells, depletion of Scribble may block differentiation (Fig. 5). The exact step at which differentiation is halted has yet to be determined. This possibility warrants further investigating as promoting cell survival would add novel functionality for this polarity protein. In *Drosophila*, the opposite phenotype has been shown to occur, whereby loss of Scribble results in cell overgrowth and tumor formation [2]. Moreover, the over-expression of Scribble in mammalian epithelial cells can inhibit cell proliferation [2].

The loss of Scribble in mammalian T-cells and epithelial cells inhibits polarized migration [3]. Therefore, it is of interest to determine whether loss of Scribble affects lymphocyte progenitor movement within bone marrow *in vivo*, and whether the lack of B-cell precursor survival is related to an inhibition of cell migration. Scribble interacts with a number of different proteins, beta-PIX (an exchange factor for the small GTPase RAC), which is intimately involved in cell migration [2]. It also associates with Vangl, a planar polarity protein [2]. Establishing whether either of these binding partners links Scribble to B-cell survival may help resolve important questions for its role in mammalian systems. B-cells are important in the overall function of the immune system. Further understanding of Scribble's involvement in B-cell survival pathways may aid in the future study of some immune disorders.

In conclusion, our results show that a decrease in the expression of the polarity protein Scribble leads to a negative effect on the survival of the B-cell lineage over a three-week period. However, silencing Scribble did not inhibit the commitment of progenitor cells to the B-cell lineage. Further work is required to determine the exact function of the Scribble protein in the survival, differentiation, and migration of lymphocytes.

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JULS

Research Article

Conformational analysis of N-protonated-β-fluoro-β-vinyl-ethylamine, a protonated adrenaline model: an exploratory first-principle computational study

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Abstract

The conformational landscapes of N-protonated-β-fluoro-β-vinyl-ethylamine, a fragmented model of L-adrenaline, were explored by full geometry optimization. The study was performed using restricted Hartree-Fock (RHF) formalism with a 3-21 contracted Gaussian (3-21G) basis set (RHF/3-21G level of theory). A complete conformational study of N-protonated-β-fluoro-β-vinyl-ethylamine was carried out from first principles of quantum mechanical computations using the Gaussian 03 (G03) program. The present study was designed to analyze the conformational characteristics of the molecule, a prerequisite to any full investigation of the adrenaline molecule. The relative energies of the various stable conformers of N-protonated-β-fluoro-β-vinyl-ethylamine were calculated for the R and S stereoisomers. For the R stereoisomer, the g^-g^+ was the most stable and the g^+g^+ conformer was the least stable. For the S stereoisomer, the g^+g^- and g^-g^- were the most and the least stable conformers, respectively. Through the combination analysis of both point and axis chirality, the enantiomeric and diastereomeric relationships of the twelve structures investigated were established. This study presents a preview of all existing stable conformers of adrenaline. Future studies must aim to computationally dock these conformers to the active site of the adrenergic receptor in order to determine which conformer has the greatest biological effect.

Keywords: adrenaline model; conformational study; first-principle quantum chemistry; β-fluoro-β-vinyl-ethylamine; molecular computation

Introduction

Historical Aspects

In May 1886, William Bates reported in the New York Medical Journal that he discovered a substance produced by the adrenal gland [1]. Less than a decade later, in 1895, the Polish physiologist Napoleon Cybulski isolated this substance and identified it as adrenaline (Fig. 1). Within the next five years, adrenaline was also isolated independently isolated by John Jacob Abel and Jokichi Takamine [1, 2]. Within four years, it was synthesized for the first time by the German chemist Friedrich Stolz [3]; adrenaline was the first hormone to be crystallized [4].

Biological Aspects

Adrenaline is a biogenic amine that acts as both neurotransmitter and hormone [5]. Adrenaline is synthesized primarily in the adrenal medulla [6]. The major effects of adrenaline include increased heart rate and stroke volume, dilation of pupils, and constriction of arterioles. The latter results in increased oxygen and glucose supply to the brain and muscles. [6]. In addition to its effects on the cardiovascular and nervous systems, adrenaline increases plasma glucose levels by directly enhancing glycogen and fatty acid breakdown and, furthermore, inhibiting insulin and stimulating glucagon secretion [7, 8].

Adrenaline mediates nerve pulses to efferent organs. Consequently, catecholamines and their analogs, such as adrenaline, are widely studied [9]. The release of adrenaline from the adrenal medulla is one of the first responses to stress and fear, and thus adrenaline is capable of regulating cardiovascular function during exercise.

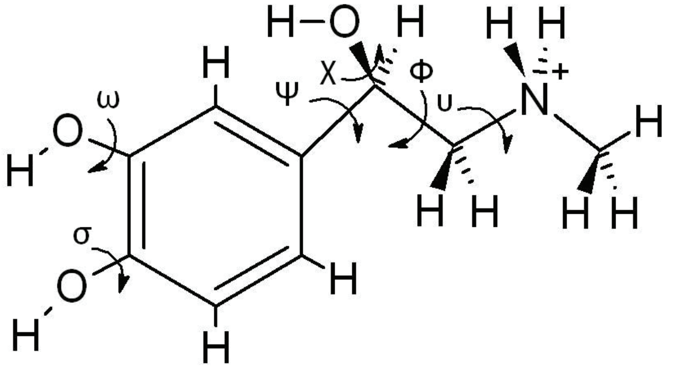


Figure 1. Protonated adrenaline and six dihedral angles as conformational variables.

Recent experimental and computational studies of adrenaline have indicated that the conformation of catecholamine neurotransmitters autoregulate receptor binding and thus directly influence the rate of metabolism [10]. It is necessary to study the energetically accessible conformation of adrenaline in order to fully understand the hormone's function [10]. The majority of conformational studies conducted to date have been performed in apolar solvents or in the gas phase because most receptor sites are strongly hydrophobic. The intramolecular hydrogen bonds are favoured in these hydrophobic environments [11]. Consequently, such an apolar environment of the receptor site greatly contributes to controlling the high concentrations of internally hydrogen-bonded adrenaline conformers [12].

Overview of conformational analysis

Conformational potential energy change as a function of dihedral angles is the basis of conformational analysis. Minima, usually *gauche*$^+$ (g^+), *anti* (*a*), or *gauche*$^-$ (g^-), are stable structures whereas maxima are unstable. Typical conformations are shown in Figure 2.

The shape of the potential energy curves vary with the degree of substitution and with the rotation of the CH_3 group; the energy curves have 3 equivalent minima corresponding to g^+, *a*, and g^-. The rotation of a prochiral centre CH_2Y leads to a curve in which g^+ and g^- are equal in energy, while *a* is of lower energy. Finally, the rotation of a chiral center CHRY produces a potential curve with three unique energy minima. These relationships are illustrated in Figure 3.

Non-superimposable mirror-image stereocenters (enantiomers) produce non-superimposable potential energy curves as shown in Figure 4.

In the case of double rotors, consisting of two independent dihedral angular motions instead of potential energy curves, one may obtain potential energy surfaces which result in a pattern of nine minima (Fig. 5). Since the component PEC has three degenerate minima (g^+, *a*, and g^-) the surface itself will have nine identical minima. Thus, the conformational landscape shown in Figure 5 has a rather regular, symmetrical pattern.

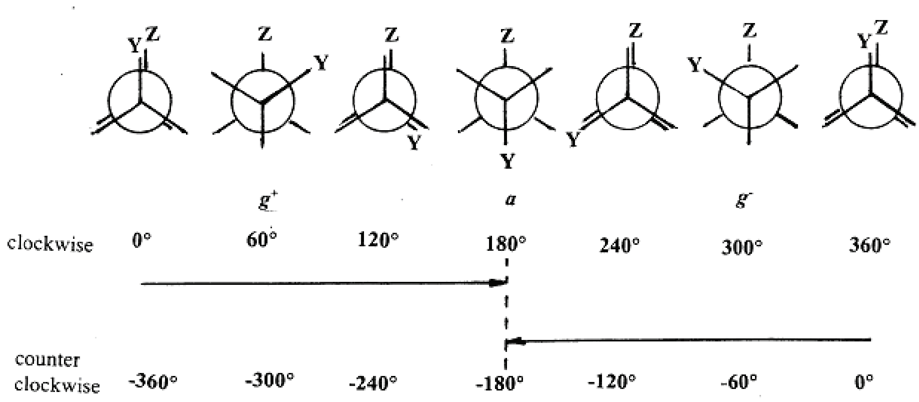


Figure 2. Conformational changes of disubstituted ethane ($Y—CH_2—CH_2—Z$) [13]. The four C—H bonds are marked as four dashes and therefore, thus the presence of the H atoms is understood but not shown explicitly (for ethane Y = Z = H, for *n*-butane Y = Z = CH_3). The fluorine atom was omitted from N-protonated-β-fluoro-β-vinyl-ethylamine. For the modified molecule, the dihedral angle Φ is the dihedral angle for the rotation about the $–CH_2–CH_2–$ single bond. Y = $–NH_3^+$ and Z = $–CH=CH_2$.

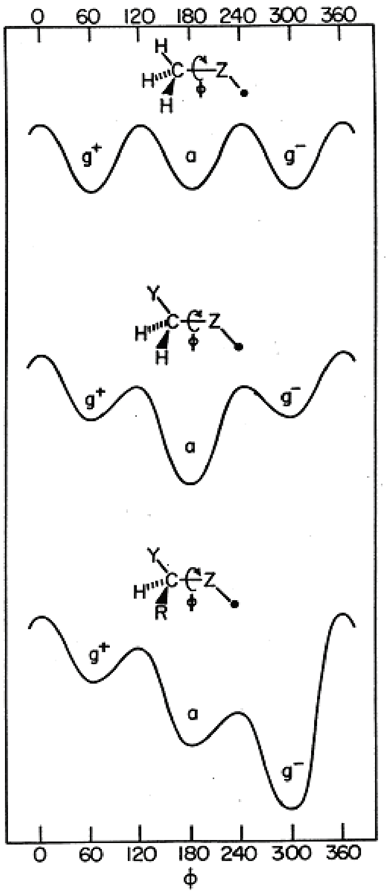


Figure 3. Modification of the conformational potential energy curve by increasing degree of substitution [13]. The top curve is like the conformational potential energy curve (PEC) of ethane ($Z=CH_3$) and the middle curve may be exemplified by *n*-butane (Y= $–CH_3$ and Z= $–CH_2–CH_3$). The bottom curve provides an example of the conformational potential energy curve of N-protonated-β-fluoro-β-vinyl-ethylamine (Y = $–CH_2–NH_3^+$, R = –F, and Z = $–CH=CH_2$).

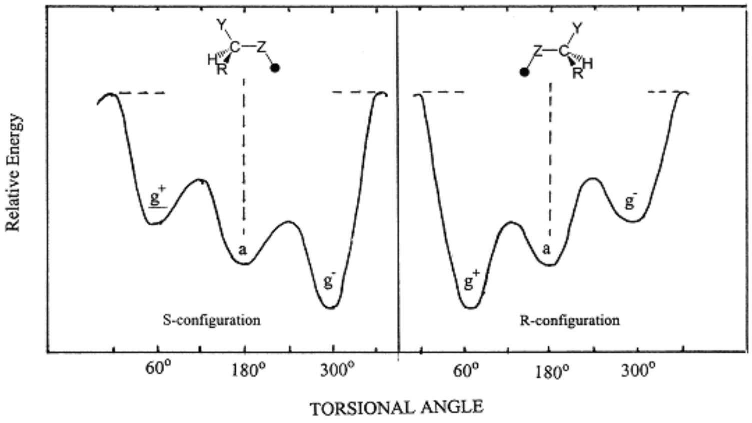


Figure 4. Anticipated mirror image potential energy curves for enantiomers such as the R and S stereoisomers of N-protonated-β-fluoro-β-vinyl-ethylamine [13]. (Y = $–CH_2–NH_3^+$, R = –F, Z = $–CH=CH_2$).

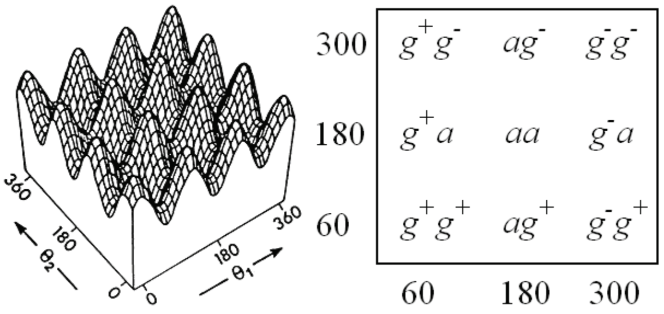


Figure 5. Conformational potential energy surface (PES) of propane corresponding to a double rotor [13]. $E = f(\vartheta_1, \vartheta_2)$ with 9 equivalent (degenerate) minima.

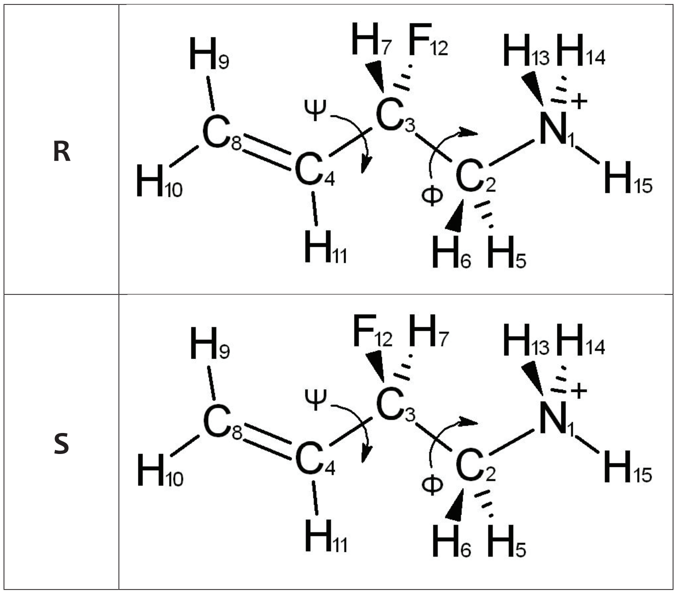


Figure 6. Two enantiomers of N-protonated-β-fluoro-β-vinyl-ethylamine.

With today's hardware and software developments, it is possible to compute molecular energy as a function of dihedral angles for using first principle computational methods.

Scope

The purpose of this conformational study of N-protonated-β-fluoro-β-vinyl-ethylamine was to determine the preferred conformational structures of adrenaline (Fig. 6) by *ab initio* Restricted Hartree-Fock (RHF) computation. Conformational analysis provides information of energy change as a function of a single or several variables:

$E = f(\Phi)$ potential energy curve
$E = f(\Phi, \Psi)$ potential energy surface

The two dihedral angles, Φ and Ψ, are the two principle single bonds about which internal rotation generates distinctly different conformations. This research focused on the variation of Φ and Ψ torsional angles in order to determine the relative stabilities of the various conformers existing on the potential energy surface. In general, the g^+, a, and g^- conformations are considered to be stable conformers.

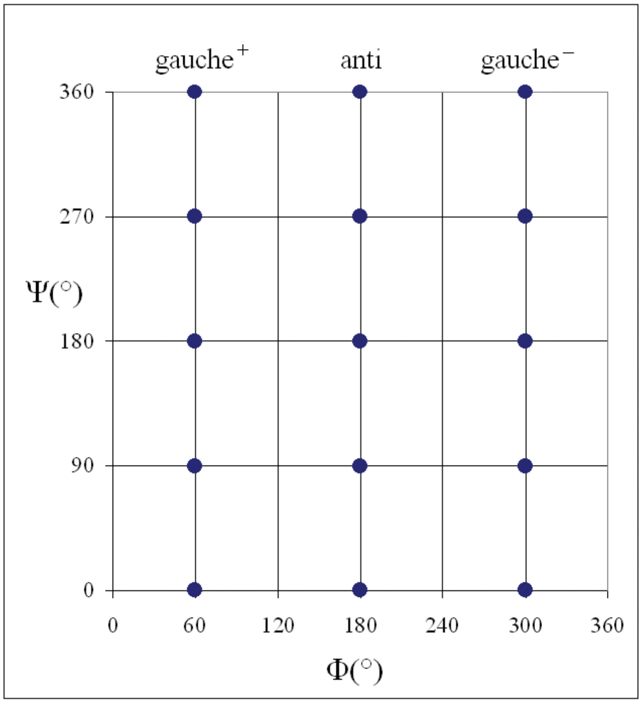


Figure 7. Topological pattern of anticipated conformers of N-protonated-β-fluoro-β-vinyl-ethylamine. The Φ and Ψ axis are the dihedral angles specified in Figure 6.

The dihedral angles (Fig. 6) were defined as follows: $\Phi = N_1C_2\text{-}C_3C_4$ and $\Psi = C_2C_3\text{-}C_4C_8$. To explore the conformational space of the adrenaline model studied, the variation of the following dihedral angles were investigated: Φ equals *gauche*$^+$ (60°), *anti* (180°), and *gauche*$^-$ (-60°), while Ψ could be at *syn* (0°, 180°), *gauche*$^+$ (90°), and *gauche*$^-$ (-90°). The presumed conformers to be optimized are depicted in Figure 7 in a topological pattern. The specific stabilities of the various conformers studied directly result from the characteristics of these angles.

Materials and Methods

All computations were carried out using the Gaussian 03 program package (G03) [14]. Each structure was initially optimized using the *ab initio* restricted Hartree-Fock (RHF) method [15] using the standard split valence 3-21G basis set that is incorporated in G03 [14]. Total energies were given in hartrees, and the relative energies were given in kilocalories per mole (with the conversion factor: 1 hartree = 627.5095 Kcal·mol^{-1}). The energy was obtained using the Schrodinger Equation. The Many-Electron-Schrodinger-Equation was first converted to multiple one-electron equations called *Hartree-Fock* equations. These were solved with the *restriction* that each molecular orbital would accommodate two electrons: one electron with an α-spin and the other electron with a β-spin. The orbital basis for the calculation was 3 Gaussians per atomic shells (K, L). For the K-shell (1s), all three Gaussians were contracted while for the L-shell (2s and 2p), two Gaussians were contracted and one was left loose.

For the full conformational study, optimized geometries and energies were computed from the first principles of quantum mechanics using G03. The conformational potential energy surfaces of N-protonated-β-fluoro-β-vinyl-ethylamine were analyzed in the form of landscapes and were explored by full geometry optimization at the RHF/3-21G level of theory. The relative energies of the different stable conformations of N-protonated-β-fluoro-β-vinyl-ethylamine were then calculated. The Z-matrices associated with the two enantiomeric global minima are described in the supplementary material published online.

Results and Discussion

Due to the considerable size of the adrenaline molecule, a smaller "fragment model" of the protonated adrenaline was studied in order to characterize the major conformational space of the hormone. The smaller model has fewer electrons to accommodate and therefore, fewer molecular orbitals are required for the calculations. Also, as the degrees of freedom are smaller, fewer bond lengths, bond angles and dihedral angles need to be optimized.

In order to gain an understanding on the topology of the potential energy surface of the fragmented adrenaline model, cross-sections corresponding to $\Phi = 60°$, 180°, and 300° were scanned with 30° intervals along the variable Ψ. This was done for both the R and S enantiomers (Fig. 7). The six cross-sections are depicted in Figure 8.

Analysis of the graphical characteristics of the cross-sections revealed that the *anti* conformers ($\Psi = 180°$) are always maxima. Minima, as determined for Ψ were detected between the range of 60° to 120° and the range of 240° to 300°. Consequently, the results suggest that only six conformations exist for the adrenaline fragment (Fig. 9).

The highest and lowest energy conformations of the R and S configurations are shown in Figure 10. Each of the R stereoisomer conformers listed in Table 1 has a unique energy value because the conformers have a diastereomeric relationship. The same observation applies to the six conformers of the S configuration. However, when the energy values of the R configuration were compounded

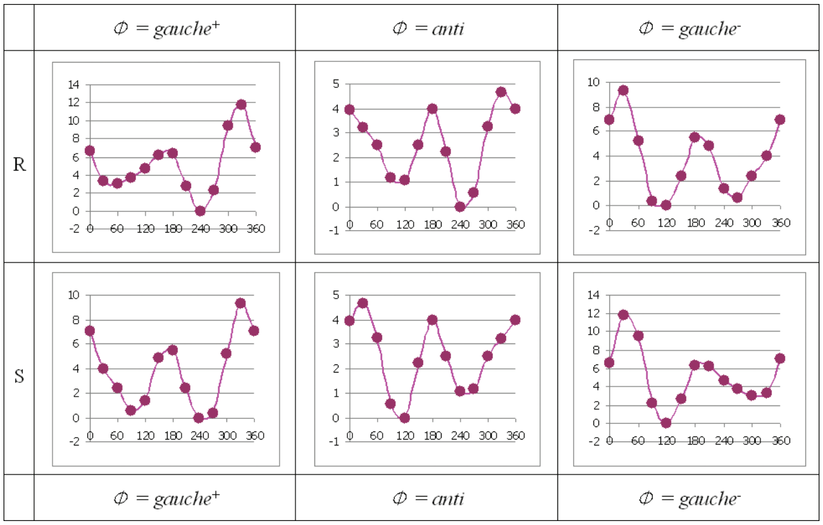


Figure 8 (Left). Relative energy ΔE in Kcal/mol (y-axis in all graphs) versus the dihedral angle Ψ in degrees (x-axis in all graphs). The following cross-sections of the R and S stereoisomers of N-protonated-β-fluoro-β-vinyl-ethylamine are shown: $\Delta E = f(\Psi)$, at $\Phi = 60°$, 180°, and 300°. The points were computed by solving the molecular Schrodinger equation at the RHF/3-21G level of theory.

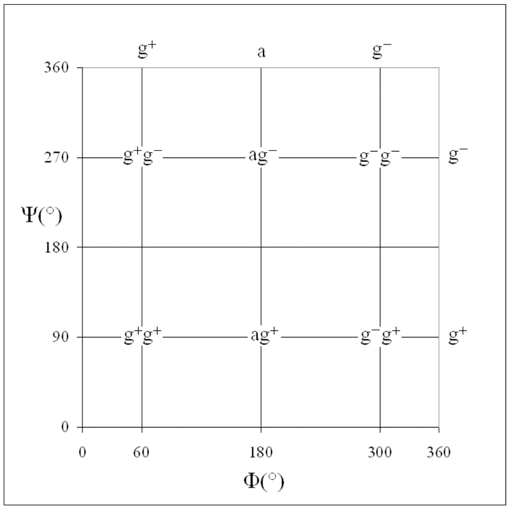


Figure 9 (Above). Topological pattern of the conformers of N-protonated-β-fluoro-β-vinyl-ethylamine to be optimized at RHF/3-21G level of theory.

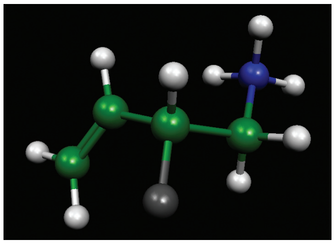
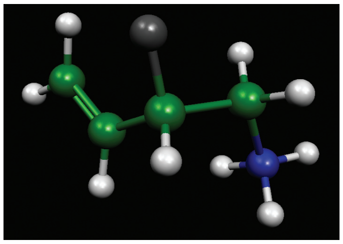
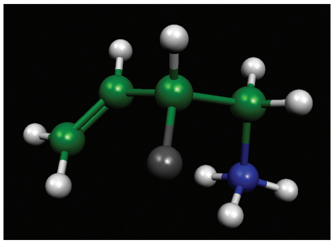
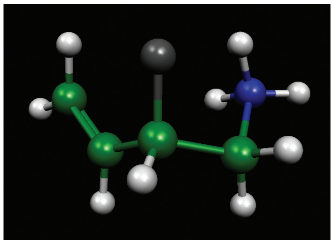
	R **stereoisomer**	***S*** **stereoisomer**
Highest Energy		
	$\Delta E(\Phi,\Psi) = \Delta E(g^+,g^+) = 9.94$ Kcal/mol	$\Delta E(\Phi,\Psi) = \Delta E(g^-,g^-) = 9.94$ Kcal/mol
Lowest Energy		
	$\Delta E(\Phi,\Psi) = \Delta E(g^-,g^+) = 0.00$ Kcal/mol	$\Delta E(\Phi,\Psi) = \Delta E(g^+,g^-) = 0.00$ Kcal/mol

Figure 10 (Left). Highest and lowest energy conformations of the R and S configurations (Note that each row has a pair of enantiomers that are equal in energy).

Table 1. Molecular conformations for N-protonated-β-fluoro-β-vinyl-ethylamine optimized at RHF/3-21G level of theory

	Φ	Ψ	Φ	Ψ	Energy (Hartree)	Rel E. (Kcal/mol)
S	g+	g+	65.06	48.83	-308.657717	2.94
	a	g+	161.71	106.71	-308.656985	3.40
	g-	g+	-59.28	107.05	-308.647870	9.12
	a	g-	166.13	-110	-308.658659	2.35
	g+	g-	67.67	-116.5	-308.662408	0.00
	g-	g-	-52.21	-97.14	-308.646556	9.94
R	g+	g+	52.2	97.13	-308.646556	9.94
	g-	g+	-67.66	116.54	-308.662408	0.00
	a	g+	-166.1	109.95	-308.658658	2.35
	g+	g-	-59.24	-107	-308.647870	9.12
	a	g-	161.7	-106.7	-308.656986	3.40
	g-	g-	-65.22	-49.15	-308.657716	2.94

Table 2. Correlation between relative conformational energy and the shortest F---H-N distance

	Φ	Ψ	Rel E. (Kcal/mol)	Shortest F---H-N Distance (Å)
S	g+	g+	2.94	2.01
	a	g+	3.40	1.95
	g-	g+	9.12	3.87
	a	g-	2.35	2.00
	g+	g-	0.00	2.05
	g-	g-	9.94	3.91
R	g+	g+	9.94	3.91
	g-	g+	0.00	2.05
	a	g+	2.35	2.00
	g+	g-	9.12	3.87
	a	g-	3.40	1.95
	g-	g-	2.94	2.01

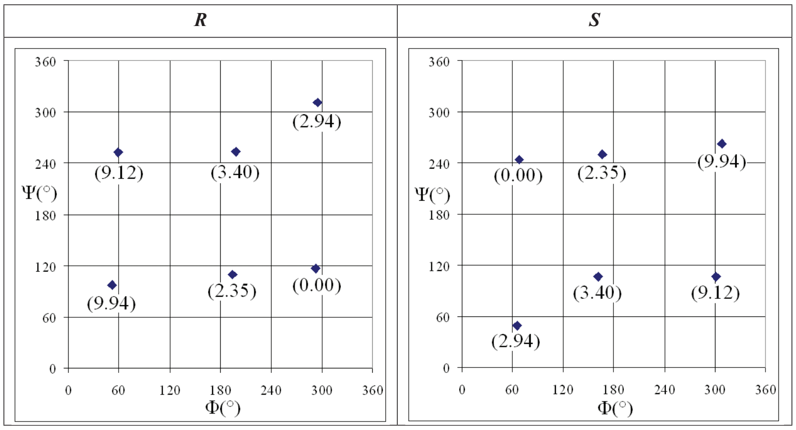


Figure 11. Topological representation of the optimized geometries for R and S stereoisomers of N-protonated-β-fluoro-β-vinyl-ethylamine. Relative energies are given in units of Kcal/mol.

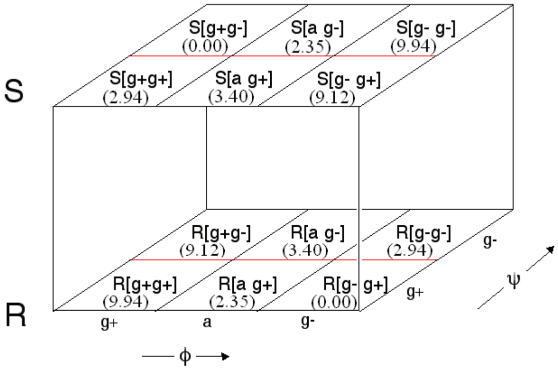


Figure 12. Topological representation of the combination of point and axis chirality for N-protonated-β-fluoro-β-vinyl-ethylamine. The global minimum energy conformers for the two enantiomeric structures are S[g^+g^-] and R[g^-g^+]. The conformer pairs have identical energies (see Table 1). Relative energies are given in units of Kcal/mol.

with the values of the S configuration, a matching pair was found for each case (Fig. 11). These identical relative energy values are listed in the last column of Table 1.

The R and S pairs have enantiomeric relationships. The chirality of adrenaline depends not only on the presence of the stereocenter (point chirality), but also on the clockwise or counterclockwise rotation about Φ and Ψ (axis chirality). Rotation in the plus (P) direction, *gauche*$^+$, and the minus (M) direction, *gauche*$^-$, and the R and S stereoisomerism of the molecule were shown to be equivalent as depicted in {Equation 1}.

