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ON THE COVER

Designed by DEBBIE GELTNER

In recognition of the 200th anniversary of Darwin's birthday and the 150th anniversary of his seminal treatise on evolution, *The Origin of Species*, our cover depicts a variety of organisms from three of the taxonomic kingdoms as proposed by Prof. Robert H. Whittaker in 1969. On the back cover is a dodecahedron, a shape made up of 12 pentagons and one of the five Platonic Solids that has preoccupied notables such as Plato and Kepler. The background image is a photo taken by the Hubble Telescope of the M 78 reflection nebula found in the Orion constellation complex.

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The editorial board would also like to extend its gratitude to the professors, post-doctoral fellows and students who participated in our peer-review for their time and expertise. The strength of our editorial board relies on their valuable input.

Finally, we wish to thank the student contributors for their tireless efforts and dedication to the scientific process.

HM: A Legacy in Neuroscience

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"The problem of neurology is to understand man himself." Walking by the Montreal Neurological Institute (MNI) and Hospital, one might give brief regard to these words coined by Wilder Penfield and prominently displayed on the edifice of the building. It is unlikely, however, that most passersby would have an accurate grasp of the groundbreaking work that has been undertaken by scientists at this institute – research that has delved into the inner workings of the human mind. During the 1930s, Penfield developed the surgical treatment of epilepsy by means of temporal lobe resection, and this procedure became a standard among many neurosurgical centres (Greenblatt, Dagi, et al. 1997). The MNI is also where Brenda Milner began her groundbreaking work in the field of neuropsychology, most notably with a patient who until recently was simply known as HM.

Nowadays, it is a rare occurrence that one can attribute significant advances in science to a single individual, especially one with no post-secondary education or formal training. However, no neuroscience textbook would be complete without mention of HM, a patient suffering from severe non-localized epilepsy and perhaps the most studied individual in the field (Corkin, 1984). H.M. was born in 1926, and at the age of 7 was involved in a bicycle accident that left him unconscious for 5 minutes. In addition, he suffered a laceration to the left supra-orbital region of his head (Corkin, 1984). At the age of 10, HM began exhibiting minor seizures, during which he would cross his arms and legs, open his mouth, close his eyes, and generally exhibit a lack of responsiveness. By the time he was 16, the seizures had progressed to major attacks. These general convulsions included tongue-biting, urinary incontinence, loss of consciousness, and ensuing drowsiness (Scoville and Milner, 1957). It is believed that the bicycle incident was linked to the onset of epilepsy, though the basic radiological studies and physical examinations available at the time showed normal findings (Scoville and Milner, 1957). This may, however, have only been a reflection of the state of imaging technology at the time and it is unclear whether modern neuroimaging techniques would have identified abnormalities. HM's family history shows a presence of epilepsy, which may be indicative of an unrelated causative factor (Corkin, 2002). Nonetheless, the etiology of HM's disorder still remains inconclusive.

In 1953, at the age of 27, HM underwent an experimental procedure in order to alleviate the severe epilepsy that had remained largely unresponsive to anticonvulsive drug therapy. At this point, he was having 10 petit mal seizures a day and at least 1 major seizure per week (Corkin, 1984). Electroencephalographic recordings indicated diffuse abnormalities; hence, the decision was made to surgically remove the medial temporal lobe structures, which were known to have epileptogenic qualities (Scoville and Milner, 1957). The bilateral medial temporal lobe resection extended 8cm posteriorly from the temporal tip, including the amygdaloid complex, temporal pole, and a large part of the hippocampal formation (Scoville and Milner, 1957). During the surgery, HM was fully conscious and talking (Corkin, 1984). The surgery succeeded in abating the severity of HM's seizures and he recovered from the surgery without any complications. However, there was one completely unexpected side effect of the surgery: severe anterograde amnesia, characterized by a loss of ability to formulate new memories (Scoville and Milner, 1957).

The beginning of the nineteenth century marked the onset of the memory debate in the neuroscience community. Specifically, scientists began to question where memory is stored and the extent of its localization in the brain (Greenblatt, Dagi, et al. 1997). In the early half of the twentieth century, Karl Lashley, a prominent neuropsychologist, conducted studies in rats that led to his theory of mass action. Removal of cortical areas of rat brains did not show any evidence of memory storage localization, but rather demonstrated that the extent of memory deficit is proportional to the amount of cortical tissue removed (Greenblatt, Dagi, et al. 1997). Opponents of this theory, notably Donald Hebb, proposed an alternate view. Hebb theorized that "assemblies" of cells work together to represent information and that these complexes are widely distributed. In the event of a localized lesion, the distributional nature and significant number of interconnected cells would ensure continued functioning (Milner, Squire, et al. 1998).

After performing the procedure on H.M. and recognizing the unexpected amnesic syndrome, William Scoville invited Brenda Milner to Connecticut to systematically evaluate HM's condition using neuropsychological methods. In 1957, Milner and Scoville published what would soon become a groundbreaking paper on memory. The surgeon and neuropsychologist described the results of testing of HM and nine other patients, who had been treated for psychosis using neurosurgical methods similar to those performed on HM (Scoville and Milner, 1957). HM was a unique case to consider since he did not suffer from psychosis, and his surgery was "frankly experimental" (Scoville and Milner, 1957). At the onset of extensive testing in 1955, memory deficits were immediately apparent – HM gave the date as March 1953 and his age as 27 (Scoville and Milner, 1957). Milner and Scoville (1957) also noted that "he reverted constantly to boyhood events and seemed scarcely to realize that he had had an operation." HM's IQ on the Wechsler-Bellevue Intelligence Scale actually improved from a preoperative score of 104 to a postoperative 112 due to the reduction in seizures (Scoville and Milner, 1957). In contrast, his score was determined to be 67 on the Wechsler Memory Scale, far below average for someone of his intellectual capacity (Scoville and Milner, 1957). A battery of tests confirmed Milner's suspicions: HM suffered from a complete loss of memory for all events occurring after the surgery, and a partial retrograde amnesia for three years preceding the surgery (Milner, Corkin, et al. 1968). Studies performed later in 1985 showed that this retrograde amnesia extended to include a period of 11 years prior to surgery (Corkin, 2002). Memories of his early life events and his pre-surgical personality remained unaffected.

Further testing also demonstrated the pervasiveness of HM's memory disorder. He was severely impaired regardless of the type of memory test, the nature of the stimulus, or the sensory modality through which the test was delivered (Milner, Corkin, et al. 1968; Corkin, 2002). He was unable to successfully acquire long-term episodic memory (events in a spatial/time context) or semantic memory (general knowledge and factual information); however, he had functional short-term memory (Milner, Corkin, et al. 1968; Corkin, 2002). HM was readily able to register new information within his immediate

memory span, but failed recall tests as soon as the information exceeded that time-span or his attention was diverted (Scoville and Milner, 1957). The evidence was progressively mounting for the role of the medial temporal lobe as a consolidation centre of long-term memory separate from short-term and working memory (Squire and Zolamorgan, 1991).

Since the surgery, HM has been examined and tested in a variety of other psychological domains. Milner was the first to demonstrate H.M's preserved learning capabilities in the form of motor learning tasks (Milner, 1970). This finding was achieved using a mirror-drawing task, in which HM exhibited learning without being able to recall any of the sessions in which he engaged in the learning (Milner, 1970). Additional studies have shown that HM withheld preserved residual learning capacities, such as perceptual learning and priming repetition (O'Kane, Kensinger, et al. 2004). Subsequent advancements in radiological imaging technology have allowed researchers to use magnetic resonance imaging to confirm the true extent of temporal lobe damage that HM had acquired. Corkin et al. (1997) found that the temporal lobe lesions were bilaterally symmetrical and included the amygdaloid complex, most of the entorhinal cortex, and approximately half of the hippocampal formation. The parahippocampal cortex was largely spared. The MRI results indicated that the extent of the lesions was less than Scoville estimated at the time of surgery (Corkin, Amaral, et al. 1997). Additional abnormalities included atrophy of the cerebellum and shrunken mammillary bodies. As the authors concluded, "these findings reinforce the view that lesions of the hippocampal formation and adjacent cortical structures can produce global and enduring amnesia."

HM has greatly advanced our understanding of the human brain and cognition. Studies conducted on him, along with the other patients given bilateral hippocampal zone excisions for psychosis treatment, have provided conclusive evidence that the medial temporal lobes are crucial regions for memory encoding. Given this evidence, HM has helped ensure that no other patient has had a bilateral resection from this critical area of the brain. The extent of HM's impairments due to the surgery prompted his surgeon, William Scoville, to campaign against continuation of the procedure (Corkin, 2002).

After the surgery, HM was cared for by his mother. Although quiet in social situations, he appeared at ease and still enjoyed puns and semantic ambiguities (Corkin, 2002). He would often apologize when interacting with other people for his apparent lack of manners, such as forgetting the names of individuals he was just introduced to (Milner, Corkin, et al. 1968). In 1980, HM moved into a nursing home due to the ailing health of his caregivers at home and was reported to have participated in the daily activities such as games, crafts, and poetry (Corkin, 1984). In the early evening hours of December 1, 2008, HM died as a result of heart failure at the nursing home he had lived in for decades.

It is essential that we not forget the humanity behind the person; many of the articles describing his psychological testing are also interspersed with anecdotal tales speaking to his continued sense of humour and social graces. While HM died completely unaware of his monumental contributions to the scientific community, even after his death, his brain will be preserved for further study. In this age of cutting edge technology and research it is easy to forget the humble roots of neuroscience. The loss of HM serves as a reminder of a time not very long ago when this field was in its infancy.

"Every day is alone in itself, whatever enjoyment I've had, and whatever sorrow I've had"

- Henry Gustav Molaison, aka "HM" (1926 – 2008)

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Uncovering fluctuations in atmospheric transmission using the VERITAS pointing monitors

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Abstract

Our project encompasses the creation of software that provides nightly estimates of atmospheric transmission and tracks its long-term fluctuations for the VERITAS telescopes. Using archived image files taken from the pointing monitors of the VERITAS telescope, we wrote software that selects stars of appropriate brightness and quantifies their intensity. We then plotted the star intensity as a function of the secant of the telescope angle from the zenith and observed a linear relationship. The ratio of this slope divided by its intercept has a value that is independent of the stars chosen and is proportional to the length of attenuation of light travelling through the atmosphere. By analyzing nightly data for all four telescopes, one can measure the magnitude of the fluctuations in atmospheric transmission using the ratio of the slope over the intercept. This method will allow improvements in the quality of the measurements taken by the VERITAS telescopes, by giving VERITAS control over the effects of the atmosphere on their star intensity data.

Keywords: *Gamma rays, Cherenkov Radiation, Atmospheric transmissions, VERITAS.*

Glossary

Telescope zenith angle: Angle between the vertical and the direction in which the telescope is pointing.
CCD camera: A charge-coupled device camera measures the motion of electric charges created by the passing of a photon, thus forming an image of the source of photons.
Flux: Amount that flows through a unit area per unit time.
Attenuation length: Characteristic distance required for a signal to reach of its initial strength.
Flat fielding: Calibrating the array of pixels in a CCD camera so that all pixels respond equally to excitation.

Introduction

The Very Energetic Radiation Imaging Telescope Array System (VERITAS) is an array of four telescopes, each 12 m in diameter and located at the base of Mt. Hopkins near Tucson, Arizona. A collaboration of more than 100 scientists from institutions in Canada, the U.S., Ireland and England operates VERITAS to find and measure astrophysical sources of gamma rays with energies in excess of 100 GeV. The VERITAS base camp is shown in Figure 1.

Very high energy gamma-ray astronomy relies on the measurement of Cherenkov radiation in the atmosphere. Gamma rays coming from space collide with the nuclei of air molecules in the earth's atmosphere roughly 10 to 20 km above ground level, producing an "air shower" of secondary particles—mostly electrons and positrons—that hurtle toward the ground. In this process, the entire energy of the gamma-ray is converted to the mass and kinetic energy of the secondary particles. Although the secondary particles travel slower than the speed of light in vacuum, they travel faster than the speed of light in air due to their higher energies. This results in an electromagnetic shock wave comparable to a sonic boom. The shock wave comes in the form of bluish light called Cherenkov radiation. Cherenkov radiation is emitted by all the charged secondary particles and propagates to the ground where it can be measured by optical devices (Hanna, 2007). Using optical telescopes and extremely fast cameras[1], it is possible to measure the Cherenkov radiation and generate an image of the air shower. Typically, a thin, tubular shape is observed that can be used to trace backwards to the origin of the gamma ray (Figure 2).

The image generated from a single telescope is not sufficient to determine the origin of a gamma-ray induced air shower, since there is no indication where the signal originated from along the long axis. However, multiple telescopes positioned in an array allow researchers to view the air shower from several perspectives, resulting in images with different orientations (Figure 3). By superimposing images of the same air shower, the source of the gamma rays can be determined by the intersection of the lines drawn through the long axis of each image.

M.K. Daniel (2007) has shown that the atmospheric regions surveyed by VERITAS display substantial changes in atmospheric transmission both daily and annually. Fluctuations in atmospheric transmission alter the levels of scattering or absorption of Cherenkov radiation, which affects telescopic measurements. To improve gamma-ray detection from the ground, we hope to quantify the effect of atmospheric transmission from archived images taken by the VERITAS pointing monitor, a CCD camera directed towards the telescope's field of view. From these images, it is possible to determine the telescope zenith angle and the intensity of the star that is being examined. By relating these two values, we can calculate the amount of atmosphere that a star's light will have to penetrate before reaching the telescope. Since the amount of atmosphere that the Cherenkov light must travel through increases with the zenith angle, we expect the extinction curve to display a decrease in intensity as a function of the telescope's angle from the zenith.

Methods

Analysis Procedure

Our software finds individual stars in images that are taken from the telescope's pointing monitors every fifteen minutes. The software then filters those stars and selects the fifteen brightest stars from the image, excluding stars that have pixel values above the highest value that the camera can measure (Figure 4). For each star that is chosen, a histogram is created of all the pixels that contribute to its background noise. Each of these histograms is used to measure the mean pixel noise in the neighbourhood of that star.

The mean pixel noise is subtracted from the sum of all

1. The VERITAS cameras contain 499 photo-multiplier tubes (PMT) that are digitised at a rate of 500 mega-samples/sec (Swordy and Brocious, 2007). Each PMT is capable of measuring a single photon and can determine its arrival time to within a few billionths of a second.

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Figure 1: The VERITAS telescope array, located at the base of Mt.Hopkins, Tucson, Arizona

the pixel values within a given radius to obtain the intensity of the star. To determine the most accurate radius to use, the intensity is plotted as a function of the radius. The radius that demonstrates the least change in intensity will best describe the star's actual intensity. The error in the star's intensity can be computed using the error in the radius.

The software tracks the intensity of each star through many images and plots its intensity values as a function of the secant of the telescope zenith angle. Theoretically, it can be shown that the flux of light from a star, Φ (1), follows the relation:

$$\Phi(l) = \Phi_0 - \left(\frac{\Phi_0 h}{l_0}\right) \sec \theta$$

Eqation (1): where θ is the telescope's angle from the zenith, h is the height of the atmosphere, lo is the attenuation length of light in the atmosphere and Φ is the flux as measured above the atmosphere (Hanna, D. 2008). Thus, a plot of star intensity versus the secant of the telescope zenith angle should be linear (Figure 5).

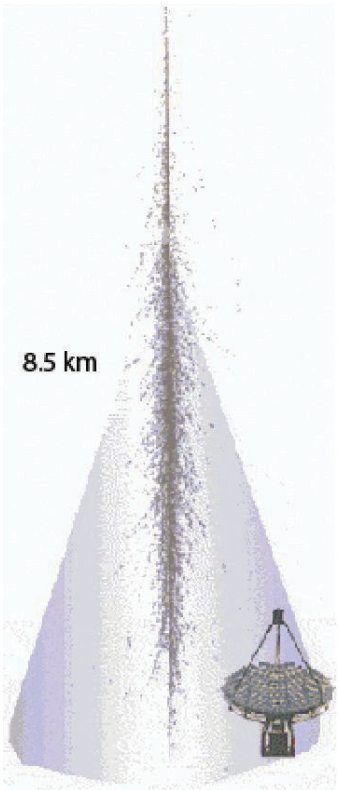

Figure 2: An air shower produced by an incoming gamma ray colliding with the nucleus of a molecule in the atmosphere. (Courtesy of the VERITAS Education Website, veritas.adlerplanetarium.org)

Results

From Figure 5, we see that the intensity of the stars in the pointing monitor images follows a linear relation when plotted against the secant of the telescope's angle from the zenith (Equation 1). Moreover, the ratio of the slope over the intercept of this relation, which is - h/l_0 , is independent of the stars used (Figure 6). In Figure 7 all of the , - h/l_0 values that were extracted from each night's images are plotted with their date (yy/mm/dd) on the x-axis. These are the weighted sums of each value from all four telescopes. The few points with very large errors in Figure 7 are cases where too few stars were found to fit the plots of intensity versus secant of the telescope zenith angle meaningfully, so only a few of these plots were considered in the averaging of data. Figure 7 clearly shows significant fluctuations in - h/l_0 which implicates daily and monthly fluctuations in the atmosphere, as supported by previous research (Daniel, 2007). Due to the large variation in intensity values as a function of the secant of the telescope zenith angle, we cannot be certain that the variations in - h/l_0 , are strictly from fluctuations in atmospheric transmission.

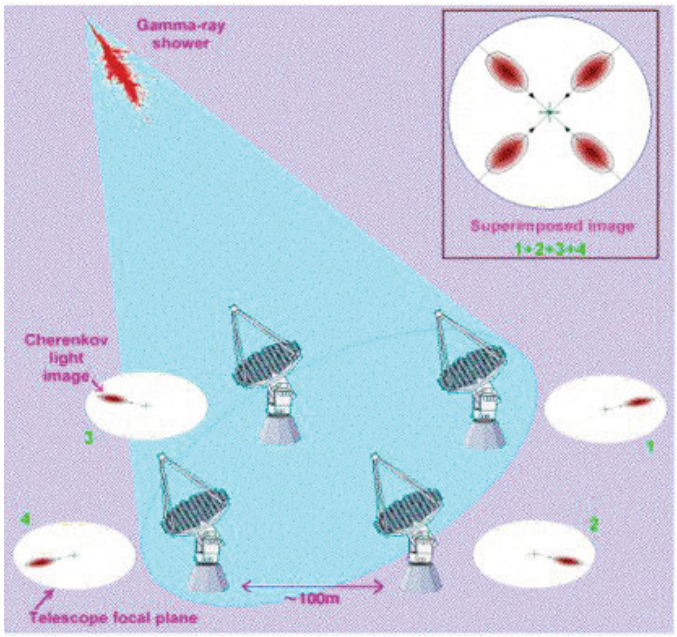

Figure 3: Stereo imaging of a gamma ray induced air shower with multiple telescopes. The turquoise colour represents the Cherenkov radiation. (Courtesy of the VERITAS Education Website, veritas.adlerplanetarium.org)

Discussion

The systematic error in the intensity

In the course of our research, we discovered that the pointing monitors capture images every two seconds and store the star's peak pixel value and its coordinates in a database for the 30 brightest stars in the image. In Figure 8, we see the peak pixel value of one star for four hours as a function of time in minutes. The blue triangles represent times when the telescope changed positions with respect to the star to get a sample of its background in a process called wobbling. We see that when the star changes positions in the image, its peak pixel value jumps significantly. This is emphasized by the plot in Figure 9, where we see a particular star's intensity versus secant of the zenith angle plot compared to the same star sampled every two seconds in the database. The jumps in intensity follow a pattern that is characteristic of a star moving in the image of a non-flat-

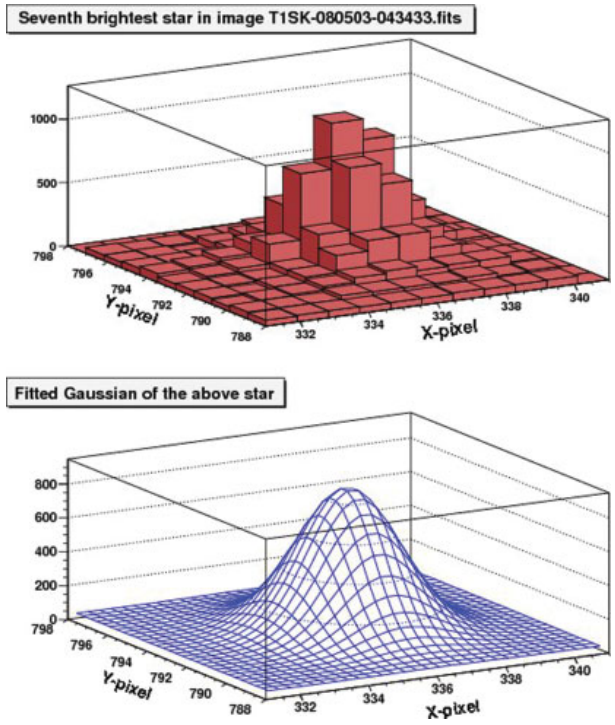

Figure 4: A two dimensional histogram of a star in an image taken by the pointing monitors, along with a fitted Gaussian function. Each bin in the histogram corresponds to a pixel in the image.

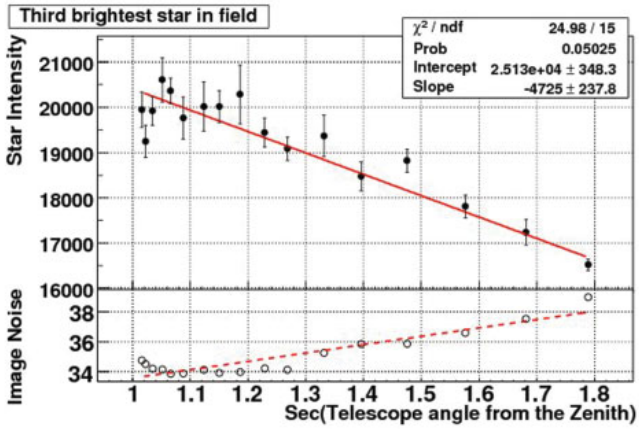

Figure 5: The upper plot in this figure is the star intensity as a function of sec θ, where θ is the zenith angle of the telescope, for a star in a group of 17 images. The smaller plot below is the entire image noise as a function of sec θ.

