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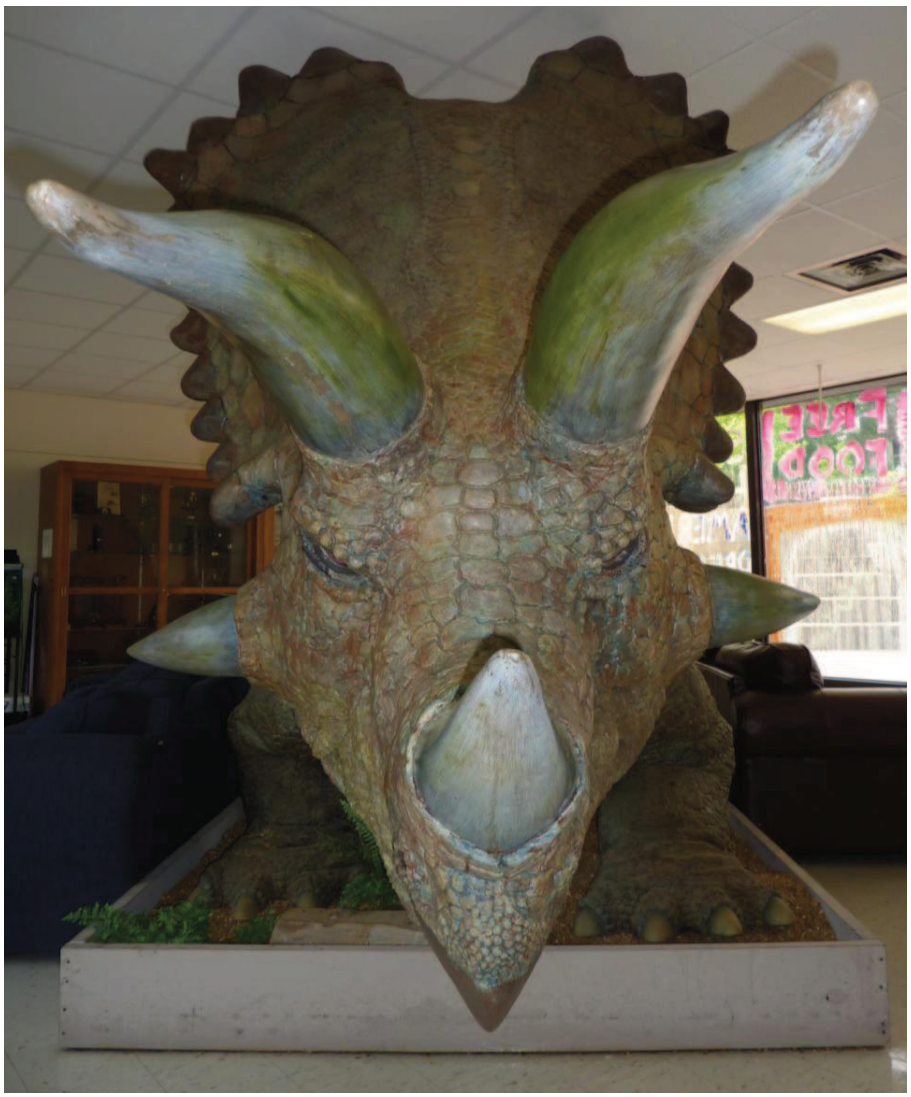

# Rhodes Journal of Biological Science

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# About this Issue

### Statement of Purpose

The Rhodes Journal of Biological Science is a student-edited publication which recognizes the scientific achievements of Rhodes students. Volume XXIV marks the sixth year since the journal was brought back into regular publication by Mark Stratton and Dr. David Kesler in 2006. Founded over twenty years ago as a scholarly forum for student research and scientific ideas, the journal aims to maintain and stimulate the tradition of independent study among Rhodes College students. We hope that in reading the journal, other students will be encouraged to pursue scientific investigations and research.



### Photo Credits

Front and back cover photos taken by Greg Palm '11.


### Acknowledgements

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## Editorial Staff

**Caroline Lee' 11 (Senior Editor)** is a Chemistry major from Mandeville, Louisiana. She is a member of βββ Biological Honor Society, Gamma Sigma Epsilon chemistry honors society, and Phi Beta Kappa honor society. She is currently working with Dr. Mauricio Cafiero on protein-ligand binding in the serotonin and dopamine synthesis pathways. In addition to doing research at Rhodes, she has worked at the Tulane National Primate Research Center studying T cell polyfunctionality in relation to AIDS. In her free time she enjoys volunteering at the MED, reading, and traveling. After graduation she plans to pursue a medical degree at Louisiana State University in New Orleans.

**Greg Palm '11 (Senior Editor)** is a biology major from Merrimack, NH. He is a member of Mortar Board honor society, βββ Biological Honor Society, Chi Alpha Sigma athletic honor society, and Omicron Delta Kappa honor society. He is also the captain of the varsity track and field team at Rhodes. Two summers ago he worked on a nerve regeneration study in the Vacanti Lab at Massachusetts General Hospital in Boston. Last summer he received the Cognitive Aging Research Fellowship to continue analyzing data and writing a manuscript on semantic priming of homophones with Dr. Katherine White in the psychology department. He is currently interning at Campbell Clinic in Memphis, working as a research assistant in the Cognition and Aging Lab, and serving as the Honor Council corresponding secretary. Greg enjoys volunteering at the MED, Regional Trauma Center and donating time to other community oriented activities. Following graduation, he plans to pursue a career in medicine.