(+) i.e. P → S

{Equation 1}

(-) i.e. M → R

When the axis chirality that results from internal rotation and point chirality (R and S) occur together, then the Enantiomeric (E) and Diastereomeric (D) relationships of adrenaline emerge (Fig. 12). As illustrated in Figure 12, conformers that occur at opposite corners of the topological representation of the combined chirality, such as R[g^+g^+] and S[g^-g^-], were shown to have an enantiomeric relationship.

Although the large relative conformational energy values (>9 Kcal/mol) are normal for compounds such as adrenaline, the low energy separations detected (<3.5 Kcal/mol) were found to be a direct result of hydrogen bonding between the –F atom and the –NH_3^+ moiety as shown in Table 2 and Figure 13.

The results generated using the fragment model of adrenaline are expected to closely emulate the characteristics of the entire molecule. Replacement of the fluorine atom (F) by a hydroxyl (OH) in order to more closely model adrenaline would unlikely yield fundamentally different results for the protonated species. The extra rotational freedom gained by making this modification, however, would result in three additional conformations (*g*$^+$, *a*, and *g*$^-$). The additional freedom to rotate about the C-O bond would result in 18 conformers for the R stereoisomer and 18 conformers for the S stereoisomer.

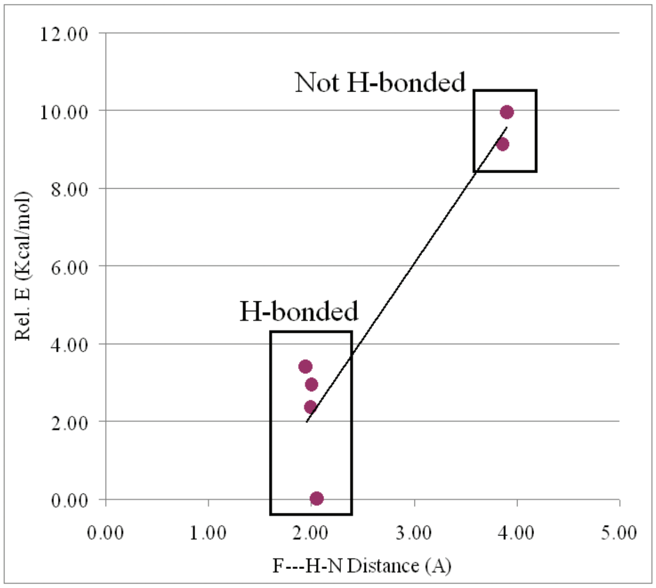


Figure 13. Correlation between relative conformational energy and shortest F---H-N distance.

Conclusion and suggestions for further research

The fragmented molecular structure constructed and analyzed in this study provides invaluable data that will undoubtedly form the basis of future conformational studies of adrenaline. The assessment of both point and axis chirality is important for analyzing the total structural behaviour of any molecule. Clearly, the application of higher level theory such as density functional theory, for example B3LYP/6-31G (d,p), will be very desirable in any future study. Once all the conformers are optimized using the Gaussian 03 Program, it would be possible to carry out an *in silico* experiment to examine the docking of each of the conformers onto the adrenaline receptor. Such a process would be a prerequisite for a drug design project.

Acknowledgements

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JULS

Conformational analysis of ethyl mercaptan as a model of glutathione and its oxidation by peroxide: an exploratory first principle molecular computational study

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Abstract

First principle quantum molecular computations were carried out using Restricted Hartree-Fock method utilizing the 3-21G level of theory (RHF/3-21G) on ethyl mercaptan (Et-SH) and diethyl disulfide (Et-SS-Et) to study their full conformational space. The axis chirality associated with the rotation about the S-S linkage has been related to the potential energy hypersurface of diethyl disulfide. The energetics of the redox reaction Et-SH + H_2O_2 + HS-Et $\rightarrow$ $2H_2O$ + Et-S-S-Et was chosen to be a mimic of the biologically important redox reactions of glutathione (G-SH +H_2O_2 + HS-G $\rightarrow$ $2H_2O$ + G-S-S-G). Using first principle molecular computations, the redox reaction of Et-SH $\rightarrow$ Et-S-S-Et has been found to be exothermic. Depending on the conformers involved, the exothermicity computed at the RHF/3-21G level of theory ranges from -62.0 kCal•mol^{-1} to -64.2 kCal•mol^{-1}.

Keywords: conformational analysis; diethyl disulfide; ethyl mercaptan; glutathione model; redox reaction

Introduction

Oxidative stress

According to the following quotation, it has been known for more than half a century that oxidative stress is dangerous to all aerobic species.

> *"Very few individuals, if any, reach their potential maximum lifespan; they die instead prematurely of a wide variety of diseases – the vast majority being 'free radical' diseases."*
> *Denham Harmon, M.D., Ph.D., who proposed the first free radical theory of aging, 1954* [1]

The explanation for the above is due to the fact that about 5% of the O_2 retained by the body escapes the full reduction process to H_2O (Fig. 1).

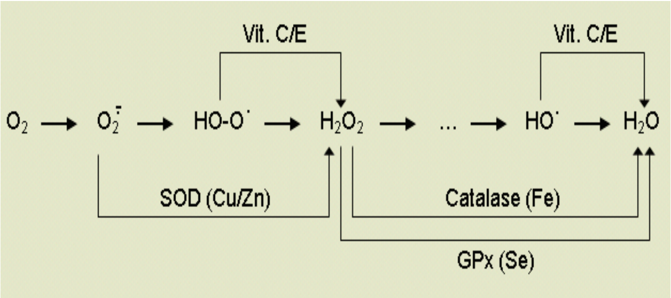


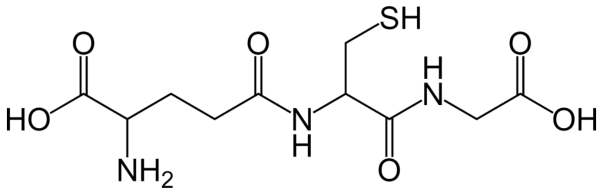
Figure 1. Schematic representation of the enzymatic reduction process of O_2 to H_2O in the mitochondria. This process produces free radicals, such as: •O_2^- (superoxide anion), HO-O• (hydrogen peroxy radical), HO• (hydroxyl radical), as well as H_2O_2 (hydrogen peroxide), collectively referred to as reactive oxygen species (ROS).

Some of the intermediate free radicals, as well as the intermediate H_2O_2, may cause biological tissue damage in a process referred to as oxidative stress. Today, it is believed that oxidative stress is the common cause of over sixty degenerative diseases [2]. Among these, oxidative stress is implicated in the initiation of highly prevalent diseases such as cancer, Alzheimer's disease, and type 2 diabetes [1,3]. Antioxidants, such as Vitamin C and Vitamin E, can trap free radicals such as HO• and HO-O• and prevent these from damaging living cells. Other intermediate reactive oxygen species (ROS) are removed enzymatically as illustrated in Figure 1. One of the enzymes that can remove hydrogen peroxide is glutathione peroxidase in which the selenium atom of the selenocysteine residue, located at the active site of the enzyme, temporarily picks up an OH from H_2O_2 and uses its oxygen to oxidize glutathione (Fig. 2). Thus, as shown by the stoichiometry in Figure 2, glutathione ultimately reduces hydrogen peroxide to water. In all enzymatic reactions, the docking process occurs for a particular conformation favored by the enzymatic active site. For this reason, it is important to know the 3D structures of all possible conformers.

Molecular structure

The tripeptide glutathione (GSH, I) is one of the most abundant (0.1-10mM) intracellular nonprotein thiols [4].

GSH has an unusual amino acid sequence (L-γ-glutamyl-L-cysteinylglycine) [5]. Its structure is unusual as it forms a peptide bond involving a glutamic acid using its side chain COOH moiety rather than the usual carboxylic acid attached to the α-carbon. The molecule consists



I

of two peptide bonds, two carboxylic acid groups, one amino group, and one thiol (-SH) group.

GSH was first isolated from yeast in 1929 [6] but the full synthesis as well as biological reactions came several decades later [7,8]. The most important aspect of this molecule is its thiol functional group [4], which may be oxidized to a disulfide linkage according to {Equation 1}:

G-SH +H_2O_2 + HS-G → $2H_2O$ + G-S-S-G {Equation 1}

This reaction represents the most important biological function of GSH as an antioxidant. GSH is a white, crystalline solid that is highly soluble in water, but is also soluble in dilute ethanol, liquid ammonia and dimethylformamide, due to its many hydrophilic functional groups and low molecular weight of 307.33. GSH has a melting point range of 192-195°C (decomposition) [4]. Its specific rotation is $[\alpha]_D^{25}$-19˚ (c=4.653, H_2O), which is lower than that of the corresponding disulfide [2]. Oxidized GSH (GSSG), most commonly available as a hydrate, has a melting point range of 178-182°C (decomposition) [4] and a molecular weight of 612.63. GSSG is a white, crystalline, water-soluble solid, with a specific rotation of $[\alpha]_D^{25}$-108˚ (c=2, H_2O) [4]. Several quantum mechanical computations have been published on a limited number of glutathione models as well as mimics of the enzyme catalyzed redox reaction [9-11]. However, the disulfide product has not yet been studied.

The final product is also important because the energy change associated with the reaction is the driving force for the reaction to occur. The more exothermic the reaction, the greater the driving force for the reaction to proceed. For the reaction to take place, the catalyst must lower the activation energy of the reaction, however enzymes cannot catalyze a reaction when the process is endothermic.

Biological synthesis

GSH is synthesized intracellularly by two consecutive reactions [12]. The first step is the production of the L-glutamyl-L-cysteine dipeptide, which is catalyzed by the γ-glutamylcysteinyl synthetase {Equation 2}:

L-glutamic acid + L-cysteine + ATP → L-glutamyl-L-cysteine {Equation 2}

The second step involves the formation of the complete GSH molecule catalyzed by GSH synthetase {Equation 3}:

L-glutamyl-L-cysteine + glycine + ATP → GSH {Equation 3}

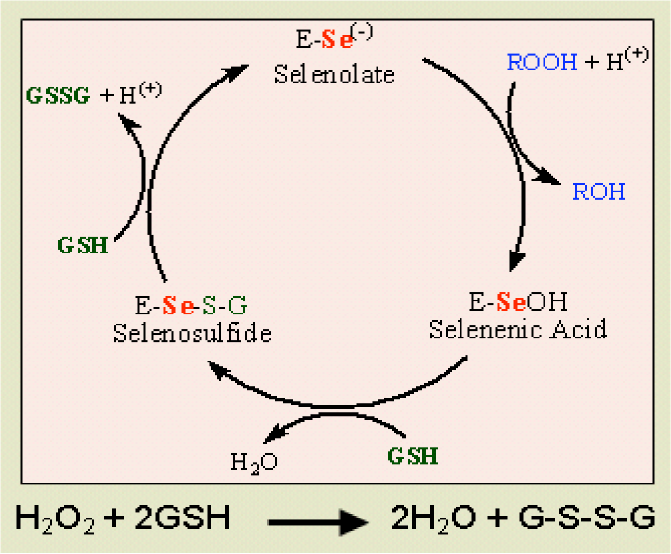


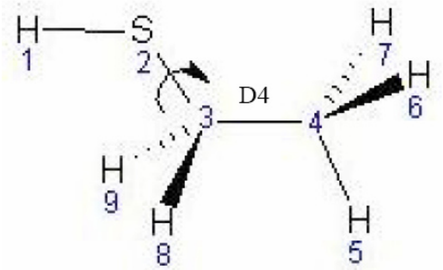
Figure 2. Catalytic cycle of glutathione peroxidase. The oxidative mechanism of GSH to GSSG is shown on the top and the oxidative stoichiometry of the reaction is shown in the lower portion of the figure.

Scope

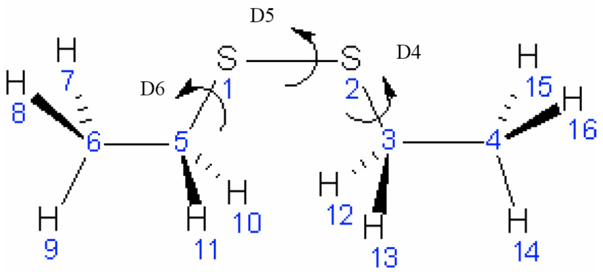
This present study aims to mimic the initial and final states of the GSH to GSSG redox reaction by using ethyl mercaptan (Et-SH) as a model compound as GSH is comparatively large. It is hoped that this model system {Equation 4} will reveal many of the intricacies of the GSH to GSSG redox reaction.

Et-SH + H_2O_2 + HS-Et → $2H_2O$ + Et-S-S-Et {Equation 4}

The atoms in the molecular structures of Et-SH (II) and its oxidized form, Et-S-S-Et (III), were numbered in the following fashion (Fig. 3):



II



III

Figure 3. Atomic numbering system for Et-SH (II) Et-S-S-Et (III) used in the definition of the dihedral angles.

It should be noted that both ethyl mercaptan (Et-SH) and the diethyl disulfide (Et-S-S-Et) are regarded as model compounds for the reduced and oxidized form of glutathione (GSH) as shown in Figure 3. Such simple models may well mimic the bioactive molecules, GSH and GSSG because in the redox reaction, the thiol group is undergoing the greatest amount of chemical change during the oxidation process. The less important of the groups attached to the thiol moiety is clearly illustrated in Figure 4.

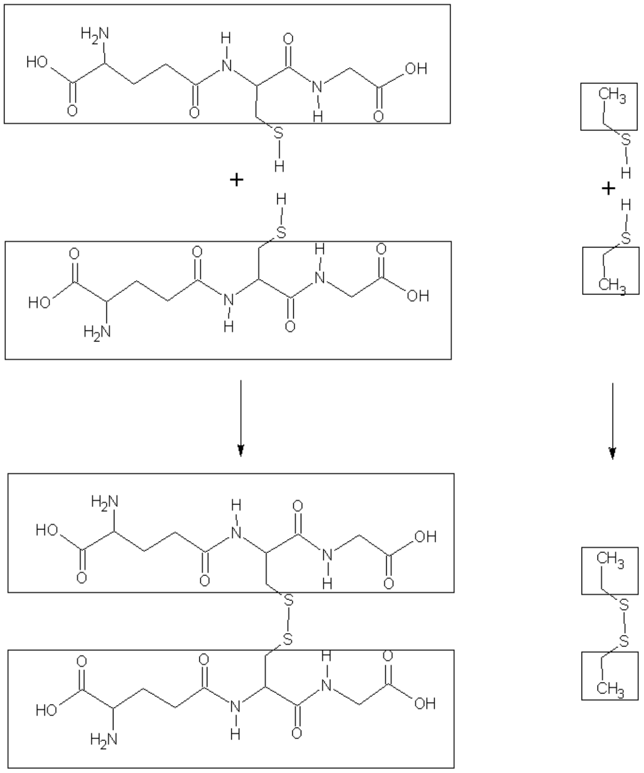


Figure 4. A comparison of glutathione oxidation and its ethyl mercaptan model emphasizing the functional change from SH to SS functionalities during the reaction.

Materials and Methods

All calculations were carried out using the Gaussian 03 (G03) program [13]. The dihedral angles for II were taken as H_1S_2-C_3C_4 (D4) S_3C_3-C_4H_4 (D5) and for III (Fig. 3) as S_1S_2-C_3C_4 (D4), C_3S_2-S_1C_5 (D5), S_2S_1-C_5C_6 (D6) in these preliminary truncated models which were sampled at *gauche*$^+$ (g^+), *anti* (*a*) and *gauche*$^-$ (g^-) to search for all probable conformers at gas phase, zero Kelvin, and in vacuum. The various conformers are illustrated in Figure 5 for the full angular range of -180° to +180° for S_1S_2-C_3C_4 (D6).

The structures were calculated within Hartree-Fock (HF) or *ab initio* molecular orbital (MO) formalism [14] using a standard RHF/3-21G basis set incorporated in G03 [13]. The geometries and self consistent field (SCF) energy values were extracted from the optimized structures.

In the case of Et-SH, the potential energy curve (PEC) of the form {Equation 5} was investigated:

$$\Delta E = f[D4] \qquad \{\text{Equation 5}\}$$

The potential energy hypersurface (PEHS) of Et-S-S-Et can be described by {Equation 6}:

$$\Delta E = f[D4, D5, D6] \qquad \{\text{Equation 6}\}$$

Two-dimensional constructions (i.e. PES) of {Equation 7} were investigated with dihedral angle D5 fixed at either +90° or -90°.

$$\Delta E_{D5} = f[D4, D6] \qquad \{\text{Equation 7}\}$$

Input Z-matrices for Et-SH and Et-S-S-Et, showing the geometrical parameters, can be found in supplementary material available online.

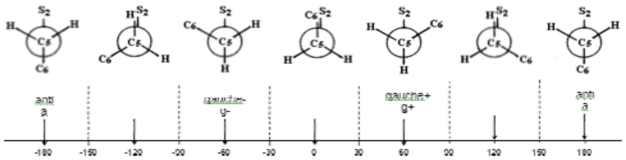


Figure 5. Conformational designations of various conformers in a Newman projection as exemplified by D6 = S_1S_2-C_3C_4.

Results and Discussion

Conformation analysis of ethyl mercaptan

In ethyl mercaptan (Et-SH), the terminal methyl group always occupies the same spatial orientation. Therefore, rotation about only the C-S bond represents an independent variable for conformational change as indicated in {Equation 5}. The variation of energy with dihedral angle D4 associated with the rotation about the C-S bond is shown in Figure 6. The actual variation of the total energies is summarized in Table 1. Total energies are given in hartrees, and relative energies were calculated in kilocalories per mole.

The *gauche* conformation is more stable than the *anti* in contrast to the general understanding of molecular conformation that *anti* arrangements are usually the global minima (Tables 1 and 2, Fig. 6). There are known examples in the literature whereby the *gauche* structure is more stable than *anti*, and this is usually referred to as the *gauche* effect [15,16]. It takes place in the case of carbohydrates where two oxygen atoms (or two different hetero-atoms) are connected to the anomeric carbon (C_1) of hexoses. In such cases, the phenomenon is usually referred to as the anomeric effect. It also happens occasionally for larger hetero-atoms such as sulfur [15,16].

Conformational analysis of diethyl disulfide

In diethyl disulfide (Et-S-S-Et), the terminal methyl groups always occupy the same spatial orientation.

Table 1. Scanned energy values computed at RHF/3-21G level of theory for conformational change involving rotation about the C-S bond (D4) in ethyl mercaptan using the following conversion factor: 1 hartree = 627.5095 kCal•mol^{-1}

D4	Energy (Hartree/Particle)	Relative Energy (kCal/mol)
-30.0°	-474.346465	0.89
0.0°	-474.345252	1.65
30.0°	-474.346464	0.89
60.0°	-474.347884	0.00
90.0°	-474.346991	0.56
120.0°	-474.345759	1.33
150.0°	-474.346440	0.91
180.0°	-474.347254	0.40
210.0°	-474.346440	0.91
240.0°	-474.345759	1.33
270.0°	-474.346991	0.56
300.0°	-474.347884	0.00
330.0°	-474.346465	0.89
360.0°	-474.345252	1.65
390.0°	-474.346464	0.89

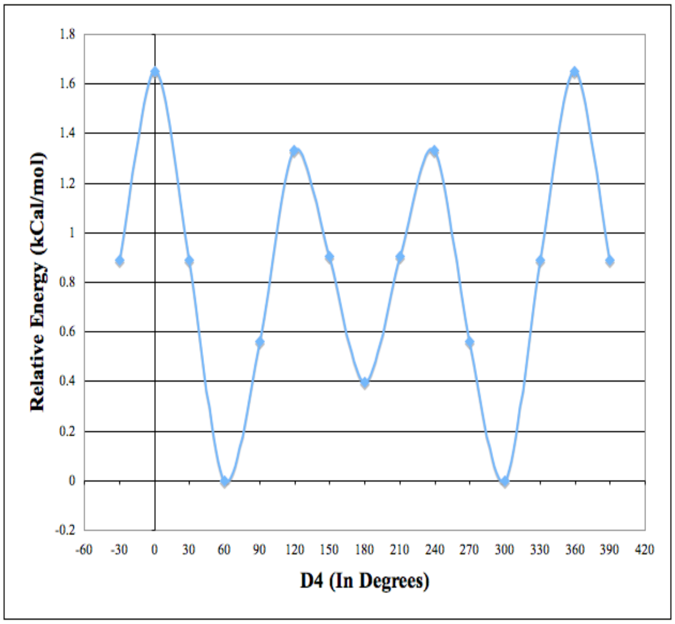


Figure 6. Potential energy curve (PEC) of ethyl mercaptan. The dependant variable is energy difference (kCal•mol^{-1}) relative to the global minimum whereas the independent variable is D4=H_1S_2-C_3C_4. Points plotted were optimized at RHF/3-21G.

Table 2. Minimum energy geometries of ethyl mercaptan optimized at RHF/3-21G level of theory

D4	Optimized D4	Energy SCF (Hartree/Particle)	Relative Energy (kCal/mol)
g^+	63.49°	-474.347903	0.00
a	180.00°	-474.347254	0.41
g^-	-63.51°	-474.347903	0.00

Therefore, only three dihedral angles (D4, D5, D6) are independent variables for conformational changes according to {Equation 6}. The topological arrangement of the potential energy hypersurface, represented by {Equation 6}, is shown in Figure 7.

Since D5 is associated with the rotation about the S-S bond, which usually assumes either +90° or -90° [16], the potential energy hypersurface can be represented with two cross sections corresponding with D5=+90° and D5=-90° (Fig. 7). The optimized structures and their energy values of the 18 (2 × 9) conformers are summarized in Table 3 and also shown graphically in Figure 8. It should be emphasized that the two levels shown in Figures 7 and 8 correspond to nine enantiomeric pairs. This is to be expected since clockwise or counterclockwise rotation represents chiral motion leading to axis chirality, also known as atropisomerism. In this context, axis chirality is also responsible for helical chirality. Thus, the rotation to (+) such as g^+, i.e. P, corresponds to right-handed helicity, while (-) such as g^-, i.e. M, corresponds to left-handed helicity. The P and M classification of helicity can be conveniently related to S and R point chirality, as shown in {Equation 8} [17,18].

$$(+)\text{ i.e. } \boldsymbol{P} \rightarrow \boldsymbol{S} \text{ and } (-)\text{ i.e. } \boldsymbol{M} \rightarrow \boldsymbol{R} \qquad \{\text{Equation 8}\}$$

Figure 9 shows the highest energy conformers $[g^+(g^+)g^-]$ and the lowest energy conformers $[g^+(g^+)g^+]$ of diethyl disulfide. It should be noted that S-S bond length is somewhat longer in the crowded high-energy conformer and such a stretch of the S-S linkage appears to be necessary to reduce the steric repulsion between the two ethyl groups.

Energetics of the redox reaction

Although the present level of theory applied is not expected to give thermodynamic accuracy, the energy change associated with the redox reaction may nevertheless be indicative of the endothermacity or exothermacity of the process. There are a number of ways to calculate the energy changes associated with the reaction. Starting with the global minimum of the reactant and ending with the global minimum may be the most appealing but it will not necessarily yield the most or the least exothermic number. For this reason, calculation starting either with the *gauche* or *anti* conformation of Et-SH and choosing the lowest energy (L) and highest energy (H) conformers of the diethyl disulfide product will yield four different

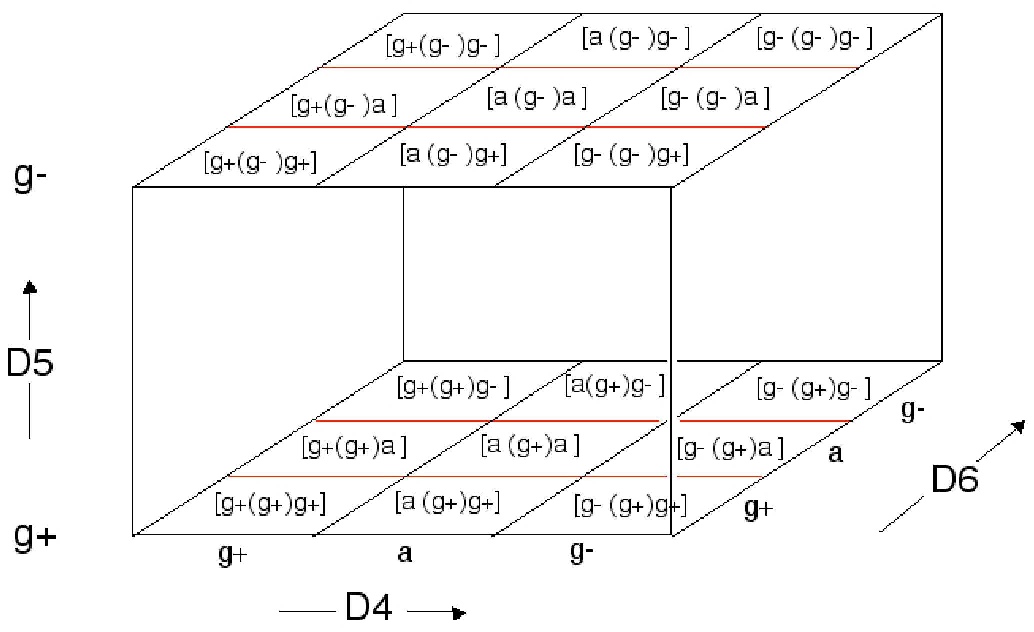
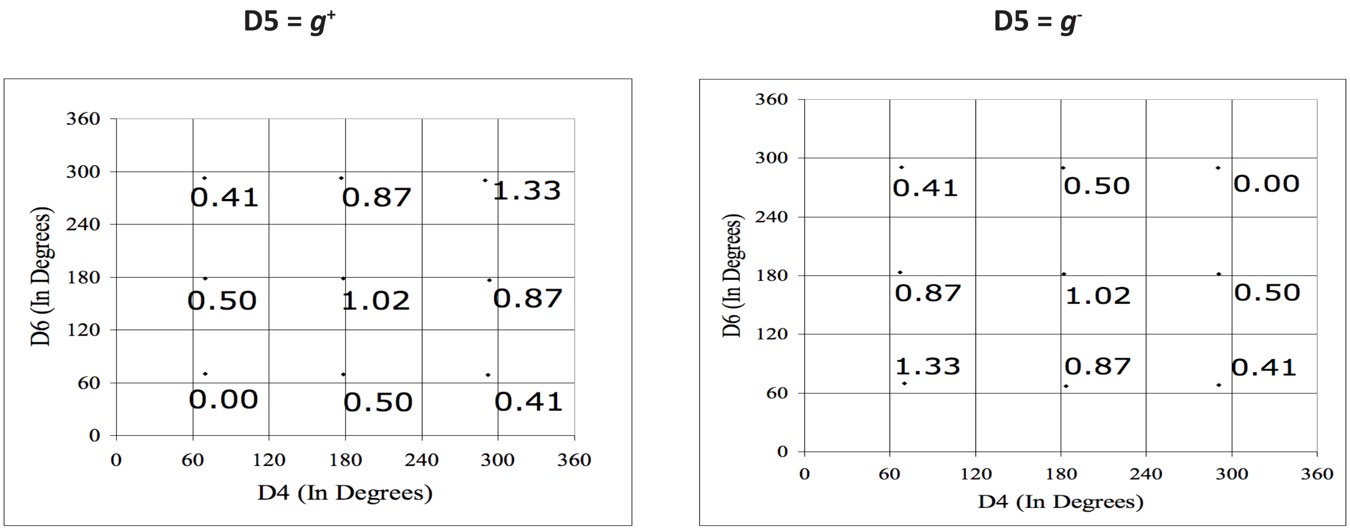


Figure 7. A topological representation of the potential energy hypersurface (PEHS) according to {Equation 6}. Each floor corresponds to a different rotation about the disulfide bond. The conformers of opposite position, such as $[g^+, g^+, g^+]$ and $[g^-, g^-, g^-]$, are in enantiomeric relationship and therefore have identical energies.

Table 3. Minimum energy geometries of diethyl disulfide optimized at RHF/3-21G level of theory. Note that the central conformer measured by D5 is associated with the clockwise (g^+) or counterclockwise (g^-) rotation of the disulfide bond, which is in the vicinity of either +90° or -90°.