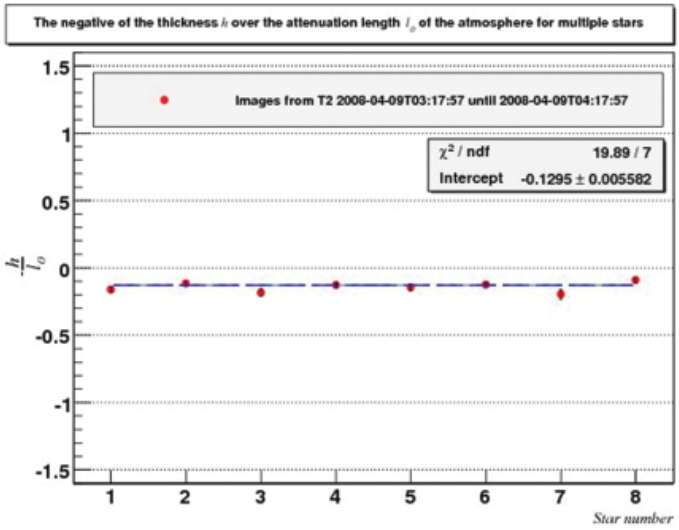

Figure 6: The ratio of the slope over intercept, $-h/l_0$, of linear fits as that in Figure 5. The blue dashed line represents the best fit of the constant that we expect.

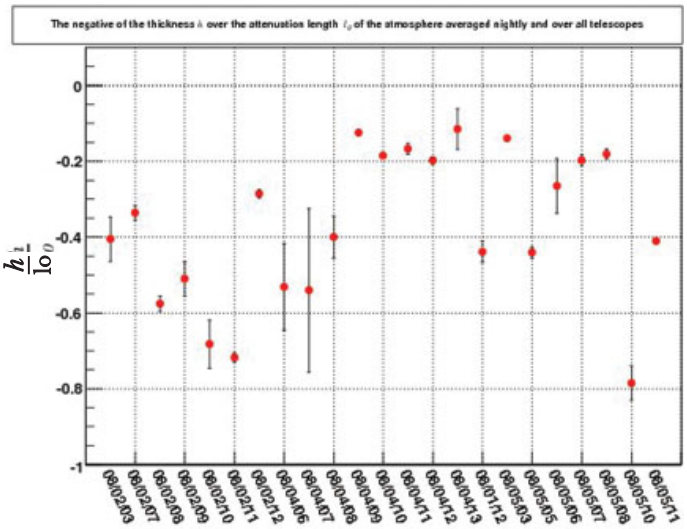

Figure 7: The ratio of the slope over the intercept, $-h/l_0$, weighted for each night by fitting a constant to all the values obtained from that night (as in Figure 6) and then further averaged over all four telescopes.

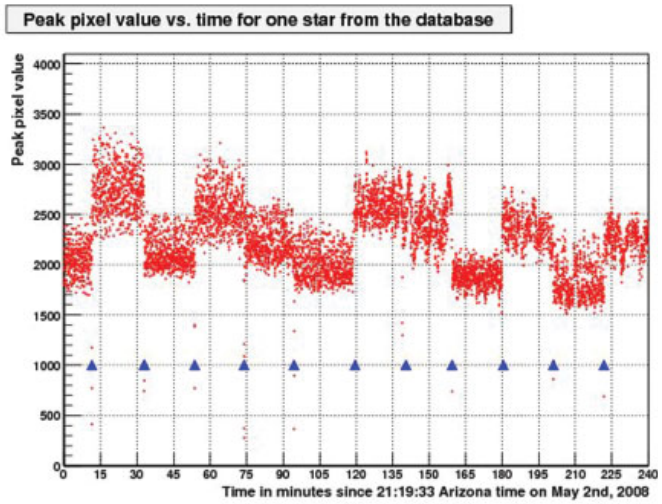

Figure 8: The peak pixel value from the database, plotted against time for a single star tracked for four hours. The blue triangles represent times when the telescope wobbled, which corresponds to the star making a jump to a new location in the image.

fielded CCD camera (Roper Scientific, 2006) and provides a reason for the large spread in intensities. As a result of our research, measures are being taken to flat-field the CCD cameras so that this process of measuring the atmospheric fluctuations may be further studied.

Conclusions

By analyzing the pixel intensity for the stars chosen by our software for the VERITAS telescope array, we have found that the relation between the intensity and the secant of the telescope zenith angle is linear. We have also found that the ratio of the slope over the intercept of this relation is independent of the star used to measure this data. Since we have shown that the fluctuations of star intensity values are due to wobbling, this suggests that our method of measuring the atmospheric effects on the telescope data can quantify atmospheric transmission. Remaining work includes flat-fielding the VERITAS pointing monitors and implementing our software at the level of the base camp. This would allow more sophisticated values that our software extracts from the images to be stored at two second intervals in the database by the pointing monitors, including each star's pixel coordinates and relative and absolute intensities. In the long term, this would provide VERITAS scientists with a robust and precise method of quantifying and correcting for atmospheric effects on their gamma-ray measurements.

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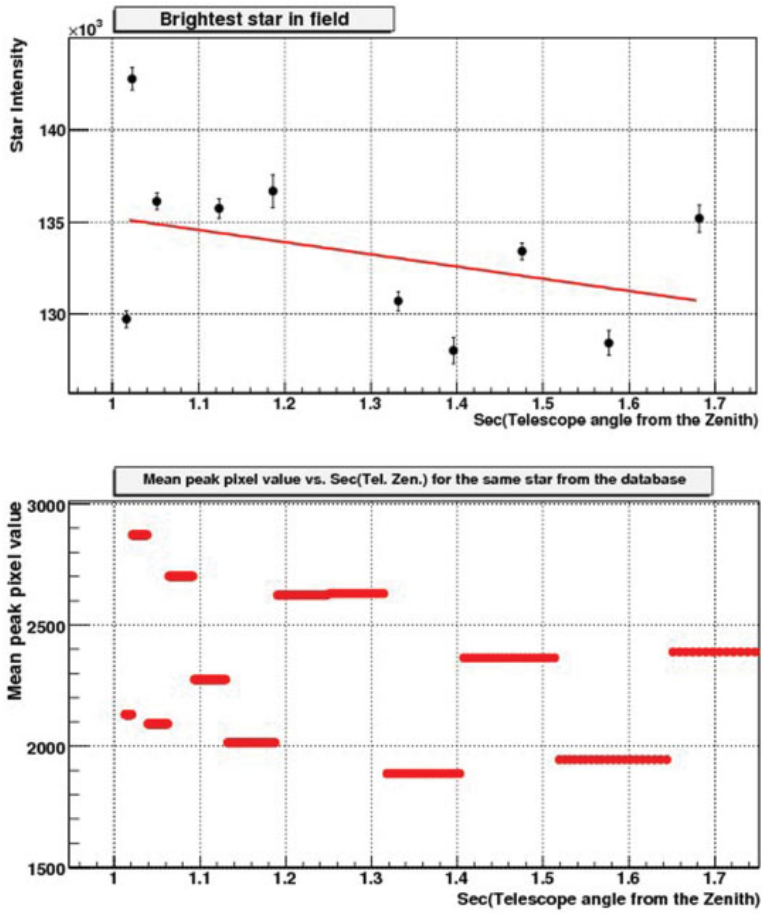

Figure 9: The top plot is the extinction curve for a star tracked through the pointing monitor images. Below is the peak pixel value of the same star shown above but sampled every two seconds from the database values.

Culture and the Aging Brain

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Glossary
Working memory: Refers to the structures and processes in the brain used to temporarily store and manipulate information.
Neurocognitive system: A term used to describe the particular brain areas, neural pathways and networks responsible for specific behaviour and thought processes.
Cognitive pragmatics: A term used to describe knowledge that has been acquired through a lifetime. It differs for people who are from different backgrounds and raised in different environments
Binding process: The ability to connect a particular object to its background

Research in the cognitive neuroscience of aging has revealed several significant changes that alter the functioning of the brain over an individual's lifetime. Particularly, some of these discoveries have contributed to our understanding of age-related degenerative diseases and cognitive decline. Most of these studies have, however, been conducted in Western (North American and Western European) populations, casting doubt on the universality of these findings. Cross-cultural investigations allow for the distinction between changes in the brain that are a consequence of aging and those changes that are due to the impact of life experiences. Research suggests that different cultures place emphasis on distinct aspects of information and use different strategies for processing this information (Nisbett and Masuda, 2003). Therefore, performing cognitive studies taking into account cultural context may provide an effective avenue for differentiating between age-related neural changes that persist across cultures and those that are driven by culture specific life experiences.

For the purposes for this paper, culture is defined as behavioural patterns, beliefs and experiences shared by individuals from a similar geographic region. This paper describes different cognitive processes, such as perception and working memory, and examines studies that reveal cultural differences in these cognitive processes. Furthermore, this paper provides a glimpse of the interplay between experience (through culture) and neurobiology (through aging) that moulds the neurocognitive system. Neuroimaging techniques such as functional Magnetic Resonance Imaging (fMRI) have allowed scientists to visualize the differences between an aging brain[1] and a young adult's brain, revealing that, relative to the younger brain, the former is constantly changing and adapting to its diminishing efficiency (Reuter-Lorenz, et al;2005). Research on neurocognitive aspects specific to culture should ideally distinguish whether the continual adaptations occurring in the brain of the aging adult follows an intrinsic neurobiological design or whether the brain is responding to life experiences that alter its circuitry.

An effective way to understand the impact of culture on behaviour and the organization of neural pathways is to compare cultures that are hypothesized to be different in some fundamental neurocognitive process (Norenzayan, 1999). We will focus primarily on comparisons between East Asian and Western cultures, mostly since the majority of current research into culture and its effects on neurobiology compare these two ethnic groups. This is a particularly relevant comparison because there are documented differences in the techniques these populations use to process information. These differences include categorization, reliance on rules, and the use of logic (Norenzayan, 1999). While it has been suggested that the differences in cognitive processes can be attributed to differences in perception as well as differences in what aspects of the environment receive more attention, it has been demonstrated that East Asians and Westerners do in fact engage different networks in the ventral visual cortex, a section specialized for processing different elements of a scene (Norenzayan, 1999). East Asians place more emphasis on the contextual relationships between objects, whereas Westerners tend to give undivided attention to individual focal objects (Chua et al.; 2005). It is believed that the differences in perception, cognition, and attention can be attributed to differences in social structures and practices. The notion that East Asians emphasize the role of social relations and harmony could be explained by the fact that agriculture has played an important role in East Asia for a greater period of time than in Western culture. Agricultural settings encouraged cooperation between farmers, because it was essential for sustained crop production (Nakamura, 1964). The economy in ancient Greece[2] was quite different from that of East Asia; the land did not lend itself to agriculture due to the mountainous terrain. Common Greek occupations, such as hunting, fishing, and domestic gardening, did not require extensive social collaboration. The individual nature of the professions rendered minimal need for interaction between individuals, depending entirely on personal skill. Consequently, attention could be maintained on a focal object. Essentially, Western societies were motivated by individual successes, while East Asian cultures gave more value to the prosperity of the community. These different values may have been perpetuated throughout many generations and are now reflected in the way East Asians and Westerners perceive their environment, making it plausible to explore the divergence in their cognitive and perceptual processes (Nakamura, 1964).

Research on the behavioural aspects of aging indicates a decline in the efficiency of basic cognitive processes, such as speed of thought, working memory, and long term memory; in contrast, knowledge[3] is preserved and in some cases increases (Park, et al; 2002). In order to investigate the joint impact of culture and aging on cognition it is important to make a distinction between the different domains of cognition. Park, et al. (1999) proposed two domains of cognition – basic cognitive hardware or mechanics, such as speed and working memory, and cognitive software or pragmatics, comparable to acquired knowledge. Using these definitions, one might expect that differences in the basic cognitive processes (hardware) seen in young adults would be minimized in older adults since age-related reductions in capacity limit the flexibility of mental operations. This would render experimental results increasingly similar across cultures as the age of the participant increases. Research on old and young Americans and Chinese has supported this model. An experiment by Hedden, et al. (2002) studied the backward digit span, which assesses participants' ability to manipulate a series of numbers in working memory and then repeat the numbers back in the reverse order in which they were originally presented. Researchers found a larger cultural difference between younger adults than older adults.

1. The aging brain refers to older adults above the age of 65.
2. Ancient Greece refers to the period of Greek History lasting from 1100 BC to 146 BC.
2. Knowledge refers to information acquired throughout a lifetime.

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Moreover, in a study related to memory, in which subjects recalled the identity of the person presenting facts in a video, no cultural differences were observed; however, large differences between age groups were reported (Chua, et al. 2006). Park, et al. (1999) conducted an additional study in which multiple measures of speed and working memories of young and old Americans and Chinese were collected. The results from this study indicated that there were larger differences associated with age compared to culture.

Conversely, one might expect that culture would magnify the effects of aging on cognitive pragmatics. Cognitive pragmatics is based on acquired knowledge and the assumption that older people have more experience within a culture than younger people. This theory is supported by the findings of a study conducted by Yoon, et al. (2004). To assess differences in categorical association, groups of young and old American and Chinese were provided with the names of 105 categories – for example, kitchen appliances, fruit, and seasons of the year. They were then asked to provide five examples they associated with each category. The results indicated that of the 105 categories, only examples from 13 were the same for participants from both cultures. Namely, there was a greater similarity in the examples that were recalled between individuals from the same cultural category, regardless of age group.

Given the above results, the relatively modest impact of culture and the stronger effects of age suggest that biological changes are primarily responsible for the age-related differences in resource-demanding, strategic functions like working memory and speed of thought. In contrast, the impact of culture assumes a far greater role in the development of knowledge-based structures. With regard to the two domains of cognitive processes, these results reveal the dichotomy between the impact of biological aging and culture on the consistency and flexibility, respectively, of cognitive structures in aging brains.

We have, so far, discussed behavioural studies that have demonstrated the relative invariance in cognitive behaviour as a function of culture. Now, we look at neuroimaging studies that reveal the impact of culture on the neural structure of aging brains. Despite the relative invariance of basic cognitive behaviours as a function of culture, the underlying neuroscience of aging and culture is quite different. There is well-documented research suggesting that the aging brain undergoes constant structural and cellular changes. At the cellular level, there is a decrease in dopamine receptors with age[4] (Backman, et al. 2000), and there is evidence that a decrease in these receptors is predictive of a decline in cognitive processes (Volkow, et al. 1998). Neuroimaging techniques have greatly advanced our knowledge of the structural changes that occur in the brain and have supported theories speculating this correlation. Given the decline of multiple cognitive systems as a function of age, one might expect that neural activity would systematically decrease, paralleling the behavioural decline. However, functional neuroimaging suggests that the aging brain is a dynamic system. When behavioural performance on a working or long-term memory task is equivalent in young and old adults, the parts of the brain activated during those behaviours differ. To elaborate, when performing the same task: (a) neural activation is distributed across more brain sites and structures in old adults compared to young adults[5], (b) older adults frequently engage the same region in both hemispheres of the brain for tasks whereas younger adults activate only one hemisphere, and (c) sometimes older adults show a greater level of activation than younger adults in the same neural regions (Reuter-Lorenz, et al. 2005). Studies have demonstrated that prefrontal activity during cognitive processes, such as episodic memory, semantic memory, working memory and perception, tends to be less lateralized[6] in older adults than in young adults. This age-related increase in the use of both hemispheres for a given

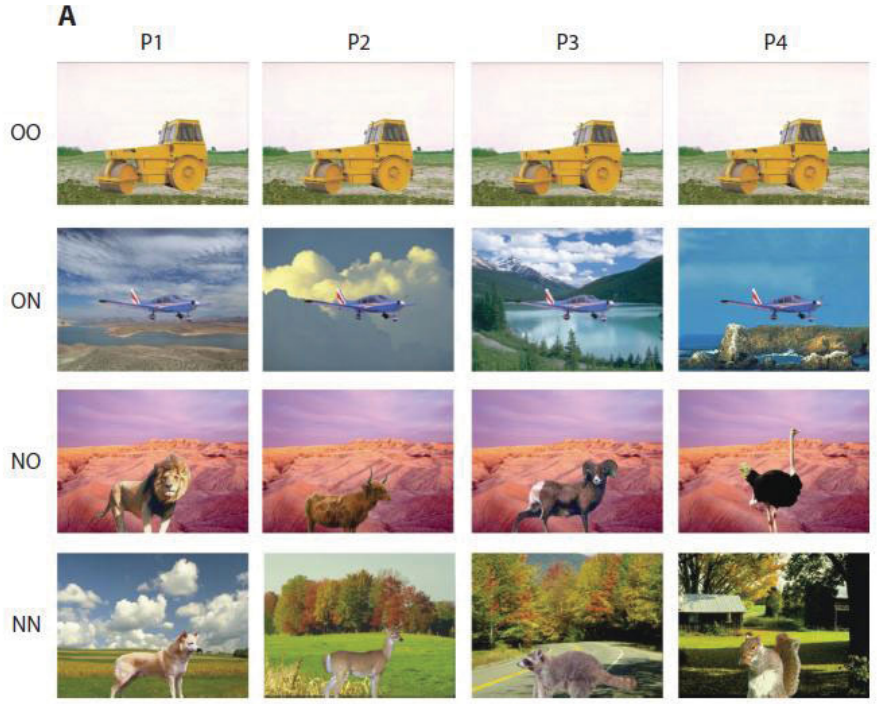

Figure 1: The four quartet conditions: four repeated objects and scenes (old object, old scene); four novel scenes with a repeated object (old object, new scene); four novel objects within a repeated scene (new object, old scene); and four novel objects with four novel scenes (new object, new scene).

4. Changes in the neurotransmitter mechanisms, including Dopamine, have been associated with normal aging. It is believed that the loss of Dopamine receptors causes alterations in frontal lobe processing and is responsible for age-related cognitive decline.

5. In this experiment, young adult participants were between the ages of 30 and 35 years.

6. When performing a specific task, lateralization refers to the localization of brain function to a particular hemisphere, right or left, of the brain.

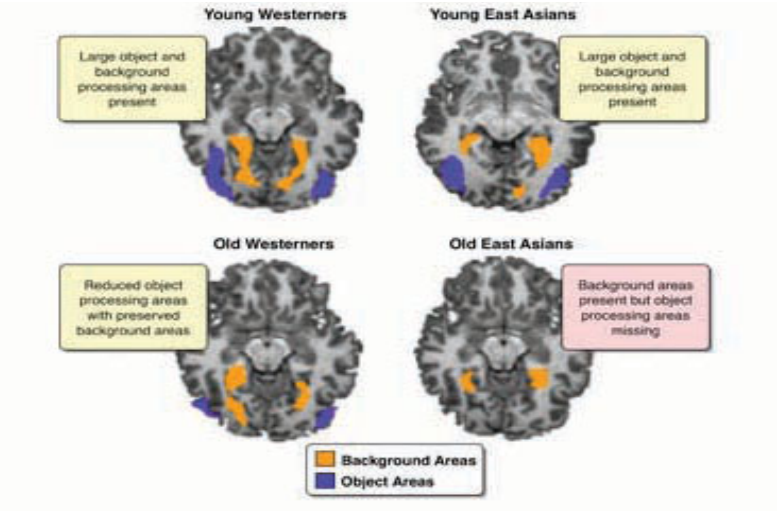

Figure 2: Mean magnitude of adaptation in young East Asians, young Westerners, elderly East Asians, and elderly Westerners. (A) Responses in hippocampal (left panel) and parahippocampal (right panel) binding regions. (B) Responses in left and right parahippocampal areas engaged in background processing. (C) Responses in left and right lateral occipital complex engaged in object processing. Standard error bars are shown.

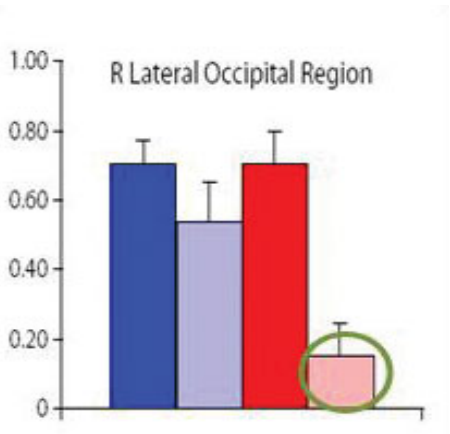

Figure 3: Object processing regions decline with age, but do so disproportionately in elderly East Asians.

task indicates the possibility that bilateral activation compensates for neural decline (Reuter-Lorenz, 2000).

Supporting evidence that the brain responds to the challenges of neurobiological aging by reorganizing, neuroimaging findings also suggest that neural structures may change as a result of prolonged exposure to stimuli. A recent study conducted by Maguire et al. (2000) has provided evidence that taxicab drivers have more posterior hippocampal volume than non taxi drivers and more importantly, within the group of taxi drivers, the subjects with more experience in the profession have a larger hippocampal volume than subjects who have been taxi drivers for a lesser number of years.

Following the notion that environment and experience may shape cognition and neural organization, we can hypothesize that differences in cultural values and customs could affect the development of neural activation patterns. In order to analyze this concept, a functional magnetic resonance imaging study (fMRI) was conducted by Goh, et al. (2007) utilizing theories from previous studies that revealed differences in visual processing among East Asians and Westerners (Chua, et al. 2005). The study investigated how culture might interact with age differences in visual processing of objects and backgrounds, as well as the contextual binding of objects to backgrounds. In order to examine this, 37 young and old East Asians and 38 young and old Americans were presented with a series of four pictures in which either the central object or the background of the picture varied; (Figure 1;).

The attenuation of the blood oxygen level dependent (BOLD) signal[7] that occurred with repetition of different elements of the pictures was measured. These measurements helped examine how specialized areas within the ventral visual cortex adapt to the repetition of different elements of a scene. That is, as subjects viewed the same images repeatedly, the object processing areas of the brain or the background processing regions of the brain adapted to the repetition. As regions adapted, they required less energy, and thus the BOLD signal in the fMRI attenuated. This method, effectively, dissociated the object processing regions, the background processing regions and binding processing regions of the brain. The pattern of results showed that adaptation in the parahippocampal gyrus is equivalent in all four groups (young and old Americans; young and old East Asians) for the processing of background information. The results also revealed that older adults in both cultures exhibited diminished binding process (Figure 2). The paper thus suggested that these findings support the theory that biological mechanisms, rather than cultural experience, are responsible for decreasing the ability of elderly people to engage their medial temporal lobe structures in binding processes to the same extent as younger adults.

The most noteworthy finding of the study by Goh, et al. (2007) is that object processing regions decline with age, but they do so disproportionately in elderly East Asians (Figure 3). The young East Asians and Westerners show relatively similar engagement of the lateral occipital cortex, but the elderly East Asians show a larger deficit of these object-processing structures than do the elderly Westerners. These findings suggest that the cultural differences in neural response are magnified over the lifespan of an individual. This data, combined with behavioural data revealing that East Asians show more eye fixations on backgrounds than on objects (Chua, et al. 2005), may indicate that after a lifetime of culturally biased information processing, the neural circuitry for looking at scenes may be sculpted in a way specific to each culture.

Research into the cultural neuroscience of aging has great potential to reveal the relative contributions of experience and biology to the process of aging. Cultural brain research may hold the key to the "use it or lose it" hypothesis – the concept that neurocognitive health is maintained by sustained intellectual engagement throughout a lifetime (Hultsch, et al. 1999). If we can identify structures that are engaged more by East Asians than Westerners, perhaps it can be postulated that these structures will maintain volume and function better in the culture that uses them more. Similarly, if certain patterns of neural recruitment are shown to

7. Almost all fMRI research uses Blood-oxygen-level-dependent or BOLD as the method to determine regions of brain activity. Neurons do not have internal reserves of energy in the form of glucose or oxygen. Thus, blood needs to release oxygen at a greater rate to active neurons than to inactive neurons. The difference in magnetic susceptibility between oxygenated or deoxygenated blood leads to magnetic signal variation which can be detected using an MRI scanner.

be universal with age across cultures, we could hypothesize that such recruitment patterns are a result of biological aging rather than experience.

Among the comparison studies that have been conducted on Western and East Asian populations, in this article we focus specifically on how they perceive their environment. The argument has long been made that Westerners, while viewing scenes, focus on the object independent of the context. In contrast, subjects from East Asian cultures are more inclined to attend to the context and to the relationship between objects and the environment. Recent findings by Masuda and Nisbett (2006) support this speculation. The authors presented Americans and Japanese subjects with two animated vignettes of scenes (e.g. a Japanese and American city) that differed in various small details. Some of the changes were made in the attributes of the salient, focal objects (such as changes the tires or the hub caps) and other changes were made in the field or context (such as the relationship between the cars and the buildings), including the background objects (such as the buildings) and location of objects. Americans detected more changes in the focal objects whereas Japanese detected more changes in the field and relationships between objects. The findings reveal subtle yet qualitatively different styles of attending to information in the environment.

Figure 4: Japanese City

Figure 5: American City

Using the results from this study, that there is a cultural difference in the viewing of scenes, we hypothesized that there must be a cultural difference in the way people navigate through their environment. To move in the environment, humans adopt different navigational strategies, which use different parts of the brain. To reach a target location, one may use a "spatial memory strategy" by learning the relationships between landmarks in the environment. This strategy is based on the formation of a cognitive map and allows a target to be reached in a direct path from any given location. This type of spatial strategy has been shown to depend on the hippocampus. Alternatively, one can navigate through the environment without knowledge of the relationship between landmarks, but instead by using a series of left and right turns. Successful repetition of this non-spatial strategy leads to a "response strategy" known to involve the caudate nucleus, a form of implicit memory or habit; (Berthoz, et al. 2001). Given the fact that we have identified two different navigational strategies that are dependent on different areas of the brain (spatial memory dependent on the hippocampus and response learning dependent on the caudate nucleus), and given what we know about cultural differences in the population regarding scenes, it can be hypothesized that we will find cross-cultural differences in navigation strategies. If a certain population is more attentive to the background, we expect them to exhibit a spatial memory strategy, i.e., using the relationships between landmarks in the environment to find a specific destination during navigation. Conversely, we expect that populations that are hypothesized to pay more attention to focal objects employ the other navigational strategy, i.e. a response learning strategy, by using a series of right and left turns from a given start position.

Cultural differences in navigation imply that a particular brain system is used preferentially over another. Results from these navigation studies have implications for neurological and psychiatric disorders, such as Alzheimer's dementia, as well as for successful aging. During the course of normal healthy and unhealthy aging, there is an overall decrease in hippocampal volume which has been associated with cognitive decline (Moffat, et al. 2006), but in patients with Alzheimer's dementia, the atrophy is more extreme and precedes the onset of dementia (Csernansky, et al. 2006). As such, a population (Japanese) using spatial memory strategy to a greater extent may have greater grey matter in the hippocampus, thus reducing the risk for dementia. Coincidently, the incidence rate of Alzheimer's disease is significantly lower in non-western countries, such as Japan, than in North America. The Honolulu-Asia aging study revealed that the prevalence rate of Alzheimer's disease among Hawaiian Japanese-Americans men is similar to that seen for North Americans in general, while far exceeding rates typically seen in Japan. They thus concluded that Japanese males who immigrate to the island of Hawaii are more likely to have Alzheimer's disease than Japanese men of the same age living in Japan, suggesting influences other than race. (White, et al.1996)

This article began by providing a glimpse into the interplay between experience (through culture) and neurobiology (through aging) that moulds the neurocognitive system. In order to further elucidate the cultural differences in cognitive processes, comparison studies between East Asians and Westerners were discussed. It was established that there are fundamental differences in the way East Asians and Westerners process information, specifically the way they perceive their

environment. Studies by Masuda, et al. showed that East Asians pay more attention to the relationships between the object and the background, whereas Westerners pay more attention to focal objects. Using this knowledge, we hypothesized that if there is a cultural difference in way people view scenes, then there should be a difference in the strategies they employ to navigate through their environment. Thus, by examining studies of cultural differences in navigation and memory, we can further understand the factors involved in degenerative diseases such as, Alzheimer's disease. The world's aging population is now living longer emphasizing the effects that age-releated diseases affect their lives. It is imperative that scientists study this novel population to expand our knowledge of age related brain changes. Ultimately, findings from age-related brain research will help us elucidate the factors involved in reducing the risks of degenerative diseases and provide answers to a fast growing geriatric population.

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Investigation of the role of heme oxygenase-1 in β-thalassemia pathophysiology

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Abstract
β -thalassemia is an inherited disorder characterized by impaired hemoglobin synthesis in developing erythrocytes due to unbalanced globin chain production, resulting in severe oxidative damage to cells. Heme oxygenase-1 (HO-1) has been shown to have antioxidant benefits and is upregulated in response to oxidative stress; thus increased HO-1 levels could serve as an indicator of β-thalassemia pathophysiology. To test this hypothesis, we created a cell culture model of β-thalassemia by decreasing β globin expression in murine erythroleukemic (MEL) cells using short interfering RNA (siRNA). An increase in HO-1 protein expression, along with a decrease in heme levels was observed in cells where β globin reduction was sustained for 96 hours. Further investigation is needed to determine the role that HO-1 plays in normal erythroid development and confirm the observed relationship between β globin reduction and increased HO-1 expression, however, our results provide evidence that this method serves as an effective and novel cell model of β thalassemia.

Keywords
Erythroid cell, hemoglobin, heme oxygenase, β-thalassemia.

Glossary
Erythroid cell: cell undergoing differentiation to become an erythrocyte (red blood cell)
Hemoglobin: the hemoprotein responsible for binding and carrying oxygen in erythrocytes
Heme oxygenase: rate-limiting enzyme in the catalytic breakdown of heme, a component of hemoglobin
β thalassemia: erythrocyte pathology involving reduced β globin chain synthesis and impaired production of functional hemoglobin.

Introduction

Adult hemoglobin is primarily composed of a single heme moiety in a complex with two α globin and two β globin protein chains (Higgs, 1993). The expression levels of each component are tightly controlled in the developing erythrocyte to prevent accumulation of excess heme or globin (Ponka, 1999; Higgs, 1993). Heme is produced in the mitochondria of aerobic cells as a complex of iron (II) sitting at the centre of a macrocyclic compound, protoporphyrin IX. Specifically, heme production in erythroid cells is limited by the uptake of exogenous iron via transferrin receptors. Iron regulates heme biosynthesis by controlling the translation of erythroid-specific aminolevulinic acid synthase (ALA-S2), the first enzyme to act in the production of protoporphyrin IX.

During the production of hemoglobin, heme regulates globin at the transcriptional and translational levels and has been found to promote β-globin transcription by blocking the activity of the transcriptional repressor Bach1 during hemoglobin synthesis (Ponka, 1999; Tahara, et al. 2004). Additionally, heme upregulates NF-E2, an erythroid-specific transcription factor involved in globin gene activation (Ponka, 1999), and is required to inactivate the heme regulated inhibitor (HRI), which inhibits proteins involved in the initiation of globin translation by phosphorylation (Ponka, 1999; Chen, 2007). These mechanisms ensure that globin is produced proportionally to the intracellular concentration of heme.

Heme transcription and translation must be tightly maintained in all cells, since increased synthesis of heme above homeostatic levels can be highly toxic (Graca-Souza, *et al.* 2005). Heme oxygenase (HO) is a ubiquitous microsomal enzyme that is responsible for catalyzing the rate-limiting step in heme catabolism: the release of iron, carbon monoxide, and biliverdin (Ponka, 1999). Iron is then recycled within the body for subsequent hemoglobin synthesis in erythroid cells. While 85% of organismal heme synthesis occurs in immature erythroid cells, most heme catabolism takes place in splenic macrophages by the HO-1 isozyme (32 000 kDa) during the recycling of senescent erythrocytes (Ponka, 1999; Abraham, *et al.* 2008). The identification of multiple transcriptional inducers and response elements suggests a complexity that could allow multiple HO-1 activation pathways, each unique to different cell types, although it is unclear what role HO-1 plays in the developing erythrocyte. The heme molecule has been demonstrated to increase HO-1 expression through interaction with *Bach1* (Alam, *et al*. 2005), which would suggest that increased heme levels would result in increased heme degradation. However, heme must accumulate in high concentrations during erythroid development in order to produce hemoglobin, suggesting that the cell must have a regulatory mechanism that prevents heme degradation by HO-1. Furthermore, it is possible that HO-1 could be active in the erythroid cell under pathophysiological conditions relating to hemoglobin synthesis, such as β thalassemia.

β thalassemia is characterized by decreased synthesis of β globin chains during erythroid differentiation, usually due to a mutation in the β globin gene that may impair transcription, mRNA processing, or translation. As a result, balanced hemoglobin synthesis and overall erythropoeisis are hampered, producing microcytic and hypochromic erythrocytes (Urbinati, *et al*. 2006). When excess hemoglobin is present in its non-tetrameric form, it becomes unstable and the α-chain precipitates on membrane structural proteins. The α hemoglobin chain also reacts with oxygen to form peroxyl radicals ($ROO^{\cdot}$), which can lead to significant oxidative damage (Urbinati, *et al*. 2006; Xie, *et al.* 2007; Sassa, 2004). Degradation of excess α-chains promotes autoxidation of hemoglobin to methemoglobin (MetHb) that contains Fe^{3}+ instead of Fe^{2}+ and thus cannot carry oxygen (Nagababu, et al. 2008). Finally, the dissociation of heme to free heme and iron, followed by their reaction with hydrogen peroxide, generates a cascade of oxidative reactions that destabilize cell membranes (Nagababu, et al. 2008; Graca-Souza, *et al.* 2005). About 365 000 infants are born each year with βthalassemia worldwide and these patients will exhibit many chronic symptoms throughout their lives, including iron over-

load from repeated transfusions (Urbinati, *et al.* 2006), making this disease of significant interest to health science researchers.

HO-1 also has great physiological importance as an antioxidant (Ponka, 1999; Abraham, *et al.* 2008). Several cellular signaling cascades are thought to increase HO-1 expression in response to oxidative stress (Maines, 2005). As well, stress response elements (StREs) within the HO-1 gene promoter have been identified (Poss, et al. 1997). Biliverdin, a product of heme degradation, has been proposed to mediate this StRE activity because it is an antioxidant (Ponka, 1999). Also, since heme has the potential to generate reactive oxygen species (ROS), its removal may be one way in which HO-1 prevents oxidative stress. However, it has recently been suggested that the anti-stress functions of the enzyme are independent of cellular heme catabolism, and that oxidative protection is conferred through a different mechanism (*Sheftel, et al.* 2007). Since HO-1 is upregulated in several cell types in response to oxidative stress, this begs the question of whether HO-1 plays any role in the pathophysiology of β thalassemia, where oxidative damage is incurred through the mechanisms described previously. Our research seeks to clarify whether HO-1 is upregulated in the β thalassemic erythroid cell in response to the destructive effects of α-Hb, metHb, free heme and iron that accumulate due to ineffective hemoglobin synthesis.

The cell culture models for βthalassemia to date have been achieved through the entrapment of heme-containing α-hemoglobin chains in erythrocytes, simulating a βthalassemic phenotype through unbalanced globin levels and partially synthesized hemoglobin (Scott, *et al.* 1990; Szuber, *et al.* 2008). Our model mimics the pathophysiological conditions of βthalassemia by promoting the degradation of β globin mRNA through the mammalian RNAi system (Shi, 2003), using short interfering RNA (siRNA) that has sequence homology to β globin mRNA. siRNA methods have previously been used in MEL cells and mouse models to decrease α and β globin levels (Voon, *et al.* 2008; Xie, *et al.* 2007). This technique should decrease β globin protein expression to simulate β+thalassemia, the heterozygous phenotype, by promoting the unbalanced expression of α and β globin and preventing proper Hb synthesis. Murine erythroleukemic (MEL) cells have been frequently used as a model for studying developing erythrocytes (Sheftel, *et al.* 2007; Voon, *et al.* 2008), and thus we used this cell line in our model of β thalassemia. We measured the expression levels of HO-1, β globin and heme in assessing this model. Decreased levels of β globin were expected due to siRNA-mediated downregulation. Converesely, HO-1 levels were expected as a result of upregulated transcription in response to oxidative stress or due to activation from heme that is not bound in a hemoglobin tetramer, so-called "uncommitted heme". Cellular heme will be measured as it is both an activator and the substrate of HO-1.

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Materials and Methods

Cell culture and Transfection

MEL cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum, to which penicillin (100 units/mL of medium) and streptomycin (100 µg/mL of medium) were added (all from Invitrogen). All experiments were conducted in uninduced MEL cells (control cells) as well as cells treated with 2% DMSO (Me_2SO_2) to promote differentiation and hemoglobinization (Friend, et al. 1971). Cells were plated with $0.5x10^6$ cells per well in 2 mL of DMEM with 10% serum and without antibiotics. 100 pmol of Hbb1 (β-globin) siRNA was transfected into MEL cells, using 5 µl of Lipofectamine™ transfecting reagent (Invitrogen), according to the manufacturer's instructions. DMEM medium without serum or antibiotics was used for the transfection. MEL cells treated with Lipofectamine™ without siRNA served as negative controls. Controls containing 'scramble' or redundant siRNA sequences were omitted from the experiments. Cells were kept in a 37° C incubator until they were harvested.

In an initial experiment, cells were either treated with DMSO 48 hours prior to transfection and harvested after 48 hours, or treated 24 hours after transfection and harvested after 48 hours after DMSO treatment. Also for this experiment only, cells were transfected with either 100pmol or 200pmol of siRNA, using 6 µl of transfecting reagent for the latter amount. In all subsequent experiments, DMSO treatment was carried out six hours after transfection.

In the second experiment, cells were harvested at 48, 72 and 96 hours to check whether the transient effects of siRNA attenuated over time.

The third set of experiments consisted of harvesting at 48 and 96 hours after transfection. The 96-hour cells were re-transfected after 48 hours, at which point DMSO was added to the cells previously treated with DMSO, to maintain a 2% concentration®.

siRNA sequence

The following sequence was used as a short interfering RNA to decrease cellular β globin mRNA levels using the RNAi system. The sequence was obtained from Thermo Scientific Dharmacon .
5'-GGGCAGGCTGCTGGTTGTCTAC-3'

Western blot

Cells were harvested and lysed using Munro lysis buffer, a solution of 10mM Hepes (pH 7.6), 3mM $MgCl_2$, 40 mM KCl, 5% glycerol, and 0.2% NP-40). Protein was determined with a Bradford assay, using a protein assay dye reagent (BioRad), and spectrophotometry absorbance of 540nm. Samples were boiled for 7 minutes prior to loading to disrupt the hemoglobin structure. Protein was separated using a 15% SDS-polyacrylamide gel at 40 mA current for 10 minutes, followed by 60mA for the rest of the separation. Gel was then transferred to a nitrocellulose blotting membrane (BioTrace™) at 200 mA for two hours at 4°C. Prestained molecular weight markers (Fermentas™) were used to estimate the molecular mass of proteins. Membranes were cut into strips according to the positions of β actin, HO-1 and globin protein in order to separately treat sections with their respective primary antibodies, and were washed in primary antibody solutions at 4°C overnight. Rabbit primary antibodies against β-actin and HO-1 (StressGen®) were used in a dilution of 1:5000. Globin rabbit primary antibody was used in a 1:10000 dilution to detect β globin protein (MP Biomedicals, Inc). Membranes were treated for two hours at room temperature with rabbit secondary antibody in a dilution of 1:20,000. The western blot was developed using HyBlot CL™ autoradiography film (Denville Scientific Inc). HO-1 and globin protein levels were normalized according to corresponding β-actin levels in each sample.

Heme measurement

MEL cells were transfected and treated with DMSO as described above. A solution of 0.1M citric acid and 0.1M sodium phosphate in a ratio of 1:2 was combined with hydrogen peroxide (1 µL/mL of solution) and 0.5g of o-phenylenediamine dihydrochloride (Sigma) per 25mL of solution. Experimental cells were harvested after 48 hours, 50 µl of each sample was

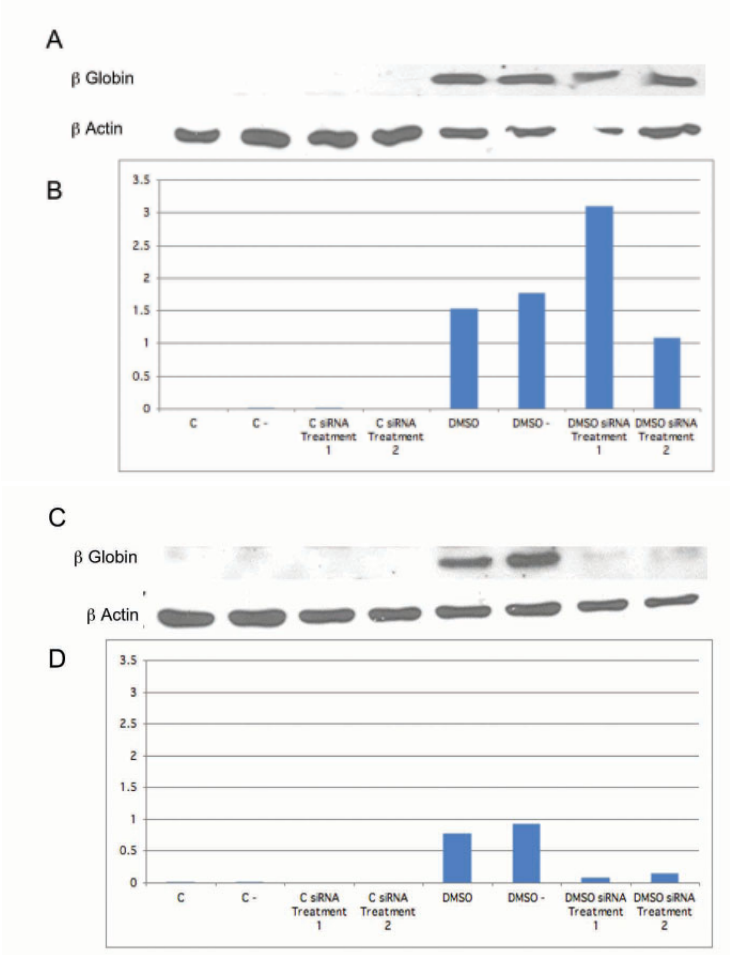

Figure 1: A) Western blot of β globin (Hbb1) and β actin levels in MEL cells treated with DMSO 24 hours prior to transfection with Hbb1 siRNA, harvested 48 hours after transfection. Figure represents the results of one experiment. B) Plot of β globin protein levels in pre-treated MEL cells normalized to β actin expression. C) Western blot of β globin (Hbb1) and β actin levels in MEL cells treated with DMSO 24 hours after transfection with Hbb1 siRNA, harvested 96 hours after transfection. D) Plot of β globin protein levels in MEL cells treated with DMSO 24 hours after transfection, normalized to β actin expression. NOTE: "Treatment 1" refers to 100 pmol of Hbb1-siRNA and 5µl of Lipofectamine™ transfection reagent. "Treatment 2" refers to cells treated with 200 pmol of Hbb1 siRNA and 6 µl of Lipofectamine™ transfection reagent (see methods).