Conformers	D4	D5	D6	Energy (Hartree/Particle)	Relative Energy (kCal/mol)
$[g^+(g^+)g^+]$	69.91°	88.17°	69.88°	-947.552076	0.00
$[g^-(g^+)g^+]$	-67.72°	103.71°	69.08°	-947.551418	0.41
$[a(g^+)g^+]$	178.2°	88.97°	69.70°	-947.551277	0.50
$[g^+(g^+)a]$	69.65°	88.95°	178.24°	-947.551277	0.50
$[g^-(g^+)a]$	-67.29°	103.36°	176.49°	-947.55069	0.87
$[a(g^+)a]$	178.11°	89.48°	178.12°	-947.550453	1.02
$[g^+(g^+)g^-]$	69.07°	103.75°	-68.1°	-947.551418	0.41
$[a(g^+)g^-]$	176.32°	103.36°	-67.21°	-947.55069	0.87
$[g^-(g^+)g^-]$	290.0°	121.71°	-70.35°	-947.549959	1.33
$[g^+(g^-)g^+]$	70.06°	-122.17°	69.91°	-947.54996	1.33
$[a(g^-)g^+]$	-176.4°	-103.36°	67.23°	-947.55069	0.87
$[g^-(g^-)g^+]$	-69.07°	-103.57°	67.83°	-947.551418	0.41
$[a(g^-)a]$	-178.12°	-89.47°	-178.11°	-947.550453	1.02
$[g^+(g^-)a]$	67.25°	-103.30°	-176.37°	-947.55069	0.87
$[g^-(g^-)a]$	-69.76°	-88.94°	-178.23°	-947.551277	0.50
$[a(g^-)g^-]$	-178.59°	-88.49°	-69.34°	-947.551276	0.50
$[g^+(g^-)g^-]$	67.96°	-103.56°	-68.99°	-947.551418	0.41
$[g^-(g^-)g^-]$	-69.74°	-87.77°	-69.80°	-947.552075	0.00


D5 = g^+

D5 = g^-

Figure 8. A topological representation of the optimized geometries of diethyl disulfide, dihedral angles D4 and D6 corresponding to the vertical and horizontal axes, respectively. Left panel corresponds to g^+ and right panel corresponds to g^- rotamer of the S-S bond (D5). Relative energies are given in units of kCal•mol^{-1}.

energy values according to the following equations:

$\Delta E_{L \rightarrow L} = \{E_{Et\text{-}S\text{-}S\text{-}Et}[g^+(g^+)g^+] + 2E_{H2O}\} - \{E_{Et\text{-}SH}[g^\pm] + 2E_{H2O2}\} = -63.33917741$ kCal•mol^{-1} {Equation 9a}

$\Delta E_{L \rightarrow H} = \{E_{Et\text{-}S\text{-}S\text{-}Et}[g^-(g^+)g^-] + 2E_{H2O}\} - \{E_{Et\text{-}SH}[g^\pm] + 2E_{H2O2}\} = -62.01073979$ kCal•mol^{-1} {Equation 9b}

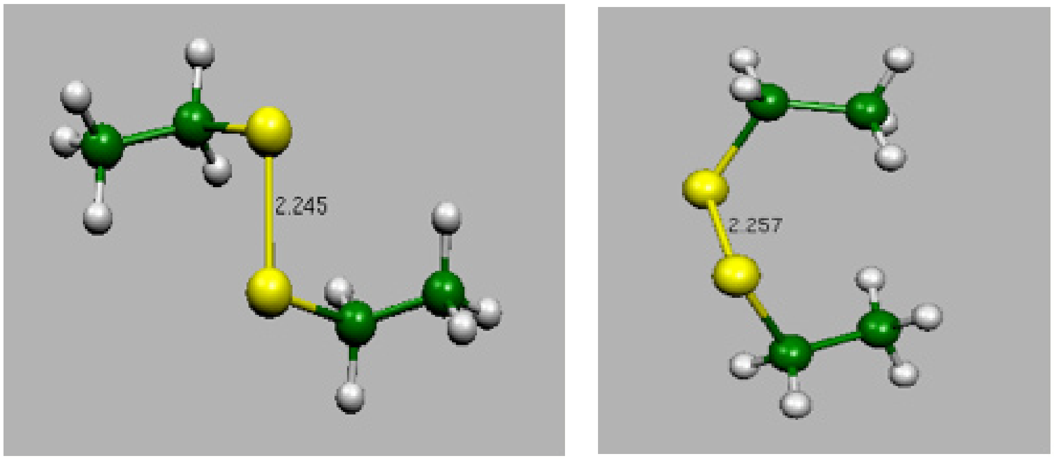
$\Delta E_{H \rightarrow L} = \{E_{Et\text{-}S\text{-}S\text{-}Et}[g^+(g^+)g^+] + 2E_{H2O}\} - \{E_{Et\text{-}SH}[a] + 2E_{H2O2}\} = -64.15368474$ kCal•mol^{-1} {Equation 9c}

$\Delta E_{H \rightarrow H} = \{E_{Et\text{-}S\text{-}S\text{-}Et}[g^-(g^+)g^-] + 2E_{H2O}\} - \{E_{Et\text{-}SH}[a] + 2E_{H2O2}\} = -62.82524712$ kCal•mol^{-1} {Equation 9d}

The total energies of the H_2O and H_2O_2 components are listed in Table 4.

From {Equations 9}, it may be seen that the lowest-to-highest energy transition (L→H) is the smallest negative energy (-62.0 kCal•mol^{-1}) and the highest-to-lowest transition (H→L) is the most exothermic (-64.2 kCal•mol^{-1}) process. The corresponding energy level diagram (Fig. 10) illustrates the various exothermicity values.

In order to determine the empirical inaccuracy of the present molecular computations at this low level of theory, experimental data [19] were used for the sake of comparison. These are summarized for the oxidation of both methyl mercaptan (CH_3-SH) and ethyl mercaptan (CH_3-CH_2-SH) in the gaseous as well as the liquid phases (Table 5). For comparison, the oxidation of Et-SH to Et-SS-Et in the gas phase was used (-334.5 kJ•mol^{-1} corresponding to -80.0 kCal•mol^{-1}). Thus, the present level of molecular quantum theory underestimates the exothermicity in the range from 15.8 kCal•mol^{-1} to 18.0 kCal•mol^{-1} for the various conformers of the reactants and those of the product. This estimate of inaccuracy neglects the small difference between enthalpy and quantum chemical energy, which is approximately RT≈0.6 kCal•mol^{-1}.



$[g^+(g^+)g^+]$

$[g^+(g^-)g^+]$

Figure 9. A stereospecific representation of the lowest and highest energy conformers of Et-SS-Et optimized at RHF/3-21G level of theory. The left-hand structure is the lowest energy (0.0 kCal•mol^{-1}) and the right-hand structure is the highest energy conformer (1.33 kCal•mol^{-1}) involving rotation from g^+ to g^- about D6. The numbers along the S-S bond refer to their respective bond lengths in Angstrom units.

Table 4. Energies of H_2O and H_2O_2 optimized at the RHF/3-21G level of theory

Molecule	Energy SCF (Hartree/particle)
H_2O	-75.5859597
H_2O_2	-149.927252

Table 5. Enthalpies of oxidations for selected mercaptans calculated from experimental enthalpies of formation[a]

Enthalpies of formations (kJ•mol^{-1})[b]	Gas	Liq.
H_2O	-241.8	-285.8
H_2O_2	-136.3	-187.8
CH_3SH	-22.9	-46.7
C_2H_5SH	-46.1	-73.6
CH_3SSCH_3	-24.7	-62.6
$C_2H_5SSC_2H_5$	-79.4	-120.1
Reactions:		
2 Me-SH + H_2O_2 → $2H_2O$ + Me-S-S-Me	-326.2	-353.0
2 Et-SH + H_2O_2 → $2H_2O$ + Et-S-S-Et	-334.5	-356.7

[a] Reference [19]

[b] 1 kCal/mol = 4.184 kJ•mol^{-1}

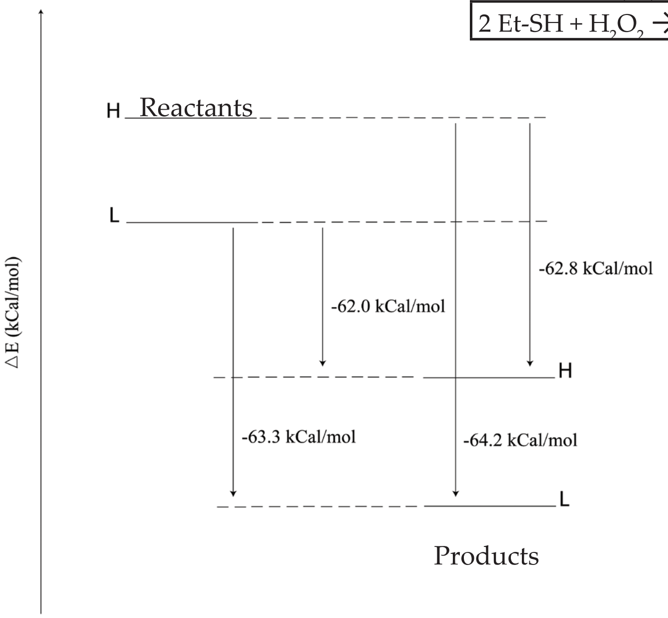


Figure 10. Ranges of exothermicity values for the redox reaction of ethyl mercaptan and diethyl disulfide. "H" represents the highest and "L" represents the lowest energy conformers. The energy values shown are for the L→L, L→H, H→L, and H→H transitions.

kCal•mol^{-1} and -64.2 kCal•mol^{-1} are more uncertain. The method used in this exploratory study was sufficiently indicative of the process, however higher level of theory using den*** calculations (density functional calculations) would be more desirable in the future. It is hoped that such simple models may be useful in mimicking complex bioactive molecules, such as GSH and GS-SG, since the thiol group undergoes the greatest amount of chemical change during oxidation. However, carrying out calculations on the oxidized form of glutathione (GSSG) would involve forty heavy atoms instead of the six heavy atoms present in Et-S-S-Et. Also, instead of two dihedral angles, as studied in this small model using Et-SH and Et-SS-Et, twenty dihedrals instead of six would have to undergo optimization.

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Supplementary material is available online at the JULS website: http://juls.sa.utoronto.ca/

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A preliminary investigation of the effects of emotional stimuli on 4-year-old children’s abstraction and cognitive flexibility on the flexible item selection task (FIST)

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Abstract

Affective decision-making increases dramatically in preschool years, but relatively little is known about the influence of emotion on young children’s executive function. This study examined the effects of emotion on four-year-olds’ rule abstraction and cognitive flexibility using the Flexible Item Selection Task (FIST). The FIST is a preschooler age-appropriate test of executive function requiring cards to be matched on certain dimensions: selection one performance is conceptualized as abstraction, while selection two reflects cognitive flexibility. In this study, four-year-olds were given a standard version of the FIST, and a modified version with happy, sad, angry or neutral faces in the background. Results showed that abstraction and cognitive flexibility performance was significantly better on the happy face version compared to the neutral face version. Furthermore, facilitative effects of positive faces on abstraction carried over to the standard version of the FIST, which was presented later. This suggests an affective learning effect that lasts beyond the original task. Results also showed that cognitive flexibility performance on the standard version was significantly better than in the neutral and angry versions. However, the relative improvement of cognitive flexibility in the standard version was not seen in comparison to the happy face version. This suggests that positive emotions mitigated the relative difficulties inherent to switching between rules in the face version. Results are consistent with the suggestion that positive stimuli promote attention and cognitive flexibility, possibly by increasing dopamine levels in the anterior cingulate cortex and the orbitofrontal cortex.

Keywords: abstraction; cognitive flexibility; emotion; flexible item selection task; “hot” executive function

Introduction

‘Hot’ executive function

Executive function (EF) refers to the cognitive control processes involved in conscious, goal-directed problem solving. Rule abstraction and flexibility in switching between rules are two such processes. Although EF is generally perceived as a domain-general functional construct, the distinction can be made between the purely cognitive “cool” aspects typically associated with the lateral prefrontal cortex (LPFC), and the relatively “hot” affective aspects of EF associated with the medial (reward) and lateral (punishment) areas of the orbitofrontal cortex (OFC) [1-3]. Traditionally, research on the early development of EF in children has focused on cool EF [4]. However, unlike many behavioural laboratory measures of cool EF (e.g., Dimensional Change Card Sort (DCCS)), most problems children face in real life likely elicit emotion, thus involving neural pathways between the emotional centers (i.e. limbic systems) and affective cognition areas (i.e. OFC) [5]. Since affective decision-making increases dramatically in the preschool years (i.e. four- to six-year-olds) [6], and relatively little is known about the influence of affect on young children’s EF and the cognito-affective mechanisms underlying hot EF, researchers are increasingly studying emotionally-significant hot executive function [7, 8].

Flexible Item Selection Task

Jacques and Zelazo developed a new behavioural paradigm to study rule abstraction and cognitive flexibility in isolation of other aspects of developing EF: the Flexible Item Selection Task (FIST) [9]. The FIST involves showing preschool children a set of three cards and asking them to first select a pair of cards that match each other on one dimension (selection one), and then selecting a different pair of cards that match on another dimension (selection two). Selection one responses primarily measure children’s ability to identify how two non-identical items might match on a particular dimension (i.e. a measure of *abstraction*) [10]. In contrast, assuming good performance on selection one, selection two responses serve as an indicator of children’s ability to switch between dimensions (i.e. a measure of *cognitive flexibility*).

Jacques and Zelazo found that three-year-olds had greater difficulty with selection one compared to four- and five-year-old children [9, 10]. The relatively poor performance of three-year-olds on suggests that they had difficulty correctly detecting a dimension that was common to two items (i.e. abstraction) when these two items were not identical. Meanwhile, four-year olds had difficulty using a second dimension to sort the cards in selection two. Instead, four-year-olds tend to continue using the card-sorting rules they applied in the first dimension. In other words, the requirement that they flexibly select two cards according to two different dimensions rendered the task difficult for them. However, by age five these students are performing at near-ceiling levels. The developing cognitive mechanism underlying this significant behavioural improvement during ages four and five is conceptualized as cognitive flexibility. Smidts and colleagues found corresponding age-related changes with the *Object Classification Task for Children* [11].

Modified FIST

The current study examined the effects of emotion on abstraction and cognitive flexibility performance in four-year-olds using a modified version of the FIST. The paradigm was a modification of the FIST, whereby the card stimuli depicted standardized faces with a consistent background emotion: happy, sad, angry or neutral. Each child was tested on a specific emotional face version of the FIST as well as a standard version of the FIST. Facial stimuli are ideal for assessing the influence of affect on DCCS performance for two reasons. First, facial stimuli vary along multiple dimensions, such as age and gender, which preschoolers can easily recognize, discriminate, and categorize [12]. Secondly, facial stimuli directly convey emotional contents such as happiness and sadness, and this emotional content is perceived by young children [13].

Emotion

Past work has suggested that emotions influence preschooler abstraction and cognitive flexibility. Wild and colleagues found that the brief presentation of happy or sad faces can induce positive and negative feelings, respectively [14]. Studies have shown that both adults and children in a positive mood are more likely to generate broader categories [15], show more creativity [16], and perseverate less [17]. This line of research suggests that the induction of a positive mood state can increase creative problem solving skills and spontaneous cognitive flexibility, [18-19]. Previous research has also shown that a positive mood can increase cognitive flexibility in school-aged children and preschoolers [15, 20], and produce improvements in tasks such as class-inclusion and Block Design [21, 22].

Several models have attempted to explain the influence of affect on cognition in terms of neurophysiology [23, 24], with particular attention given to the close association of EF with prefrontal cortex function. Ashby and colleagues proposed that mildly positive moods lead to phasic increases in dopamine in the ventral tegmental area and substantia nigra – areas that have projections into the striatum and PFC (including the anterior cingulated cortex) [18]. Therefore, mildly positive mood may facilitate functions dependent on these brain regions, such as rule selection, rule use and cognitive flexibility [18, 21, 22, 25, 27].

However, research with adults suggests that the effects of emotion on EF are complex, as positive affect may also lead to detrimental consequences on different aspects of EF. For example, positive mood may increase distractibility [17]. Phillips and colleagues further demonstrated that induced positive moods can impair performance on executive functioning tasks involving forced switching between response patterns [29]. Recently, Deveney and Deldin found that more perseverative errors were made during a positively-valenced version of the Wisconsin Card Sorting Task (WCST), indicating that adults were less flexible when presented with positive stimuli [30].

Studies on the cognitive effects of negative mood have shown that such emotions induce decreases in divergent thinking and category fluency, as well as generation of fewer novel associations [16]. Furthermore, dysphoric undergraduates and individuals diagnosed with major depressive disorder exhibit impairment on the WCST, suggesting decreased cognitive flexibility [15, 31]. Siegle and colleagues found that unhappy individuals processing negative stimuli give less attention to the non-emotional aspects of these stimuli [32]. Therefore, the poor performances on cognitive flexibility can be attributed to a failure to attend to aspects of the stimuli that are essential for good task performance. However, Qu and Zelazo found no significant effect of sad faces on preschoolers' performance on the DCCS [20]. Recent findings also demonstrated that faces with threat-related values such as anger tended to attract attention more than neutral faces [33].

Current study

Taken together, the previous literature suggests that different emotions will have different effects on cognitive performance on the FIST. It remains unclear whether emotional stimuli will influence performance on cognitive flexibility tasks in preschool children as young as four, and no studies involving the FIST have evaluated whether the emotional nature of stimuli affects the ability of individuals to abstract and switch between perceptual properties of the stimuli. The current study assesses the impact of emotional stimuli on FIST performance in four-year-old preschool children. Specifically, this preliminary study addresses whether varying the emotional nature of the stimuli impacts performance on abstraction and cognitive flexibility. To this end, each participant completed a modified version of the FIST that included faces with a specific emotion (e.g. happy, sad, angry or neutral) as well as a standard non-emotional version. The following hypotheses were made: (1) children exposed to the happy face version of the FIST will show increased abstraction and cognitive flexibility performance compared to children in the neutral face version; (2) children exposed to the sad face version of the FIST will show decreased cognitive flexibility performance compared to children in the neutral face version. No explicit hypotheses were made regarding the angry version of the FIST.

Materials and Methods

Participants

The sample consisted of 50 four-year-olds recruited from 11 local daycare centers and the Child Study Centre telephone database. Their ages ranged from 46 months to 59 months, with an average age of 53.44 months. All children received the standard version of the FIST and a face version with a particular background emotion (i.e. neutral, happy, sad, or angry). The order of version presentation (i.e. the standard and the face version) was counterbalanced. In total, there were eight conditions (i.e. standard to neutral, neutral to standard, standard to happy, happy to standard, standard to sad, sad to standard, standard to angry, and angry to standard). The variance in age and receptive language skills was controlled for in each condition. The experimental design and methods were approved by the Department of Psychology Ethics Review Committee, in accordance with the policies of the University of Toronto Ethics Review Committee.

Materials

Eight cards were used in each of the five versions: a standard version (i.e. boats and cars), a neutral-emotion face version (i.e. calm expression), a happy face version, a sad face version and an angry face version. Within the version's background attribute (i.e. vehicles or faces of a particular emotion), each card depicted a stimulus derived from a combination of three dimensions. Each dimension was represented by one of two cues. The dimensions were identity, colour and size. In the standard version, identity was represented by different vehicles: car and boat; colour was represented by red or blue shading on the upper portion of the vehicle; and size was represented by small or big (3 times the size of small). In the four face versions, identity was represented by male or female adults (i.e. 'boys and girls'); colour was represented by red or blue hair; and size was represented by small or big (3 times the size of small). The same male and female identity from the MacBrain Face Stimulus Set was used in all face versions, although the emotion varied accordingly across conditions. Overall, each condition had eight cards varying on three dimensions (Supp. Table 1). Figure 1 illustrates sample FIST card (while samples of the other cards are shown in Supp. Fig. 1A-1D).

Item identification task

All of the children received an item identification task to determine whether they could correctly identify all attributes of each dimension used in the FIST. Before presentation of the first version of the FIST, children were presented with a sheet depicting both attributes of one dimension (i.e. colour, size, face identity or vehicle identity; see Fig. 2). With each sheet, the experimenter asked the children to identify each attribute and provide feedback (e.g. "Show me red. That's right / Good try, but I think this one is red. What do you think?"). If a child misidentified an attribute, the experimenter asked them to re-identify the attribute, after they had identified the other remaining one. After both attributes of a given dimension were identified correctly, the experimenter mentioned the dimension term (e.g. "You really know your *colours!*").

FIST: demonstration, practice and test trials

Each child then received two different versions of the FIST: the standard version and a face version with a specific background emotion (i.e. neutral, happy, sad or angry). At least twelve children received each face version with a specific

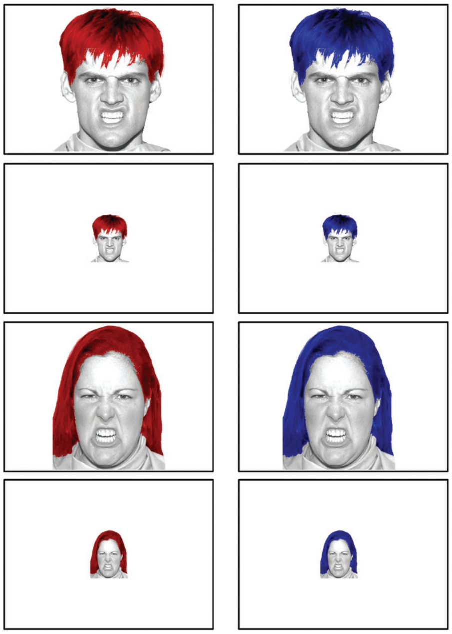

Figure 1. Angry version of FIST.

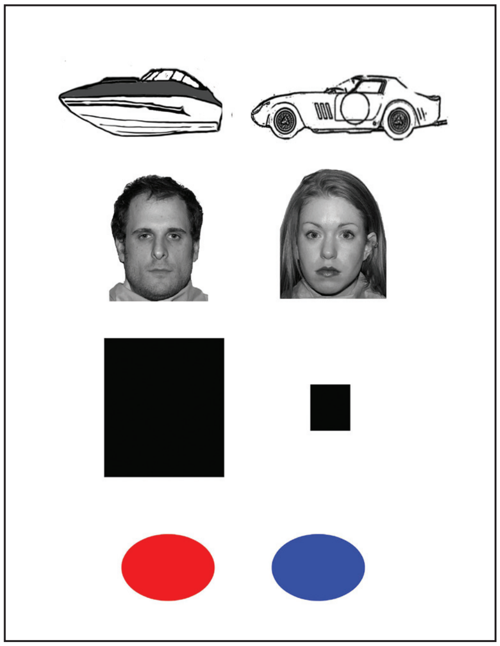

Figure 2. Item identification task. Each of the four rows was a different sheet, with each sheet depicting both attributes of each dimension. For example, row sheet 3 illustrates the big and small attributes of the size dimension.

background emotion, with half of them receiving the standard version first and the other half receiving the face version first. Each version of FIST consisted of one demonstration trial, two practice trials and twelve test trials. In each trial, two of the three cards matched on one of the relevant dimension (e.g. colour) and a different pair of cards matched on the other relevant dimension (e.g. identity) (Supp. Fig. 3). Thus, the pivotal card matched one card on the first relevant dimension and the other card on the second relevant dimension.

The experimenter introduced the FIST as a matching card game, and began by presenting a demonstration trial and two practice trials on the first version. These three trials were repeated at the beginning of the second version, with the purpose of ensuring that each relevant dimension was demonstrated to or practised by the child twice (Supp. Fig. 4). Afterwards, the children received two practice trials. These trials are presented in similar fashion as the demonstration trials except that children were asked to make each selection themselves. The experimenter provided corrective feedback (without using any dimensional or cue labels), and as in the demonstration trial, summarized both selections before proceeding to the next trial (i.e. "So you chose two pictures that go together in one way, and two others that go together in another way. Good job! Now you know how to play, so let's play the game for real now!").

After the demonstration and practice trials, each child received the twelve test trials using each specific version's stimuli set. Across versions, the test trials were identical in terms of the order of presentation, relevant-dimension pairs and test card placement (Supp. Fig. 5). Within each block of three trials, each of the three relevant dimension pairs (i.e. colour and identity, size and colour, size and identity) was presented once in a random order. Test card placements were also counterbalanced. In addition, relevant dimensions (e.g., cards matching on colour) were crossed and counterbalanced with window placements.

After receiving the first version of 15 trials, there was a three minute break. Then the child received the second version of FIST consisting of one demonstration trial, two practice trials and 12 test trials. Thus, each child received a total number of 30 trials.

PPVT

After administration of both versions of the FIST and the emotional rating scale, the experimenter gave children the Peabody Picture Vocabulary Test-Third Edition (PPVT-III), a standardized test that provides a rough estimate of receptive language skills and administered it in the standardized manner. Briefly, children were shown a picture book. Four items appeared on each page (e.g., a rope, a zipper, a rake, and a wheel), and children were asked to identify a predetermined item on each page (e.g., "Show me rope"). The task was administered until children failed to identify eight items in a set, and a raw score was then calculated on the basis of the number of items that they identified correctly.

Statistical analyses

FIST performance scores were coded in three ways. The first method used a raw score, in which a correct response was given 1 point and an incorrect response (e.g., wrong pair, same pair, all cards, or no cards) was given 0 points. Thus, the selection one and selection two scores were each scored out of 12, and the total score for each version (i.e. face versions or standard version) was out of 24. The second method used a percentage score. For selection one scores, the raw score divided by 12 (i.e. maximum score possible) to give a percentage directly equivalent to the raw score. Selection two scores were based only on trials with a correct selection one; the percentage score was calculated with the number of trials with both selections correct as a fraction of the number of trials with at least selection one correct. The rationale behind this scoring method was that a child who chose a wrong pair in selection one and then a correct pair in selection two was judged not to have correctly switched from one sorting dimension to another. The final method used sequential analyses: each trial was coded into either both selections correct, only selection one correct, only selection one correct, and neither selections correct. All statistics mentioned below used method two unless stated otherwise.

Paired sample *t*-tests were used to compare performance according to order of presentation (performance on version one compared to performance on version two). Pearson's correlation was used to determine the correlation between age and performance on the FIST. To determine differences in performance according to condition, independent samples *t*-tests assuming equal variance were used. All probabilities listed are two-tailed.

Results

Fatigue

A paired sample *t*-test showed a performance decrease on the second FIST presented compared to the first based on selection one raw scores, selection two raw scores and total raw scores (Fig. 3). Analysis showed an average decrease of 1.48 on a total score of 24 ($p > 0.01$), an average decrease of 0.78 out of 12 on selection 1 scores ($p > 0.01$), and an average decrease of 0.70 out of 12 on selection 2 scores ($p = 0.07$).

Correlation of age to flexibility performance

The results show that age, within the range of 48 months to 60 months (i.e. four-year-olds), was predictive of selection two performance, but not selection one performance. It was found that older children had higher selection two scores on the face versions ($p = 0.02$) and also higher selection two scores on the second version (according to order of presentation; $p = 0.04$), while younger children had more frequent trials with only selection one correct on both face ($p = 0.02$) and standard versions ($p = 0.03$).

Improved FIST performance on the happy to standard condition

Participants performed better on the happy face version of the FIST compared to the neutral face version, but only in the conditions where the face version was

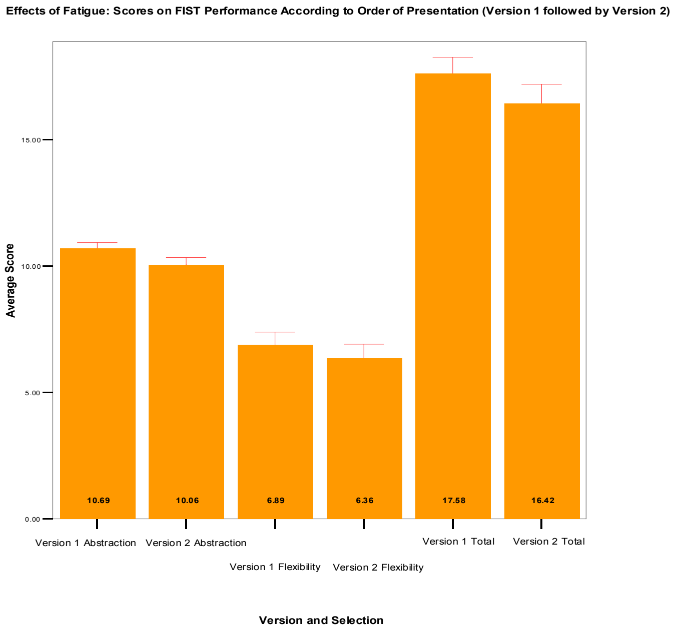

Figure 3. Scores on FIST performance according to order of presentation (version 1 followed by version 2) illustrating effects of fatigue. A paired sample t-test showed a performance decrease on the second FIST version presented compared to the first version based on selection 1 raw scores, selection 2 raw scores and total raw scores.

presented before the standard version. There was no significant difference in performance between the happy face version and the neutral face version if the standard versions were presented before the face version. There were six participants in the neutral to standard condition and six participants in the happy to standard condition. The average age of the neutral to standard condition was 54.33 (±2.87) months and the average age of the happy to standard condition was 54.50 (±2.17) months (no significant difference). Similarly, there were no significant differences in receptive language ability between the groups, although the neutral to standard condition scored slightly higher on the PPVT with an average score of 72.33 (±7.99) while the happy to standard condition scored only 65.50 (±16.33) on the PPVT.