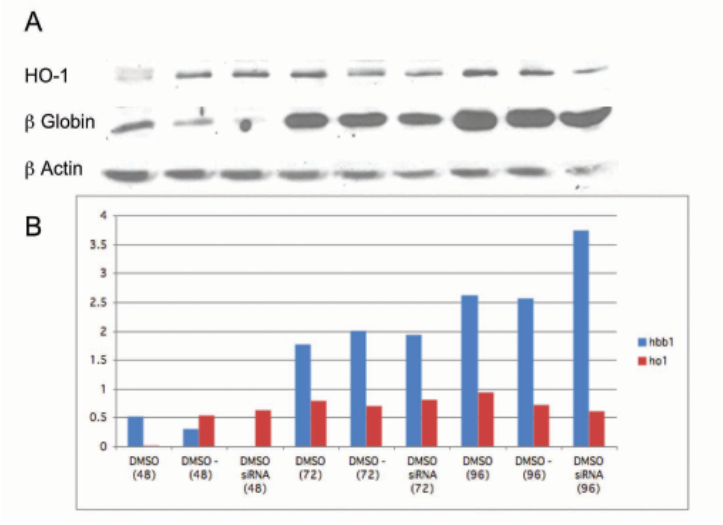

Figure 2: Cells treated with DMSO 6 hours after transfection, and harvested at either 48, 72, or 96 hours. For each time point, cells were either not transfected (DMSO), mock-transfected (DMSO-), or transfected with Hbb1 siRNA (DMSO siRNA). Control cells that were not treated with DMSO are not shown. Figure represents the results of one experiment. A) Western blot of HO-1 and β globin with β actin as a loading reference. Wells correspond to the plots labelled in B). B) Plot comparing β globin with HO-1 levels, normalized to β actin. The Y-axis uses arbitrary units.

collected and lysed by adding 125 µL of the NP-40 Munro Lysis buffer then incubating with cell lysates at 37° C for 5 minutes. Heme reacts with this solution to promote a colour change that is proportional to the level of heme in the lysates. 25 µL of 8 M sulphuric acid was added to the samples to stop the reaction. Absorbance was measured at 492 nm for all lysates.

Results

Protein expression

A single, initial experiment was conducted to determine when differentiation should be induced with DMSO relative to siRNA transfection. Figure 1 shows the results of two separate gels, the first (A, B) represents globin expression in cells pre-treated with DMSO, and the second (C, D) represents globin expression in cells that were induced to differentiate after transfection. Note that in both cases, the variation in globin expression levels in cells that have not been treated with DMSO suggests they have not significantly exceeded the basal level of MEL cell expression. A small reduction in β globin levels was observed in pre-treated cells using 200 pmol of siRNA, but this was not the case for cells treated with 100 pmol of siRNA. If cells are signalled to hemoglobinize before transfected with β globin siRNA, large amounts of the tetrameric globin protein would have been produced before the RNAi system could knock down β globin mRNA levels. However, a large reduction in β globin expression was observed when cells were transfected before DMSO treatment, using 100pmol and 200pmol of siRNA. Subsequent experiments used 100 pmol of siRNA exclusively.

For the following set of experiments, we used a time-course approach to determine the length of time that the transient activity of the siRNA can sustain a reduction in β globin. We also examined levels of HO-1 expression during the period following β globin reduction. Protein levels from cells that were harvested at three different time points were measured using a western blot. Figure 2 depicts cells that were treated with DMSO six hours after transfection. A notable reduction in globin was found in siRNA-treated cells that were harvested after 48 hours, compared with mock-transfected, DMSO-treated cells. In cells transfected after 72 hours and 96 hours, no reduction was observed, and globin levels progressively in-

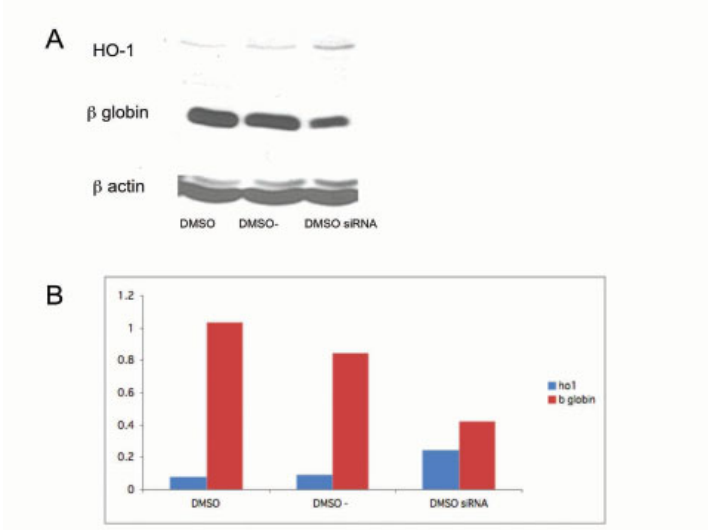

Figure 3: Non-transfected (DMSO), mock-transfected (DMSO-) and transfected cells (DMSO siRNA), harvested after 96 hours when re-transfected 48 hours after initial transfection. Controls untreated with DMSO are not shown. Figure represents the results of a single experiment. A) Western blot of HO-1 and β globin with β actin as a loading reference. B) Plot comparing HO-1 and β globin protein levels, normalized to corresponding β actin. The Y-axis uses arbitrary units.

creased. It appears that the knock-down effects of the siRNA are attenuated after about 48 hours, likely because globin production ensues due to MEL cell differentiation.

The amount of HO-1 did not increase in the same cells that showed a reduction in β globin levels. In Figure 2 B), HO-1 levels are slightly increased in the siRNA-treated cells compared to mock-transfected cells in the 48-hour and 72-hour group. However, no conclusions can be drawn from this experiment regarding the activity of HO-1 in response to β thalassemia-type conditions because globin levels were not sufficiently decreased in the 72-hour and 96-hour cells. These results do suggest that HO-1 levels do not change over time in normally differentiating MEL cells.

In order to maintain decreased levels of β globin mRNA, and to provide time for α-hemoglobin chains to accumulate within the erythrocytes, during the next set of experiments, cells were transfected a second time with siRNA 48 hours after the initial transfection, then harvested after 96 hours. Figure 3 shows a reduction in the globin levels of the transfected cells was observed when compared with mock-transfected cells. A concomitant increase in the levels of HO-1 was observed in transfected cells compared to mock-transfected and non-transfected controls treated with DMSO.

Discussion

Our siRNA approach to developing a β-thalassemic cell model represents a novel tool for investigating the pathology of this acquired disease. We conclude that in order to sustain reduced β-globin protein levels, repeated transfection is required due to the transient knock down effect of siRNA. DMSO-treated MEL cells have a limited lifespan, thus harvesting must take place no later than 96 hours. Although results from all western blots showed decreased globin levels in cells transfected with siRNA, it is necessary to quantify the amounts of α and β globin separately, to clarify whether globin chain synthesis is sufficiently unbalanced to mimic a β-thalassemic phenotype. Further experiments should also include non-specific siRNA sequences to serve as additional controls.

Heme oxygenase-1 expression did not substantially increase in knock-down cells that were harvested 48 hours after transfection with Hbb1 siRNA (Figure 2). However, after 96 hours, cells showed reduced globin levels due to siRNA treatment as well as an observable increase in HO-1 protein levels (Figure 3). Despite the fact that HO-1 protein concentrations only displayed visible increases when they were harvested after 48 hours this does not rule out the possibility that the enzyme's activity may be increasing within the cell in response to pathophysiological changes, even if HO-1 synthesis is not yet being transcriptionally activated.

Heme levels in β globin knock-down cells were distinctly lower than those of DMSO-treated controls (Figure 4). In the MEL cell model, siRNA-mediated reduction of β globin mRNA is theoretically hampering balanced hemoglobin synthesis and may explain the decrease in heme levels. Current understanding of hemoglobin synthesis places suggests heme is a regulator of globin expression (Ponka 1999), but it is possible that the regulatory pathway is more complex than current research would suggest. As well, decreases in β-globin levels may have unknown cis or trans effects on other proteins involved in heme synthesis. One way to further investigate these issues would be to culture transfected cells with $^{59}Fe_2$-transferrin and measure ^{59}Fe incorporation into cells and cellular heme (Sheftel, et al. 2007). Previous results in our laboratory demonstrate that "uncommitted" heme inhibits iron acquisition from transferrin, as well as its incorporation into heme (Ponka, *et al. 1988*). Hence, it is conceivable that heme associated with excess β-globin chains could inhibit iron acquisition from transferrin and consequently, heme biosynthesis. Another possibility is that HO-1 catalyzes the degradation of any heme proteins that are not successfully incorporated into hemoglobin tetramers. This would explain the observed increase in HO-1 expression, which could also be initiated by unincorporated heme.

Finally, it is important to note that the physiological role of HO-1 in the developing erythrocyte is not yet fully understood. Current knowledge suggests that there must be a control mechanism to prevent HO-1 from degrading heme before it reaches a concentration sufficient for hemoglobin synthesis. Therefore, if heme is not degraded by HO-1 during normal cellular differentiation, unbalanced hemoglobin synthesis could result in substantial degradation of unincorporated heme by HO-1.

The role that heme oxygenase plays during erythroid differentiation is still a onditions provides considerable possibilities for future study. Key questions include how HO-1 activity during impaired erythroid development affects the characteristics and lifespan of β-thalassemic red blood cells that enter circulation. HO-1's ability to degrade excess heme and exert

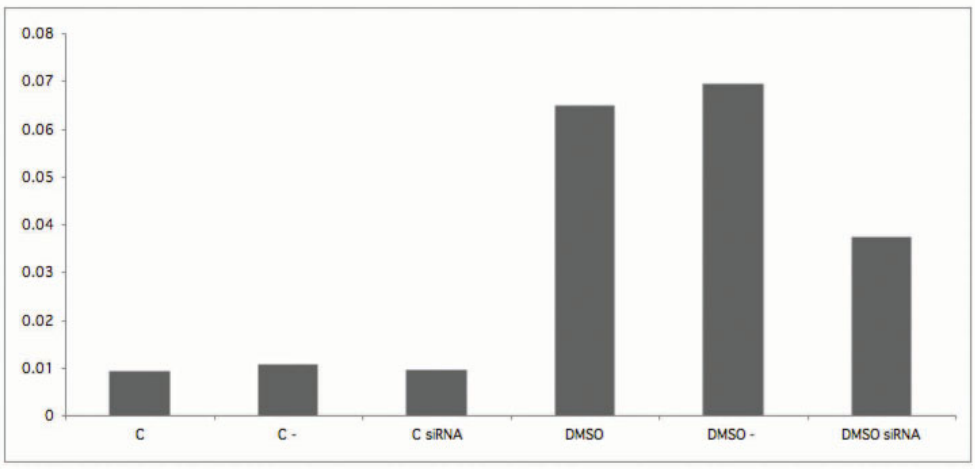

Figure 4: Plot of heme levels in cells harvested 48 hours after transfection, normalized to protein levels of corresponding cell lysates. Non-transfected (DMSO), mock-transfected (DMSO-), or transfected cells (DMSO siRNA), as well as corresponding control cells left untreated with DMSO are shown. The Y-axis depicts arbitrary units of absorbance. Figure represents the results of a single experiment.

a protective effect by reducing free radical generation and oxidative stress is another possible area of investigation. The *Hmox*1 promoter exhibits a $(GT)_n$ repeat polymorphism, which affects the degree to which it is induced in different individuals (Exner et al. 2004). Future research could investigate these polymorphisms in conjunction with the differing severities of β-thalassemic phenotypes that exist across a variety of ethnicities; it is possible that this disease phenotype could be affected by the ability to upregulate heme oxygenase-1.

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How taxonomic revisions affect the interpretation of specimen identification in biological field data

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Abstract
Cumulative revisions in taxonomy of organisms over time can create difficulties for researchers in numerous scientific fields, including conservation biology. This paper compares the taxonomic names of the *Collembola* species named in Ivan P. Vtorov's 1993 paper entitled *Feral Pig Removal: Effects on Soil Microarthropods in a Hawaiian Rain Forest*, with the more modern *Collembola* checklists. In comparing Vtorov's original graphs with recreated graphs, this study finds that out of the sixteen species Vtorov collected, three had changed generic names, two had changed species names, and one was absent from modern *Collembola* checklists. Five taxa were identified only up to genus, making it impossible to evaluate them in comparison with modern checklists. Only six species matched current moneclature of soil microarthropods. Not only were there taxonomic reclassifications, but also differences in the descriptions of species' ecological status indicating whether the species was endemic or adventive. None of the three species described as endemic in Vtorov's study were listed as such in current checklists. Additionally, Vtorov did not deposit voucher specimens, so morphological comparisons or re-identification of species named in his study are impossible. Inconsistencies due to changes in nomenclature and the species' ecological status or lack of physical documentation can, as shown here, be detrimental to researchers examining data from older studies.

Keywords:
Taxonomic revisions, Collembola, Microarthropods, Feral Pig, Sus scrofa.

Glossary
Dichotomous key: A tool used to classify organisms into respective taxa by following a series of choices; each choice presents alternative sets of descriptive morphological traits. The key eventually leads to the taxon name of the characterized organism.
Collembola: Minute, wingless insects that live in soil communities and primarily feed on detritus; common name is springtails.
Endemic species: A species with the ecological state of being unique to a particular geographic location, such as a specific island, habitat type, nation, or other defined zone.
Adventive species: These species have been introduced to a new habitat or environment that is outside of their native geographic range; they are neither native nor fully established in the new habitat or environment
Soil Microarthropods: A group denoting small invertebrates that are less than two mm in length; they are found in the phylum arthropoda; the most well known groups are the mites (*Acari*) and springtails (*Collembola*).
Cosmopolitan species: A plant or animal species that is found almost anywhere in the world.
Nearctic: Of, relating to, or denoting a zoogeographical region comprising North America as far south as northern Mexico, together with Greenland.
Holarctic: Of, relating to, or denoting a zoogeographical region comprising the Nearctic and Palearctic regions (Europe, parts of N. Africa and Asia) combined. The two continents have been linked intermittently by the Bering land bridge, and their fauna are closely related.
Voucher specimens: Any specimen that is retained as a reference for future morphological comparison. These should ideally be in a publicly accessible scientific reference collection. A type specimen is a particular voucher specimen that serves as a basis for the taxonomic description of a species through physical comparisons.
Biomass: The total mass of organisms in a given area or volume.

Introduction

Taxonomy is considered a science in its own respect, but it is also an essential tool for applied or experimental studies in many fields of biology, especially in microbiology and ecology. All biologists are involved in the identification of the particular organisms they are studying, but taxonomists produce or alter the classification of organisms as needed and provide tools by which fellow scientists can identify specimens. The number of described organisms has continually increased since the time of Linnaeus due to the discovery of new species. This growth requires methods for identification of organisms to be practical, easy, and flexible in order to efficiently adapt to changes in how organisms are classified. In addition, novel and efficient organization of data and infrastructure will have to be implemented in order for researchers to have the tools necessary to access this growing body of taxonomic data.

Brief Overview of Ivan P. Vtorov's Study

Vtorov's study was carried out in the Hawaii Volcanoes National Park (HAVO), Hawaii. The feral pig (*Sus scrofa*) is an invasive species that is problematic for Hawaiian ecosystems because it creates wallows and compacts the soil when foraging for food. The resulting higher soil density is known to negatively affect populations of endemic *Collembola*. The composition of Microarthropod communities can be used to indicate the quality of soil health (Straalen and Krivolutsky, 1996). Vtorov sampled and classified various soil microarthropod insects, but specifically focused on the presence of endemic *Collembolan* species as environmental indicators of forest succession following the removal of feral pigs. Results indicated that with ecological recovery of the sampling area, the total density of soil microarthropods nearly doubled, the biomass rose by 2.5 times and the number of species of *Collembola* doubled. The endemic population of *Collembola* increased with a restoration of populations within seven years while the number of adventive species of *Collembola* decreased: (Vtorov, 1993).

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Taxonomic Comparisons Between 2008 and Vtorov's Collembola/Feral Pig Study 1993				
Family	**Vtorov's ID**	**Our ID**	**Vtorov's Status**	**Our Status**
Onychiuridae	*Tullbergia silvicola*	*Tullbergia silvicola*	Nearctic	Adventive
Onychiuridae	*Protaphorura cryptopya*	*Allaphorura cryptopyga (Arthropod sp. Checklist 4th ed. 2002)*	Nearctic	Adventive
Isotomidae	*Folsomina onychiurina*	*Folsomina onychiurina*	Cosmopolitan	Adventive
Isotomidae	*Cryptopigus caecus*	*Cryptopigus thermophilis?**	Pacific	Adventive
Isotomidae	*Isotomiella sp.*		Endemic	likely adventive
Entomobryidae	*Sinella caeca*	Sinella caeca	Cosmopolitan	Adventive
Entomobryidae	*Homidia sauteri*	*Entomobrya (subgenus homidia) sauteri*	Holarctic	Adventive
Entomobryidae	*Lepidocyrtus inornatus*	*Lepidocyrtus inornatus*	Endemic	Adventive
Entomobryidae	*Salina maculata*	*Salina celebensis*	Endemic	Adventive
Entomobryidae	*Harlomilsia occulata*	*Harlomilsia occulata*	Tropical	Adventive
Neelidae	*Neelides minutus*	*Neelides minutus*	Holarctic	Adventive
Hypogastruridae	*Xenylla sp.*			?
Hypogastruridae	*Neanura sp.*			?
Sminthuridae	*Sminthurides sp.*			?
Onychiuridae	*Mesaphorura sp.*	*Tullbergia (subgenus mesaphorura) sp.*		?
	Parisatoma dichaeta	*? Not in any of our keys*	Pacific	?

Table 1: Species lists and taxonomic comparisons from 2007 using current Hawaiian microarthropod references, Bellinger, Christiansen, 1992 & the Microarthropod Checklist 4th ed., 2002.[1]

Here, we examine the consequences of taxonomic revisions as they pertain to the interpretation of ecological data. We re-evaluate Vtorov's study by comparing his conclusions to those made interpreting his data with current taxonomic references.

Methods

In order to evaluate the effects taxonomic revisions have on present day research, we utilized a number of methods. Ideally, voucher specimens would have been obtained and compared to museum specimens and modern taxonomic checklists. Unfortunately, Vtorov did not leave any voucher specimens so morphological comparisons of species named by Vtorov were not possible. Since we were unable to employ this method, our analysis was restricted to using Vtorov's data and comparing this with modern checklists.

First, we compared Vtorov's listed specimen inventories to modern references. Then we further examined all discrepancies between them to determine if they were due to possible taxonomic reclassifications, changes in species ecological status, or misidentifications by Vtorov. We also calculated the total number of organisms of each *Collembola* species named in Vtorov's study, reanalyzed the data graphically, and compared the results of the recreated graphs to those of his original graphs. This was done in order to determine if there were differences in the ecological status or taxonomic classifications of organisms named in Vtorov's study and to see if these differences affected the interpretation of his results.

The most recent taxonomic references for Hawaiian *Collembola* species were used to evaluate the species lists and taxonomic classifications named in Vtorov's study. These included Bellinger and Christiansen's (1992) taxonomic dichotomous key and the Microarthropod Checklist 4th ed. (2002). Vtorov's references included Bellinger & Christiansen (1989), a reference which is no longer used in the field of taxonomy because it is outdated. Vtorov's original data from his Table 1 (not seen here) entitled "Numbers and biomass of microarthropods under ohi'a trees and tree ferns at fenced sites free of pigs for 0, 2-4, and 7 years" and Table 3 (not seen here) entitled, "Restoration of springtail poulations (in percent of numbers) under ohi'a trees and tree ferns at fenced sites free from pigs for 0, 2-4, and 7 years" were used to calculate the total number of each *Collembola* species examined in his study. These results were tabulated and graphs were reconstructed using Microsoft Excel.

In his data, Vtorov listed the number of individuals of each *Collembola* species per square meter, the percentage of each *Collembola* species in the total population and the average number of *Collembola* species per square meter. We used these values to graph Vtorov's original data and further analyse any reclassification and incompatibilities in the description of ecological species status in light of modern *Collembola* taxonomic references. Vtorov did not define which species he classified as cosmopolitan leading to some ambiguity in classification (Figure 1). To compensate for this, species from Vtorov's data were analyzed graphically in two groups of ecological species status. The classification of "Cosmopolitan" was either considered alone or it was considered to comprise all species listed as not endemic, i.e., all species listed as nearctic, cosmopolitan, pacific, holarctic, and tropical.

The discrepancies revealed by the reconstructed graphs showed how changes in taxonomic names for many of the *Collembola* species were significant enough to change Vtorov's original results. If we had access to a history of the revisions of the name of each *Collembola* species since 1993, it would have been possible to clarify the reasons for the differences between the recreated graphs and Vtorov's original graphs. Unfortunately, we did not have access to precise records of Hawaiian *Collembola* taxonomic historical literature because no databases or libraries contain clear, detailed, or accessible historic records of *Collembola* reclassifications to our knowledge. However, by analyzing graphs with various combinations of ecological species status, it was possible to determine if the discrepancies were a result of ambiguous groupings of ecological species status or because of recent taxonomic revisions. This comprehensive analysis and reconstruction of data allowed careful consideration of how taxonomic incompatibilities between Vtorov's data and present day checklists might affect research on *Collembola* using more modern resources.

Results

Many taxonomic differences were found between Vtorov's species lists and the current Hawaiian microarthropod references. Several of the sixteen *Collembola* species reported in 1993 have either disappeared from the checklist altogether, have had changes in their name or have seen changes in ecological status. The status of a species can be listed as endemic to Hawaii or adventive, having been introduced to Hawaii. *Lepidocyrtus inornatus, Salina maculata*, and *Isotomiella sp*. were described as endemic in Vtorov's species list while they are listed as adventive in the most recent literature. Here it should be noted that the standard procedure for naming specimens whose genus is known but the species identity cannot be determined is to name them "sp." following the genus name; for example, an unknown species of the genus *Salina* would be labelled *Salina sp.* Vtorov had a total of five specimens that were identified in this manner, making it difficult to analyze this data. *Isotomiella sp*. is likely an

1. According to Bellinger and Christiansen (1992), the family, Isotomidae, has only one species listed under the genera, Cryptopigus (Cryptopigus thermophilis). Thermophilis is the most common Collembola near vents in Hawaii Volcanoes National Park. It is found in litter soil and bird nests from sea level to 4000 ft.

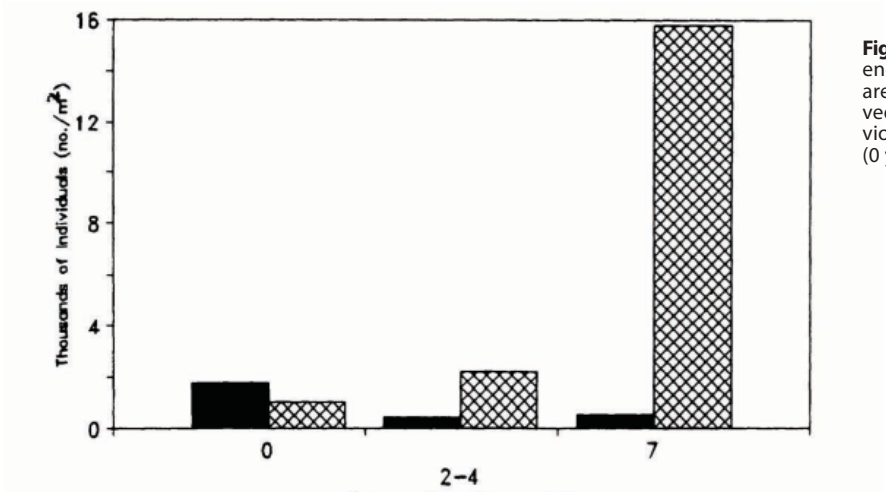

Figure 1: Changes in cosmopolitan and endemic Collembola species in fenced areas where feral pigs had been removed 7 years previously, 2-4 years previously, and where pigs are still present (0 years) (Vtorov, 1993).

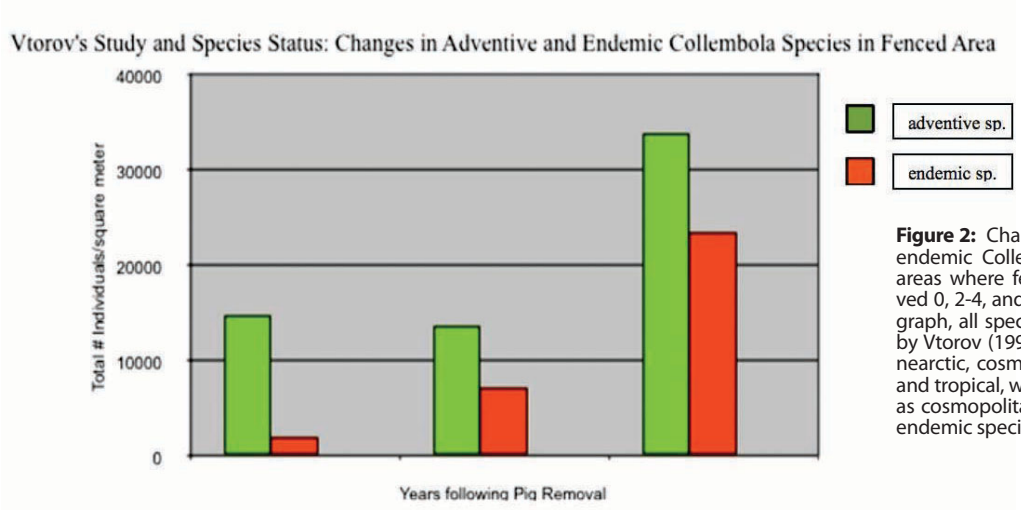

Figure 2: Changes in cosmopolitan and endemic Collembola species in fenced areas where feral pigs had been removed 0, 2-4, and 7 years previously. In this graph, all species listed as non-endemic by Vtorov (1993) i.e., all species listed as nearctic, cosmopolitan, pacific, holarctic and tropical, were combined, considered as cosmopolitan and were compared to endemic species.

adventive species because all species in this genus are listed as adventive according to Microarthropod Checklist 4th ed. (2002), although there is no way of being certain. There were additional changes in nomeclature and incompatibilities between Vtorov's taxonomic assignments and the presently accepted names of the organisms studied by Vtorov. Using a dichotomous key to identify the possible modern species names that were closest to Vtorov's names, *Protaphorura cryptopyga* was most likely identified as *Allaphorura cryptopyga* by Vtorov (Arthropod sp. Checklist 4th ed., 2002), *Cryptopigus caecus* was most likely identified as *Cryptopigus thermophilis, Homidia sauteri* was most likely identified as *Entomobrya* (subgenus *homidia*) *sauteri*, *Salina maculata* was most likely identified as *Salina celebensis, Mesaphorura sp*. was most likely identified as *Tullbergia* (subgenus *mesaphorura) sp.* and *Parisotoma dichaeta* was not present in the modern literature (Table 1). There was no way of knowing if these discrepancies were a result of reclassification or misidentifications by Vtorov as no voucher specimens were retained for comparison.

For faster and more accurate identification, Vtorov (1993) used a tiered approach to the taxonomic resolution of the species he named in his study. He identified his specimens of interest (*Collembola)* to the level of species while identifying less relevant specimens to a broader taxonomic grouping such as family or order. For example, he identified mite superfamilies (*Oribatida, Acaridida, Gamasida, and Prostimata*) and grouped several of the microarthropod specimens into even less specific classifications such as nematodes, enchytraenia worms, earthworms, butterfly larvae, molluscs, and protura, with *Collembola* being the only group classified to the level of genus and species.

Out of the sixteen *Collembola* species collected by Vtorov, six matched modern nomenclature according to the current Microarthropod Checklist 4th ed., (2002). Three species had changed genus names, two had changed species name, and one was absent from the Microarthropod Checklist 4th ed. (2002) altogether (Table 1).

In Figure 1, Vtorov examined changes in the number of cosmopolitan and endemic *Collembola* species in fenced areas, where feral pigs had been removed 7, 2-4 and 0 years prior to sampling. His results showed an overall decrease in cosmopolitan species and an overall increase in endemic species over time after the removal of feral pigs. In Figure 2, all species that were not endemic were considered to be cosmopolitan, i.e., all those species listed as nearctic, cosmopolitan, pacific, holarctic and tropical. Both adventive and endemic species populations increased with time after removal of feral pigs. Although this was similar to the original findings (Figure 1), we found a higher number of adventive species than Vtorov originally found. When we graphed the two species that Vtorov listed as cosmopolitan in comparison to endemic species, we observed an overall increase in both cosmopolitan species and endemic species (Figure 3), which was in accordance with his original findings (Figure 1). In order to clarify the trend seen in Figure 3, we created another graph with a scale of higher resolution showing only

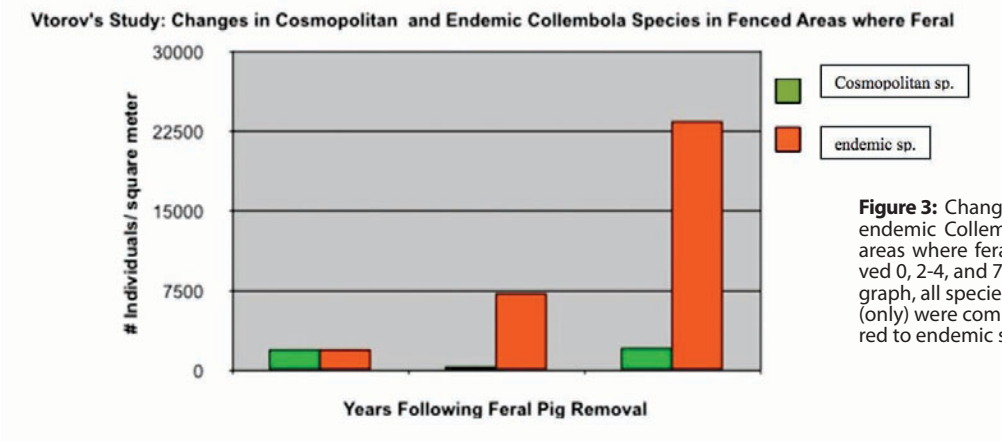

Figure 3: Changes in Cosmopolitan and endemic Collembola species in fenced areas where feral pigs had been removed 0, 2-4, and 7 years previously. In this graph, all species listed as cosmopolitan (only) were combined and were compared to endemic species.

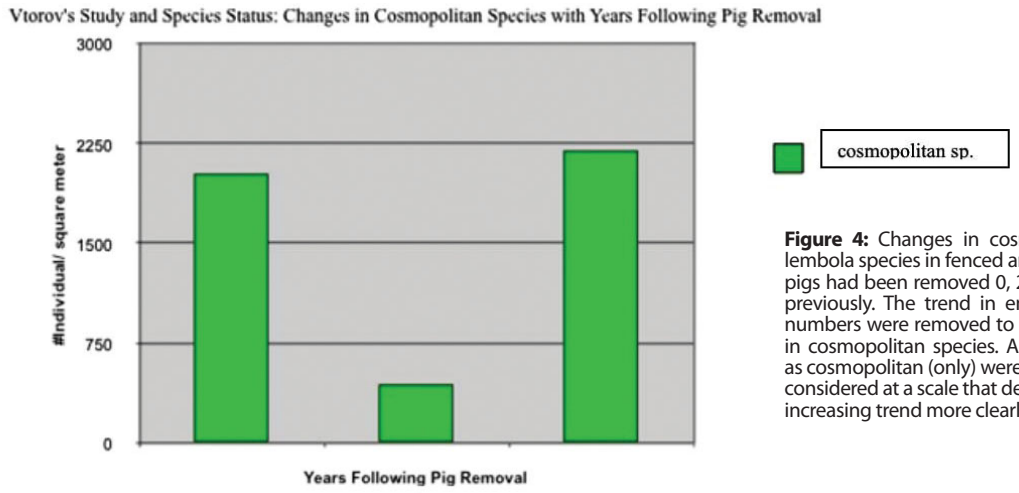

Figure 4: Changes in cosmopolitan Collembola species in fenced areas where feral pigs had been removed 0, 2-4, and 7 years previously. The trend in endemic species numbers were removed to show the trend in cosmopolitan species. All species listed as cosmopolitan (only) were combined and considered at a scale that demonstrates the increasing trend more clearly.

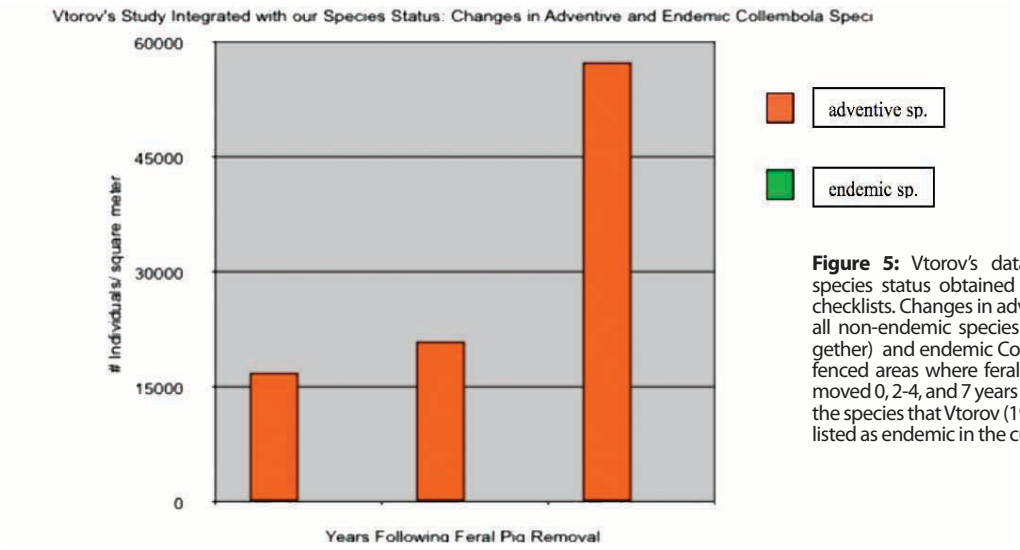

Figure 5: Vtorov's data integrated with species status obtained from modern day checklists. Changes in adventive (in this case all non-endemic species were grouped together) and endemic Collembola species in fenced areas where feral pigs had been removed 0, 2-4, and 7 years previously. None of the species that Vtorov (1993) recorded were listed as endemic in the current checklists

the effects on the cosmopolitan species. Here, it was clear that the number of cosmopolitan species increased overall with time after removal of feral pigs; data taken 0 years and 7 years after removal of feral pigs showed almost identical numbers of cosmopolitan species; there was a sharp decline in the 2-4 years after removal of feral pigs followed by another sharp increase from 2-4 years to 7 years (Figure 4).

A comparison of Vtorov's species lists with modern checklists showed no species listed as endemic in modern checklists, and the species that he listed as adventive showed a gradual increase in population over time after removal of pigs from the area (Figure 5).

Discussion

The discrepancies found in this case study include differences in *Collembola* taxonomic classification, ecological species status and the conflicting results between Vtorov's data and the revised graphs, all of which can be explained by a number of factors. Changes in taxonomic nomenclature and ecological status designations of the *Collembola* species

since 1993 had the largest effect on the discrepancies found. In addition, Vtorov did not clearly specify which species were placed in the cosmopolitan group, which complicated the interpretation of his data. The degree to which Vtorov's graphs differ from the graphs recreated in this study can be explained by the modern techniques of graphing employed. For example, there could have been different scaling or rounding off to the nearest decimal point of numbers in the Microsoft Excel program, or perhaps errors introduced by manual drafting of figures in the original study compared to more accurate computerized drafting.

The largest discrepancies are primarily due to the adoption of new taxonomic nomenclature and reclassifications since 1993. This finding emphasizes the point that undocumented or inaccessible knowledge of revisions in taxonomic names accumulate over time, making interpretation of the original data a challenge for modern research.

Though we particularly focused upon changes in taxonomy of *Collembola,* the changes in ecological status of species are also apparent in Vtorov's study, as all the species Vtorov listed as endemic are considered adventive in modern checklists (Table 1). The status of a species as adventive or endemic is of particular interest to scientists in this area of the world as an island of Hawaii's size, isolation and fragile ecosystem renders it highly susceptible to ecological invasion by alien plants and animals (Canfield and Loope, 2000).

These findings also underline the importance of permanent physical documentation of specimens in taxonomic research. Voucher specimens are collections of organisms that are kept as confirmation of the identification of species; unfortunately, there are no supplementary data or voucher specimens available from Vtorov's study to enable further investigation of the discussed taxonomic discrepancies. There is no way of telling, for example, if *Cryptopigus caecus* (Vtorov, 1993) is similar or even identical to the reclassified *Cryptopigus thermophilus* (Nishida, 2002). As primary repositories for voucher specimens, museums are associated with many kinds of biological research and play an important role in documenting biological diversity through voucher specimen databases and catalogues (Wheeler, 2004). If Vtorov had retained voucher specimens, these could have been compared to museum specimens in conjunction with modern checklists to determine their correct classification. While descriptions, drawings and photographs can supplement identifications, the actual preserved specimens are essential to compare the original species with those identified using more modern techniques.

Here, we highlight one situation in which discrepancies between original identification and modern nomenclature may have been created by taxonomic revisions introduced since the original research. Difficulties, in differentiating between technical errors and actual revisions as the cause of these discrepancies, are compounded by the lack of voucher specimens to properly compare species originally identified by Vtorov. Our findings underscore the need for centralization of taxonomic revisions and nomenclature as well as the need for the retention of preserved voucher specimens for future comparison.

Fortunately, there are current and ongoing endeavours to reduce any discrepancies caused by taxonomic revision. The centralization of taxonomic information, including current and past knowledge of revisions in taxonomic names, will make the interpretation of original data less of a challenge for modern research.

Rapid and efficient access to large amounts of taxonomic data, including past and present name revisions, is now possible through online resources and databases. Not all taxonomic groups are available through extensive and complete online databases yet, but ongoing endeavours and collaboration by museums and specialized researchers are working towards improving existing databases and the creation of new ones. Such databases will hopefully enable researchers to easily and accurately refer to scientific literature containing recently revised taxonomy.

Acknowledgements
We are grateful to Hawaii Volcanoes National Park, and to D. Foote and M. Montgomery for their guidance at the USGS Pacific Island Ecosystems Research Center, Kilauea Field Station. We are also grateful to G. Fussmann of McGill University for his comments on the manuscript.

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DJA1 and DJA2 Carboxyl-Terminal Fragments and their Role in Peptide Binding and Luciferase Refolding

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Abstract
DJA1 and DJA2 are Hsp40-family co-chaperone proteins that regulate the activity of the chaperone Hsc70/Hsp70. The N-terminal J domains of DJA1 and DJA2 promote ATP hydrolysis and polypeptide binding by Hsc70. Both co-chaperones are also thought to bind unfolded polypeptides in their central to C-terminal regions. Yet, the two are functionally distinct, possibly due to divergence in the connections between their functional domains. Here, we constructed C-terminal fragments of DJA1 (A1-C, residues 254-397) and DJA2 (A2-C, residues 254-412), expected to contain the homodimerization site but not the main polypeptide-binding region. The fragments were expressed in *E. coli* under optimized conditions, and purified. Co-precipitation experiments with the pure fragments suggested that A1-C retained partial binding to model polypeptides, while A2-C showed less binding. The fragments were then transfected into HeLa cells, in which overexpression of full-length DJA1 and DJA2 had previously been found to increase the folding of a co-transfected model protein. Both A1-C and A2-C increased folding moderately, less than the full-length co-chaperones, but notably the fragments did not inhibit folding. These results suggest that these C-terminal domains may provide a second site for polypeptide binding, contributing to the substrate specificity or overall activity of the co-chaperones.

Glossary
Hsp70: Heat shock protein 70kDA; a chaperone
DJA: DnaJ domain-containing co-chaperone of Hsp70
A1/2-C: Protein fragment of corresponding DJA protein containing only the C-terminal domain
NEF: Nucleotide Exchange Factor

Keywords
Chaperone, DnaJ co-chaperones, Hsp40, Hsp70, DJA1, DJA2, DNAJA1, DNAJA2

Introduction

Molecular chaperones are considered key players in a variety of events related to protein metabolism. Chaperones display increased expression under conditions of cellular stress and are believed to mediate folding and prevent aggregation of proteins. They also play important roles in protein translation, translocation and degradation.

One of the most important chaperones, Heat Shock Protein 70 (Hsp70), is a ubiquitous chaperone that appears to be particularly important in genital development (Dix, *et al*. 1996). Hsp70 chaperones bind to hydrophobic regions of unfolded polypeptides and prevent the formation of non-productive aggregates while promoting polypeptide folding. Although the exact mechanism by which it promotes folding is still under contention, it is believed that Hsp70 acts as a molecular wrench to pull protein segments apart during folding to allow the polypeptide to re-nature the hydrophobic region that is affected (Goloubinoff, *et al.* 2007). A cycle of ATP binding, hydrolysis and nucleotide exchange controls this refolding activity (Qiu, *et al*. 2006). When bound to ATP, Hsp70 has a low affinity for unfolded polypeptides, but hydrolysis of ATP to ADP leaves the Hsp70:ADP complex in a state competent for polypeptide binding. Previous work suggests that nucleotide exchange factors (NEFs) are responsible for triggering Hsp70 dissociation from ADP (Cyr, *et al.* 2008), while the J-domain of co-chaperones from the DnaJ/Hsp40 family appears to stimulate hydrolysis of ATP (Tzankov, *et al.* 2008). The first biochemical and genetic evidence to support this mechanism of ATPase regulation by DnaJ proteins was identified in *Escherichia coli*, however, much of our understanding of J-protein function comes from work performed on Ydj1, the DJA1 equivalent in *Saccharomyces cerevisiae*.

Between lower and higher eukaryotes, there is significant sequence conservation in both the J-domain and the C-terminal domain (Figure 1) (Cheetham, *et al.* 1998). Crystal structures of Ydj1 segments suggest that the C-terminal domain of DnaJ proteins is involved in dimerization (Wu, *et al.* 2005) and may also play a role in binding short peptide sequences (Li, *et al.* 2003). As well, research on Ydj1's functional domains has proven valuable in elucidating the diverse spectrum of roles that the human DnaJ homologs play within the cell (Ramos, *et al.* 2008).

One structural modification that is shared between DNAJA1, DNAJA2 and Ydj1 is a farnesylation motif. Located at the C-terminus, the farnesyl group is covalently attached to a cysteine residue by a thioester bond and mimicks the hydrophobic qualities of a lipid due to its highly aliphatic, conjugated structure (Wright and Philips, 2006). Although the significance of this modification in Hsp40 homologs is still not clear, farnesylation is often used to target proteins to membranes. A recent paper by Flom, *et al.* suggests that disruption of farnesyltransferases disrupts Ydj1 interaction with Hsp90 chaperones, however, this data has yet to be confirmed in mammalian cells (Flom, *et al.* 2008).

More than 20 DnaJ proteins have been reported in humans and these can be divided into three groups based on the presence or absence of certain functional domains. Type I Hsp40 proteins have the same structural design as DnaJ; a J-domain located at the amino-terminus, followed by a linker region rich in glycine and phenylalanine, a zinc-finger region and a carboxyl-terminal homodimerization domain. Type II proteins lack the zinc-finger region, while Type III proteins consist only of a J-domain. In bacteria, DnaJ is the only J-domain co-chaperone of DnaK, the Hsp40 homolog. In humans, however, there are three cytosolic Type I co-chaperones, DJA1, DJA2 and DJA4. DJA4 and the Type II co-chaperones appear to have either specialized functions or increased levels of synthesis under stress conditions, whereas DJA1 and DJA2 are expressed constitutively in all cells (Terada, *et al.* 2000). Accordingly, DJA1 and DJA2 appear to be the main regulators of Hsp70.