An independent samples *t*-test with two-tailed significance assuming equal variance showed that participants in the happy to standard condition performed significantly better than participants in the neutral to standard condition on selection 1 of the face version ($p < 0.01$) and selection 2 of the face version ($p = 0.03$). This result was also significant when the data was analyzed using different score coding methods: participants in the face-first conditions scored an average of 1.66 (raw score out of 12) higher on selection 1 of the happy face version compared to the neutral face version ($p < 0.01$), and 3.5 (raw score out of 12, affected by score on selection 1) higher on selection 2 of the happy face version compared to the neutral face version ($p < 0.01$; Fig. 4).

A comparative analysis of the standard FIST version performance between the neutral to standard condition and the happy to standard condition shows that the positive emotions of the happy face version had lasting effects on abstraction beyond the original task. In these conditions, the standard FIST version was presented after 3 demonstration and 12 test trials of the face version (i.e. neutral face or happy face) and a 3 minute break. An independent samples *t*-test with two-tailed significance assuming equal variance showed that participants in the happy to standard condition performed significantly better than participants in the neutral to standard condition on selection 1 of the *standard* version ($p = 0.05$; see graph 9). Thus, the improvement in abstraction performance shown by participants in the happy group transferred to the subsequent standard version. When playing the standard version after different face versions, children who were first tested on the happy face version did significantly better on abstraction than those who were first tested on the neutral face version ($p = 0.05$). However, children in the happy to standard condition did not perform significantly better on selection 2 of the standard version compared to the children in the neutral to standard condition ($p = 0.18$), even when analysis was based on raw scores ($p = 0.10$).

In the opposite counterbalance conditions whereby the participants were first exposed to the standard version before the face version, FIST performance on the happy version (of the standard to happy condition) was not significantly better than FIST performance on the neutral version (of the standard to neutral condition). The participants in these conditions had no significant difference in age and PPVT score.

Performance on the condition showing the happy version first was also compared to face versions other than the neutral version. Using independent-sample *t*-tests with two-tailed significance, participants in the happy to standard condition performed better than participants in the sad to standard condition on selection one of the face version ($p = 0.06$). In addition, participants in the happy version scored on average 2.2 (or 13%) higher on selection two compared to participants in the sad version, although this difference was insignificant due to the relative large variance in selection two scores of the sad to standard condition. Participants in the happy to standard condition also performed significantly better than participants in the angry to standard condition on selection two of the face version ($p = 0.01$) and marginally better on selection 1 of the face version ($p = 0.08$).

Significant differences in cognitive flexibility in the standard version vs. face versions

FIST performance was also analyzed by looking at only the first version presented in each condition. Under this restriction, independent samples *t*-test showed that participants given the standard version had significantly higher scores in selection two compared to participants given the neutral version (*p* = 0.01) and the angry version (*p* = 0.04). In addition, children in the standard version made on average of 1.7 (or 14%) more successful trial switches than children in the sad version, but the difference was insignificant due to the high variance in selection 2 scores of the sad to standard condition (Fig. 5). On the other hand, children in the happy version showed no significant differences in selection 2 scores when compared to children in the standard version on (*p* = 0.94). There were no significant differences in age or PPVT score across the conditions. In fact, the average age of the participants given the standard version first, instead of the face versions, was the lowest. Finally, when analysis was based only on the second versions presented, no significant differences were found, presumably due to high variances (Supp. Fig. 7).

Lack of differences between sad, neutral and angry face versions

An independent-sample *t*-test examining performance between the neutral, sad and angry versions found no significant differences in either selection one or selection two scores.

Within-group: no differences between standard and face version

Paired-sample *t*-tests show that there were no significant differences between the face versions to the standard version within any group.

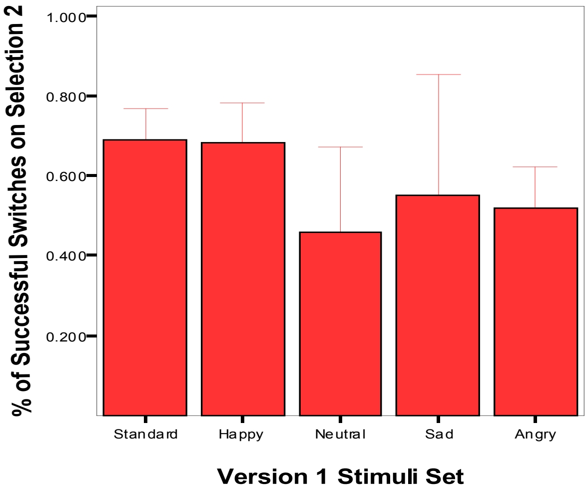

Figure 5. Differences in selection 2 performance based on first version of FIST presented. Participants given the standard version had significantly higher scores in selection 2 compared to participants given the neutral version (p = 0.01) and the angry version (p = 0.04). Children in the standard version made on average of 1.7 (or 14%) more successful trial switches than children in the sad version, but the difference was insignificant due to the high variance in selection 2 scores of the sad to standard condition. Children on the happy version showed no significant differences in selection 2 scores when compared to children in the standard version on (p = 0.94).

Discussion

General findings regarding the FIST

Our results show that age, within the range of 48 months to 60 months (i.e. four-year-olds), was predictive of selection two performance, but not selection one performance. This is consistent with Jacques and Zelazo's finding that four-year-olds perform at near-ceiling levels on abstraction, but show age-related difficulties with cognitive flexibility [9]. The findings also indicate declining performance on the second FIST version presented compared to the first FIST version. This replicates Jacques and

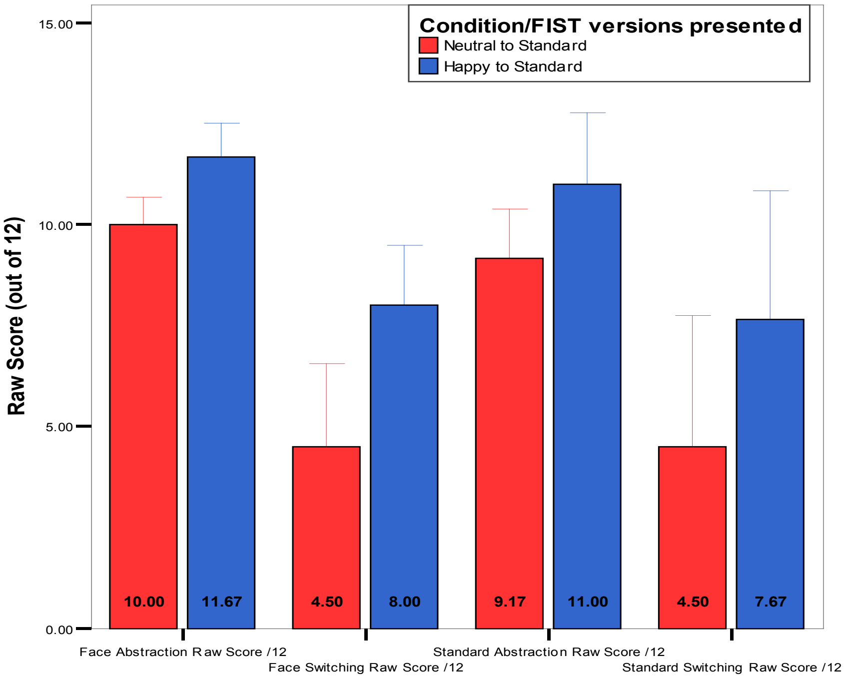

Figure 4. Comparison of FIST performance between happy to standard condition vs. neutral to standard condition. Participants in the face-first conditions scored an average of 1.66 (raw score out of 12) higher on selection 1 of the happy face version compared to the neutral face version (p < 0.01), and 3.5 (raw score out of 12, affected by score on selection 1) higher on selection 2 of the happy face version compared to the neutral face version (p < 0.01).

colleagues finding of declining performance in 4-year-olds during an 18-trial FIST task – an effect hypothesized to be due to fatigue [10].

The effect of fatigue is a possible explanation for the high variance in scores on the FIST presented second regardless of condition (Supp. Fig. 7). Fatigue may play a significant role in mitigating significance of mood effects induced by the emotional valence of the FIST presented first. However, the large variances suggest that a larger sample size is needed to identify differential performance at a significant level.

Facilitative effects of happy faces

The current investigation aimed to expand the understanding of emotional effects on abstraction and cognitive flexibility in young children using a modified version of the FIST paradigm. Our results suggest that the negative affect of sad and angry faces do not have a significant influence on abstraction and flexibility that is detectable with the current experimental design. However, it was also shown that children exposed to the happy face version performed significantly better on abstraction and cognitive flexibility compared to children exposed the neutral face version. Furthermore, children exposed to the happy face version first continued to perform better in the subsequent standard task compared to children exposed to the neutral face version first. Further analyses showed that these differences were not due to participant age or receptive language ability.

It is possible that the presence of happy faces in the FIST induced a transient, mildly positive mood in children, and that this mood, in turn, improved children's abstraction and cognitive flexibility. Previous research has shown that mild positive mood increases cognitive flexibility in adults and school-aged children [15-20]. Our results add to these findings and indicate that the facilitative effect of positive facial stimuli may be observed even in four-year-olds using a well-documented measure of EF. This finding among children corroborates Qu and Zelazo's finding that happy faces improve children performance on the DCCS [20].

The improvement in abstraction and cognitive flexibility on the happy face FIST version may be due to a domain-general mechanism. One hypothesis is that the happy faces serve as positive feedback to reinforce attention and reduce fatigue [34]. From an adaptive–evolutionary perspective, it can be assumed that emotional faces have a priority role in biasing the allocation of attention resources given their significance as indicators for another person's mood state. An alternative interpretation of this improvement is that positive emotions lead to increased compliance to the task demands [35].

A neurophysiological explanation for improvement in performance may be attributed to increased activation of the orbitofrontal cortex (OFC) due to dopamine release in the reward limbic system. The OFC receives information about the sight of objects and faces from the temporal lobe cortical visual areas [36] The OFC is further linked to stimulus-reinforcement association, learning, and working memory [36]. With this understanding, the increase in functional activation of orbitofrontal cortex neurons and stimulus-reinforcement association learning may underlie the flexible interpretation and reinterpretation of the stimuli dimensions. Happy facial expressions also lead to increased brain activity in the left anterior cingulate cortex (ACC) [37]. This is a brain region previously implicated in emotion processing tasks and the role of performance monitoring in children's flexible rule use [38, 39]. In addition to the OFC and the ACC, another region to explore in future research is the retrosplenial-posterior cingulated areas, which may encode the emotional salience of stimuli such as happy or angry faces [40].

Distinct mechanisms: abstraction and cognitive flexibility

The improvement of abstraction performance (but not cognitive flexibility) on standard FIST versions following the happy face version suggests that distinct cognitive mechanisms and their connections with the limbic system may underlie the two cognitive processes suggested above. In particular, the significant facilitative effects of positive emotion on abstraction last beyond the original task. One explanation for this is attention: happy faces tend to capture attention more readily than other faces and objects [34]. Increased attention towards positive stimuli may help with abstraction, but its facilitative effects on cognitive flexibility may be restricted by other basic processes specific to selection two.

Affective learning

The lack of significant within-group differences suggest that child participants identified both versions of the FIST (i.e. the standard and the face version) as the same game. Furthermore, there was a lack of performance differences among conditions in which the standard version was presented first. In particular, the differential effects of background emotion on the face versions (i.e. facilitative effects of happy faces) were mitigated when children were exposed to the standard version first. This suggests that the initial learning environment (i.e. the specific stimulus set and background emotion) affects performance on both the original and later tasks that are similar. With this understanding, only affective learning (i.e. initial exposure to the task used emotional stimuli) will lead to significant facilitative or detrimental effects on cognitive performance depending on the emotion. This suggests that initial exposure to the task may be more important than subsequent exposures.

Furthermore, the lack of significance also suggests that knowledge gained through affective learning was *transferred* from the first version to the second version. For example, if happy faces led to a good performance, the standard versions that followed would also show improved performance; if sad faces led to declines in performance, this would be also be manifested on the standard version that followed.

General difficulty of face version compared to standard

By restricting analysis to only the first FIST version presented, we found that cognitive flexibility performance on the angry and neutral face versions was lower compared to the standard version. However, the children in the happy face version showed similar flexibility scores to children in the standard versions. Together, the findings suggest that the face version of the FIST is more difficult than the standard version of the FIST in terms of cognitive flexibility (i.e. switching to another rule to pair cards), but the positive emotions induced by the happy face version contribute to the difficulties inherent to the face version.

This can be explained by the fact that participants show less extinction in attention to faces compared to other shapes [41], causing them to perseverate and focus on one aspect of the face (i.e. colour). Interestingly, the positive emotions induced by the happy face version moderate the difficulties inherent to the face version. This may be due to the facilitative effects on the limbic system specific to happy emotions, which compensate for this perseveration of attention or memory by also activating prefrontal cortex areas associated with rule-switching and flexibility. As Bechara and colleagues observed, the emotional mechanism that biases rule-use is different from the emotional mechanism related to memory [42].

Limitations and future research

The focus of our study was to investigate the impact of the emotional content of the stimuli on task performance; however, we did not induce mood states in our participants. According to Kensinger and Corkin [43], investigations of the impact of emotional state on specific cognitive processes may be different than those that evaluate the impact of the emotional content of stimuli on those same cognitive processes. The difficulty with standard mood induction procedures is that very transient changes in mood can be small relative to the normal variability of young children's affective states [44]. Moreover, the results of this study and others suggest that young children have difficulty reflecting on and describing their mood, making it difficult to confirm the efficacy of mood manipulations [45]. There is also evidence to suggest that a degree of stimulus processing can still take place independent of attention and awareness, and this pre-attentive processing may serve to preferentially guide attention to salient events [46].

Given that participants were never directed, nor instructed to pay attention to the emotional expression of the faces, the differential cognitive flexibility performance as a result of stimuli valence is striking. It may be useful to use a test design more similar to Qu and Zelazo's "hot" DCCS study, where participants had to sort by emotions [20]. This will direct greater attention, and conscious awareness, towards the emotional content. However, the current FIST modification was designed to adhere as closely as possible to the standard FIST, and therefore the emotional content of the words was irrelevant to actual task demands.

As this is the first study to investigate abstraction and cognitive flexibility of preschoolers while varying the emotional content of the stimuli, further research is necessary to clarify the exact nature of the impact of emotional stimuli on executive function. It may also be useful to use a broader framework to explore executive function and the influences of emotion. One possibility is the somatic marker hypothesis. The key idea here is that 'marker' signals, such as emotional faces, influence the processes of response to stimuli at multiple levels of operation, some of which occur consciously [47]. Finally, for an exploratory study, the number of participants in each condition is considered low. While this is an issue, it also demonstrates that the performance differences due to emotional effects is very large, given that only 6 participants in each condition were able to demonstrate significance less than 5% two-tailed probability.

Conclusion

The current study suggests that stimuli with positive emotional content improve preschooler performance on a rule abstraction and cognitive flexibility task, compared to stimuli with no emotion. The facilitative effects of happy emotions on abstraction further appear to last beyond the original task to influence later performance on a non-emotional standard version of the task. Our results also suggest that the face version of the FIST used in this experiment is more difficult than the standard version of the FIST in terms of cognitive flexibility (i.e. switching to another rule to pair cards) for four-year-olds, but the positive emotions induced by the happy face version mitigate the difficulties inherent to the face version.

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Supplementary material is available online at the JULS website: http://juls.sa.utoronto.ca/

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Understanding and managing refractory septic shock in the intensive care unit

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Abstract

Refractory septic shock is due to a dysregulated immune response that is caused by a bacterial or a fungal infection in the blood. Endotoxins like lipopolysaccharide (LPS) from bacteria stimulate the release of pro-inflammatory mediators including tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1). These cytokines activate leukocytes resulting in the release of additional inflammatory mediators promoting widespread inflammation. This leads to refractory septic shock, which adversely affects the cardiovascular, respiratory and renal systems. In this review, current therapies to manage septic shock are examined and novel treatments are proposed to make sepsis more manageable in the clinical setting. ICU patients diagnosed at an early stage have the highest rate of survival as they can be effectively treated to prevent the onset of septic shock. However, patients that have progressed into later stages of sepsis have complications due to septic shock. We suggest treating these patients with cytokine inhibitors and anti-LPS molecules (cationic lipids and poly-L-histidines) in addition to therapeutics for organ-localized complications. This two-pronged approach treats the adverse effect due to inflammation that has already occurred and prevents further inflammation, resulting in an improved clinical outcome.

Keywords: cytokines; Gram-negative bacteria; lipopolysaccharide; refractory septic shock; sepsis

Introduction

Though bacteria are commonly associated with pathogenicity and disease most interactions with humans are symbiotic. The intestinal epithelium is highly dynamic and is home to remarkably diverse microbial flora, which help digest plant carbohydrates and assist in vitamin synthesis. However, if pathological perturbations occur, these helpful bacteria in the gut translocate into the blood resulting in bacterial infection [1, 2]. Once bacteria have translocated into the circulatory system the innate immune system is activated, resulting in an inflammatory response. As bacteria continue to spread throughout the circulatory system the bacterial infection affects physiological functions and results in the development of refractory septic shock. This results in symptoms that include tachycardia, tachypnea, refractory hypotension, decreased or elevated body temperature and a leukocyte count out of the normal range (<4x109cells/L or >12x109cells/L) [3]. The prevalence of refractory septic shock is widespread in intensive care units (ICUs) and is the leading cause of death in non-coronary ICUs [4]. This occurs due to the multitude of adverse clinical symptoms that result during the development of refractory shock, making this disease very difficult to manage [5]. In order to improve the clinical prognosis for these patients that develop refractory shock, in this review we suggest alternative treatments along with the evaluation of current treatments and a background on the mechanism of the disease.

Development of refractory septic shock

There are two phases in the development of refractory septic shock. The early phase consists of bacteremia and sepsis. This paves the way for the late phase which results in severe sepsis, followed by septic shock and finally refractory septic shock. Sepsis is initiated with the occurrence of a Gram-negative bacterial infection. As bacteria enters the blood it undergoes autolysis, where the outer membrane disintegrates releasing a component from the cell membrane known as lipopolysaccharide (LPS) [6]. The presence of bacteria in the blood (bacteremia) and the release of LPS into the blood represents stage one in the development of refractory septic shock. LPS in blood then binds to a serum protein known as LPS binding protein (LBP) [7]. As this occurs there are no clinical symptoms; however a positive blood culture is present when measured through a diagnostic test. Sustained formation of the LPS-LBP complex in blood then results in the progression to the next stage–known as sepsis.

During the sepsis stage, there is widespread inflammation due to the release of pro-inflammatory mediators from monocytes, which include tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6). In addition, the first clinical symptoms occur at this stage with an elevation of body temperature. Prolonged inflammatory mediator release then causes the patient's condition to deteriorate resulting in severe sepsis [7]. Throughout severe sepsis TNF-α continues to activate other circulating monocytes thus increasing inflammation through the continuous release of additional pro-inflammatory mediators (platelet activating factor, tissue factor, and leukotrienes) [7]. This widespread inflammation results in decreased tissue perfusion, and simultaneously causes an increase in the release of secondary inflammatory mediators. These mediators cause decreased tissue perfusion, ultimately leading to acute organ failure. As this occurs, the disease progresses into the next stage known as septic shock. The abundant release of secondary inflammatory mediators causes a significant decrease in arterial blood pressure resulting in additional circulatory shock symptoms [8]. Furthermore, complications with integral body systems such as respiratory and hematological systems may cause other components of organ systems to become compromised. Such complications include the development of acute respiratory distress syndrome (ARDS) and disseminated intravascular coagulation (DIC). Managing DIC is especially challenging, since it simultaneously causes thrombosis and thrombolysis. This results in increased hemorrhaging and paradoxically increases the risk of stroke or heart attack because of concurrent thrombosis.

Once individual components of organ systems are adversely affected this results in the transition into the final stage known as refractory septic shock. This transition usually occurs due to prolonged symptoms that cause whole organ systems to fail resulting in multiple organ failure (renal system, hepatic system, cardiac dysfunction).

Current treatments used to manage septic shock

Treating septic shock is a complex clinical task that requires an accurate assessment of the patient's clinical stage on the bacteremia–refractory septic shock continuum. Current therapies are tailored to the distinct stages involved in refractory septic shock development. In the first stage–bacteremia, antibiotics are used as a first line of defense to control the infection [9]. Although this is administered early, it has a very limited efficacy in controlling immune dysregulation that occurs due to the bacterial infection.

The majority of current therapeutics target the late phase of refractory septic shock development. In the late stages, vasopressor therapy is routinely used however recent literature has shown that although this method can restore systemic blood flow it also results in decreased microcirculatory and mesenteric perfusion. This causes blood flow to divert away from the jejunum and pancreas [10], which leads to an adverse effect as decreased blood supply to the gastrointestinal system results in cell death. In addition, other therapeutics used to treat severe sepsis include the use of antioxidants, which have proven to be more effective due to their limited side effects [11]. Investigations of antioxidant vitamin therapy by Carlson et al. showed that administration of Vitamins A, C, and E resulted in a significant decrease in the activation of the innate immune system (mediated by the proinflammatory transcription factor family, NF-κB). This in turn, decreased cytokine release, thereby minimizing inflammatory damage and resulting in improved myocardial contractile function. Other adjuvant therapies are also used, such as recombinant human activated protein C (rhAPC). A study by Looney et al. showed that administering rhAPC had an anti-coagulant, anti-inflammatory, anti-apoptotic and profibrinolytic effect, but the exact mechanism through which rhAPC exerts its benefit in severe sepsis is unclear [12].

Therapies used during the septic shock stage are targeted at restoring systemic circulation and treating failing components of individual organ systems. Fluid resuscitation is first used to maintain blood flow to organ systems and prevent further damage [13]. In addition, mechanical ventilators are used to assist the patient in breathing if the respiratory system fails resulting in acute respiratory distress syndrome (ARDS) [14]. Furthermore, complications associated with the hematological system (such as disseminated intravascular coagulation) are treated with anti-thrombotic medications to prevent blood clotting [7].

If the symptoms of sepsis are prolonged, the patient's condition continues to deteriorate and the patient ultimately develops refractory septic shock. During refractory septic shock localized therapies are administered to increase tissue perfusion however prognosis at this stage is bleak and most patients undergo multiple organ failure.

Possible treatments to manage refractory septic shock

Early management in the bacteremia—refractory septic shock pathway is crucial in order to effectively prevent the development of refractory septic shock. Upon identification of a positive blood culture, anti-LPS molecules can play a key role in preventing the progression into the next stage. Bosshart et al. used cationic lipids and poly-L-histidines to successfully prevent the formation of the LPS-LBP complex [7]. As cationic lipids or histidine coils surround and sequester LPS, the LPS molecule is unable to bind LBP, and is therefore inactive and unable to stimulate the release of TNF-α. This prevents the activation of additional pro-inflammatory mediators which can cause further injury through the release of tissue factors (namely leukotrienes and platelet activating factors).

During the septic stage therapeutic interventions might also improve the clinical outcome. A study by Ranmana et al. recently noted that inflammatory signaling and cytokine generation during sepsis is dependent on the enzyme aldose reductase [15]. They concluded that down-regulation of this enzyme using interfering ribonucleic acid (RNA) or a pharmacological inhibitor (sorbinil) decreases the activation of the NF-κB nuclear factor and prevents TNF-α release. In addition, Coimbra et al.

noted similar results with the use of a phosphodiesterase inhibitor, pentoxifylline (PTX). These *in vitro* and *in vivo* studies demonstrate the use of novel methods to reduce the release of TNF-α which is the main contributor to the development of refractory septic shock. Integrating these methods into the clinical setting may allow for a better outcome in patients that are affected with sepsis.

An improved clinical paradigm: a mix of current and future therapies

Current clinical therapies primarily target the late phase of refractory shock development after adverse clinical symptoms are detected. Although, this approach may work to stabilize the patient at that stage, targeting early stages are more likely to improve clinical outcome. As noted, the release of inflammatory mediators that cause life-threatening clinical symptoms in the later stages are produced early in refractory septic shock development. We believe that the use novel treatment methods developed by Bosshart et al. [7], Ramana et al. [15] and Coimbra et al. [16] have the potential to significantly decrease the inflammatory response, thereby resulting in improved patient outcome. It is important to note that these treatment modalities have only demonstrated efficacy in *in vitro* and *in vivo* models, and clinical trials are necessary to assess the effects in a patient population. Ultimately, the use of current therapies in conjunction with the proposed therapies would treat the entire milieu of adverse clinical symptoms, preventing further inflammation and improving clinical outcomes for septic patients.

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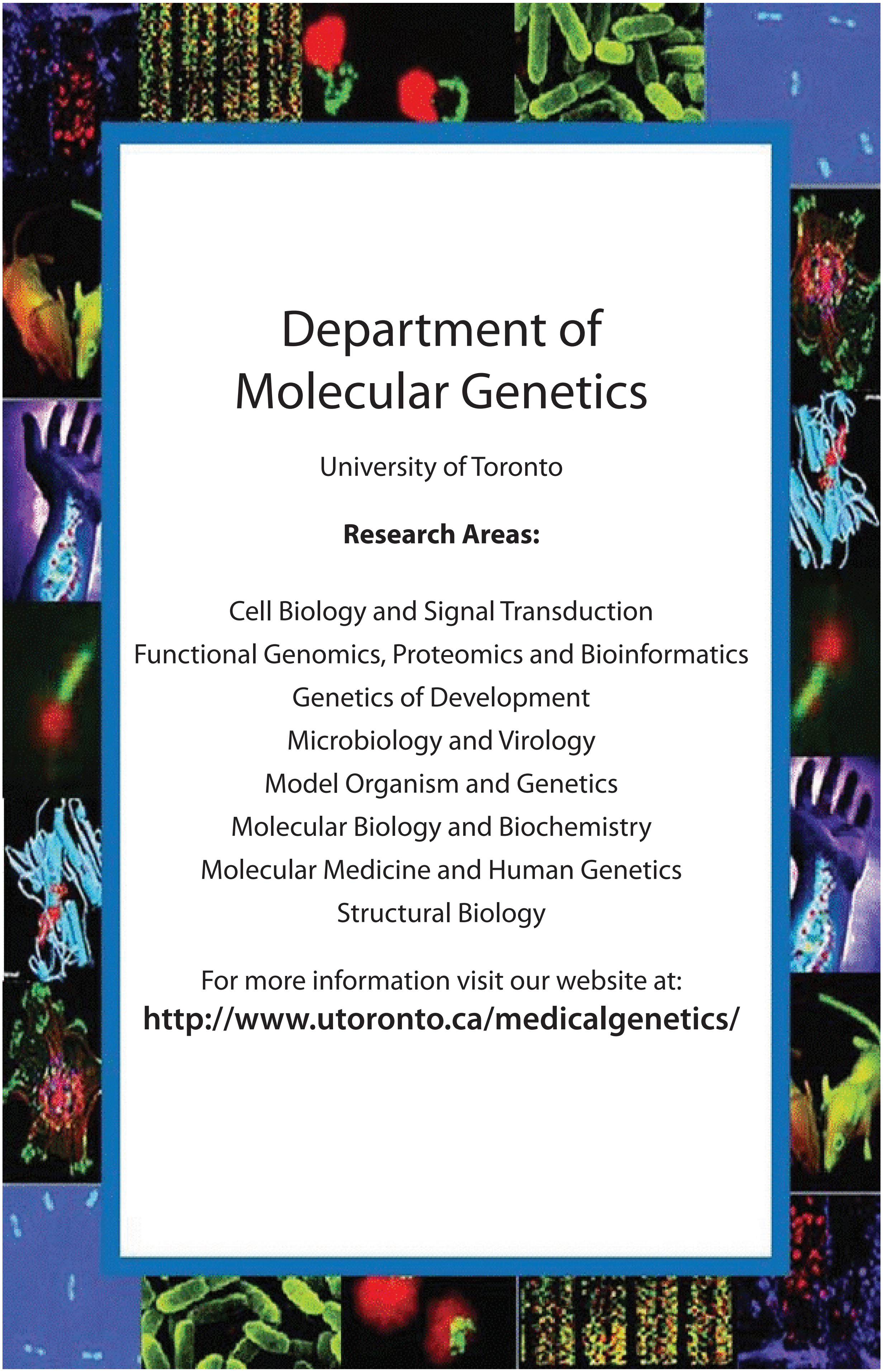

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Review Article

Molecular oncogenesis from a microRNA perspective

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Abstract

MicroRNAs (miRNAs) are a class of small, non-protein-coding RNA molecules that negatively regulate gene expression primarily through post-transcriptional repression. They have been implicated in the regulation of a variety of fundamental cellular and physiological processes relevant to cancer development. By acting as oncogenes ('oncomirs') or tumour suppressors, miRNAs play a crucial role in oncogenesis and cellular transformation. This review highlights the association of miRNAs with a number of cancer types and their mode of action, where available, will also be discussed.