Recent work from this laboratory suggests a more complex Hsp70/Hsp40 relationship than the DnaJ/DnaK presen-

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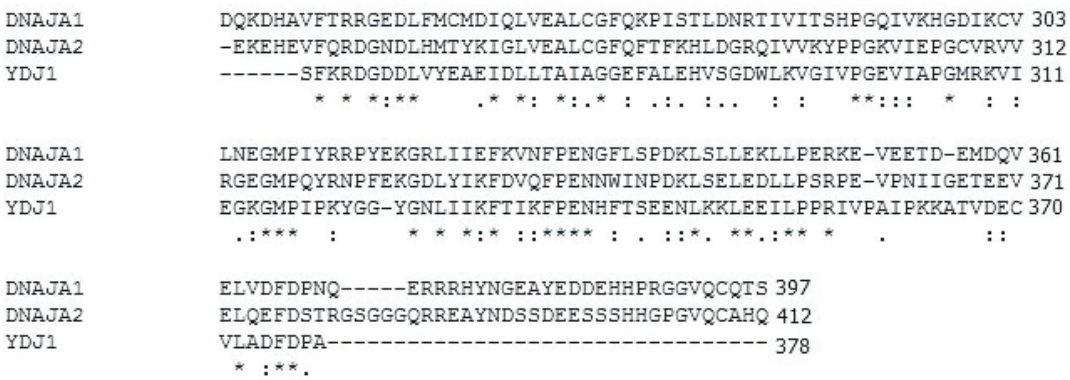

Figure 1: Protein sequence alignment of C-terminal dimerization segments of human DJA1, DJA2 and yeast Ydj1, the homolog of the human DJA1. DJA1 and DJA2 share 48% homology and are 33% and 38%, respectively, homologous with Ydj1 in their C-terminal dimerization sequences.

ted in the bacterial model. Clear variations in biochemical properties have been shown amongst DJAs. DJA1 appears to activate the ATPase domain of Hsc70 the strongest and has the highest binding affinity to preproteins. Furthermore, DJA1 function *in vivo* has been shown to be the most effective at promoting luciferase renaturation and the mitochondrial import of phosphate carrier protein (PiC). DJA2 promotes luciferase refolding with the slightly less efficiency than DJA1. A DJA2 mutant lacking the J-domain (ΔJ) inhibits binding of Hsc70-preprotein most severely (Bhangoo, *et al.* 2007). Previous results from our laboratory suggest that this partial specialization allows assistance of a wider range of substrates and, hence, provides the cell with a greater advantage over a cell possessing a single Hsp40 co-chaperone.

It has since been determined that DJA1 and DJA2 are functionally distinct with reference to their ATPase activating domains and peptide-binding domains, although contributors to these differences have yet to be established (Tzankov, *et al.* 2008). The differences could be due to divergence in the connections between their functional domains. The C-terminal homodimerization domains of the DJAs may be important for functional connections and, furthermore, may have additional interactions with substrate polypeptides or even other chaperones. To investigate these ideas, C-terminal fragments of DJA1 and DJA2 carboxyl-terminus mutants were studied for their ability to bind to different polypeptides and to affect luciferase folding in live cells.

Methods

Plasmids

Sequences encoding DJA1 C-term (amino acids 254-397) and DJA2 C-term (amino acids 254-412) were amplified by PCR using pcDNA3.1 DJA1-mycHis and pcDNA3.1 DJA2-mycHis as templates. The following specific primers were used (Integrated DNA Technologies, Inc.); DJA1 244C Bam up (ccggccggatccgatgGATCAGAAGGACCATGCTG), DJA1 MHC dn (cggccagcggccgcagaggtctgacactgaacacc), DJA2 254 Bam up (ccggccggatccgatgGAGAAAGAACATGAGGTATTTC), and DJA2 MHC dn (cggccagcggccgcctgatgggcacactgcactcc). PCR products were digested with restriction enzymes, BamHI and NotI (New England Biolabs) and inserted into cut pcDNA3.1 mycHis C with T4 ligase. Plasmids were transformed into DH5α cells and DNA was extracted using a QIAprep Spin Miniprep Kit (250). To identify positive clones, the DNA was cut with BamHI and NotI, and resolved on a 1% polyacralymide gel. DNA purification using QIAGEN Plasmid Midi Kits gave final DNA concentrations of 1.03 µg/µl and 1.09 µg/µl for DNA1 and DJA2 mutants, respectively. The same procedure was used for cloning C-terminal fragments into pPROEXHTA (Invitrogen), with the following exceptions: primers used were DJA1 PRO new dn (ggccggctcgagtcaagaggtctgacactgaac) and DJA2 Sal dn (ggccgggtcgacttactgatgggcacactgc), and PCR products and vectors were digested with BamHI, XhoI, and BamHI, SalI for DJA1 and DJA2 mutants respectively. Final DNA concentrations were 0.14 µg/µl and 0.13µg/µl for DJA1 and DJA2 mutants, respectively.

Full length mycHis-tagged DJA in pcDNA 3.1 (Invitrogen), DJA ΔJ mycHis-tagged mutants in pcDNA 3.1, along with PiC in pGEM-SP6 were as previously reported (Bhangoo, *et al*. 2007; Tzankov, *et al*. 2008).

Bacterial Expression

Cultures of DJA1 and DJA C-term in pPROEXHTA plasmids were incubated at 37˚C until the measured OD of 0.8-1.0 was reached in BL21DE3 cells (Promega). IPTG was added to a final concentration of 1mM to induce expression and cultures were grown at 37˚C for 30, 60 and 90 minutes and 30˚C for 1, 2 and 3 hours. Once the induction time was completed, cells were harvested at 8000xg for 10 minutes, followed by resuspension in DJA equilibration buffer (750mM, 60mM imidazole, 20mM KH_xPO_4 pH 7.5) (Bhangoo, *et al.* 2007) with Complete Protease Inhibitors (Roche Diagnostics).

Small Scale Condition Experimentation for Protein Purification

Aliquots were lysed by addition of lysozyme to a final concentration of 0.5mg/mL, followed by 30 minutes of incubation on ice. Tween-20 and DNase1 were added to final concentrations of 0.1% and 33µg/mL, respectively, then allowed to incubate on ice for 30 minutes. Samples were pelleted at 20,000 x *g* for 1 hour and rocked with 50µL nickel-Sepharose columns (GE Healthcare) at 4˚C for 30 minutes. After washing with DJA equilibration buffer, two fractions were collected with an appropriate elution buffer (1M imidazole, 0.5M NaCl, 20mM KH_xPO_4 pH 7.5) (Bhangoo, *et al.* 2007).

Full Scale Purification

Aliquots of suspended bacterial pellet were lysed by cavitation in a french press then pelleted at 20,000 rpm for 1 hour. The supernatant was run through a 5mL high-performance nickel-Sepharose column and proteins were eluted using an elution buffer. Fractions containing protein peaks were loaded onto a Superdex 200 Hi-Load 16/60 column (GE Healthcare) and eluted with Hi-Salt buffer (500 mM NaCl, 20 mM HEPES-KOH, pH 7.5, and 5 mM $MgOAc_2$). Yield of peak fractions was determined by BCA assay.

Coprecipitation Experiments

The same assay as previously outlined for quantifying binding of pre-proteins was used (Bhangoo, *et al.* 2007, from Young, *et al.* 2003; Fan, *et al.* 2006). Purified DJA1, DJA2 and their respec-

tive C-terminal mutants at final concentrations of 5μM were bound to nickel-Sepharose in buffer H (500mM NaCl, 20mM HEPES-KOH at pH 7.5, 5mM $MgOAc_2$) for 30 minutes at 4˚C. Rabbit reticulocyte lysate expression systems were used to translate the phosphate carrier (PiC) and oxoglutarate carrier (OGC) (Palmisano, A. *et al.* 1998) with SP6 polymerase, diluted 1:20 into buffer GTI (20mM imidazole, 0.1% Triton X-100, 500mM NaCl, 20mM HEPES-KOH pH 7.5, 5mM $MgOAc_2$) with 2mg/mL ovalbumin. Protein reactions were terminated with 0.1U/μL apyrase after 2 minutes at room temperature. DJA-protein complexes were recovered on beads for 30 minutes at 4˚C then washed with buffer GTI. All washing steps were conducted with Buffer G (100mM KOAc, 20mM HEPES-KOH pH 7.5, 5mM $MgOAc_2$). Complexes were eluted using Laemmli loading buffer and 50mM EDTA, then analyzed on 12% SDS-PAGE and Typhoon Phosphimager (GE Healthcare).

Construct Transfection and Luciferase Assays

HeLa cells were maintained in DMEM containing 4.5 g/l glucose, 36 mg/l pyruvate, 2mM glutamine, and 10% fetal bovine serum (Invitrogen). Cells were grown to a density of 2.5 x 10^5 cells/ml, and co-transfected using 2 μl of Lipofectamine 2000 PLUS (Invitrogen) in twelve-well plates with either 1.6 μg of DJA C-term mycHis, DJA ΔJ mycHis, the full length mycHis DJA, or vector alone, and 0.4 μg of HA-tagged luciferase. All of these DNA samples were cloned in pcDNA 3.1. Twenty-four hours after transfection, cells were harvested with 0.4mL 1X PBS, centrifuged at 600 x *g* for five minutes and lysed using 80 μL of 1% Triton-X 100 in 1X PBS. Lysates were left at 4°C for four minutes and cleared by centrifugation at 20,000 x *g* for five minutes. Lysates were assayed for luciferase activity using luciferase reagent (Promega) in a 1:5 dilution and RLU/sec measurements were recorded using a SIRIUS Luminometer V3.2. Equal amounts of lysate were resolved by 12% SDS-PAGE and analyzed with immunoblots using α-HA (Upstate Cell Signaling Solutions) and 9E10 α-c-Myc (Covance) as primary antibodies, and α-mouse-HRP (Stressgen Bioreagents) as secondary antibody. Signals were observed on HyBlot CL autoradiography film using ECL reagent (GE Healthcare) with various exposure times, ranging from three seconds to three minutes. Protein concentration was assayed using BCA reagents (Thermo Scientific) and 10 μL of cell lysate in a 1:500 dilution. Optical Density (OD) measurements at 562 nm were taken using a Beckman Coulter DU 730 UV/Vis Spectrophotometer; these readings were used to calculate the normalized luciferase activity.

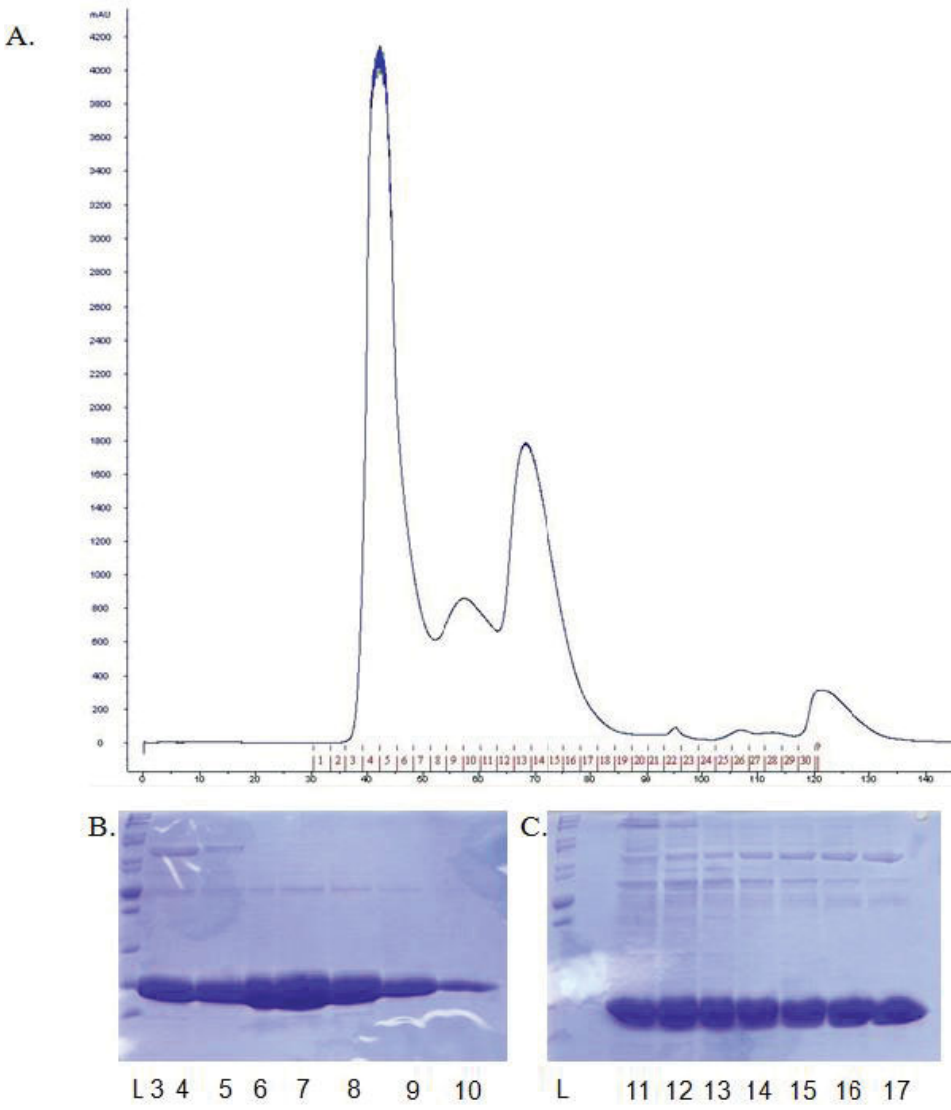

Figure 2: (A) The UV profile of DJA1 C-term elution fractions from Superdex Size-Exclusion chromatography column and SDS-PAGE analysis of fraction content from fractions (B) 3-10 and (C) 11-17.

Results

Isolation of DJA1 and DJA2 C-terminal Mutants

The predicted C-terminal homodimerization domains of DJA1 and DJA2 (A1-C and A2-C, respectively) are well conserved, although not identical (Figure 1). To study their possible functions and properties, protein fragments of the C-termini of DJA1 and DJA2 fused to N-terminal His-tags were first expressed in *E. coli*, then purified.

To determine the optimal conditions for bacterial expression, induction at 37°C and 30°C was tested at several time points. At each temperature/time point, the bacteria expressing A1-C and A2-C were lysed and single step purifications on nickel-Sepharose were carried out. SDS-PAGE gel analysis of pellet, flow-through, wash and two eluates was conducted for both C-terminal proteins. Expression profiles were selected for magnitude of the expected product band and significance of contaminating agents in eluates. In most conditions, the proteins appeared to express well, although there were differences in amount and number of contaminants after the trial purification. For A1-C and A2-C, it was determined that the optimal conditions for large scale purification were 30˚C for 2 hours and 37˚C for 1 hour, respectively.

The A1-C and A2-C proteins were next purified on a larger scale to produce amounts sufficient for *in vitro* experiments. After expressing the proteins under the conditions described above, a first purification step using a nickel-Sepharose column was performed. As expected, the proteins eluted in a narrow peak and appeared relatively pure. These fractions were pooled and loaded on a Superdex 200 gel filtration column, producing broad elution profiles for both A1-C and A2-C. Some aggregated material eluted in the void volume, but one distinctive peak in the separation range of the column was indicated by the UV absorbance profiles. Interestingly, a smaller earlier peak was observed for both A1-C and A2-C upon gel filtration (Figure 2, and data not shown), suggesting possible formation of tetramers. Because SDS-PAGE gel analysis showed that both peaks contained the correct proteins, fractions combining both peaks were pooled and concentrated. Final concentrations of A1-C and A2-C were determined to be 1.22mg/mL and 0.803mg/mL, respectively, when concentrated to 2mL.

Coprecipitation Experiments

It was possible that the C-terminal fragments of the DJAs contained secondary binding sites for unfolded polypeptides, in addition to the main binding site in the middle domains. To address this, an established assay to test polypeptide binding to the purified full-length His-tagged DJAs was used. The DJAs were known to bind certain unfolded mitochondrial precursor proteins, which depend on the chaperone system for their import, including the phosphate carrier (PiC) and oxoglutarate carrier (OGC). A1-C and A2-C were bound to nickel-sepharose beads in small columns then incubated with RRL translation reactions expressing radiolabelled PiC and OGC. The amounts of radiolabelled polypeptide co-precipitating with the DJA proteins were analyzed. Similar trends were observed for co-precipitation experiments with both preproteins. Positive control experiments using full-length DJA1 and DJA2 were as expected from previous results (Tzankov, *et al.* 2008; Bhangoo, *et al.* 2007). DJA1 displays the strongest binding affinity for PiC at nearly four-fold binding compared to the negative control, whereas full-length DJA2 displays about half the binding efficiency (Figure 3). The C-terminal mutants have decreased PiC binding efficiency relative to their full-length proteins, at levels similar to the negative control. Interestingly, co-precipitation with OGC revealed a different trend to PiC. At just under three-fold binding efficiency relative to negative control,

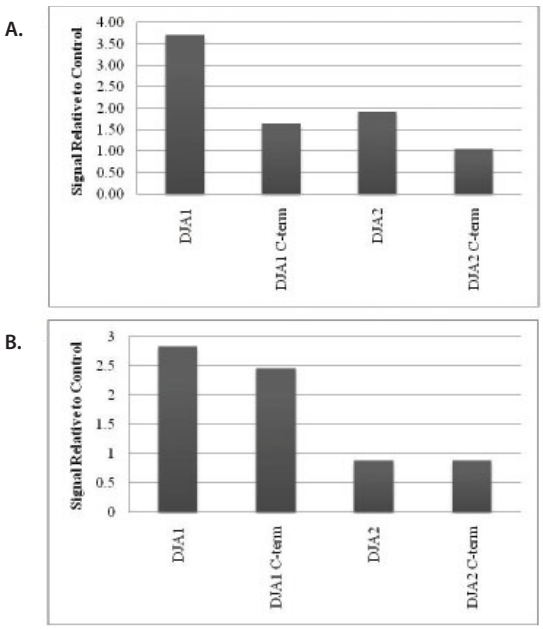

Figure 3: Coprecipitation of (A) PiC and (B) OGC with DJA1, DJA2 and their C-terminal mutants relative to a control containing no purified protein. All binding reactions used final protein concentrations of 5μM.

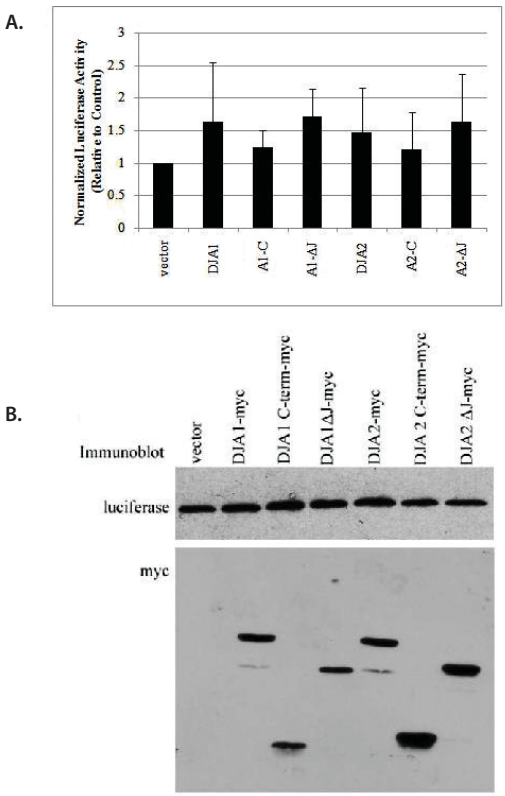

Figure 4: DJA Mutant function in vivo. HeLa cells were transfected with plasmids expressing luciferase, and either empty vector or the indicated myc-tagged DJA proteins. Twenty-four hours after transfection, cells were harvested. (A) Equivalent amounts of lysate were analyzed for luciferase folding activities. To compare folding activities, the average activities of cells transfected with luciferase and empty vector were set to one. (B) Equivalent amounts of lysate were analyzed by immunoblots to detect myc-tagged DJA proteins, ΔJ fragments, C-terminal fragments and HA-tagged luciferase.

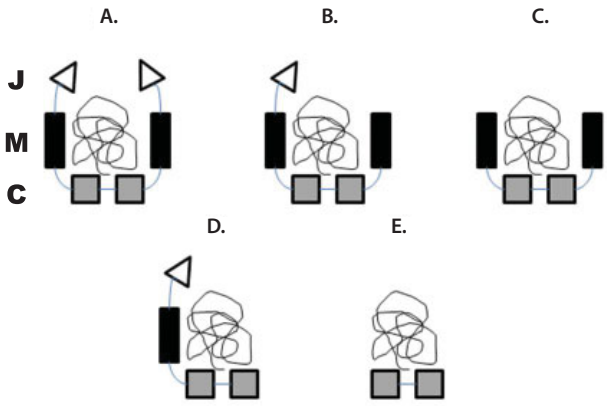

Figure 5: Model of DJA and fragment structure in vivo and in vitro. (A) Full length DJA1 and DJA2 are present as homodimers with three distinct domains; the J-domain, the middle domain and the C-terminal domain. When transfected in cells, it forms heterodimers with (B) the ΔJ fragments and (D) the C-terminal fragments. Our paper and previous research suggest that when purified, the homodimers formed by (C) the ΔJ DJAs and (E) the C-terminal fragments may both have the ability to bind substrate.

DJA1 exhibits lesser affinity for OGC relative to PiC, but both DJA1 and the A1-C C-terminal fragment appear to be significantly better than DJA2 and A2-C at binding OGC.

DJA Mutant Function in vivo

Previous experiments have demonstrated the ability of DJA co-chaperones to promote polypeptide folding in the native cellular environment (Bhangoo, *et al*. 2007). In this study, we examined the effects of overexpressing DJA1 and DJA2 as well as their C-terminal fragments on the folding of firefly luciferase in HeLa cells. When the wild type DJAs were overexpressed they both appeared to display an increased activity relative to the control (Figure 4). Wild type DJA1 displayed an average relative normalized luciferase activity of 1.63, somewhat less than has been reported by a previous publication from this laboratory (Bhangoo, *et al*. 2007). Also, DJA1 was shown to be more effective at promoting luciferase activity than DJA2 (Bhangoo, *et al*. 2007; Tzankov, *et al*. 2008) and DJA2 produced a 1.47 fold increase over the control. Interestingly, overexpression of the DJA C-term mutants resulted in similar increases in activity. The DJA1 C-term mutant produced a relative luciferase activity of 1.25, while the DJA2 C-term mutant produced a result of 1.21 relative to control. As a further comparison, mutants of DJA1 and DJA2 lacking the N-terminal J domains, but having complete central domains, were also tested in the HeLa cells. These fragments, A1-ΔJ and A2-ΔJ, also promoted luciferase activity at 1.71 and 1.63 fold over the control. Overall, it appears that the C-term DJA mutant co-chaperones are capable of promoting polypeptide folding *in vivo*.