Keywords: apoptosis; cancer; miRNA; oncogenesis; translational repression

Introduction

The transformation of cells from normalcy to malignancy is complete when cells proliferate disregarding the limits of cell division and invade and colonize the surrounding tissue. This process involves the acquisition of six aberrant traits: (1) self-sufficiency in growth signals, (2) disregard for the natural limitations of proliferation, (3) ability to evade apoptosis, (4) capacity to avoid replicative senescence and differentiation, (5) facility to sustain angiogenesis and (6) capacity to metastasize [1]. Recently, microRNAs (miRNAs) have been implicated in promoting malignancy [2, 3]. miRNAs are small, non-protein-coding, endogenous RNAs that negatively regulate gene expression through sequence-specific translational repression and degradation of target mRNAs. They can function either as oncogenes [4, 5] or tumour suppressors [6, 7]. The upregulation of the former and downregulation of the latter may enhance the development of cancer. This review will discuss the aetiology of human cancers from a miRNA perspective, focussing on the mechanisms by which they deregulate cellular homeostasis. This mechanistic understanding will allow for more accurate diagnosis and classification of cancers and identification of potential targets for cancer therapy.

miRNAs

Biogenesis and function

miRNA biogenesis is initiated when RNA polymerase II transcribes endogenous genes into long primary RNA transcripts (pri-miRNA). Pri-miRNAs are characterized by imperfectly base-paired secondary hairpin structures of approximately 70 bases [8, 9]. These hairpin structures are released from the pri-miRNAs following cleavage by the RNase III enzyme Drosha and the double-stranded RNA (dsRNA) binding protein, DGCR8 in the nucleus [10]. The resulting pre-miRNAs are then transported to the cytoplasm, where further cleavage by the RNase III enzyme Dicer produces an imperfect double-stranded miRNA/miRNA* (passenger strand) duplex. Each duplex consists of mature miRNAs of 22 nucleotides and subsequently becomes incorporated into an RNA-induced silencing complex (miRISC). The strand with the weakest base-pairing at its 5′ terminus is designated the guide strand and the other strand is designated the passenger strand [11].

The miRNA performs its regulatory role of translational repression and mRNA degradation when the miRISC binds to specific target sites within the 3′ untranslated region (UTR) of a protein-coding mRNA. Upon binding to the target, the passenger strand is removed and degraded as mediated by miRISC [12, 13]. The mode of negative regulation by the guide strand is based on the degree of complementarity between the target sequence and the miRNA. A near-perfect complementarity to the target sequence brings about endonucleolytic cleavage and degradation of the target mRNA via the short interfering RNA (siRNA) pathway in a miRNA-directed fashion [45, 46]. This is the same mechanism exploited by RNA interference (RNAi). A less strict complementarity causes translational repression and partial mRNA decay [14]. Interestingly, recent studies have shown that imperfect base pairing between target sequences and some miRNAs such as let-7 and miR-124 can also result in mRNA degradation [15, 16].

As miRNAs regulate many cellular process and are expected to regulate thousands of target genes [17], inappropriate miRNA action can have detrimental effects on cellular homeostasis, such as transformation into malignant cells.

Implications in cancer

In recent years, many studies have demonstrated that miRNAs are involved in the regulation of diverse biological processes such as cell proliferation and differentiation, developmental timing, apoptosis and maintenance of stem cells [18]. Moreover, given that most miRNAs have multiple targets, and that many of these targets are themselves regulators of basic cellular functions [17], it is therefore not surprising that a small perturbation in miRNA expression or function can disrupt cellular processes and eventually contribute to oncogenesis [18].

By profiling the expression levels of 228 miRNAs in six different solid tumours, Volinia et al. observed that 21 miRNAs were overexpressed in three unique tumours types [19]. Upon investigating the miRNA expression in colon, kidney, prostate, urinary bladder, lung and breast cancers, Lu et al. reported a global decrease in the expression of 217 miRNAs. In addition, more than 50% of the aberrantly expressed miRNA genes were found to be located within cancer-associated regions and at fragile sites in the genome [3]. While it may be difficult to determine whether the expression of miRNA undergoes a general increase or decrease in cancers, it can be established that alterations in miRNA expression is likely to be causally related to oncogenesis. Using array-based comparative genomic hybridization, Zhang et al. detected copy number changes in 41 unique miRNA-containing regions in breast cancer, ovarian cancer and melanoma samples [14]. It is evident from these studies that disturbances in the normal expression of miRNAs are associated with cancers due to the far-reaching effects of miRNA regulation in many cellular processes. A number of these processes are discussed below and the miRNAs involved are correspondingly categorized (Table 1).

Apoptosis

In vivo cell populations are maintained by a delicate balance of proliferative and apoptotic factors. In addition, potentially cancerous cells, such as those that have become stressed or damaged, are actively eliminated by apoptosis. It follows that those miRNAs that upset this homeostatic balance by facilitating proliferation or inhibiting apoptosis are likely to promote oncogenesis. An extensively studied example of such oncogenic miRNAs is the miR-17-92 polycistron. This miRNA is transcriptionally activated by c-myc, a proto-oncogene known to be upregulated in several human malignancies [4, 20, 21]. While the overexpression of c-Myc in mice resulted in highly apoptotic B-cell lymphomas, mice that also expressed a truncated form of the miR-17 cluster miR-17-19b developed lymphomas without extensive apoptosis [4]. These findings provide compelling evidence for the proapoptotic activity of miR-17-92.

O'Donnell et al. further demonstrated that c-Myc binds directly to the genomic locus encoding the miR-17-92 polycistron and subsequently activates the transcription of two miRNAs: miR-17-5p and miR-20 [22]. These miRNAs are known to repress the translation of E2F1, a transcription factor with both proproliferative and proapoptotic properties. As E2F1 is known to be transcriptionally activated by c-Myc, the simultaneous increase in miR-17-5p and miR-20 expression results in a feedback mechanism that attenuates the proapoptotic function of E2F1 and favour its oncogenic properties [23]. Collectively, miR-17-92 and c-Myc cooperativity enable lymphomas to proliferate and circumvent apoptosis [4].

In contrast to miR-17-92, miRNAs such as miR-15 and miR-16 act as tumour suppressors by inhibiting the activity of oncogenes; these two miRNAs are often downregulated or deleted in cancer. [24]. Cimminio et al. reported that these miRNAs bind post-transcriptionally to their respective binding sites within the 3' UTR of the BCL2 mRNA transcript and subsequently inhibit the activity of this prosurvival and antiapoptotic protein [7]. Supporting the regulatory role of these miRNAs, the expression levels of both miR-15 and miR-16 correlate inversely with BCL2 levels in patients diagnosed with chronic lymphocytic leukaemia (CLL). Therefore, the loss of miR-15 and miR-16 may result in increased Bcl expression and uncontrolled B cell proliferation in CLL.

The family of let-7 miRNAs target another well-known proto-oncogene, RAS. The dysregulation of Ras is a critical event in the pathogenesis of lung cancer [25]. Accordingly, let-7 is expressed at lower levels in lung carcinomas than in normal tissues [26]. In addition, the expression level of let-7 correlates inversely with Ras protein levels but not Ras mRNA levels, proving that let-7 represses translation [25]. Importantly, unlike protein-

Table 1. MicroRNA target prediction for genes involved in cancer

miRs	Target genes	Tumour types	References
miR-17-92	E2F1	Breast, B-cell lymphomas	4, 20, 21, 22, 23
miR-15, miR-10	Bcl2	CLL	7, 24
let-7	Ras	Lung	25, 26
miR-21	Timp3	Breast	27
miR-221/222	KIT	Thyroid	5, 19, 28, 29
miR-125a, miR-378	VEGF	Breast	38, 39
miR-9	E-cad, B-catenin	Breast, lung	30, 31, 32
miR-32, miR-92	Integrins		25
miR-126*	Prostein	Prostate	40, 41
miR-10b	HoxD10	Breast carcinomas	42, 43

coding tumour suppressors whose function is usually abolished in cancers, the tumour suppressing activity of miRNAs simply decreases below a threshold level. No longer able to offset the effect of oncogenic protein accumulation, a malignant phenotype is likely to develop.

Angiogenesis

For a solid tumour to attain a size greater than 2 mm, the formation of new blood vessels is necessary. This is the size threshold at which oxygen and nutrients become limiting for the interior cells of the tumour [27]. In addition to helping the tumour grow, the newly-recruited blood vessels may also facilitate the migration of metastatic cells. Tumour angiogenesis is stimulated by the increased production of angiogenic factors such as vascular endothelial growth factor (VEGF) and its receptor, VEGFR. Because it is very likely that a subset of miRNAs assist in promoting angiogenesis, it is hypothesized that angiogenic factors or the regulators of these factors serve as miRNA targets. For example, the 3′ UTR of the tissue inhibitor of metalloproteinase-3 (TIMP-3), a gene implicated in preventing the binding of VEGF to VEGFR2, has been shown to contain potential binding sites for miR-21, miR-17/20/106, miR-221/222 and other miRNAs known to be upregulated in certain cancers [5, 19, 28, 29]. Binding of these miRNAs to TIMP-3 may inhibit its antiangiogenic function and therefore indirectly promote angiogenesis and tumour progression.

Similarly, increased expression of VEGF can also contribute to angiogenesis. miR-378 binds to the 3′ UTR of VEGF in competition with miR-125a which has a repressive effect on the expression of VEGF [38]. As both miRNAs share the same binding site, binding of miR-378 is able to abolish the effect of miR-125a completely and instead upregulate its expression, resulting in increased vessel diameter in tumours [38]. A more recent study has implicated miR-378-mediated repression of the tumour suppressor protein suppressor of fused (Sufu) in enhancing angiogenesis [39]. However, the relationship between VEGF and Sufu remains to be elucidated.

Cell migration

E-cadherin is a transmembrane protein that plays a crucial role in cell-cell adhesion at epithelial surfaces. It acts by coordinating with the actin cytoskeleton via β-catenin [30]. In cancer, the E-cadherin/β-catenin adhesion system is often disrupted when epithelial cells undergo epithelial-mesenchymal transition (EMT). In addition to upsetting cell-cell adhesion, EMT also facilitates the metastatic dissemination of epithelial tumours [30]. Recently, it was discovered that E-cadherin/β-catenin mediated adhesion is also disturbed by certain oncogenic miRNAs. Both E-cadherin and β-catenin have been shown to contain miRNA binding sites; miR-9 and miR-200a binding sites exist with the transcripts of E-cadherin and β-catenin, respectively [31, 32]. The presence of the miR-9 binding site is particularly significant, as previous experiments have demonstrated that this miRNA is upregulated in both breast and lung cancers [19, 33]. Just as E-cadherins are important in cell-cell adhesion, integrins are indispensable in mediating cell-matrix adhesion. Cancer cells are known to often overexpress the integrin $\alpha_v\beta_3$ at the invasive front of the tumour [34]. Therefore, specific changes in integrin expression levels can impede metastasis by inhibiting both tumour cell migration and the attachment of tumour cells to the extracellular matrix. There is some evidence that miRNAs may serve this very role: the α_v subunit transcript found in multiple integrins including $\alpha_v\beta_3$ contains potential binding sites for miR-32 and miR-92 [25]. In addition, the β_3 subunit transcript contains potential binding sites for let-7. As described earlier, let-7 has been implicated in Ras regulation [25]. It is therefore noteworthy that all three miRNAs have been observed to be downregulated in tumours [16].

However, cell adhesion molecules are not the only determinants of the metastatic ability of cancer cells. Prostate cells express a high level of a prostate-specific antigen, prostein, which has an apparent function in promoting the migration and invasiveness of prostate cancer cells [40, 41]. Musiyenko et al. have shown that prostein could be a natural target for miR-126*, an intronic product of the epidermal growth factor-like domain 7 gene (Egfl7) [40]. The 3′ UTR of the prostein transcript contains two sites to which miR-126* binds specifically, thereby inhibiting its translation. The natural deficiency of miR-126*, then, may be causally related to the abundance of prostein and thus the invasiveness of prostate cancer. A novel regulatory pathway for invasion and metastasis also seems to exist in breast carcinomas [42]. In this pathway, the expression of miR-10b is induced by the transcription factor Twist through its direct binding to the putative promoter of miR-10b. The induced miR-10b then inhibits the synthesis of the homeobox D10 protein (HOXD10), resulting in the expression of an established pro-metastatic gene, RHOC [43]. In contrast to the direct control exerted by other miRNAs on their target mRNAs to repress expression of either a pro-metastatic or anti-metastatic gene, this new pathway has an added layer of complexity.

Differentiation and senescence

In addition to regulating cell migration, miRNAs have been implicated in mediating both differentiation and senescence. For example, the deletion of specific component proteins in the miRNA biogenesis pathway, such as Dicer-1 and Argonaute in Drosophila, resulted in early lethality and the abolition of certain stem cell populations [35-37]. These results suggest that miRNAs may be important in maintaining these stem cell populations. Similar to stem cells, cancerous cells are also able to avoid differentiation and replicative senescence. Therefore, an understanding of the regulatory mechanisms employed by miRNAs may prove helpful in elucidating how miR-

NAs endow normal cells with malignant properties.

Future directions

In this review, the potential roles of miRNA in causing malignant phenotypes were briefly discussed. Undoubtedly, miRNAs are key regulators in oncogenesis but much work is still required to dissect their intricate relationship with potential targets. Many of the currently identified miRNA target sequences are predictions based on computer simulations, and must be validated experimentally. The tumours studied to date are often a heterogeneous mixture of different cell types. miRNA profiling should be carried out at a higher resolution to obtain cell type specific expression maps. This will greatly augment the use of miRNAs in cancer classification, diagnosis and prognostication.

More importantly, because miRNAs have many potential targets and regulate multiple oncogenic pathways, they are likely targets or means for therapeutic intervention. For example, miRNAs upregulated in cancers could be specifically silenced *in vivo* by the use of antisense nucleotides for miRNAs or 'antagomirs' [44]. Eventually, the successful exploitation of miRNAs in cancer treatment depends on a thorough understanding of their molecular mechanisms and technical developments such as the safe and efficient delivery of miRNA-based therapeutics. Future research is likely to focus in this direction.

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JULS

Review Article

Appropriate maze methodology to study learning in fish

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Abstract

Using various sorts of mazes, researchers have gained much insight into the cognitive psychology of fish, particularly spatial and visual discrimination learning, along with the biochemical, morphological and ecological aspects of learning. Fish are known to orient themselves using landmarks, and, in some cases, create mental maps of geometric relationships using several landmarks. It is possible that by understanding the mapping mechanisms of fish we may one day uncover the possible evolutionary relationships in higher vertebrates. This is possible because the telencephalic structures became specialized early in the evolution of vertebrates for learning map-like representations of the environment. This review investigates the use of mazes (open-field, Y-maze, T-maze, radial, and multi-chamber) to learn more about spatial learning and memory in fish. To date, it has been shown that mazes are the most efficient way of studying the spatial capabilities of fish. Videographic experimentation in the natural environment would help to draw definitive conclusions on the cognitive capacities of fish.

Keywords: conditioning; fish; learning; mazes; telencephalon

Introduction

We owe the majority of our knowledge about the learning mechanisms in fish to controlled maze experiments conducted over the last decade. Spatial memory, which develops as an organism collects and processes sensory information from its surroundings, records one's spatial orientation in the environment. In the case of fish, it is used to help select cues that help them effectively perform a series of tasks, including navigating through unpredictable environments, foraging, avoiding predation and reproduction. Fish are known to orient themselves using single landmarks, and have the capacity to create mental maps of geometric relationships between numerous landmarks [1]. Depending on environmental conditions, fish develop preferences for specific sensory cues to maximize efficiency [2].

Spatial memory has been attributed to the telencephalon. This structure (constituting the anterior forebrain and the cerebral hemispheres) became highly-specialized early in the evolution of vertebrates. Its function is believed to have been refined through the use of map-like representations of the environment [3]. In fact, such use of map-based representations can help us uncover possible evolutionary relationship among higher vertebrates. Using various maze designs, researchers have gained insight into the cognitive psychology of fish: particularly the biochemical, morphological, and ecological aspects of spatial and visual discrimination learning.

Maze Set-up and Controlled Conditions

Experimental maze architecture is primarily categorized into five research models: the open-field maze, Y-maze, T-maze, radial maze and multi-chamber maze. The simplest structure is the open-field maze – a circular or rectangular arena lacking any barriers, but containing food rewards or landmarks depending on the task [4]. Y-mazes are simply a joining of three channels in the form of a Y, just as the T-maze is built in the form of a T. Connecting two T-mazes together forms a four-armed radial maze. Radial mazes consist of a central area branching off into as many as eight arms of equal length. Finally, a multiple chamber maze is an open-field maze sectioned off into several compartments by walls and doors, often built from opaque white Perspex® as opposed to transparent plastic that would allow vision through walls [5].

Learning behaviour is experimentally reinforced using food rewards. The food is administered using floating rings [6] or electronic feeders or tubes through which food is dropped [7]. Petri dishes containing Vaseline® are used on occasion to hold lodged bait that becomes visible when fish approach [4, 8].

Experimenters often use artificial illumination with fluorescent tubes in a twelve hour light-dark cycle when lighting the test room. However, light and dark conditions have been shown to affect learning [9], and must be accounted for.

Extra-maze cues are normally excluded by hanging curtains around the maze to rule out the effects of global cues in spatial learning when testing the significance of intra-maze cues [8]. In maze trials involving turn decision-making, fish will develop one of two strategies. An egocentric strategy entails learning spatial relationships relative to the organism, and is therefore easily disrupted by habitat alteration [10]. For instance, memorizing specific turns relative to one's body is a strategy susceptible to disruption by shifting silt or foliage in the environment. On the contrary, utilizing an allocentric strategy requires the fish to learn spatial relationships all around the maze environment, using physical landmarks or extra-maze cues [10]. Place and cue strategies are general terms that refer to allocentric strategies using extra-maze and intra-maze cues, respectively. At times, it is hard to distinguish between the strategies exercised by the fish. This is easily resolved by conducting probe tests, which manipulate available cues to observe how performance may be correspondingly disturbed.

Mazes uncover different learning mechanisms

Spatial learning

There is growing evidence that fish are capable of formulating cognitive maps to orient themselves within their respective environments. Open-field mazes are being used to understand the cognitive capabilities of fish based on environmental cues, since any kind of cue can be used by the fish in its spatial orientation – intra-maze, extra-maze and sensory cues. Saito and Watanabe confirm the advantages of an open-field maze in analyzing spatial learning [4]. In their experiment, goldfish were initially trained to find a particular baited hole among the 16 laid out throughout a circular maze containing a dead bloodworm food reward sunken in Vaseline®. Different situations were created to learn more about the utilization of different cues. These included changing the location of the baited hole (to analyze spatial learning), rotating the wall and floor (to examine for intra-maze cue use), putting up curtains (to exclude extra-maze cue use), and cutting of the olfactory tract or eye enucleation (to determine sensory cue use). It was found that goldfish relied mostly on extra-maze visual-sensory cues. An open-field maze was also used to study the blind Mexican cave fish and their ability to encode shape and size into a cognitive representation [5]. It was shown that blind Mexican cave fish can detect geographical changes, though this population warrants future study.

Schluessel and Bleckmann investigated the use of cognitive maps in stingrays using a radial maze [11]. Before proceeding with any trials, the fish underwent two controls within a T-maze. In the first, food was offered to the fish to confirm their knowledge of impending rewards, while the second assessed the stingrays' innate sense direction or place preferences by judging the frequency of left and right turns. Fish were then trained either egocentrically or allocentrically in a radial maze to locate a food reward at the end of an arm. Egocentric training simply used constant start and end positions. On the other hand, allocentric training involved setting up a constant end position with varying start positions in different arms to encourage the use of cues. The stingrays were subsequently put through four probe tests using novel start positions while the maze either remained stationary or moved within the room (thereby eliminating single or sets of extra-maze cues in case the fish rely on overall spatial arrangement or single cues). Extra-maze cues were eliminated using white sheets. The authors found that stingrays utilized visual information and constructed cognitive spatial maps using different navigating methods even within groups, perhaps simultaneously, to solve the spatial task [11].

Visual discrimination learning

Another important learning skill used by many fish species to navigate through the wild is the ability to discriminate visual cues. Visual learning is primarily tested using T-mazes and radial mazes. Colwill *et al*. demonstrated the ability of zebrafish to employ visual discrimination learning by conditioning the fish to enter the correct colour region of a T-maze with two different coloured arms in order to obtain a food reward [12]. Additional trials were conducted by swapping the colours. They found that zebrafish were capable of reversing their previously-learned discrimination by learning to choose a colour that was formerly incorrect or by ignoring an incorrect one that was previously rewarded.

Additionally, in two radial maze experiments, Hughes and Blight worked with corkwing wrasse and 15-spined sticklebacks in an effort to illustrate the use of visual association learning in foraging behaviour [13, 14]. In the second of these experiments, an eight-arm radial maze was used to investigate algorithmic behaviour and spatial memory employed in feeding [14]. First, all arms were loaded with a food source and experiments were conducted in the presence and absence of visual cues. Visual cues were presented with different colours marking each arm, and it was found that food rewards were discovered much faster compared to controls when coloration was used. The authors then restricted the consumption of food sources to three arms. Once all other arms were opened, the avoidance of previously-visited arms using spatial memory and visual cues was observed. A significant increase in arms revisited after repositioning of colour cues was also noted. However, when the maze was rotated while preserving the relative spatial configuration, there were no increases in revisited arms. Though promising, these results suggest that more research is required to determine the algorithmic behaviour and spatial memory in these and other fish.

Biochemical aspects of learning

To better understand the underlying mechanisms of learning in fish, researchers have studied the related neurotoxicology. The T-maze is most frequently used in such studies, an example of which is Creson *et al*'s study of black molly fish [15]. The study examined the correlation between chronic lithium treatment and spatial memory impairment. Previous work has shown that lithium treatment can have an adverse side effect on memory, and has warranted further study [15]. A place-learning task was given to four different dose regimen groups, including a control. A food goal was placed at one end of an arm. Start positions were rotated and a different arm of the four-armed maze was blocked each time in order to form a T-maze. The highest-dosage group took a significantly longer time to learn spatial task when compared to controls, suggesting that lithium treatment can have an adverse effect on spatial cognition.

Morphological aspects of learning

Studies investigating the morphological aspects of learning have been conducted using open-field and radial mazes. In the previously-described study by Saito and Watanabe, the goldfish were also tested using an open-field maze [8]. To compare the spatial learning functions of the dorsolateral and dorsomedial areas of the telencephalon, goldfish were first trained to locate a single baited hole in a circular tank of 16 sunken holes. The subjects were then given a lateral or medial telencephalon lesion and learning capabilities were examined. The first test analyzed the ability to use extra-maze cues to create a spatial map, while a second test assessed the subject's ability to use a landmark, in cases where the position of baited hole and landmark were fixed or varied (while maintaining the same distance relative to one another). Although no significant changes were observed between presurgical and postsurgical tasks, damage to the dorsomedial area significantly impaired spatial learning in the maze. The dorsomedial telencephalon, therefore, is crucial to the spatial learning capabilities of fish.

In a related study, Lopez *et. al,* looked at the effects of surgical excision of the telencephalon on place and cue learning [10]. Two groups of goldfish, one with intact telencephala and the other with telencephala removed, were trained to find a food reward in a T-maze that contained colourful visual cues. Two sets of experiments were performed. In the first, the four arms of the maze were blocked and intra-maze cues were altered in order to distinguish between place and cue strategies. In the second, extra-maze cue reliance was assessed by removing intra-maze cues and using novel start positions, while intra-maze cue reliance was examined by surrounding the maze with a curtain and using novel start positions. The results indicated that intact fish learn both place and cue strategies, whereas telencephalon-excised goldfish rely exclusively on a simple egocentric turn-strategy.

Ecological impacts on spatial learning

Research has shown that the type of spatial memories that fish use is significantly dependent on the environment in which a species lives. A recent study used a multi-chamber maze to study the influence of predation pressure and interspecific competition on spatial learning in poeciliid fish [6]. Four doors leading into equally-sized partitions were coloured using different markers. It was found that fish that experienced low predation pressure located the reward patch faster than highly-predated fish. The authors inferred that this was the result of relying on extra-maze cues and the coloured cues within the maze. It was also noted, however, that the close proximity of the sites from which the fish subjects were obtained for the study were not likely to cause differences in visual cue usage among the fish. The roles of predation pressure and interspecific competition in shaping spatial learning in fish of different habitats demand further scrutiny.

The multi-chamber maze has also been manipulated to compare the use of visual cues for orientation in fish from permanently turbid eutrophic ponds and clear water ponds [5]. A eutrophic pond is characterized by high mineral and nutrient content that promotes the growth of plant life, especially algae, resulting in a reduction in dissolved oxygen content. Once again, a maze with four compartments was used, with coloured tiles marking the doors leading into each. The subjects were required to find the contained shoal either with or without the landmarks. A shoal, or school of fish, is used as a reward due to the resulting social contact with other fish. The markers were then repositioned to generate test the use of intra-maze cues and algorithmic strategies. It was shown that fish from turbid environments followed global cues more effectively; however, both populations were adept at using visual landmarks in spatial tasks.

Use of the T-maze has also shown that habitat instability generates unreliable visual cues in the three-spined stickleback and other species [16]. River or pond habitats were used for sampling. River fish were found to more frequently employ a turn response in which the fish turned the same direction regardless of the changed landmark position.

Advantages of diverse maze utilization

Mazes are the most efficient way of studying the spatial capabilities of fish. The 60º arm-branching of Y-mazes have made them amenable to testing preference reactions. However, Hughes and Blight postulated that choosing a 4-armed starburst configuration instead of a Y-maze can offer distinctions between algorithmic alternations in foraging behaviour and behaviour guided by changing visual cues [13]. A starburst formation only has arms 1, 2, 3 and 6 open out of a radial 8, leaving arms 2 and 6 perfectly-aligned. It was also designed to allow experimenters to renew food sources unseen by the fish during tasks.

The T-maze is quite similar to the Y-maze in shape, but is popularly used to set controls prior to experimentation with more complex radial mazes. Such a maze can be used to familiarize the fish with a spatial task, confirm their knowledge of an impending award, and check for innate direction and place preferences [11].

Open-field mazes offer the advantage of enabling subjects to move freely in open space [8]. Spatial learning can thus occur due to multiple cues, and internal spatial maps can be formulated. It has been suggested that fish are less likely to form spatial maps in other kinds of mazes [8], such as radial mazes, where fish perceive goals as being fixed relative to extra-maze cues due to the identically-branching arms. In spite of this, radial mazes have been used successfully to test the complex behavior of cue memorization due to the number of arms and choices. One study used such a maze to assess foraging productivity – the memorizing of cues associated with quality of potential food sources at the end of the arms [14]. The maze formation allowed researchers to continually rotate the starting position. Overall, given that fish do not use the same strategy in different mazes, maze structure must be carefully selected according to investigational purpose.

Mazes are diverse in their abilities to challenge numerous types and aspects of spatial learning and memory. Each type of maze (open-field, Y-maze, T-maze, radial, and multi-chamber) is conventional in shape and investigational potential. Nevertheless, the structures are amenable to manipulation in order to create unique set-ups to optimize results and investigate a broader range of learning behaviour in fish. The results of learning studies have been largely inconsistent, making it difficult to draw definitive conclusions on the cognitive capacities of fish. Videography should be considered as a feasible next step. This would allow for more applied results pertaining to the daily living pressures on fish that make spatial learning indispensable.

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JULS

Functional importance of adult hippocampal neurogenesis

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Abstract

The hippocampus is crucial for memory formation and spatial processing. It is no surprise that the discovery of postnatal neurogenesis in the dentate gyrus of the hippocampus has inspired an extensive amount of research on its functional contribution to the adult brain. Correlational evidence reveals that neurogenesis is a dynamic process subject to modulation by a variety of factors, such as environmental enrichment and learning. Electrophysiological experiments show that young neurons constitute a distinct neuronal population within the dentate gyrus. Unlike mature neurons, they are not inhibited by gamma-amino-butyric acid (GABA) and require less stimulation to induce long-term potentiation. Furthermore, there is now strong evidence that new neurons can be functionally integrated into memory networks. However, significant debate still persists about the functional necessity of neurogenesis. To resolve this issue, several techniques have been used to ablate neurogenesis, such as X-ray irradiation, administration of anti-proliferative drugs and genetic knockdowns. Unfortunately, these methods are not equally effective, are invasive and can cause adverse physiological effects. This article offers an overview of the functional significance of hippocampal neurogenesis and presents a critical review of current research methodology. Methods of improving traditional techniques of neurogenesis ablation are also explored.

Keywords: behaviour; development; irradiation; methylazoxymethanol acetate (MAM); neurogenesis

Introduction

The hippocampus makes a vital contribution to human consciousness [1]. It is important for declarative memory, spatial learning, navigation, and relating items in time and space. It is also known for its high degree of functional plasticity, which is characterized by synaptic changes and required for learning [2]. However, recent evidence shows that the hippocampus also exhibits structural plasticity in the form of neurogenesis – a very rare phenomenon in the adult vertebrate nervous system [3-11]. Although it has been known since 1965 that postnatal hippocampal neurogenesis occurs in rodents [3], convincing evidence for this process in humans was only found in 1998 by Eriksson and Gage [6]. Following that discovery, a large and steadily growing amount of research has been invested into elucidating the functional contribution of neurogenesis to hippocampal plasticity, and consequently, behaviour.

Hippocampal neurogenesis takes place in the dentate gyrus, the first relay station of the hippocampus that processes incoming information (Fig. 1) [12-14]. Under basal conditions, approximately 9,000 new neurons are produced each day in the adult rat, with up to 250,000 by the end of the month [15]. Although this contribution seems minor in the context of the total number of neurons in the entire brain, it amounts to 10% of the neuronal population in the dentate gyrus. However, less than half of newborn cells reach maturity, as selection is highly dependent on neuronal activity [15].

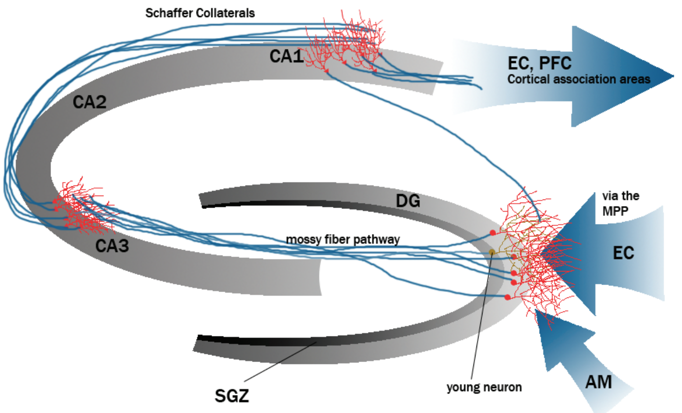


Figure 1. The adult hippocampus. The hippocampus is composed of two distinct areas, the Ammon's Horn with its three subfields (CA1 to CA3, CA for *cornu ammonis*) and the dentate gyrus, which contains the neurogenic subgranular zone (SGZ). On the simplest level, these form a trisynaptic circuit. Incoming information, primarily from the entorhinal cortex (EC) and to a lesser extent, the amygdala (AM), is carried by the medial perforant path (MPP) to the dentate gyrus. The granule cells of the dentate gyrus project to pyramidal neurons of the CA3 subfield via the "mossy" fiber tract, which in turn project to the CA1 subfield via the Schaffer collateral pathway. The CA1 subfield is the main output station of the hippocampus, which projects back to the entorhinal cortex, the prefrontal cortex (PFC), to a lesser extent, back to the dentate gyrus.

Neurogenesis is a highly dynamic process, and has been shown to increase in response to several environmental stressors, suggesting that it may be an important adaptive mechanism [14, 16, 17]. Nonetheless, significant debate centers on its functional contribution to the hippocampus. Noting that levels of mammalian hippocampal neurogenesis are negatively-correlated with brain complexity, it is not out of the realm of possibility that it is simply an evolutionary vestige. However, recent studies show that neurogenesis may be necessary for optimal performance on a range of hippocampus-dependent tasks. Resolving this debate requires an examination of neurogenesis at the levels of individual cells, neuronal networks, and behaviour [18].

Physiology

The subgranular zone (SGZ) of the dentate gyrus provides a vascular niche for thousands of neural progenitor cells (NPCs) [19]. NPCs have stem cell-like functionality *in vitro*, self-replicating and developing into all cell types of the central nervous system (CNS) [20]. However, such multipotency has not been observed *in vivo*. This is likely due to highly specific cell signaling within the SGZ, where astrocytic communication and growth factor concentrations are tightly regulated [14]. NPCs undergo three mitotic divisions before finally exiting the cell cycle

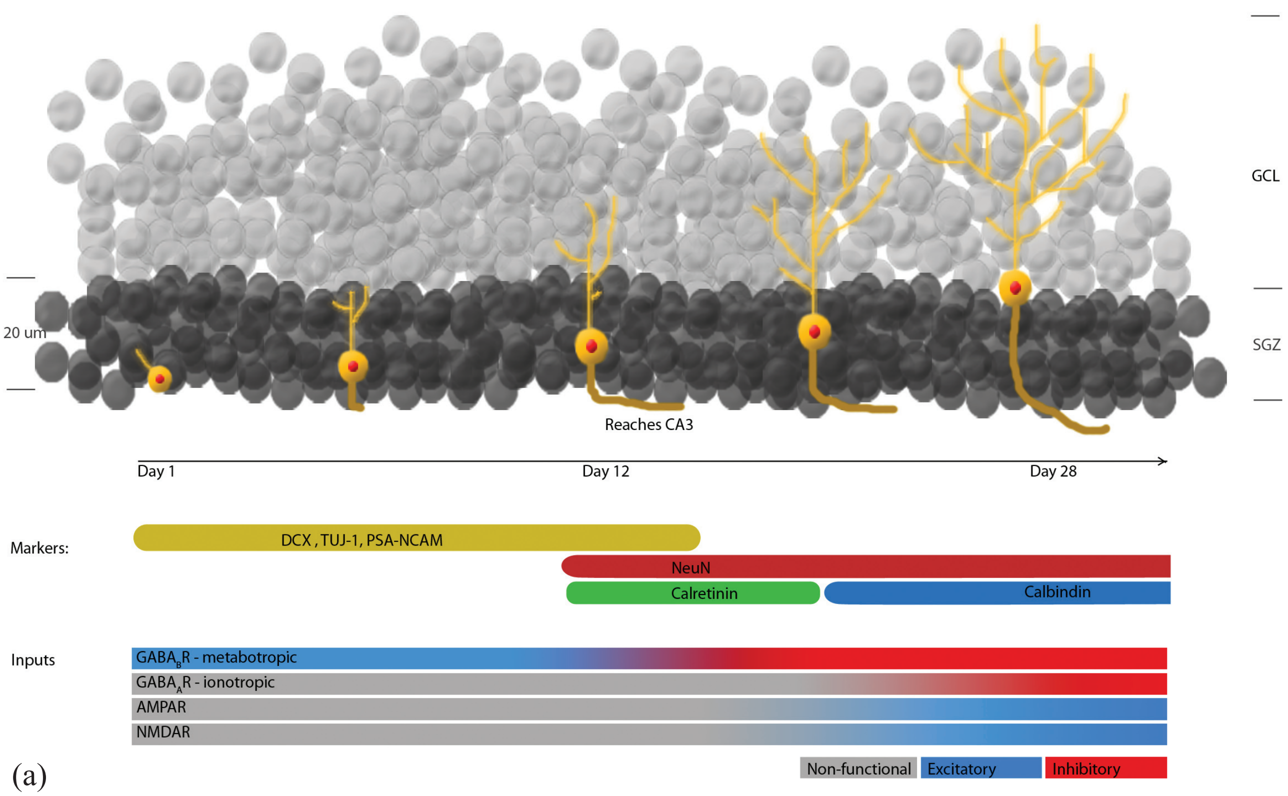
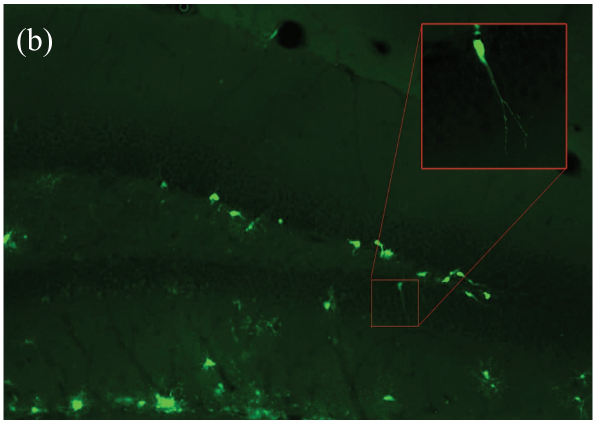


Figure 2. (a) A diagrammatic representation of a maturing, adult-generated neuron in the SGZ. There are several stages of maturation, each of which can be identified by specific markers listed. Doublecortin (DCX), microtubulin-1 (TUJ-1) and polycialic acid neural cell adhesion molecule (PSA-NCAM) are common markers for immature neurons in the initial stages of growth. The NeuN protein is expressed approximately by day 12 and continues through the life of a neuron. Calretinin and calbindin staining can further distinguish the developmental stage of a neuron. As can be seen from the figure, functional glutamateric excitatory inputs via α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPARs) and N-methyl-D-aspartic acid receptors (NMDARs) develop later than γ-amino-butyric acid receptor A ($GABA_AR$) inputs, despite the presence of all of these receptors from a very early age. It is notable that metabotropic $GABA_B$ inputs in developing neurons are excitatory during the first two weeks and not inhibitory as in the rest of the nervous system. **(b) a 10-day-old neuron stained with Green Fluorescent Protein (GFP).**



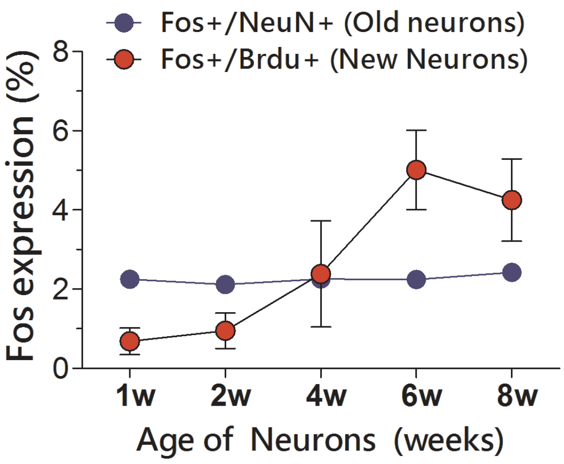


Figure 3. Preferential recruitment of mature neurons into memory networks. As neurons mature, they become preferentially recruited into memory networks. *c-fos* is a neural activity marker and is used here to visualize cells participating in spatial memory networks. Bromodeoxyuridine (BrdU) is a thymidine analogue and labels dividing cells at the time of injection. Hence, the age of neurons corresponds to the time between BrdU injection and perfusion. NeuN is a marker of mature neurons. Cells staining for both NeuN and Fos (Fos+/NeuN+) represent approximately 2.5% of mature (NeuN-labeled) cells, suggesting that only 2.5% of existing granule cells in the dentate gyrus participate in spatial memory networks. Conversely, recruitment of young neurons (Fos+/BrdU+) is dependent on their age. 1- and 2-week old neurons are suboptimally recruited. However, when neurons reach 4 weeks of age, they reach parity in recruitment when compared with the mature neuronal population. At 6 weeks of age and older, young neurons are preferentially recruited over existing ones into memory networks, with 5% of BrdU cells expressing *c-fos*, in contrast to 2.5% in the control (NeuN) group.

and becoming neuroblasts [21].

It takes at least two months for a hippocampal adult-generated neuroblast to develop into a mature granule cell (Fig. 2a) [18, 21]. Maturation spans several morphologically-distinct stages, and is slightly slower than its counterpart in the developing brain. The initial days are mainly spent increasing cell body size (Fig. 2b). By day 12, axons reach the CA3 area, and by day 16, there is emphasis on spine growth and synapse formation [21]. During the first two weeks after birth, synaptic currents are exclusively mediated by gamma-amino-butyric-acid (GABA) neurotransmitter. Contrary to its traditional inhibitory role in the CNS, GABA has a slow, excitatory effect on young neurons [22]. This paradoxical behaviour is likely caused by the relative insensitivity of ionotropic $GABA_A$ receptors to GABA and is thought to facilitate initial development. If the neuron has survived by week four, it now has an equal probability of being recruited into a memory network as a mature cell, despite having a different electrophysiological signature [15, 23]. At this age, neurons develop functional excitatory glutamatergic and inhibitory GABAergic inputs. However, due to the relative paucity of inhibitory stimulation, the induction threshold of long-term potentiation (LTP) is low [24-27]. Of note, six-week-old neurons have been found to be more preferentially integrated into memory networks relative to mature cells (Fig. 3) [23]. Therefore, as a population, immature neurons make a unique contribution to hippocampal function.

Neurogenesis is a dynamic process that is very sensitive to external factors [17, 28]. For instance, running doubles cell proliferation and enhances neuron survival, both of which are correlated with markedly improved learning and LTP in animal models [29-32]. Similar effects have been observed after learning hippocampus-dependent tasks [33-35] and environmental enrichment [17, 29, 36-38], whereby animals have access to running wheels, tunnels, and mazes. Surprisingly, even social hierarchy affects neurogenesis, whereby dominant animals show enhanced neuronal proliferation relative to subordinates [39]. Furthermore, neurogenesis is upregulated following ischemia [40] and seizure onset [41, 42], suggesting that it also has a restorative function [43]. Notably, balancing mature cell death and refining of neural networks optimizes the hippocampus for learning in computational models [14, 44, 45].

On the other hand, increased levels of adrenal stress hormones inhibit neurogenesis [46-48] and are the likely cause of decreased neuronal production in senescence [49]. Conditions that affect circadian corticosterone regulation and sensitivity, such as social isolation or maternal separation, interfere with neurogenesis [50-53] and delay the beneficial effects of environmental enrichment and physical activity [53].

However, correlational studies akin to those described above do not provide insight into causal relationships between neurogenesis and behavior. It is challenging to dissociate the effects of neurogenesis from other physiological factors on cognitive function. For instance, environmental enrichment fine-tunes neural networks by increasing dendritic branching and acetylcholinesterase activity [17]. Furthermore, the occurrence of cognitive improvement within a week of enrichment or physical activity suggests that other factors are important, as newly-generated neurons are relatively immature. Experimental approaches that actively manipulate neurogenesis without affecting other variables are necessary for the determination of a precise role for adult-generated neurons in hippocampal function. To date, several loss-of-function techniques that ablate neurogenesis have been used, and results have been intriguing.

Blocking neurogenesis with anti-proliferative drugs

Some of the earliest attempts to find a causal relationship between neurogenesis and behavior employed the cytostatic drug, methylazoxymethanol acetate (MAM). MAM is the active component of cycasin, a naturally-occurring carcinogen [54, 55] that methylates guanine residues at the C7 position during the S-phase of mitosis and prevents DNA duplication [56, 57]. As a result, proliferating cells become apoptotic [58]. MAM has been shown to select for neuronal lineages in the CNS, while leaving other CNS cells largely unaffected [58, 59].

Using MAM, Shors et al. managed to reduce hippocampal neurogenesis in the rat hippocampus by approximately 80% [60, 61]. Consequently, they reported deficits in two hippocampus-dependent tasks: trace-eyeblink and trace-fear conditioning, impairments that were reduced upon recovery of neurogenesis. However, performance on other hippocampal-dependent tasks, such as the Morris water maze and contextual fear conditioning, was not affected, suggesting that new neurons may function to retain temporal associations [62]. Furthermore, Bruel-Jungerman et al. found that ablation of neurogenesis removes the beneficial effects of enrichment, suggesting that it is solely the increased number of neurons that is responsible for improved performance on behavioural tests [63]. However, this conclusion is largely contrary to what has been reported in the literature [17].

Caution must be exercised in interpreting these results. It is likely that MAM administration approximately four weeks before behavioural testing eliminated relatively immature neurons which do not participate in learning. In addition, the second of the two studies by Shors et al. used a slightly higher dose (7mg/kg) of MAM than the first one (5mg/kg), as the smaller dose yielded inconsistent results. Originally, however, the 7mg/kg dose was rejected because it was associated with a decline in general health [60]. In follow-up studies, doses of 7mg/kg and higher were associated with impaired motor balance and reduced locomotor activity [64]. Furthermore, follow-up studies failed to substantially block neurogenesis with the same dosage, yielding only a 30 % reduction in cell proliferation [65]. Ciaroni et al. also showed that administration of MAM enhances survival of post-mitotic neurons, perhaps because of a general homeostatic mechanism. Thus, it becomes increasingly challenging to dissociate effects of reduced neurogenesis from non-specific effects of MAM treatment on behavioral performance.

Ablation of neurogenesis with X-ray irradiation

A more effective approach to blocking neurogenesis is brain irradiation. This procedure often results in acute and profound ablation of proliferative activity and spares mature cells. In addition, it can be manipulated to increase spatial specificity by targeting the desired area of the brain, a vast improvement over cytostatic drug administration [24]. Snyder and Kee were among the first to use this technique to comparatively analyze plasticity in hippocampal slices. They reported that LTP in the presence of GABA requires the presence of young neurons [26]. This finding has been corroborated by subsequent studies [24, 25, 66].

Behavioural analysis of irradiated animals is done at least two months after the procedure to allow for inflammation to subside [24]. This also allows the elimination of adult-generated neurons that could actively participate in learning. However, while several studies show impairments in trace and contextual fear conditioning [24, 67], the results of studies exploring performance on spatial tasks are discrepant. Some groups report impairments in the Morris water maze on later trials [68, 69], suggesting less than optimal memory retention, while others do not [24, 70, 71]. It is possible that these results may simply reflect the low power of the test to detect differences between groups, preventing observation of any appreciable impairment in the absence of neurogenesis. On the other hand, spatial learning in the Barnes maze is impaired upon irradiation [70], suggesting that this test may have a slightly different neural substrate.

Surprisingly, Meshi et al. have shown that hippocampal neurogenesis is not necessary for the effects of environmental enrichment. Enriched animals showed improvements in several behavioural indicators, regardless of whether they were irradiated or not. Importantly, irradiated animals did not differ from their sham counterparts in anxiety tests[71], contrary to what has been reported elsewhere[72]. Thus, upregulation of neurogenesis following enrichment is likely a by-product of a much broader physiological process..

Although irradiation is a significant improvement over cytostatic drugs for blocking neurogenesis, it still has several serious flaws. Monje et al. showed that irradiation profoundly alters the neurogenic microenvironment by inducing chronic inflammation and aberrant vascularization [73]. As such, it is necessary to consider non-specific effects of irradiation on cognitive function when evaluating behavioural performance. In a follow-up study, the group also showed that irradiation itself is not sufficient to ablate neurogenesis and requires inflammation with an increase in microglial activity, which markedly change hippocampal physiology [74]. Though irradiation has shown promise, further research is required to refine the approach.

Genetic ablation of neurogenesis

Only one reliable transgenic model for neurogenesis ablation has been used to date. Glial fibrillary acidic protein (GFAP)–thymidine kinase (TK) transgenic (TG) mice express TK receptors on neurons. Administration of antiviral prodrug ganciclovir (GCV) kills proliferating cells, while sparing existing ones. This technique affords a high degree of spatial and temporal specificity, as GCV must be delivered by a stereotaxic injection to the hippocampus. In accordance with irradiation studies, this method of neurogenesis blockade results in impaired contextual fear conditioning, but improves working memory [24, 25]. However, non-specific effects of GCV on TG mice have not yet been elucidated. It can be inferred that GCV may eliminate cell proliferation in the SGZ, thereby preventing the development of astrocytes and glial cells, and adversely affecting hippocampal function.

Future directions

The study of neurogenesis has provided great insight into structural plasticity within the hippocampus. While an abundance of correlational evidence implies that adult neurogenesis may have broad behavioural ramifications, studies attempting to find a causal link between the two have been hard-pressed to produce convincing results. It is perplexing as to why this has occurred – after all, young neurons do make a unique contribution to hippocampal plasticity on the cellular and network levels. Two reasons may account for this discrepancy. First, behavioural tests may not be powerful enough to detect effects of altered neurogenesis. Secondly, ablation techniques require further refinement, as they cause physiological complications in their current state. The solution to these problems will likely emerge from the development of better transgenic models. While the results of Saxe et al with GFAP-TK transgenic mice have been promising, their method of ablation stops all proliferative activity, regardless of cell type. A more suitable approach would be to develop a model in which developing neurons transiently express TK by coupling its transcription to one of the many markers expressed during the maturation process. This would allow specificity in the type of cell being targeted, as well as highly precise temporal and spatial control. At the present time, however, the relationship between neurogenesis and hippocampal function remains among the most intriguing mysteries in neuroscience.

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JULS

Retroviral vectors: new solutions to old problems

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Abstract

Gene therapy is a powerful new therapeutic approach that targets disease at the genetic level. While gene therapy encompasses a vast range of molecular practices and methods, viruses are often selected as vectors due to their intrinsic nature and specialized mechanisms of cell entry. The retrovirus family, *retroviridae*, is particularly useful in gene therapy as a vector for introducing genetic material into cells as they have the capacity to integrate genetic material into the host genome. This characteristic is desirable as it can facilitate stable and long-term expression of the transgene. However, although having the capability to introduce genetic material into deficient cells is highly beneficial, insertional mutagenesis and oncogenesis may result from uncontrolled integration. Such risks are a major safety concern when considering the use of retroviral vectors in a clinical setting. Mal-integration can disrupt the host's genetic integrity resulting in diseases such as cancer. Fortunately, strategies aimed at limiting transgene integration to predetermined sites are in development. Furthermore, modified lentiviral vectors (LVs) have been constructed to transfer genetic elements without any integration. Thus, new solutions that minimize the risk of mal-integration have made retroviral vectors a prospective therapeutic tool which may have a great impact on the landscape of modern medicine.

Keywords: biotechnology; gene therapy; integration; retrovirus; viral vectors.

Introduction

Gene therapy is a novel therapeutic strategy that aims to target genetic diseases at the molecular level. With the rapid development of molecular genetics, this strategy is becoming increasingly more feasible. Conceptually, gene therapy requires the insertion of a corrective genetic element which can alleviate or deter the progression of a disease [1, 2]. Gene therapy has the potential to treat many inherited and acquired diseases. However, a major obstacle in the development of such treatment strategies is finding a suitable, efficient, and effective vector to deliver corrective genetic material into cells [1, 3, 4].

Currently, there are two main classes of vectors used for gene therapy: non-viral and viral [3]. As suggested by the terminology, the former class includes methods that do not require viruses. The direct injection of DNA and the use of polylysines or liposomes to ferry the DNA across the cellular membrane are included in this

Table 1. Major characteristics and application of vector systems.

Vector	Main Advantage	Main disadvantage	Applicable disease treatment
Gammaretrovirus (MuLV)	Integration leading to stable and long-term gene transfer	Malignant transformation due to insertion Requires dividing cells	ADA-SCID, X-SCID, FH, Cancer
Lentivirus	Integration leading to stable and long-term gene transfer Can infect non-dividing cells	Malignant transformation due to insertion Risk of reversion to competent HIV	HIV, MPS-type VII
Adenovirus	Can infect non-dividing cells High efficiency	Innate immune response Transient gene expression	CF, mesothelioma, colon cancer
Adeno-associated Virus	Can infect non-dividing cells Minimal innate response	Small packaging limit Adaptive immune response to capsid	CF, hemophilia B, AAT deficiency
Herpesvirus	Can infect non-dividing cells High efficiency	Adaptive and innate immune responses	Brain tumours, colon cancer, Intractable pain
Non-Viral	Can infect non-dividing cells Large packaging capacity	Innate immune response Transient gene expression	CF, DNA vaccines, metastatic cancer

ADA = adenosine deanimase; X-SCID = X-linked severe combined immunodeficiency; FH = Familial hypercholesterolemia; HIV = Human Immunodeficiency Virus; MPS = Mucopolysaccharidosis; CF = Cystic fibrosis category [5]. However, most studies today employ viral vectors. Viruses, which have evolved highly sophisticated mechanisms to deliver genetic material into cells, are an easy and effective way to transduce cells [6]. Although the natural diversity of viruses has provided a large spectrum of feasible vectors, each with their own advantages and disadvantages (Table 1), the unique characteristics of retroviruses have encouraged their extensive use and study [4].

This review will describe the biological properties of retroviruses including their composition and lifecycle. In addition, an analysis of the key characteristics of the retroviral vector will reveal that retroviral integration, a very desirable characteristic of the vector, is also the critical factor limiting its use in clinical settings. Research in preventing malintegration has yielded two potential solutions: integrase zinc finger fusion proteins and non-integrating retroviral vectors. While further research is warranted, the prospect of eliminating insertional mutagenesis and oncogenesis will not only make retroviral gene therapy therapeutically viable, but also open doors to treating illnesses previously considered incurable.

Biology of the retrovirus

The majority of retroviral vectors currently utilized for gene therapy are based on the well-characterized gammaretrovirus, murine leukemia virus (MuLV) [3, 4]. Unlike other viral vectors, retroviruses have an RNA genome that consists of three genes, denoted as *gag*, *pol* and *env* (Fig. 1). Prior to transcription, these genes are flanked on the 5′ end by an R-U5 sequence and on the 3′ end by a U3-R sequence. After transcription, the genes become flanked by two terminal U3-R-U5 sequences known as long-terminal repeats (LTRs). LTRs contain both the promoter and enhancer sequences necessary for the expression of viral genes and also play an essential role in the reverse transcription of the viral and host genome integration. In addition to these LTRs, the viral packaging "Ψ" is critical for the virus to successfully complete its life cycle. Ψ is responsible for incorporating genetic material into new viral particles [1, 3].

The viral proteins encoded by the three genes *gag*, *pol* and *env* are necessary for the structure, activity, and virulence of the virus. In the MuLV model, the enzymatic gene products of *pol*, such as protease, reverse transcriptase, and integrase, are encapsulated along with two copies of the RNA genome. The viral capsid that encloses these proteins is composed of the *gag* gene products; this capsid is itself enclosed by a lipid envelope containing anchored viral glycoproteins encoded by the *env* gene [1].

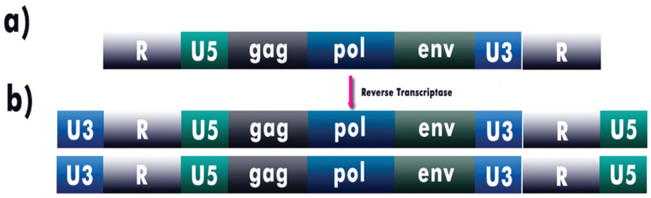

Figure 1. Conversion of RNA retroviral genome into proviral dsDNA. (a) Retroviral RNA genome consists of three genes, *gag*, *pol* and *env*, flanked on the 5' end by an R-U5 sequence and on the 3' end by a U3-R sequence. (b) In the host cell, the RNA genome becomes reverse transcribed into dsDNA which possesses a U3-R-U5 sequence. These elements are known as long terminal repeats (LTRs).

The glycoproteins encoded by *env* play an essential role in host cell recognition and fusion. Upon interacting with the host cell receptor, the *env* glycoproteins mediate cell recognition, cell surface binding, and initialization of the retroviral lifecycle (Fig. 2). Ultimately, this interaction mediates virus-host cell fusion which in turn allows the retroviral capsid to enter the host cell. After entry, the viral capsid dissociates and releases the RNA genome and the viral enzymes into the host cell cytoplasm. Subsequent reverse transcription of the RNA genome by the viral reverse transcriptase creates proviral double stranded DNA (dsDNA). This dsDNA is then transported into the nucleus where with the activity of the viral integrase becomes stably integrated into the genome. Upon successful integration, RNA transcripts encoding *gag*, *pol* and *env* are made. The full unspliced viral RNA transcript containing Ψ will be packaged into new viral particles, while spliced transcripts without Ψ are used to translate proteins. The translated *gag* and *pol* proteins assemble into the viral core and become packaged with viral RNA. This complex then localizes to the *env*-containing portions of the cellular membrane. In the final stage of the viral life cycle, the mature viral particles bud off from the host plasma membrane, gaining a lipid envelope in the process [1,3,6].

MuLV, like many classes of retroviruses, requires host cell-mediated breakdown of the nuclear envelope in order for the viral dsDNA to reach the nucleus [1]. This breakdown most often occurs during mitosis. Consequently, these retroviruses are only able to infect dividing cells.