Discussion

In this paper, we have explored the roles of DJA1 and DJA2 both *in vitro* and *in vivo* by comparing them to peptide fragments containing only carboxy-terminal segments. Although it may seem that stimulation of Hsp70 with a DnaJ protein lacking its J-domain would be impossible, our research suggests that C-terminal DJA1 and DJA2 still retain some of the stimulation potential of their full-length proteins. We have also outlined an effective purification method for the C-terminal DJA fragments based on methods outlined by Bhangoo, *et al*.

The DJA1 and DJA2 C-terminal mutants have been shown to display decreased binding affinity for peptide substrates when compared to the full-length proteins. This is an interesting result because previous studies on Ydj1, the yeast homolog of DJA1, suggest that the cysteine rich region and the G/F regions may also contribute to some binding specificity (Yan, *et al.* 1999). This implies that the C-terminal portions must have at least some partial capacity to bind substrate and are, therefore, likely to determine peptide specificity. Although further research needs to be done before drawing any definite conclusions, the differences between DJA1 and DJA2 binding to PiC and OGC support the theory that DnaJ homologues may carry peptide specificities. Further studies should aim to determine what specific sequence within the C-terminus is responsible for specificity, and whether this specificity can be controlled by selectively mutating residues. Being able to control Hsp70 binding partners in cells could be an invaluable tool for therapeutics targeting neurodegenerative diseases.

The transfected DJA C-terminal mutants appear to have reduced refolding of firefly luciferase when compared to the full-length DJA proteins in HeLa cells, but they still increase luciferase activity. They do not act as dominant negatives by inhibiting luciferase folding, as might be expected. This could be explained if heterodimers of the DJAs form upon overexpression of the C-terminal fragments, and these proteins having a single J domain and central region are sufficient for co-chaperone activity (Figure 5). This is supported by the results presented here and by Tzankov, *et al*., suggesting that a heterodimer of a DJA and its fragment lacking a J-domain are sufficient for stimulating activity of Hsp70 in luciferase refolding. We interpret these results as evidence that DJA1 and DJA2 do not homodimerize out of any functional necessity, since this structural feature has shown to be unnecessary for stimulating Hsp70 activity. Instead, the DJAs form dimers to increase their ability to activate Hsp70 ATPase function. However, as evident from the error present in Figure 4, these conclusions should only be used as guidelines for future research. We will need to elucidate whether heterodimers can form between DJA proteins and other proteins by increasing the reproducibility of the *in vivo* data.

We have also outlined the experimental basics necessary to implement the C-terminal DJA1 and DJA2 proteins as experimental models for co-chaperone research. As researchers continue to deconstruct the still poorly understood physical framework of DnaJ proteins, creating a library of mutant and fragmented proteins will ensure they have the tools necessary to experiment effectively. Although further research is required to clearly elucidate the potential of the C-terminal fragments as co-chaperones, we have presented a clear description of their isolation and methods for investigating their peptide binding and luciferase refolding activity.

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The Evolution of Algorithms to find Prime Numbers

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Prime numbers are what is left when you have taken all the patterns away. I think prime numbers are like life. They are very logical but you could never work out the rules, even if you spent all your time thinking about them.
- Mark Haddon, *The Curious Incident of the Dog in the Night-time*

Introduction

Prime numbers, and the deterministic formulas used to find them, have garnered considerable attention from mathematicians, professionals and amateurs alike. A prime number is a positive integer, excluding 1, whose only divisors are 1 and itself. For example, 23 is a prime number as it can only be divided by 1 and 23. A number that is not prime is called a composite number.

While prime numbers under 100 are fairly abundant, they become less frequent and difficult to find in a systematic manner as the digits in the number increase since they do not appear to follow a predictable distribution. So why do researchers keep studying them? For over 150 years, mathematicians have attempted to uncover a deterministic formula to identify prime numbers. If such a formula existed, all numbers could be factored relatively quickly using computers. Paradoxically, much of electronic data today is encrypted by taking advantage of the fact that it is difficult and time consuming for a computer program to factor a large composite number. A formula to find all prime numbers would be a significant breakthrough in mathematics, but severely detrimental to data security.

A simple algorithm to find prime numbers: The Sieve of Eratosthenes

As early as 200 BCE, in their efforts to determine the first few prime numbers, Greek mathematicians developed an algorithm requiring relatively easy calculations. All integer numbers greater than 1 can be uniquely factored as a product of prime numbers; this is the Fundamental Theorem of Arithmetic (Andrews, 1994). Consequently, it is a corollary that any composite number must have at least one factor smaller than or equal to its square root. For example, consider the factorization of 118: $118=2\cdot59$, and the factor 2 is less than $10.86=\sqrt{118}$.

Since all composite numbers have prime factors smaller than or equal to their square roots, it follows that prime numbers, which cannot be factored, do not. This idea prompted the Ancient Greek mathematician Eratosthenes to conceive of the Sieve of Eratosthenes to find small prime numbers (Ore, 1988). In order to find prime numbers less than 100, for instance, Eratosthenes would remove all factors of 2; then all factors of 3; then since 4 is not a prime, having already been removed as factors of 2, remove all factors of 5; etc. as seen in Figure 1.

The Sieve of Eratosthenes is an example of a deterministic algorithm used to unearth all prime numbers, but is only practical for "small" prime numbers, those less than 10,000,000 (Ore, 1988). Beyond that boundary, it is too resource-consuming for a computer to perform such a calculation.

The sporadic, but never-ending, primes: the Prime Number Theorem and the Infinity of Primes

Even though the Sieve of Eratosthenes offers an effective algorithm for finding small prime numbers, it gives little insight into the distribution of prime numbers. Carl Friedrich Gauss was the first to notice the only clear distributive property of prime numbers: they get scarcer as numbers get larger. Among the first 10 integers, 40% are prime; among the first 100, 1 in 4 is prime. This pattern continues, such that in the first 100,000 integers, 1 in 10.4 is prime (Peterson, 1996). In fact, Gauss wrote that "this frequency is on the average inversely proportional to the [natural] logarithm" (Tschinkel, 2006), so the approximate number of primes below a number *n* follows Equation(1):

$$\int \frac{dn}{\ln(n)}$$

Equation 1: Gauss' equation of the distribution of prime numbers

The French mathematician Adrien Marie Legendre independently developed a similar equation just a few years later. The result is known as the Prime Number Theorem, which while giving no definitive equation to find prime numbers, provides an approximation of the distance between prime numbers within any given interval. In fact, it states that the average distance between two consecutive primes near some number n is close to the natural logarithm of n (Peterson, 1996). For example, since ln(1000)=6.91, near 1000, approximately every seventh number should be prime.

As the density of prime numbers decreases, it might be expected that eventually prime numbers get so scarce that there exists a single largest prime number. However, the infinitude of prime numbers has been known since 300 BCE when it was established in Euclid's *Elements*. Euclid's proof hinges on the Fundamental Theorem of Arithmetic: if there is a single largest prime number, there would be a finite set of prime numbers. This theorem implies that all composite numbers could then be factored into these prime numbers. However, Euclid found a number that could not be divided by any of these prime numbers, thus necessitating the existence of another prime number (Ore, 1988). By induction, prime numbers are thus infinite. For a concise proof and examples, see Figure 2.

Despite providing insight into the distribution of prime numbers over the real number line, the Prime Number Theorem did not contribute to creating a definitive formula to find prime numbers.

Finding a faster algorithm: Euler's formula, the Riemann hypothesis, and a polynomial-time algorithm

Although the Sieve of Eratosthenes is a foolproof method to find prime numbers, this primitive algorithm is very time consuming, and mathematicians have devoted their efforts to finding a faster method.

Leonhard Euler spent many years working on a deterministic formula for finding prime numbers and eventually developed the equation seen in Equation 2. However, this equation only works for restricted inputs and does not determine consecutive prime numbers, meaning that its use as a test of primality is limited.

$$f(x) = x^2 + x + 41 \quad \text{for} \quad 0 \le x \le 39$$
$$\text{For example, } f(4) = 4^2 + 4 + 41 = 61 = \text{prime}$$

Equation 2: Euler's prime generating function

Multiples of 2 are underlined, of 3 are *overlined*, of 5 are **bolded**, and of 7 are *italicized*.
The primes are boxed.

1	2	3	4	5	6	7	8	9	10
11	12	13	14	15	16	17	18	19	20
21	22	23	24	**25**	26	27	28	29	30
31	32	33	34	**35**	36	37	38	39	40
41	42	43	44	45	46	47	48	*49*	50
51	52	53	54	**55**	56	57	58	59	60
61	62	63	64	**65**	66	67	68	69	70
71	72	73	74	75	76	*77*	78	79	80
81	82	83	84	**85**	86	87	88	89	90
91	92	93	94	**95**	96	97	98	99	100

Figure 1: The Sieve of Eratosthenes

For primes p_i, $(p_1p_2p_3...p_n + 1)$ has remainder 1 when divided by all primes p_i, so cannot be decomposed by the finite set of primes. This contradicts the Fundamental Theorem of Arithmetic. Then another prime must exist, either $(p_1p_2p_3...p_n + 1)$ or a smaller number which divides it.

For example, this clearly holds on small primes:
$2 \cdot 3 + 1 = 7 = prime$
$2 \cdot 3 \cdot 5 + 1 = 31 = prime$
$2 \cdot 3 \cdot 5 \cdot 7 + 1 = 211 = prime$
$2 \cdot 3 \cdot 5 \cdot 7 \cdot 11 + 1 = 2311 = prime$
etc.

Figure 2: Short proof of the infinitude of prime numbers

Euler also developed the zeta function, relating a sum of fractions to a product of prime numbers, shown in Figure 3. Bernhard Riemann extended it into what is now known as the Riemann Zeta function (Ivic, 2003). In 1859 Riemann published his results and hypothesized that a function that has a zero root uniquely defines a prime number. Recently, in 2004, Gourdon and Sebah verified the Riemann hypothesis for the first 10 trillion zeroes (Crandall and Pomerance, 2005); nevertheless, the hypothesis remains unproven.

The discovery of prime numbers is simplified by computer programs, where the main challenge is finding a more rapid algorithm. Computer algorithms are usually compared using runtime analysis, which determines the worse case runtime given an input of length n. The runtime of a program is a function of the length of the input, and can be a polynomial, logarithmic or exponential equation. As the input size increases, an exponential runtime will always be longer than a polynomial runtime, which in turn will be longer than a logarithmic runtime.

The Sieve of Eratosthenes is an exponential algorithm to find prime numbers, which renders it ineffective in finding exceptionally large prime numbers. Since Eratosthenes, all deterministic algorithms to find primes have been exponential, so it was remarkable when a relatively simple deterministic polynomial algorithm was finally discovered in 2002 by Agrawal, Kayal and Saxena (AKS). The AKS algorithm, based on Fermat's Little Theorem and other proven mathematical assumptions, is an improvement but may still have an extremely long runtime, rendering it impractical. The search for an expeditious yet deterministic formula to find prime numbers is still underway, and there is no doubt that computers will continue to provide mathematicians with the ability to make further improvements.

$$\begin{aligned}
\zeta(s) &= 1 + \frac{1}{2^s} + \frac{1}{3^s} + \dots \\
&= \sum_{n=1}^{\infty} \frac{1}{n^s} \\
&= \prod_{primes\ p} \frac{1}{1 - p^{-s}} \\
&= \prod_{primes\ p} \frac{p^s}{p^s - 1} \\
&= \left(\frac{2^s}{2^s - 1}\right)\left(\frac{3^s}{3^s - 1}\right)\left(\frac{5^s}{5^s - 1}\right)\dots
\end{aligned}$$

If the function has a root, then

$$\begin{aligned}
0 = \zeta(s) &= \left(\frac{2^s}{2^s - 1}\right)\left(\frac{3^s}{3^s - 1}\right)\left(\frac{5^s}{5^s - 1}\right)\dots \\
0 &= \frac{p^s}{p^s - 1}
\end{aligned}$$

for some prime p

Figure 1: The Riemann-Zeta function

The use of prime numbers in Cryptography and the consequences of a deterministic formula for finding prime numbers

Cryptography, the science of encrypting and decrypting messages for transmission between a sender and an intended recipient, may at first glance seem unrelated to the discovery of prime numbers. Beginning in Roman times, information was frequently encrypted using a private-key, meaning that the sender and the recipient had to define a decryption codec that would allow the recipient to decode the message. This practice became impractical as technology evolved, since there was often no secure way to communicate the private-key, especially over considerable distances. To deal with these challenges, public-key cryptography was developed. Presently, the most common encryption methods in use rely on the difficulty of efficiently finding prime numbers.

Whereas prime numbers are known up to several thousands of digits, it is much harder to factor composite numbers with several thousands of digits, especially if they are composed of large prime numbers; a deterministic exponential time algorithm could take centuries to factor the composite number. This is the basis of public-key cryptography using the RSA encryption method created in 1978 by Rivest, Shamir and Adleman. Two large prime numbers p and q decide a large composite number N=pq as well as the encryption key e using an equation. From p, q and e, the decryption key d is determined by the same equation. An individual who wants to receive information securely makes public N and e, allowing anyone to send them the information. If the information is intercepted, then knowledge of d, which can only be determined if p, q and e are known, is required for decryption. This means that in order to decrypt the intercepted information, the individual, who only knows N and e, must factor N back into p and q, which is a restrictively time consuming process. On the other hand, if prime numbers could be found quickly, then composite numbers could be factored much more swiftly, and the RSA method would fail, rendering electronic public-key encryption insecure.

A similar threat to secure encryption is quantum computing, which is based on the principle that quantum properties could be used to represent data and perform operations as a traditional computer does. If quantum computing evolves beyond the experimental stages where it currently is, it presents the possibility of performing computations in record time. The same algorithms could be used, but would be executed much faster. For example, while it may take centuries to break an RSA code using a traditional computer, a quantum computer could take just seconds or minutes.

Conclusion

The field of prime numbers is ever changing, with new prime numbers discovered every few years. Early mathematicians dealt with the distribution and infinitude of prime numbers, whereas modern mathematicians aspire to find a deterministic formula to identify all prime numbers. Algorithms that quickly generate small prime numbers already exist, and the recent AKS polynomial time algorithm will even allow large prime numbers to be found quickly. Although finding prime numbers with ease would be a seminal accomplishment in mathematics, it would also create new challenges for the safety of electronic data encryption. Are the benefits of a deterministic prime generator to mathematics worth the destruction of the most common form of data encryption in computer science? Only time will tell.

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The Roles of Dopamine D1 and D2 Receptors in Working Memory Function

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Abstract
Dopamine has been implicated in the modulation of working memory via its interactions with circuits located in the prefrontal cortex of rodents and non-human primates. However, the role that pathways triggered by dopamine receptor subtypes play in affecting processes of working memory remains unclear. In humans, the evidence for dopaminergic modulation of working memory is controversial and the neurological substrates for dopamine's modulatory effects are not fully understood. This paper will review the major animal and human studies that implicate synaptic dopaminergic transmission in working memory function and will outline a new framework to clarify the specific contribution of dopamine D2 receptors to the performance of this cognitive function. Specifically, it is proposed that activation of hippocampal dopamine D2 receptors by chemical agonists could result in the enhancement of spatial working memory.

Keywords
Working memory, Dopamine, D1 receptor, D2 receptor, Hippocampus, Dorsolateral prefrontal cortex.

Introduction

Working memory is an important cognitive process mediated by circuits involving the prefrontal cortex (PFC) and posterior cortical areas. In patients with schizophrenia, altered synaptic dopaminergic transmission in the dorsolateral PFC is related to deficient working memory (Abi-Dargham, *et al.* 2002). Since this deficit is thought to underlie the extensive cognitive impairment and negative symptoms present in schizophrenia (Green, 1996; Abi-Dargham and Moore, 2003), much research has been devoted in the past couple of decades to determining the neurobiology of working memory. Despite these efforts, however, there are presently no evidence-based treatments available to ameliorate the highly debilitative cognitive symptoms of schizophrenia. The goal of this paper is to review the roles of dopamine D1 and D2 receptors in working memory and to highlight a potential new direction for research in humans that could further improve our understanding of the neurobiological underpinnings of this important cognitive function.

Overview of the Prefrontal Cortex and Working Memory

The prefrontal cortex is a collection of distinct cortical areas located anterior to the frontal eye fields in the frontal lobe. In humans it is larger relative to lower mammals such as monkeys, cats, dogs and squirrels. This anatomical difference is thought to account for some of the pronounced differences in cognitive ability that are observed between species (Squire, *et al.* 2003). The PFC can be separated into three major subdivisions based on general differences in cytoarchitecture and connectivity: ventromedial, ventrolateral and dorsolateral (Fellows, 2004; see Figure 1). The PFC is the substrate for the sophisticated cognitive processing that serves to organize and guide complex behaviour. Specific processes governed by the PFC include decision making, working memory, planning, emotional regulation and reward processing. Collectively, these processes are called executive cognitive functions.

The ventrolateral and dorsolateral areas of the PFC, in unison with posterior cortical areas, such as the posterior parietal cortex, are involved in working memory. While it is widely accepted that these broad cortical areas are critically involved in working memory, there has been less agreement about the precise contributions of each area to this cognitive function. In a seminal paper in 1988, Goldman-Rakic outlined a domain-specific theory of working memory, hypothesizing that the dorsal PFC processes spatial working memory information and the ventral PFC processes non-spatial working memory information. More recently, Petrides (1995, 1996) put forth a process-specific model or two-stage hypothesis of working memory function. In this model, information becomes progressively processed along a pathway leading from the ventral lateral to dorsal lateral PFC. Specifically, the mid-ventral lateral PFC retrieves task-relevant information from posterior cortical association areas and transmits it to the mid-dorsal lateral PFC which is involved in the monitoring of information. Manipulation of task-relevant information, another important facet of working memory, is conducted in the posterior parietal cortex (Champod and Petrides, 2007). Emerging neurophysiological evidence generally supports the domain-specific theory over the process-specific model (for a review, see Tanji and Hoshi, 2008).

Dopamine Receptors

The mesocortical dopamine system projects from the ventral tegmental area of the midbrain to various areas of the PFC, including the orbitofrontal, medial, dorsolateral and cingulate regions (Abi-Dargham and Moore, 2003). Dopamine receptors are found on both pre and post-synaptic neurons (Siegel, *et al.* 2006). There are five different types of dopamine receptors, typically classified into D1-like receptors (D1 and D5) and D2-like receptors (D2, D3 and D4) (Siegel, *et al.* 2006). D1-like receptors cause increases in cyclic AMP concentrations, whereas D2-like receptors cause decreases in cyclic AMP concentrations and calcium channel activity and increases in potassium channel activity (Siegel, *et al.* 2006). The five different dopamine receptors show unique patterns of regional distribution in the human brain (for a review, see Joyce and Meader-Woodruff, 1997; Abi-Dargham and Moore, 2003). D1 receptors are present in high densities in cortical areas, including the PFC, and the striatum. D2 receptors are present in high densities in the striatum, but at very low densities in the PFC. D3 receptors are particularly dense in the ventral striatum while D4 receptors are located in the PFC and hippocampus. D5 receptors are present in the hippocampus and entorhinal cortex.

Animal Studies: How is dopamine involved in working memory?

Studies conducted in rodents and non-human primates suggest that dopamine exerts its modulatory effects on working

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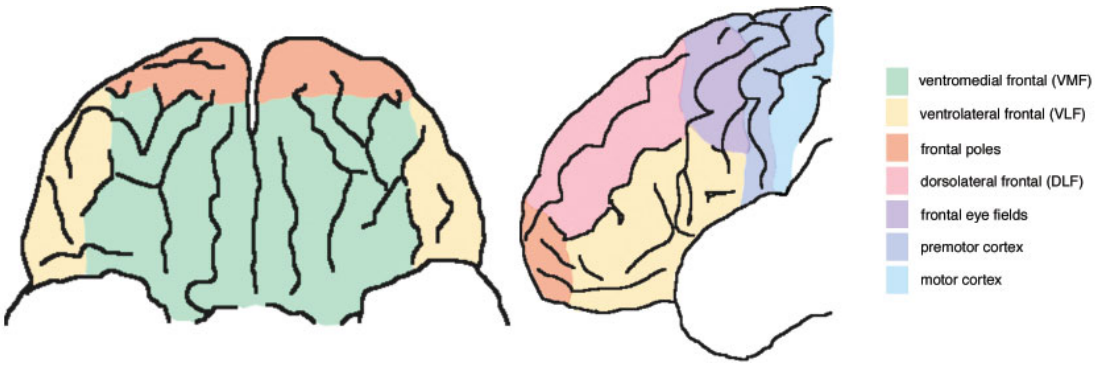

Figure 1: Subdivisions of the prefrontal cortex (PFC).The PFC is composed of all regions anterior to the frontal eye fields, namely the ventromedial, ventrolateral and dorsolateral frontal regions and the frontal poles. Reproduced with permission from Fellows (2004).

memory primarily via the D1 receptor in the PFC. A seminal study by Brozoski, *et al*. (1979) showed that selective depletion of dopamine in the dorsolateral PFC of rhesus monkeys significantly impaired their reaction on a delayed spatial alternation task. In this task, one of two wells was baited with food. After selecting the food, the subject waited over a period of delay ranging from 7 to 60 seconds until the next trial commenced. Then, the subject had to select the well that was not baited in the previous trial, i.e., it had to alternate its selection of wells on successive trials. In the same experiment, Brozoski, *et al.* (1979) also ablated the dorsolateral PFC of one subject and observed a decrease in performance on the delayed spatial alternation task that was similar in magnitude to the dopamine-depleted subjects. Specifically, the performance of both subject types on this task following a 20 second delay fell to between 40% - 60% accuracy after the experimental manipulation compared to between 70% - 100% accuracy prior to the experimental manipulation. Notably, the impairment induced by dopamine depletion was subsequently reversed by administration of levodopa (the immediate precursor to dopamine in the synthetic pathway) or apomorphine (a combined D1 and D2 receptor agonist). However, Brozoski, *et al.* (1979) posited that the location of action for the levodopa-induced reversal of impairment was not confined to the dorsolateral PFC because the drug was administered systemically via an intraperitoneal injection. Nonetheless, this study suggested that dopamine receptors in the PFC could play an important role in the modulation of working memory and spurred further research into the mechanisms underlying this relationship.

Subsequent studies conducted in rodents and non-human primates sought to clarify the behavioral effects of altering synaptic transmission at different dopamine receptors and to determine the cellular mechanisms underlying these effects. The results of several behavioral studies involving pharmacological manipulation of dopaminergic transmission suggested that D1 receptors in the PFC may critically influence performance accuracy on various working memory tasks (Amico, *et al.* 2007; Cai and Arnsten, 1997; Gonzalez-Burgos *et al.*, 2005; Henze, *et al.* 2000; Kobori, *et al.* 2006), though D2 receptors can also play a role under some drug and task conditions (Druzin, *et al.* 2000; Karl, *et al.* 2006). Moreover, there may be an optimal range of D1 receptor stimulation in the PFC that underlies this effect, such that working memory performance and synaptic dopamine concentrations in the PFC follow an inverted-U function (Kroner, *et al.* 2007; Vijayraghavan, *et al.* 2007; Williams and Goldman-Rakic, 1995; Zahrt, *et al.* 1997).

In two classic studies by Sawaguchi and Goldman-Rakic (1991, 1994), monkeys were tested with an oculomotor delayed response task after receiving intra-cerebral injections of either D1 and D2 receptor antagonists or saline control injections. In this task, subjects fixated their gaze on a central spot of a screen and remained fixated on this spot while a visual stimulus was presented somewhere else on the screen, within their peripheral visual field. After a delay period of 1.5 to 6 seconds, the stimulus at the fixation point was turned off, instructing subjects to shift their gaze (make a saccade) to the point on the screen where the visual stimulus was previously located. Compared to injections of saline and D2 receptor antagonists, various D1 receptor antagonists induced a delay and dose-dependent impairment in performance accuracy on the occulomotor delayed response task. Non-specific effects of the antagonists on motor function and visual perception were controlled for by using a separate oculomotor task that required the monkeys to make a sensory-guided saccade rather than a memory-guided saccade. Arnsten, *et al*. (1994) complemented these results by demonstrating that monkeys were impaired on a delayed response task after systemic administration of a D1 receptor antagonist, while their performance improved after systemic administration of a D1 receptor agonist. In each trial of this task, a well was baited with food while the monkey watched. Then, all of the wells (two to four) were covered with plaques. For the delay period (0 to 40 seconds), an opaque screen was lowered. After the delay period, the monkey had to recall which well had been previously baited with food and select it. In another study performed on monkeys, Castner, *et al.* (2000) effectively reversed working memory impairments on a delayed response task by selectively stimulating D1 receptors. Previously, it has been demonstrated that D1 receptors are down-regulated in the PFC of primates after chronic administration of antipsychotic drugs that antagonize D2 receptors (Lidow and Goldman-Rakic, 1994). In order to replicate this effect at the D1 receptor, Castner, *et al.* (2000) chronically administered the potent D2 receptor antagonist haloperidol to monkeys. After a period of one year on this regimen, the monkeys were given a D1 agonist with or without haloperidol and their performance was assessed on a delayed response task. The number of correct responses on this task increased significantly over time in monkeys given the D1 agonist, effectively reversing the performance decline observed during and after long periods of haloperidol administration. Considered together, these studies suggest an important role for D1 receptors in PFC working memory processes.

The modulatory influence of dopamine on working memory is not limited to the dorsolateral region of the PFC. A study conducted by Seamans, *et al*. (1998) showed that dopaminergic modulation of the pre-limbic region of the PFC in the rodent

brain could also affect spatial working memory performance. They used delayed and non-delayed versions of the spatial win-shift task to assess spatial working memory after injection of D1 or D2 antagonists via a cannula into the pre-limbic region of rodents. In this task, the animal is placed in an eight-armed maze (spatial aspect). In a training session, the animal obtains food in four out of eight arms of the maze, while the other four out of eight arms are blocked by barriers. In the delayed version, the animal is placed back in the maze after a delay of 30 minutes and must now locate food in the four out of eight arms that were previously blocked. In the non-delayed version, the animal must immediately locate the food in the same manner as described above. After obtaining food in one arm of a maze, the animal must switch to a different arm to obtain another piece of food (win-shift aspect). Half of the arms are baited with food, while the other half are empty. After infusions of a D1 receptor antagonist into the pre-limbic region of the PFC, rodents were significantly impaired in the delayed version of the spatial win-shift task, but not in the non-delayed version. Interestingly, injection of a D2 receptor antagonist did not affect performance of this task relative to the baseline. These results further support the theory that D1 receptors have a greater influence on the functioning of working memory in the PFC than D2 receptors.

While D1 receptors may contribute significantly to the dopaminergic modulation of working memory, recent studies posit that dopamine also exerts its effects through other receptors. Von Huben, *et al*. (2006) found evidence for D2 receptor modulation of working memory in monkeys after systemic administration of raclopride (a D2 antagonist) and SCH23390 (a D1 antagonist). In this study, accuracy on a self-ordered spatial search task was significantly decreased in monkeys administered raclopride compared to those administered SCH23390. In this task, two, three or four coloured rectangles were presented randomly in different locations on a dark screen. The subject had to select each of the coloured rectangles once, without reselecting any of the coloured rectangles. It is important to note, however, that the negative linear association observed between D1 agonist administration and accuracy on the self-ordered spatial search task was not statistically significant. Although this contradicts other statistically-substantiated studies that demonstrate a preferential modulatory effect of D1 receptor agents over D2 receptor agents when administered systemically (Arnsten, *et al.* 1994; Castner, *et al.* 2000), it does raise questions concerning relative influences of D1 and D2 receptors.

Human Studies: Are D2 receptors less important than D1 receptors for working memory?

Studies in humans involving the selective modulation of D1 and D2 receptors also exhibit a spectrum of results. Several papers have demonstrated facilitation of working memory following administration of D2 receptor agonists (Kimberg, *et al*. 1997, 2001; Luciana, *et al*. 1992, 1998; Luciana and Collins, 1997) while others show impairment of working memory after administration of D2 receptor antagonists (Luciana and Collins, 1997; Mehta, *et al*. 2004). In addition, some studies have reported no effect of D2 receptor modulation on working memory (Kimberg, *et al*. 2001; Muller, *et al*. 1998). Ellis, *et al.* (2005) also published data suggesting that combined stimulation of D1 and D2 receptors in dopamine-depleted participants results in an impairment of working memory. Thus, the relative contributions of D1 and D2 receptors to working memory function in humans remains unclear.

Work by Muller, *et al*. (1998) suggests that D2 receptors may play a less significant role than D1 receptors in modulating working memory in humans. In this study, a pharmacological subtraction paradigm was used to compare the relative contributions of D1 and D2 receptors on working memory since a chemical agonist with specificity for D1 receptors in humans has yet to be discovered. Participants were tested on two separate days after administration of comparable doses of pergolide (a mixed D1 and D2 receptor agonist) or bromocriptine (a D2 agonist). It is a "subtraction paradigm" because the effect of stimulating D1 receptors is examined indirectly by subtracting the effect of a mixed D1 and D2 receptor agonist (pergolide) from the effect of a D2 agonist (bromocriptine). Working memory was tested using a visuospatial delayed matching task in which participants had to remember the location of seven points on a screen, over a delay period of 2, 8 or 16 seconds, and then determine if a newly presented pattern matched the previously viewed pattern. The results showed that working memory performance was improved by administration of pergolide, but not bromocriptine. Since the drugs were administered at comparable doses in an attempt to provide a similar level of D2 receptor stimulation, the authors concluded that only D1 receptor stimulation was responsible for the observed performance enhancement on the working memory task. Conceivably, it is possible that stimulation of both D1 and D2 receptors resulted in working memory improvement because of a common action on a downstream molecule. In the case of the D2 receptor stimulation alone, this downstream molecule may not have been adequately affected. However, this is not a likely explanation for the observed effect in the study by Muller, *et al.*(1998), given that D1 and D2 receptors generally have different effects on downstream molecules, such as cyclic AMP (Siegel, *et al.* 2006).

While it is likely that D1 receptors also prominently modulate working memory in humans, evidence has emerged that suggests D2 receptors could play a specialized role in facilitating spatial aspects of working memory. In a series of studies using the D2 receptor agonist bromocriptine in healthy human volunteers, Luciana, *et al*. (1992, 1997, 1998) observed ameliorated performance on a visuospatial delayed response task and no effect on the visuospatial non-delayed response task or other tasks assessing attention, arousal, and motor and perceptual functioning. At the beginning of each trial of the visuospatial delayed response task, the participant initially fixated their eyes on a central point of a computer screen. A black circle subsequently appeared somewhere on the screen in their peripheral vision. After a delay period of 500, 4000 or 8000 milliseconds, participants were prompted to touch the screen with a pen to indicate where the black circle was situated prior to the delay. In the visuospatial non-delayed response task, however, participants selected the location of the black circle immediately after it was presented instead of after a delay period. Specifically, Luciana and Collins (1997) showed that the D2 antagonist haloperidol impaired performance on this task more severely when subjects were asked to indicate the location of the circle after a short delay compared to those who were asked to recall the location of the circle immediately. Moreover, it was observed in the same study that antagonism and agonism of the D2 receptor modulated performance of memory tasks requiring spatial coordination but did not alter performance of working memory tasks lacking a spatial component. Thus, it is possible that D2 receptors could play a specialized role in controlling the spatial components of working memory.

Hippocampal D2 Receptors: A potential substrate for the facilitation of spatial working memory?

The juxtaposition of studies by Luciana, *et al*. (1992, 1997, 1998) and Muller, *et al*. (1998) serves to highlight the continuing controversy surrounding the involvement of D2 receptors in working memory. Various explanations have been offered to account for these contradictory results, including differences in the working memory tasks used across studies (Von Huben, *et al.* 2006), D2 receptor involvement in the focusing of attention via a striatal mechanism (Mehta, *et al.* 2004), D2 receptor facilitation of goal-directed behaviour (Luciana, *et al.* 1992) and D2 receptor contributions to the alteration of PFC inputs (Abi-Dargham, *et al.* 2003). None of these possible explanations can be discarded because all studies in humans, and some studies in animals, have used systemic administration as a means of targeting drugs to dopamine receptors, resulting in indiscriminate distribution of these drugs to the entire brain. Still, these theories do not adequately account for the selective facilitation of spatial working memory by drugs modulating D2 receptors observed in both monkey and human studies (Luciana, *et al.* 1992, 1997, 1998; Mehta, *et al.* 2004; Von Huben, *et al.* 2006). While animal studies overwhelmingly suggest that D1 receptors rather than D2 receptors critically modulate working memory processes subserved by the PFC, findings from human studies suggest that D2 receptors outside the PFC could potentially contribute to this cognitive process. Based on this proposition, and emerging evidence from animal studies and recent human neuroimaging and post-mortem studies, an alternative framework for the influence of D2 receptors outside of the PFC on working memory function will be outlined.

Post-mortem studies in humans have shown the precise anatomical location and relative distribution of D2 receptors in the brain. Using radioactively labeled D2 receptor agonist [3H]CV 205-502 and the D2 receptor antagonist [3H]Spiroperidol to label D2 receptors in post-mortem human brain tissue slices, Camps, *et al*. (1989) found moderate densities of D2 receptors in areas CA1 and CA3 of the hippocampus. Kohler, *et al*. (1991) used the radioactive D2 receptor ligand 125I-NCQ 298 and found that the highest densities of D2 receptors in the human brain tissue samples were located in the outer layers of the presubiculum and hilus of the dentate gyrus, in the hippocampus. Comparatively, Cortes, *et al*. (1989) showed that high densities of D1 receptors were located primarily in the striatum, while intermediate densities were located in the cerebral cortex, amygdala, mammillary bodies and area CA1 of the hippocampus of post-mortem human brain tissue slices. It can be inferred from these anatomical findings that moderate levels of D2 receptors are present in different regions of the hippocampus and directly adjacent brain tissue as well. Additionally, higher densities of D1 receptors relative to D2 receptors were found in the PFC. This evidence supports the proposition that D2 receptor modulating agents could exert their effects on spatial working memory outside of the PFC, specifically in the hippocampus.

Previous findings in animals have similarly suggested that dopaminergic transmission plays an important role in the function of normal spatial working memory (Beatty and Rush, 1983; Oades, 1981; Bushnell and Levin, 1993; Kim and Levin, 1996; Korz and Frey, 2007; Lopes Aguiar, *et al.* 2008; Simon, *et al.* 1986; Wilkerson and Levin, 1999; Wisman, *et al.* 2008), most likely through a pathway located in the hippocampus. In two separate studies with rats, Packard and White (1989, 1991) found that the D2 receptor agonist LY 171555 facilitated spatial working memory performance as assessed by the win-shift paradigm. Although it was found that intra-hippocampal injection of the D1 agonist SKF-38393 also facilitated spatial working memory performance in rats (Packard and White, 1991), this could have been due to the different drug administration methods used in the two studies. In the first study (Packard and White, 1989), rats received subcutaneous injections of each drug into the systemic circulation, while in the second study (Packard and White, 1991), rats received direct injections into the dorsal hippocampus. Previous research has indicated that the effect of D2 receptor modulation on spatial working memory is more robust when experimental manipulations target the ventral hippocampus specifically, rather than in combination with other areas (Wilkerson and Levin, 1999). This could account for the increased facilitation of spatial working memory that was observed after subcutaneous injection of a D2 receptor agonist compared to direct injections into the dorsal hippocampus.

Recent human neuroimaging studies further support the proposition that D2 receptors in the hippocampus could modulate cognitive functions subserved by the PFC. For example, Takahashi, *et al.* (2007, 2008) found a positive correlation between performance on tasks assessing memory, verbal fluency and executive cognitive function and D2 receptor binding in the hippocampus. The authors suggest that D2 receptors in the hippocampus influence memory functions subserved by this structure as well as other cognitive functions executed in the PFC. It should be noted, however, that the task used in these studies to assess "frontal lobe function" (the Wisconsin Card Sorting Task) does not explicitly test working memory function. Additionally, the authors highlight several specific neurochemical and neurophysiological processes initiated by D2 receptors in the hippocampus and suggest that they are at least partly responsible for influencing the dopamine receptor-cognitive function relationships demonstrated in their studies. These findings suggest that hippocampal D2 receptors influence circuits between this structure and the PFC and this could have an effect on executive cognitive functions, including working memory. The present framework extends this notion by suggesting a specific construct that is modulated by D2 receptors in the hippocampus, namely spatial working memory.

Smialowski and Bijak (1989) proposed that facilitation of spatial working memory is a direct result of changes to levels of excitatory and inhibitory stimulation and to long-term potentiation of hippocampal CA1 neurons. This hypothesis was derived from electrophysiological studies with rat hippocampal tissue slices that demonstrated a modulatory effect of D2 receptor agonists and antagonists on the firing rates of CA1 pyramidal neurons. Specifically, it was shown that D2 receptor agonists and antagonists caused increases and decreases, respectively, in CA1 hippocampal neuron firing rates in the rat brain. Conversely, they found that agonist-activation of the D1 receptor in the same hippocampal slices decreased the rate of CA1 neuronal firing. These results suggest that stimulation of D2 receptors in the hippocampus facilitates excitation of CA1 pyramidal neurons, while D1 receptor stimulation elicits the opposite response. A more recent study by Hammad and Wagner (2006) suggests that drugs that stimulate the D2-like family of receptors could increase the firing rates of CA1 pyramidal neurons by decreasing the effects of their local inhibitory signals. Other studies have also demonstrated that activation of D2 receptors in CA1 neurons of the hippocampus increases effects associated with long term potentiation (Swant and Wagner, 2006; Thompson, *et al.* 2005). While these findings point to a direct role for hippocampal D2 receptors in modulating spatial working memory functions, it is possible that these are indirect effects that are actually due to the increased acetylcholine release associated with D2 receptor stimulation (Imperato, *et al.* 1993; Umegaki, *et*

al. 2001). Nonetheless, extrapolating these findings to humans suggests that hippocampal D2 receptor activation by chemical agonists is at least partly responsible for the facilitation of spatial working memory seen in humans.

Conclusion

More work is needed to specify the nature of the relationship between hippocampal D2 receptors and components of working memory in humans. Specifically, future neuroimaging studies need to determine if hippocampal D2 receptor binding correlates with performance on tasks that explicitly test spatial and non-spatial working memory, such as the visuospatial delayed response task and the n-back task, respectively. If, contrary to expectations, a dissociation between these two constructs is not observed, it will be necessary to conduct subsequent functional and receptor neuroimaging studies in humans with tasks that separate working memory into putative component processes, such as retrieval, monitoring and manipulation. This would provide insight into the specific role that D2 receptors play in affecting specific component processes related to working memory. Additionally, the development of a clinically-viable D1 agonist would allow for a direct comparison of its effects on working memory to those initiated by a D2 agonist, such as bromocriptine. This approach could be used in unison with functional and receptor neuroimaging designs to enhance our understanding of the respective contributions of these receptor subtypes to working memory in humans. Furthermore, it will be necessary to elucidate the specific neurochemical and neurophysiological mechanisms that underpin the suspected relationship between hippocampal D2 receptors and spatial working memory.

Most research into the effect of modulating synaptic dopamine transmission on working memory has focused on the roles of D1 and D2 receptors in the PFC. While evidence in rodents and non-human primates has overwhelmingly pointed to D1 receptors as the most important receptors in the modulation of PFC-associated working memory functions, evidence from studies in humans is less conclusive. This paper has reviewed the major findings in the animal and human literature and has proposed a framework that implicates synaptic dopamine transmission at hippocampal D2 receptors in the modulation of spatial working memory. A fuller understanding of the neurochemical and neurophysiological mechanisms that underlie working memory could one day lead to clinically-relevant advances in the treatment of the debilitating cognitive impairments associated with neuropsychiatric diseases such as schizophrenia.

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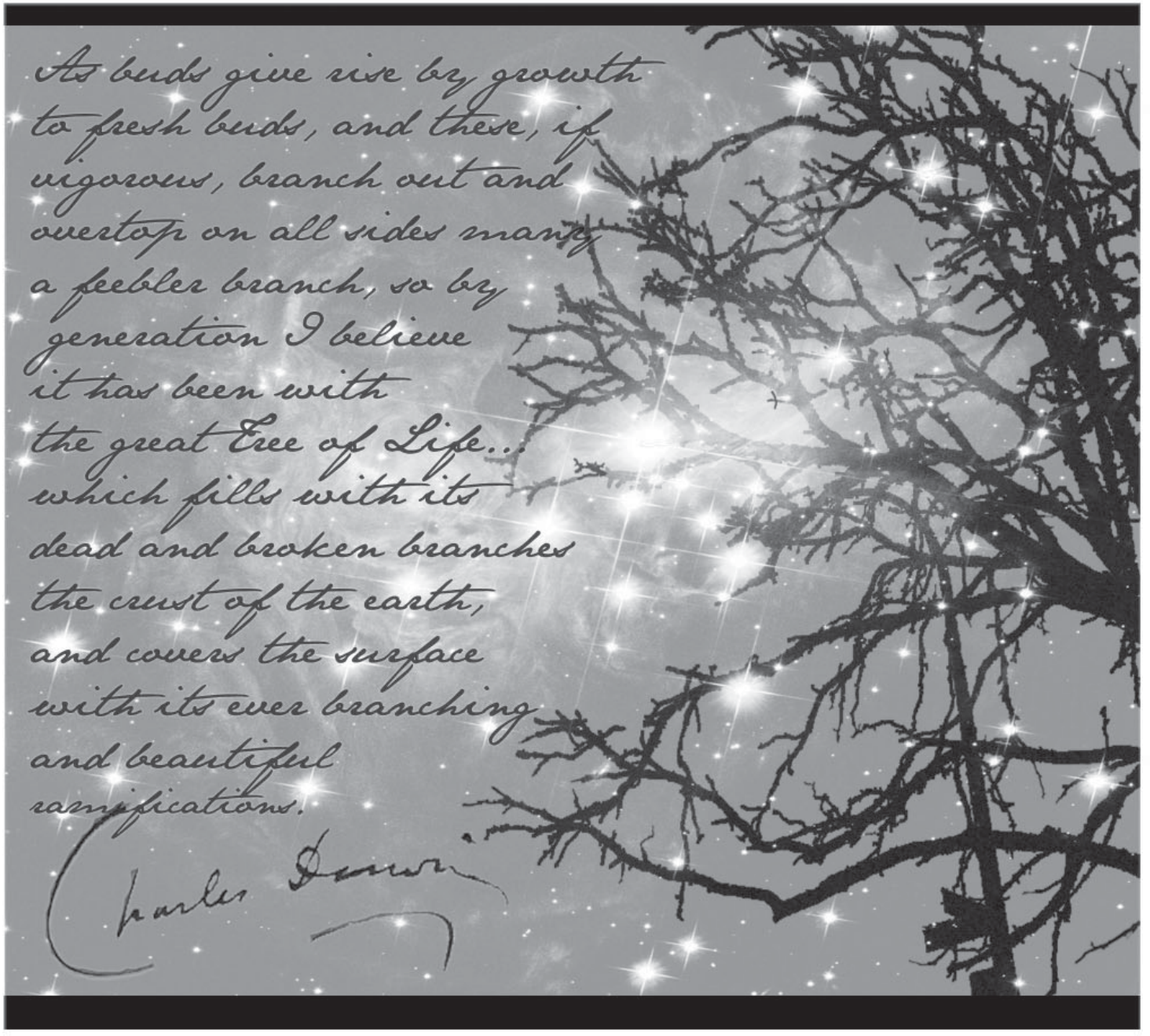

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