Retroviruses and gene therapy

As vectors, retroviruses have many favourable characteristics. First, they are defective vectors, as successful delivery and expression of the transgene does not require the expression of any viral genes [2, 7]. Therefore, the degree of the host immune response will be less severe since it is presented with fewer foreign particles. Second, almost all viral genes, except the LTRs and the packaging signal Ψ, can be replaced with foreign sequences of interest [1,3]. Furthermore, while commonly used gammaretrovirus-derived vectors (e.g. the MuLV model) can only infect dividing cells, lentiviruses (LVs), such as the human immunodeficiency virus (HIV), can infect both dividing and non-dividing cells [3, 4]. Most

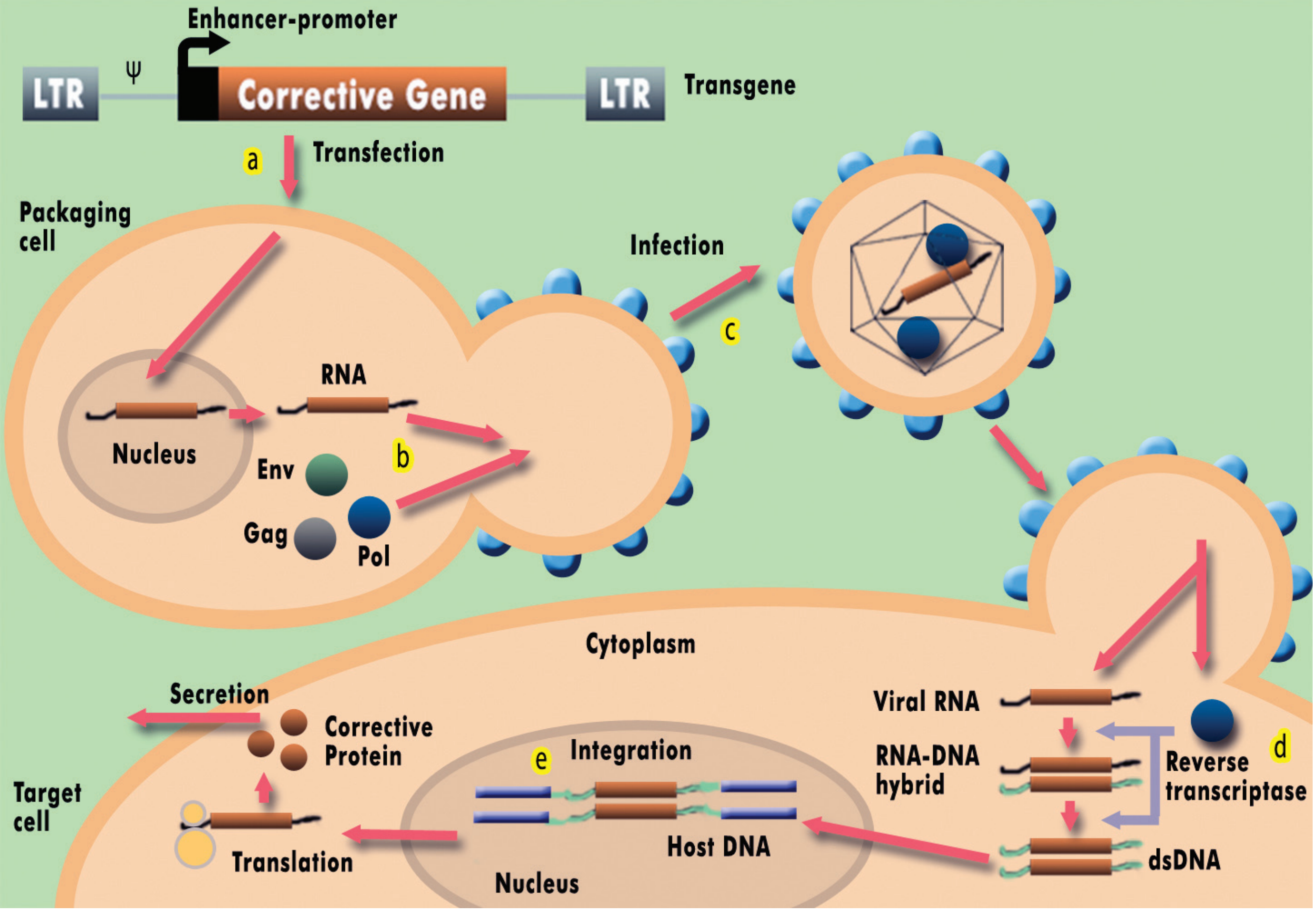

Figure 2. The production of retroviruses through packaging cells and the exploitation of the natural retroviral life-cycle in gene therapy. (a) The corrective transgene is transduced into packaging cells expressing viral gene products (*gag*, *pol* and *env*). (b) The incorporation of the DNA into the nucleus induces transcription, leading to production of vector RNA. This RNA becomes packaged into new retroviruses, budding from the cell. (c) Upon infection, the lipid envelope of the viral vector fuses with the host cellular membrane allowing vector RNA and viral proteins to enter the cell. (d) Vector RNA becomes reverse transcribed by the viral enzyme reverse transcriptase into double-stranded DNA. (e) This DNA product is then integrated into the host genome resulting in the expression of the corrective gene and its product.

importantly, retroviral vectors are able to integrate the foreign sequence of interest into the host genome. For the purposes of gene therapy, this integration is useful as it facilitates long-term and stable transgene expression [1, 3, 4, 6].

Despite the many clear benefits of using retroviral-based vectors for gene therapy, the capacity of these viruses is small and thus limits the size of the transgene to be inserted. The majority of retroviral vectors can accept a transgene that is between 6 and 8 kb. As retroviruses can only carry a small number of genes, they cannot be used to treat diseases that require the regulation of multiple genes [4]. In addition, it is difficult to obtain a high titre of retroviruses, further restricting their potential as a therapeutic option [8]. The tendency of retroviruses to integrate into the host genome also causes a much greater concern: mal-integration [6]. As retroviruses integrate their genetic material into the host cell genome randomly, the viral genome may insert within a functional gene and thereby render the host gene non-functional. More worrisome, however, is that by perturbing the regulation of certain genes, cancer may manifest. [3, 6, 9].

Currently, most gene therapy studies utilize retroviral vectors in *ex vivo* transduction (Fig. 2) [2, 3, 6]. In this method, cells of interest, usually stem cells, are extracted and infected with the retroviral vector *in vitro*. After treatment, the cells are reimplanted into the body.

As MuLV-derived vectors are generally limited to *ex vivo* therapy, their treatment capacity is restricted. In contrast, LVs are able to infect non-dividing cells such as muscle and neural cells and can therefore be used for *in vivo* therapeutics. The most common LV used for *in vivo* gene therapy is a disabled HIV vector [1, 21]. Thus, retroviral vectors have great therapeutic potential in both *ex vivo* and *in vivo* settings.

The importance of integration in gene therapy

The benefits of employing retroviral-derived vectors in gene therapy must be carefully weighed against the evident costs of this strategy. In 2000, a retroviral-based gene therapy was developed to treat human severe combined immunodeficiency (SCID)-X1 disease. The results of the clinical trials for this treatment clearly illustrated both the severe repercussions of malintegration and the necessity to avoid such events [10].

SCID is a genetic disease where cells in the body are unable to express interleukin(IL)-2 receptors due to a non-functional γ_c encoding gene [11]. As IL-2 is critical for the differentiation and development of T-cells, the immune systems of those afflicted with this disease are greatly compromised. If left untreated, SCID is ultimately fatal.

In the clinical trial performed by Fischer et al., *ex vivo* retroviral gene therapy was used to treat young males diagnosed with SCID [12]. Fischer's team aimed to treat the disease by integrating the functional γ_c gene into the genome of autologous hematopoietic stem cells (HSCs). HSCs were extracted, infected with retroviruses carrying the functional γ chain, and re-implanted. Shortly after treatment, the T and NK cells of all patients were shown to express γ_c [10]. However, although the initial followup studies suggested that the treatment had effectively corrected the disease phenotype, long-term followup studies revealed that two subjects had developed leukemia [13, 14]. Upon further investigation, it was found that the retroviral vector had integrated near the promoter of the proto-oncogene LMO2 in both cases. The subsequent overexpression of LMO2 caused the development of leukemia [15, 16]. These tragic results had profound repercussions on the use of retroviral gene therapy in clinical settings. The treatment protocol was temporarily banned in many countries including the United States [14]. Evidently, if gene therapy is to become a safe and effective treatment method, the risk of insertional mutagenesis must be completely eliminated.

Recent developments in retroviral integration

Zinc finger proteins – integrase fusion proteins

Immediately following the results of the Fischer et al. trials, much work was initiated to investigate the mechanisms of viral integration and its potential solutions. It is expected that by understanding the molecular basis of insertional mutagenesis, this event can be prevented. Two very recent studies have demonstrated that by site-specific integration the prevention of mal-integration is possible [18, 21].

The first such approach is based on the hypothesis that if sequence specific DNA-binding proteins are fused with viral integrase, the resulting fusion protein should direct site-specific integration; the novel protein should both recognize and bind to its target site. Upon binding, the fusion protein is expected to spatially confine integrase activity to the genomic region near or adjacent to the binding site. Tan et al. were able to prove this hypothesis using a fusion protein that consisted of the HIV-1 integrase coupled to a polydactyl zinc finger protein [18]. This construct effectively targeted transgene integration to specific DNA sequences. Zinc finger motifs are classified as helix-turn-helix motifs, a common DNA-binding pattern found in eukaryotes [19]. The Cys2-His2 zinc finger motif used by Tan et al. is composed of an α-helix and a β-sheet held together by a zinc atom [20]. Typically, these 30 amino acid domains bind to a 3bp DNA sequence. In contrast, polydactyl zinc finger proteins are composed of many zinc finger domains and are therefore able to specifically bind and recognize DNA sequences of greater specificity. By utilizing the polydactyl zinc finger E2C, Tan et al. were able to construct a fusion protein capable of binding and recognizing a specific contiguous 18bp sequence on the human chromosome 17. Through PCR-based assays, the authors demonstrated that the HIV-1 integrase-E2C fusion protein can direct retroviral integration to predetermined sites. They also noted that fusion proteins with E2C tethered to the N- or C-terminus of the integrase resulted in similar binding, catalytic and integration activities. Although integration was primarily localized around E2C binding sites, they observed a considerable number of integration events outside E2C recognition sequences. Tan et al. accredited these unexpected occurrences to the non-specific DNA-binding activity of the HIV-1 integrase. Thus, a better understanding of the interactions between viral integrase and host DNA interaction is required if the non-specific DNA-binding activity of the viral integrase is to be effectively suppressed.

Non-integrating LVs

In addition to the development of traditional integrating retroviruses, many researchers are currently attempting to develop non-integrating lentiviral vectors (NILVs) derived from HIV. In contrast to MuLV, these vectors are able to infect both dividing and non-dividing cells. Moreover, NILVs do not pose the risk of malintegration [21, 22]. As opposed to integrating DNA into the genome, NILVs are able to transduce cells by introducing episomal forms of DNA [21, 23]. As episomal DNA is diluted with cell division, NILV can be used to achieve transient transgene expression in dividing cells. Non-dividing cells infected with NILVs, however, show stable long-term expression of the gene. Thus, for the purposes of the genetic modification of post-mitotic cells, NILV may be safer than integrating viral vectors [22]. Although no integration event occurs, integrase cannot be completely deleted because of its role in reverse transcription. Therefore, to prevent the integration activity of the integrase and the risk of insertional mutagenesis, researchers have induced mutations within the viral integrase gene in a recent study [21]. Apolonia et al. evaluated different methods of inhibiting LV integration and the stability of transgene expression in non-dividing cells *in vitro* and *in vivo*. They demonstrated that vectors carrying integrases with induced point mutations (D64V, N120L, and W235E) integrated 10^3-fold less frequently as compared to vectors carrying the wild-type integrase. Furthermore, introducing various combinations of mutations did not have an impact on reducing the integration efficiency. These results indicated that these mutations operate in a "limiting step" mechanism such that the integration efficiency is limited by the most hindering mutation.

Apolonia et al. also infected neonatal mice myocytes both *in vitro* and *in vivo* with NILVs and an integrating vector for comparison. After enhanced green fluorescent protein (eGFP) microscopy and viral DNA viral DNA quantification assays, they noted prolonged transgene expression (> three months) in the non-dividing muscle cells *in vitro* and *in vivo*. In addition, NILVs successfully transduced muscle cells at an efficiency rate similar to integrating vectors. However unlike cells transduced with integrating vectors, NILV transduced cells displayed a more scattered pattern of integration. The researchers suspected that this may be due to the dilution of the viral episome that is caused by the proliferation resulting from the development of neonatal muscle tissues. Thus, further studies, including trials with adult mice muscle tissues, must to be conducted to understand the nature of this distribution pattern. Nonetheless, the safe stable and long-term expression of the transgene suggests that NILVs have significant clinical potential.

Summary

Although retroviral vectors are an easy and effective method of delivering genes into cells and transduce stable and long-term expression of transgenes, various safety factors such as the risk of insertional mutagenesis and oncogenesis prevent its widespread use in gene therapy. The development of leukemia resulting from retroviral gene therapy emphasizes the hazards of mal-integration. However, more recent studies have discovered potential solutions to this problem. Both integrase zinc fusion proteins and non-integrating LVs can effectively mediate safe and stable transgene expression. The prospect of retroviral vectors in clinical settings must be reassessed as a safe retroviral vector has immense potential in future therapeutic applications.

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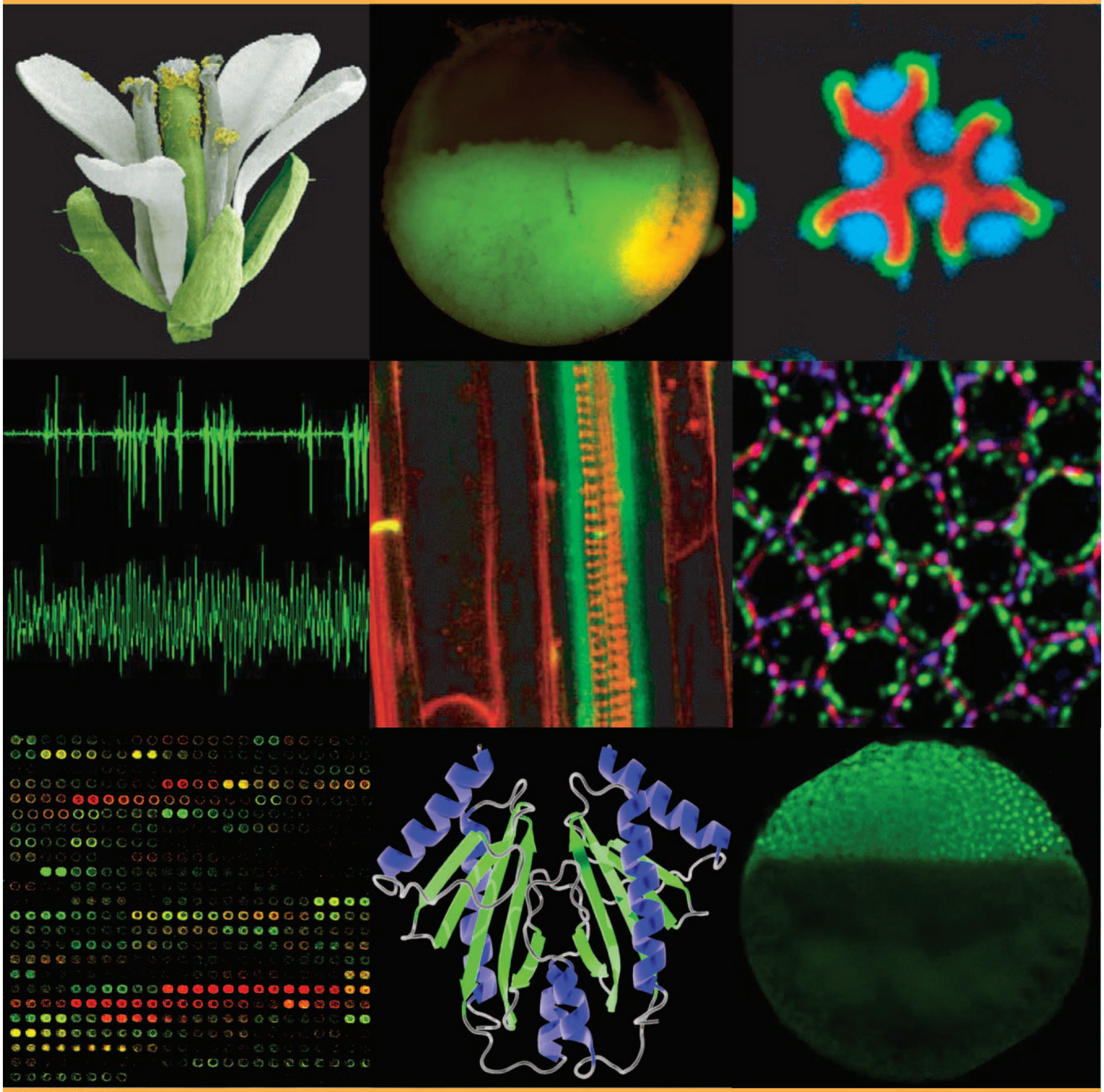

JULS

Mechanisms of eukaryotic translation initiation factor 4F (eIF4F) inhibitors in combating cancer

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Abstract

The survival and proliferation of cancer cells depend largely on the elevation of oncogene expression. Oncogenic expression can be affected by alterations in translation, or protein synthesis. Translation initiation, the rate limiting step of translation, closely regulates protein synthesis which both healthy and cancerous cells require for division and growth. Inhibitors of the eukaryotic translation initiation factor 4F (eIF4F) have been shown to selectively repress the expression of oncogenes in multiple *in vitro* studies. Although chemotherapeutic application of translation initiation inhibition is in its early stages, promising *in vitro* results demonstrate the potential of eIF4F inhibitors for use in clinical settings. This review offers a brief overview of the mechanisms that underlie eIF4F inhibitor activity in combating cancer.

Keywords: apoptosis; chemotherapy; cytotoxicity; signalling; translation

Introduction

Cell division is governed by the cell cycle and is fundamental for the growth and regeneration of tissues within all living organisms. Regulated by a specific set of genes, cell division will only take place when these genes are expressed within the cell. Moreover, when subjected to mutation, the regulatory genes are often overexpressed, leading to a deregulated cell cycle. This mutation process contributes to uncontrollable cell proliferation and ultimately tumorigenesis or even cancer [1]. Such cancer-causing genes are called oncogenes. As the expression of oncogenes is dependent on replication, transcription and translation, the effective inhibition of one or more of these processes may induce apoptosis and could therefore facilitate the eradication of cancerous cells. In fact, this antineoplastic approach is one of the underlying principles of chemotherapy [2].

The large majority of chemotherapeutic agents target the replication and transcription of oncogenes. Such treatments aim to suppress mitosis in an attempt to hinder cell division or induce apoptosis in rapidly dividing cancer cells. Although most genes must undergo translation prior to being expressed, only recently has the inhibition of translation been considered in the development of novel cancer drugs. This is because the mechanism of translation is poorly understood in comparison to that of replication and transcription. Therefore, this process has not been a focus of drug development in combating cancer. However, in response to the promising results of recent *in vitro* studies published by Jiang et al. [3] and many others, interest in translation inhibition is increasing.

The development of translation inhibitors as novel chemotherapeutic agents has largely focused on targeting and inhibiting eukaryotic translation initiation factor 4F (eIF4F). Composed of the subunits eIF4E, eIF4G and eIF4A, eIF4F regulates translation initiation, the rate limiting step of protein synthesis from mRNA [4]. In cancer cells, such as HeLa cells [5] and breast carcinoma cells [6], the aberrantly elevated expression of eIF4E acts to increase eIF4F availability [7]. In turn, increased eIF4F augments oncogene translation and thus contributes to excessive cell proliferation [8]. It follows that by inhibiting the interaction between eIF4F and the mRNA of various oncogenes, cancer cell proliferation may be slowed or halted [3]. Here, the mechanism of eIF4F function and eIF4F inhibitors will be reviewed in an attempt to shed light on this promising new area of cancer drug development.

Mechanism of Translation Initiation

Gene expression in eukaryotic cells is tightly regulated by translation efficiency [9]. As translation initiation is rate limiting, this step is of greatest interest for the investigation in the mechanism of translation regulation [4, 8]. Specifically, the cap-binding protein eIF4F mediates translation initiation by first associating with the 5′ cap of the mRNA that undergoes translation. Following the subsequent recruitment of the 40S and 60S ribosomal subunits, the complex initiates protein synthesis at the first “AUG” codon (start codon) (Fig. 1) [10]. This mode of translation which requires binding to mRNA cap structure is termed “cap-dependent”. Almost all eukaryotic proteins are synthesized by eIF4F mediated cap-dependent translation [11].

Each eIF4F subunit plays a unique and vital role in initiating cap-dependent translation. The 25 kDa eIF4E subunit binds to the 5′ m^7GTP cap of mRNA with high affinity. Due to its scarce availability, eIF4E confers rate limiting properties onto translation initiation [4, 8]. Following cap binding, eIF4E associates with the scaffold protein eIF4G. The dimer then facilitates the recruitment of free cytosolic eIF4A (eIF4Af) (Fig. 1). Interaction among all three subunits is critical for eIF4F function and therefore the trimer formation is regulated under various biological conditions in order to modulate translation. Under cellular conditions in which the rate of translation is low, eIF4E is prevented from binding to eIF4G by eIF4E binding protein (eIF4E-BP) [4, 12]. However, translation may begin with an appropriate translation stimulus, such as an activated PI3K/Atk/mTOR pathway (Fig. 2), which will enhance the phosphorylation and subsequent dissociation of eIF4E-BP from eIF4E [13]. The delicate interaction between eIF4E and eIF4G or eIF4E-BP is dependent on a conserved Tyr-X-X-X-X-Leu-X recognition moiety (X: Leu, Met, or Phe) that exists within the N-terminal region of eIF4G and eIF4E-BP [14-16]. Although this motif allows both eIF4G and eIF4E-BP to attach to a conserved region of eIF4E, the binding of eIF4E to eIF4G is much stronger as the interaction is not limited to the conserved recognition moiety [15, 16]. This strong interaction and subsequent co-folding of eIF4E and eIF4G mediate the allosteric induction of conformational changes in the active site of eIF4E. These conformational changes greatly enhance eIF4E cap binding and hence eIF4F function [14, 16].

Although it is evident that both eIF4E and eIF4G are crucial for cap-dependent translation initiation, eIF4A is also essential for proper eIF4F function. The most abundant eIF4F subunit, eIF4A, has been demonstrated to have RNA helicase activity [17, 18]. Using the energy derived from ATP hydrolysis, eIF4A contributes to eIF4F function by unwinding various secondary structures in the 5′ untranslated region (5′ UTR) of the mRNA undergoing translation [4,17]. In addition, following the coupling of eIF4A and eIF4G, specific domains within the tertiary structures of the eIF4A/eIF4G dimer facilitate the recruitment of the 43S ribosomal subunit to both capped and uncapped mRNAs. eIF4G contains two eIF4A binding domains (Fig. 3). The carboxyl terminal domain (CTD) and amino terminal domain (NTD) of the eIF4A are able to bind to the middle region and CTD of eIF4G, respectively [17]. Moreover, when bound to eIF4G, the conformation of eIF4A is shifted from the inactive dumbbell-shaped conformation to the active closed conformation. This conformational shift promotes the binding of both ATP and mRNA to eIF4A. Hence, the helicase activity of eIF4A is significantly enhanced [17, 18]. Evidently, each subunit of eIF4F is essential for the proper initiation of translation and therefore, all subunits serve as attractive targets for the development of novel chemotherapeutic agents.

Mechanisms of eIF4F inhibitors

Direct interaction with eIF4F

Due to the fundamental role of eIF4F in translation initiation, it is expected that the inhibition of one

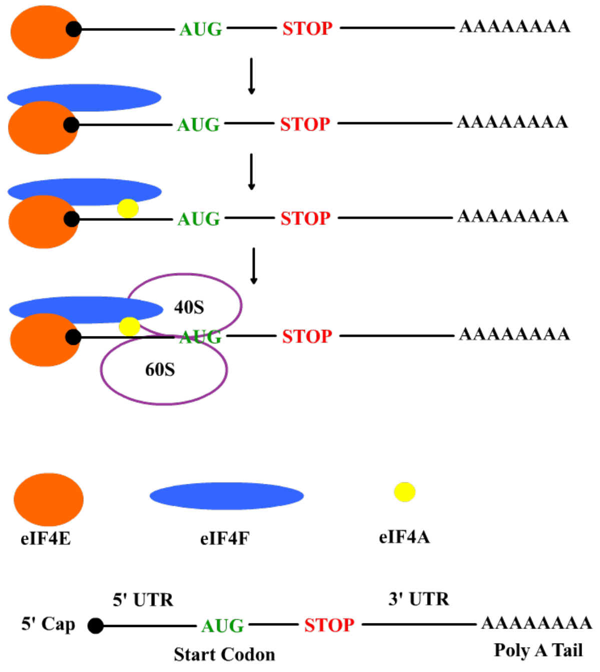

Figure 1. Mechanism of eIF4F mediated translation initiation. eIF4E binds to 5’ cap structure of mRNA. eIF4G binds to eIF4E and recruits eIF4A. Association of eIF4G and eIF4A promotes the recruitment of ribosomal subunits. eIF4A acts as a helicase and unwinds secondary structures in 5’ UTR.

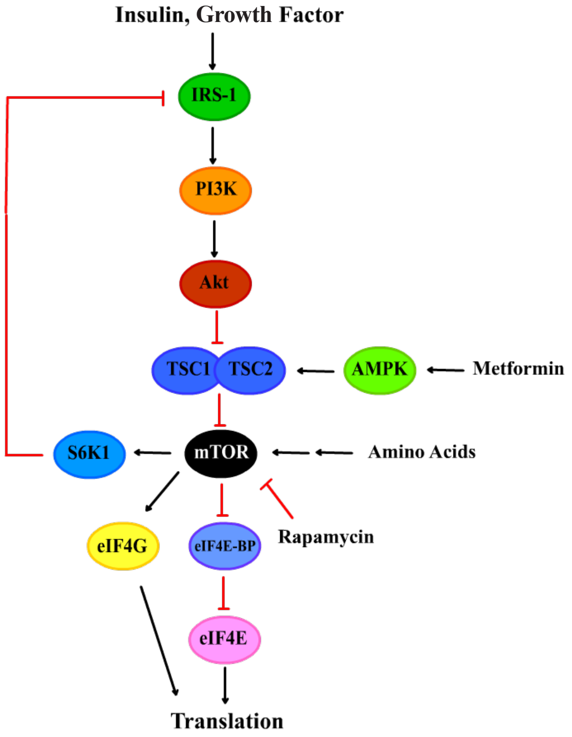

Figure 2. IRS1/IP3/Akt/mTOR pathway targeting eIF4E. Activation of IRS1 leads to downstream signalling of eIF4E-BP hyperphosphorylation by mTOR. Rapamycin can target mTOR and inhibit its kinase activity. Thus eIF4E-BP’s binding affinity for eIF4E is enhanced, resulting in translation inhibition.

or more eIF4F subunits will downregulate eIF4F availability and ultimately inhibit translation. Therefore, the development of eIF4F inhibitors involves the screening of various compounds that target and inhibit eIF4F subunit activity.

Small molecule eIF4F inhibitors are expected to suppress the expression of oncogenes because the mRNAs of multiple oncogenes require significantly greater levels of eIF4F as compared to other types of mRNA [19]. For example, oncogenes such as c-myc and Bcl-xL express mRNAs with long and highly structured 5′UTRs [19]. Because of this unique property, these mRNAs require high eIF4A levels in order to trigger protein synthesis [20]. Clearly, as these mRNAs sequester eIF4A and thus diminish overall eIF4F availability, rapidly dividing cancerous cells must overexpress eIF4F for cell growth and maintenance.

In 2006, Bordeleau et al. identified hippuristanol as a promising candidate eIF4F inhibitor that selectively targets eIF4F and cap-dependent translation [21]. Their study in Krebs-2 extracts demonstrated that hippuristanol can inhibit cap-dependent translation by more than 60% at a dosage of merely 1 μM, while not affecting prokaryotic protein synthesis, which is cap-independent, in *E. coli* S30 extracts at a concentration as high as 50 μM [21]. Using poliovirus, Mazroui et al. further confirmed hippuristanol's targeting selectivity [22]. As an extract from the coral *Isis hippuris,* hippuristanol is a cytotoxic polyoxygenated steroid. Bordeleau et al. demonstrated that hippuristanol weakens the RNA-binding activity of eIF4A [21]. It was hypothesized that hippuristanol binds reversibly to multiple conserved motifs within the C terminus of eIF4A (motifs IV, V and VI). Motif IV is thought to couple mRNA binding with ATP hydrolysis. Motifs V and VI are believed to assist in the formation of the active closed conformation of eIF4A. Since ATP binding, mRNA binding and an active conformation are required for eIF4A function, hippuristanol may directly or allosterically impair the mRNA binding and helicase activity of eIF4A [21].

Similar to hippuristanol, pateamine A also targets eIF4A. However, pateamine A utilizes a mechanism that is both distinctive and moderately counterintuitive [23, 24]. Interestingly, pateamine A enhances the ATP/mRNA binding and helicase activity of eIF4A; it is the only translation inhibitor that is also an agonist of eIF4A activity. However, in addition to enhancing eIF4A activity, pateamine A also induces eIF4Af (free cytosolic eIF4A) to unwind cytosolic mRNAs that do not participate in translation [23, 24]. Furthermore, pateamine A causes eIF4A and mRNAs to remain tightly bound so that these eIF4As cannot regain their free cytosolic form. By reducing the amount of eIF4Af available to be incorporated into eIF4F complexes for translation initiation, pateamine A effectively interferes with protein synthesis at the cellular level [23-25]. In comparison to hippuristanol, pateamine A has a higher efficacy under similar conditions [22, 23]. However, scientists are currently struggling to construct a framework within which pateamine A operates. This is because of its irreversible binding to eIF4A, which could potentially lead to eIF4A degradation and permanent impairment of the translation initiation machinery [23].

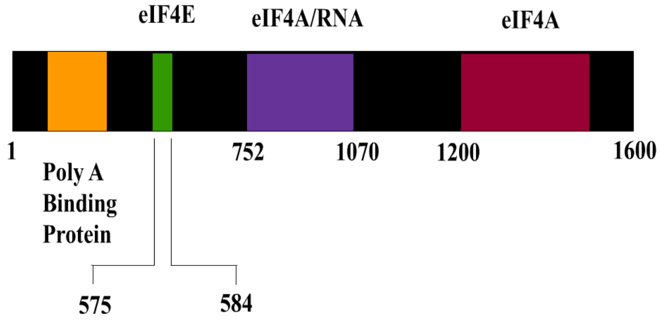

Figure 3. eIF4G binding sites for eIF4E and eIF4A.

Although eIF4A is the most common target of all known eIF4F inhibitors, eIF4E conveys rate limiting properties onto translation initiation and has therefore become the focus of many protein translation researches. eIF4EGI-1, a compound recently discovered by Moerke et al., is among a small number of molecules that are able to impair the activity of eIF4E [14]. Moerke et al. demonstrated that one of the two eIF4EGI-1 isomers preferentially and reversibly binds to eIF4E. By clustering around the eIF4G binding site of eIF4E, eIF4EGI-1 is able to displace eIF4G from eIF4E; this property of eIF4EGI-1 was demonstrated both *in vitro* and *in vivo*. Interestingly, as the interaction between eIF4EGI-1 and eIF4E also enhances the binding of eIF4E-BP to eIF4E, it was determined that eIF4EGI-1 does not interact with the Tyr-X-X-X-X-Leu-X motif of eIF4E. In addition to disrupting eIF4F function, eIF4EGI-1 was also found to promote both DNA and nuclei fragmentation in Jurkat cells and may therefore effectively induce apoptosis [14]. Since only a limited number of eIF4E inhibitors have been well characterized, it is difficult to make valid comparisons between eIF4EGI-1 and its counterparts.

Indirect interaction with eIF4F

The number of signaling pathways that indirectly regulate eIF4F activity is significant. Activation of the majority of these pathways results in the phosphorylation and subsequent release of eIF4E-BP from eIF4E. One such pathway is the IRS-1/PI3K/Akt/mTOR pathway (Fig. 2), which has been implicated in the regulation of cell proliferation [26]. When IRS-1 is stimulated, mTOR, a 289kDa Ser/Thr kinase, is activated and subsequently hyperphosphorylates eIF4E-BP [27]. This phosphorylation cascade results in the dissociation of eIF4E-BP from eIF4E and the formation of a functional eIF4F complex.

As the compound rapamycin was determined to interfere with mTOR activity, this molecule shows promise as an anti-cancer agent and is being evaluated in clinical trials [28, 29]. One recently published phase III trial revealed rapamycin's potential in combating advanced renal cancer, which is a condition that usually limits patients' median overall survival to no more than six months. Rapamycin was able to improve the median overall survival to 10.9 months [30].

Although no eIF4F inhibitor has yet to transition from the laboratory to the clinic, a small subset of approved drugs has demonstrated the capacity to inhibit translation. As these approved drugs are often designed to treat conditions other than cancer, it is becoming increasingly evident that non-chemotherapeutic agents may also be successful in the treatment of cancer. For example, metformin has been used to indirectly inhibit translation in, and the proliferation of, MCF-7 breast cancer cells *in vitro* [31]. Also, Schneider et al. had previously observed similar inhibitory effects of metformin in pancreatic cancer progression in a hamster model [32]. The effects of metformin on cancerous human subjects have yet to be studied. Originally designed to treat type II diabetes, metformin acts by elevating hepatocyte glucose output while simultaneously counteracting the insulin resistance of insulin receptor tyrosine kinases [31, 33]. By activating 5'AMP-activated protein kinases (AMPK), metformin facilitates the phosphorylation of mTOR and subsequent activation of TSC2. Upon mTOR phosphorylation, eIF4E-BP phosphorylation and therefore eIF4E activity are inhibited [31]. Apparently, an appropriate eIF4F inhibitor need not directly interact with eIF4F itself.

Future Research

Translation is a crucial component of cancer cell proliferation. Although the development of translation inhibitors for the purpose of chemotherapy is a recent phenomenon, this strategy in combating cancer has quickly attracted attention from the scientific community. Although the most common method of translation inhibitor development is small molecule screening, the purification and storage methods of many such compounds are arduous. Moreover, the natural sources of these compounds may be scarce. For these reasons, research into the *de novo* synthesis of eIF4F inhibitors is increasing. The synthesis of episilvestrol, an eIF4F inhibitor that mimics the action of its natural but scarce stereoisomer, silvestrol, is testament to the potential of synthetic drug development [34]. The synthesis of pateamine A is another product of eIF4F inhibitor synthesis advancement [24]. Regardless of the method of development, the number of currently identified eIF4F inhibitors is extremely limited. Therefore, researchers are attempting to identify more compounds, such as eIF4EGI-1 mentioned previously, that are able to interrupt the interaction between eIF4A, eIF4G, and eIF4E.

Despite the fact that thousands of translation inhibitors are screened and examined *in vitro* on a daily basis, the concept of translation inhibition is still new to the field of cancer research. In addition, the availability of appropriate animal models for the *in vivo* analysis of such compounds is limited. Although numerous and extensive *in vitro* and *in vivo* studies must be performed prior to the successful transition of eIF4F inhibitors from the laboratory to the clinic, the increasing demand for novel cancer treatments is motivating the development eIF4F inhibitors as novel chemotherapeutic agents.

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Magnetometrical dike hunt: a Grenvillian expedition

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Abstract

A dike is an intrusion into pre-existing rock by an igneous body. Since the magnetism of the pre-existing rock is usually significantly different from that of the intrusion magnetometers can be used to detect the presence of a dike. The aim of this project was to detect the presence of dikes at two locations in Ontario near the French River. The first location was along Highway 11 near Trout Creek where a dike is visible in the recent rock-cut. This location was chosen to act as a control for the second location on Highway 69 near Nobel. Identifying a dike here would allow for the westward extension of a known dike system. A Proton Precession Magnetometer (PPM) was used. It measures the total strength of the Earth's magnetic field at a single point. The Earth's magnetic field is affected by magnetic bodies in the subsurface. Therefore, a disturbance of the magnetic field strength allows us to infer the presence of a magnetic body at various depths. Size and other properties of the magnetic body can be obtained by fitting the measurements to computational models. The Trout Creek dike appeared to be a clear magnetic anomaly, 90m wide with an amplitude of 1.7×10^3nT. This fits a model of a thin dike 75m wide extending 85m deep. At Nobel we did not confirm the existence of a dike, only finding small anomalies of less than 1.3×10^3nT, likely caused by artifacts such as water pipes. This research was conducted to better understand mountain formation and plate movement.

Keywords: dyke; magnetism; geophysics; grenville; dyke-swarm

Introduction

As magma seeps toward the Earth's surface and hardens, it can take on a variety of large-scale shapes. When magma hardens inside a fissure, the resulting formation is called a dike. A dike's size ranges generally from less than a metre to tens of metres across a small dimension [1]. Dikes often are made up of soft rock, which leaves a valley upon erosion. Clusters of dikes formed together are known as swarms; such swarms feature prominently across the Canadian Shield.

A dike swarm is a localized event, temporally and physically, giving a geologist an array of points fixed in time and space, which one can use as a reference in the study of mountain building and plate movement [3]. Better knowledge of plate motion and mountain building is critical in the understanding of natural disasters, such as landslides and floods, when combined with research into the origin of life and local ecology [3].

The dike swarm we investigated partially covers the Grenville Province on the southeastern edge of the Canadian Shield. The Grenville Province is an eroded mountain belt formed almost 1 billion years ago [1]. The dike swarm is approximately 500km long, and may be related to the resulting mountain-building process or tectonic activity [2].

Magnetometry is used to determine the existence or size of a buried dike if the dike's magnetism varies from that of the surrounding rock. We used this technique to conduct our measurements at a valley near Nobel, Ontario along Highway 69 to examine the region for dikes. Were one to be found, it would belong to the Grenville swarm, which is shared with a family of dikes near Trout Lake, Ontario.

Materials and Methods

For our magnetic survey we used a Proton Precession Magnetometer (PPM). The PPM used was a GSM-19G Overhauser rented from Gem Systems (Supp. Fig. 1). The instrument carries a GPS system, which synchronizes every reading with the appropriate location. A PPM reads the total magnetic field at a point in space. This measurement is the sum of the earth's magnetic field, solar-influenced variation, and the magnetic fields of nearby bodies. The latter can alter the magnitude of the total magnetic field; this alteration is called an anomaly.

The device is attached to a backpack that elevates it to approximately two meters above the ground level to prevent recording small surface anomalies – such as pop tins and magnetic rubble. At a regular walking speed, this results in readings slightly over a metre apart. The PPM was set to record one measurement every second.

The PPM also contains a hydrogen-rich fluid. The magnetic dipoles of this fluid naturally align along the direction of the magnetic field to be measured. A current then induces an artificial magnetic field, causing the dipoles to realign along this new direction. When the current is turned off, the dipoles revert. In the same way that a pendulum does not immediately rest at equilibrium upon release and swings past it, the dipoles do not align perfectly, but continue oscillating. This oscillation induces a current in the wires surrounding the liquid that is proportional to the magnetic field strength at the point where the reading is taken

To measure the magnetism of a dike, we mapped out the area to be surveyed and made note of any potentially-magnetic objects like guard rails or drain pipes that might influence our reading. Drain pipes give readings similar to that of a dike, making it crucial to note their existence. Another consideration was the daily fluctuations in the Earth's magnetic field caused by the sun, known as diurnal variations. These can also have a considerable impact on the data, especially if the anomaly produced by the object is small.

Magnetic susceptibility is the degree to which a body will create a magnetic field when under an external magnetic field. It is obtained either experimentally from a sample, or approximated after identifying the rock type and considering the susceptibility of similar rocks. Remnant magnetization is the property of a rock to partially retain a component of its previous magnetization. This is particularly useful when geoscientists track plate movement, despite its inherent problems in determining total field strength [1]. The remnant magnetization may also influence the shape of the anomaly. However, unless the body is made of metal, this effect is usually small and does not always demand consideration [4].

The Earth's molten core creates a magnetic field surrounding the Earth similar to a bar magnet, which is a dipole with a positive and negative pole. This is the Earth's background magnetic field. Due to the way the magnetization is created, the field is not completely uniform and changes over time. The background magnetic field can be calculated online using your exact position and inputting that into the International Geomagnetic Reference Field (IGRF) calculator on the USGS website [5, 6].

Theoretical models are useful in geomagnetics because they allow for easier interpretation of the data. The model used in this study [7] requires knowledge of the variables discussed above to produce an anomaly of an arbitrary 2-dimensional object. An infinitely long two-dimensional approximation is usually appropriate given that the ends of a dike have practically no effect on the field anywhere but near the ends. Dikes of varying width and depths may have differences in their predicted field strength [7] (Supp. Fig. 2). The magnetic anomaly of a body is typically represented by a positive value accompanied by a negative one because the magnetic field is a dipole field [8]. A deeper dike has greater maximum positive field strength and less of a negative field. A wider dike creates a wider anomaly with similar field strength. Changes in magnetic susceptibility also increase with width and magnitude.

Results

Our research was conducted over a period of two days in early November 2007 at the Nobel and Trout Creek locations.

At Nobel, surveys were taken along both roadsides (Fig. 1). The first 250 m of each run resulted in small or brief anomalies. At about 275m, both runs detected a magnetic disturbance: -815.12 nanoTesla (nT) on the east side, and +1858.8nT on the west, relative to the background field. Beyond 300m, near the end of the survey, both observers detected strongly-negative fields (-1500nT on the east, -2000nT on the west).

There was agreement among every survey of the Trout Lake area of a positive field at 225m (+1164.8 nT) and a negative field at 325m (-258.7 nT) (Fig 2). Smaller anomalies (appearing at 115m and 265m) exist within the data and can be superimposed onto the significant anomaly. These smaller anomalies also appear in the Nobel data, most notably the curve at the 100 m mark.

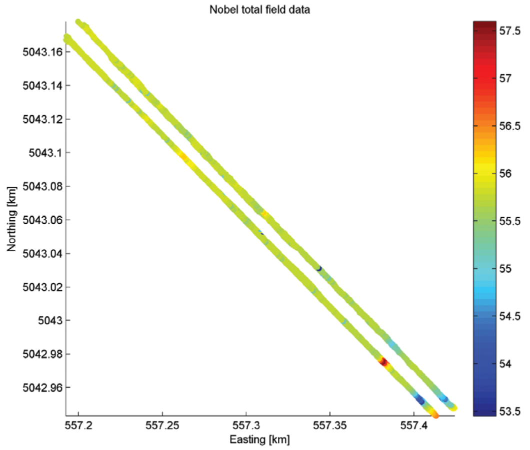

Figure 1. Map of readings taken at Nobel; total field strength at each location is denoted by colour. The colour bar on left is in 10^4 nanoTesla (nT), and the axes are based on the UTM coordinate system. We surveyed both sides of the road.

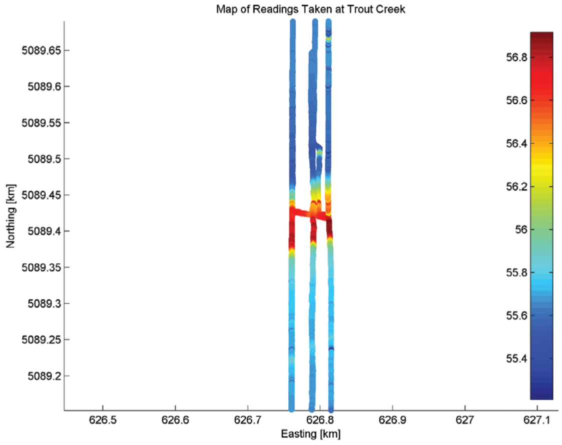

Figure 2. Map of readings taken at Trout Creek; total field strength at each location is denoted by colour. The colour bar on left is in 10^4 nanoTesla (nT), and the axes are based on the UTM coordinate system. Here, we surveyed both sides, as well as the grassy median of the highway.

Discussion

Our data show anomalies resulting from factors other than the dike itself. These need to be identified in developing a clearer model of the dike. Any sharp spike observed over a relatively short distance was likely caused by a vehicle driving past since we took only one reading every second. Thus, a car passing by at a speed of 30m/s would only graph one or two data points, thereby creating a sharp spike. On the other hand, an underlying pipe would produce a reasonably small, smooth anomaly; walking over it at a speed of about 1 m/s would allow several data points to be graphed. These appear as anomalous-looking disturbances superimposed onto the larger anomaly, similar to two waves passing through each other.

The diurnal variations were the first we considered. They are considered significant if they are similar in amplitude to the anomalies we want to interpret. The readings taken at Nobel and Trout Creek were superimposed on the diurnal variations of the days of our study (Supp. Fig. 3). The resulting changes (at most 19.1nT) were insignificant compared to the change caused by the dike (more than 1700nT), suggesting that the data need not be corrected for diurnal effects.

Next, a model closely matching the field generated by the dike was created [7]. The GPS coordinates and height, as recorded by the PPM, were used along with a United States Geological Society (USGS) application to determine the total background field, inclination, and declination as predicted by the IGRF model [6]. The dike swarm under investigation is largely made of mafic intrusions; therefore we considered the average susceptibility of basalt as an approximation [9]. When creating the model, the width of the anomaly was fitted first, and then the depth of the dike was adjusted to fit the amplitude of the field as closely as possible. Achieving an exact fit is unlikely due to immeasurable variables (such as remnant magnetization and more precise susceptibility, among others). As a simplifying assumption, only rectangular shaped dikes were considered. The closest fit was achieved for a dike 70m wide and 85m deep.

Small anomalies were found at Nobel (Supp. Fig. 4). Though there were some promising changes in magnetic fields, others were attributed to smaller anomalies (such as a drainpipe). Measurements beyond our range of study are required to affirmatively eliminate the possibility of dike presence in the area.

Conclusion

By employing the use of magnetometric techniques, we were able to estimate the depth of the known dike at the Trout Creek location. This dike is approximately 70m wide and 85m deep. Conversely, we were unable to confirm the presence of a dike at the Nobel location. There is a need for further investigation in the area, since strong magnetic fields lacking readily explainable sources were observed near the end of our trials.

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Supplementary figures are available online at the JULS website: http://juls.sa.utoronto.ca/

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Ground-penetrating radar survey: the search for the foundation remnants of the first Huron Street Public School

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Abstract

Ground-penetrating radar (GPR) uses the principle of scattering of electromagnetic waves to locate buried objects. This is a non-destructive and non-hazardous technique which aids in assessing archaeological, forensic, and geological sites. The purpose of this study was to detect the sub-surface remnants of the old Huron Street Public School which had been demolished in 1956. We assumed that the foundation of the old building still remains in its original location, which has since been turned into a playground. Our experiment was used to evaluate the feasibility of GPR for the required archaeological assessment of terrain before any construction can occur. GPR was used to image the subsurface of the Huron Street Public School's playground with an approximate area of 30 metres by 60 metres. The total area was divided into grids incorporating parallel lines approximately 10 metres apart that marked the starting point of GPR measurement. We found several prominent subsurface reflections that we concluded mark the foundation of the old building. Our results confirmed that the asphalted area of the basketball court differed significantly from the grass area in the radar sections. Small diffraction hyperbolas could be correlated to the position of trees and thus interpreted as roots. A strong reflector coming at 120 nanoseconds along one of the West-East lines of the study area was most likely the remnant of the old building's foundation, since its position appeared similar to that determined from a photograph of the school taken in the 1890s. The photograph was not used as conclusive evidence but rather as a guide. These findings of the school playground were promising and contributed directly to the main aim of this study. We suggest an in-depth survey covering a larger area to map the full extent of the old school building.

Keywords: archaeology; foundation; geophysics; remote sensing; urban environment

Introduction

Ground-penetrating radar (GPR) is used to image the shallow subsurface by propagating radar energy downward into the ground from a transmitting antenna and collecting the reflected energy from subsurface boundaries between media possessing different electromagnetic (EM) properties by a receiving antenna. The fundamental property most valuable in a GPR survey is commonly known as the dielectric constant of a material [1]. It is a critical parameter because it controls the propagation velocity of electromagnetic waves through a material and the reflection coefficients at interfaces, as well as affecting the vertical and horizontal imaging resolution [2]. Therefore, knowing dielectric constant values of materials helps in planning GPR surveys and in better understanding and interpreting GPR images. GPR is non-destructive, yet it is effective in many areas of field research such as detection of underground structures, fracture zones, archaeological remnants and subsurface oil contamination [2]. Strong GPR data gathered for various subsurface structures like sedimentary rocks and even archaeological settings are presented in research done by Grasmueck et al., who developed a 3D image of such structures [3].

In this study, we surveyed the Huron Street Public School at Huron and Lowther Streets in downtown Toronto. Supplementary Figure 1 shows a view of the old school building which was torn down in the late 1950s [4]. The new school was built just to the south of the old school. We expected that the remains of the foundation were still buried under the school playground. GPR was the most suitable approach in locating the foundation of the old school because of its speed and the expectation of a strong dielectric contrast between the soil and the remaining foundation. Our research would substantiate the process of construction and help in the pre-construction analysis of a particular area in a manner which is simple, safe and cost-effective in the long run.

Materials and Methods

GPR works in a similar fashion to ultrasound imaging in medicine; however, the latter process uses lower frequency sound waves [5]. In comparison, GPR uses radar waves to image the subsurface. The transmitter emits short pulses of electromagnetic waves, with frequencies between 25 MHz to 1000 MHz, which penetrate into the ground where they are reflected by any object with contrasting electrical properties. The receiver detects and registers these reflections side-by-side as radargrams in real-time on the screen of the control unit [6]. Usually a GPR instrument is moved along the ground, rather than being fixed; this allows it to acquire responses at regular intervals.

Because a radargram shows reflections coming from different directions, we often see artefacts in single radargrams as well as in radargram sections, for example diffraction hyperbolas (Supp. Figs. 2, 3) [2].

The reflection time (two-way time) for the wave to be transmitted and reflected back is shown instead of depth, because knowledge of the wave velocities is necessary to convert the two-way times into depths [6]. Thus, the dielectric constant for the medium is important since it affects the propagation velocity of the wave. Water saturation dramatically raises the dielectric content of the soil because the dielectric constant for water is high compared to other materials [7]. This leads to a large increase in two-way time values with increasing water saturation, thereby giving rise to background noise. Due to the previous factor, the reflected EM wave might not even be recorded by the GPR system [2]. Since the playground was damp prior to our study we had to take this factor into account. After our investigation, we made changes to the settings of the Geophysical Survey Systems Inc. (GSSI) software used to process and analyze the data from the GPR unit. A soil sample of known mass was taken from the study site and a procedure similar to distillation was applied. Water accumulated in a separate beaker as the distillate at about 100˚C and the water:soil mass ratio was determined. These values were inputted in the GSSI software which then altered the two-way times by removing the effect of water and thereby reducing the background noise. This is the best approximation the software can achieve under the given circumstances.

The GPR system (Supp. Fig. 4) used in this field experiment was a model SIR3000 with a pair of 400 MHz antennas made by GSSI. The GPR unit was set up to send a pulse every 5 cm of distance covered to provide maximum accuracy. To attain a representative subsurface image of the Huron Street Public School playground, the GPR system was moved in straight lines across the target portion of the playground that included a section of the grass field and a small portion of the asphalt-covered basketball court (Supp. Fig. 5). The lines were created by attaching a string from the origin to the other end of the field in order to keep our GPR steady. A member of our team worked with the GPR cart and another member assisted in keeping the cart along as straight of a line as possible (Supp. Fig. 4). Thus, five lines in the north to south direction and six lines in west to east direction were collected; each was saved as a new file.

Water content changes the dielectric properties of the ground. We noted the fact that the ground at the site was very wet from previous rain spells. Radar velocities for different materials are previously described [7]; knowledge of the velocity allowed the estimation of a depth of reflection on the radargrams.

Results

The sets of results are shown in Supplementary Figures 6 (north to south) and 7 (east to west) in the form of radargrams. These show 250 nanoseconds of data on the vertical scale, while the horizontal scale marks approximately 30 metres for west-east lines (Supp. Fig. 7) and 60 metres for north-south lines (Supp. Fig. 6). Strong reflections are circled and labelled on the radargrams.

The asphalt and gravel layer located on the southeast section of the playground was obvious on the radargrams (Supp. Fig. 7). It produced a unique double reflection (two parallel dark bands marked 1) on the lines 112, 124, 125 and 126 (Supp. Figs. 6, 7). Also, there was a continuous reflector stretching along most of line 113 at about 120 nanoseconds (marked A on Supp. Fig. 6). On the east sections for maps 123, 124, 128, 129, 130 and 131, there were small and sharp hyperbolas (labelled 2); deeper hyperbolas appeared broader and were caused by small point reflectors. Additionally, yellow circles marked a more continuous reflection – that means one that can be followed for several meters – on 112, 114, 124 and 126 and showed larger hyperbolas that were crowded together as on the west sections of 125, 128, 129, 130 and 131. The west-east lines crossed the north-south lines and therefore the same reflectors may appear on several lines such as reflector 1. These types of reflectors provide important hints of what could have produced them.

Discussion

GPR is a technique that assists in evaluating subsurface structures prior to construction or to find any archaeological remains before digging up the land. Because some lines are crossing, and the same reflectors appear at such crossings, a three dimensional extent of these reflectors can be inferred. Such reflectors could be caused by various materials and structures. For example, the reflector labelled as 1 is caused by different surface material: an asphalt-covered basketball court as opposed to the grass cover in the rest of the area (Supp. Fig. 7). Tree roots must take up a considerable portion of the playground's subsurface in the east section as trees were located within this region. Roots, due to their small cross-section, cause sharp diffraction hyperbolas. In particular, roots should be concentrated around a large, old maple tree that is located on the northeastern corner since it is the only large plant that is visible. Indeed, sharp diffraction hyperbolas could be seen on lines 123 and 124 where the GPR equipment must have crossed these roots. Smaller trees were sitting on the eastern portions of lines 128, 129, 130 and 131. There too, sharp and small hyperbolas, marked as 2, were found and were interpreted as roots.

Reflections A, B and C were continuous and not correlated to variations in surface properties. The groundwater table could play a part in producing these reflections. However, the groundwater table would be expected to be continuous in the locations surveyed as the area is flat and underlain by glacial sediments. Thus, the water table would be excluded as a cause for those reflections.

Reflection A is likely to be the wall of the old school building. Line 113 displays this reflection as a long continuous structure in the north-south direction. By comparing how the old school was situated with regards to Huron Street and the fences before it was demolished

(Supp. Fig. 1), the location of reflection corresponded to the wall on the west side. Moreover, the reflection labelled as B in 114 could be interpreted as the main entrance of the old building. Thus, A and B together appear to mark the whole wall section of the old building that existed on the west side. By examining the scales associated with the satellite image, it can be assumed that the wall was about 7 meters away from the road.

Reflections labelled C that appear in lines 112, 124 and 126 on the radargrams may correspond to the internal structures of the old building, since they were situated in an area that correlates to a possible location of this section of the building as observed in Supplementary Figure 1.

It is possible to calculate the depth using the shape of diffraction hyperbolas from small obstacles; however, it is easier to provide a rough estimate. Propagation velocity of radar wave of wet soil is approximately 10 centimetres per nanosecond [2] and therefore 120 nanoseconds two-way time corresponds to a depth of around 60 centimetres. Therefore, it could be postulated that foundation walls of the old school building could be found by digging down about 60 centimetres along line 113.

Similar researches to this study were previously carried out on outlining building structures using GPR. In 2005, Abbas et al. [8] obtained strong GPR reflections from the foundation walls of the Islamic Museum in Cairo. More recently, Barone et al. [9] used GPR to identify the foundation of a temple in Sicily. The foundation of the temple needed to be reinforced and Barone used GPR to find its exact position rather than attempting to excavate an object without any knowledge of its location [6]. Both studies interpret strong, linear reflectors similar to the ones presented here, corroborating our own interpretation.

Conclusion

There is a great need for the ability to evaluate subsurface parameters without disturbing the ground. It is essential that any technology used to detect, identify and locate such buried material be capable of scanning large surface areas rapidly and efficiently in the presence of disturbances.

We have found strong GPR reflections beneath the playground at the Huron Street Public School that were interpreted to be foundation walls. A follow up study should include more precise positioning. More lines at shorter spacing to each other could provide a more detailed three-dimensional map of the foundation. As this study was limited to a grid of orthogonal 2D lines, much closer lines (with 10 cm spacing) should be prepared to gather full 3D data from the study area. In addition, the study would have to expand across a tennis court on the east side of the playground and closer to the present school on the south side to increase the chances of finding the other walls of the old building. Nevertheless, this study clearly confirmed the presence of the front wall of the old public school and the data is informative as it was simple to achieve and very reliable.

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Supplementary figures are available online at the JULS website: http://juls.sa.utoronto.ca/

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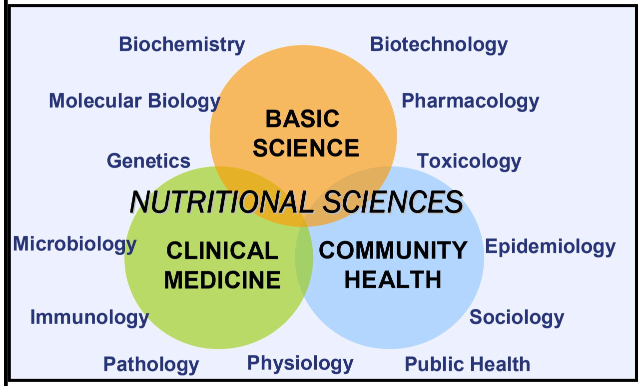

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