**Laura Atkinson '12** is a neuroscience major and film studies minor from St. Louis, Missouri. She spends most of her time serving others by volunteering in Child Life at the Church Health Center Wellness, with children at Chess Club, and at the MED in the ER. She hopes to excite others to serve by coordinating volunteers from Rhodes in the ER at the MED and in Child Life at CHC-W. She has previously done psychology research with Professor Marsha Walton and now helps out in Professor Kimberly Gerecke's Neuroscience laboratory. She is also a member of the Psi Chi Honor Society and hopes to pursue a life of service in the medical field working with the underserved. In her free time, she loves to run, listen to music, and take portraits.

**Shannon Blair '13** is a biology major and religious studies minor from Spartanburg, South Carolina. She is a member of βββ Biological Honor Society and is currently completing an Internship at the Church Health Center. Shannon also plays for the Rhodes Women's soccer team, holds an on campus position in the Residence Life office, and is active with Tuesday Fellowship on campus. In 2011-2012, she will be participating in the Rhodes Intentional Community Fellowship. After graduating from Rhodes, she plans to go into speech pathology for children.

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**Shannon Fuller '11** is a biology major from St. Louis, MO. She is a member of βββ Biological Honor Society, Mortar Board, Secretary/Treasurer of OΔK Honor Society and Founder/Co-President of the GlobeMed chapter at Rhodes. She is currently researching Malaria with Dr. Luque de Johnson in the Biology department. In her free time, she enjoys running, photography, and piano. Last summer, Shannon traveled to Madagascar to study traditional medicine and the socio-political dimensions of healthcare delivery. She will begin a Global Health masters program at the University of California, San Francisco (UCSF) this fall.

**William Hawes '12** is a biology major from Springfield, Missouri. He is the Vice President of the Catholic Student Association, a member of CHEERS, and a member of the βββ Biological Honor Society. William does research studying the role of arginine vasotocin in aggression modulation in brown anole lizards under the supervision of Dr. David Kabelik of the Rhodes Biology Department. William runs varsity cross country as well as indoor and outdoor

track and field. He volunteers at a free medical clinic, tutors local middle school students, and has volunteered in the trauma center at the Regional Medical Center of Memphis. After graduation, William plans to attend medical school.

## Student Contributors

**Jessica Fawer '11** is a Neuroscience major from New Orleans, Louisiana. She is a member of βββ Biological Honor Society and has served as dance team co-captain, vice-president of the Jewish Student Organization, and a board member of Habitat for Humanity. She has spent two years investigating the beneficial effects of exercise on neurodegeneration due to chronic stress with Dr. Gerecke. In addition, she spent last summer at LSU Health Science Center examining the effects of a topical Neuroprotectin D1 treatment on a mouse model of age-related macular degeneration. After graduation, Jessica plans to pursue a career in optometry.

**Matt McCulloch '11** is a Senior Neuroscience major from Little Rock, AR. He is a member of the Phi Beta Kappa, Mortar Board, Beta Beta Beta, and Omicron Delta Kappa Societies and served as student body President and Vice-President. As a St. Jude Summer Plus Fellow, Matt researched proteinopathies associated with Lou Gehrig's disease and related neurodegenerative disorders at St. Jude Children's Research Hospital and enjoys volunteering at the Regional Medical Center of Memphis in the trauma unit. A member of Sigma Nu Fraternity, Matt has coordinated support events for St. Jude and has contributed two spring breaks to rebuilding homes in New Orleans. He was voted "Mr. Rhodes" by his fellow students, and in his free time enjoys eating out in Memphis, traveling, camping, intramural sports, and watching movies. After graduation, Matt plans to take the MCAT and enjoy living in Memphis for a year before entering medical school in 2012.

**Andrew Millis '11** is a biochemistry and molecular biology major from Hinsdale, IL. He is the Vice President of Mortar Board and a member of βββ Biological Honor Society and OΔK Honor Society. Andrew has conducted biological research on chemotherapeutic agents for osteosarcoma models at St. Jude in the Department of Surgery. He has also pursued research/writing for health policy issues relating to American prisoner organ donation and Chinese organ procurement systems. He has been an avid volunteer for the homeless community, coordinating Souper Contact and Advocates for the Homeless, and has also been a volunteer at the MED. After graduation, he plans to pursue a Masters in Public Health and a career in medicine.

**Sarah Tchang '12** is a biology major and environmental science minor from Memphis, Tennessee. She is an active member of βββ Biology Honors Society, the Rhodes Student Associate (RSA) for the biology department, and has been a Teaching Assistant for core biology for two years. Her favorite extracurricular activity is volunteering at BRIDGES in order to advance racial, economic, educational, and environmental justice in Memphis communities. Currently, she does research on Memphis brownfields with Dr. Rosanna Cappellato. During her free time, she enjoys baking various breads, cakes, pies, and other delicious pastries. When she is not baking, she loves spending time in the great outdoors with her friends and her best canine companion named Valentine. After graduating early in December, she plans to attend graduate school in environmental science and pursue a career in sustainability research.

**Emily Woods '12** is a biochemistry and molecular biology major and Russian studies minor from Boulder, CO. She researched microRNA expression in rheumatoid arthritis while studying in Zurich, Switzerland during the Fall semester of 2011. She is a member of βββ Honor Society and is the Director of Internal Communications of GlobeMed. She is also a member of Rhodes Christian Fellowship and AOΠ. Among other things, she enjoys cooking and hiking. After graduation, she plans to attend graduate school to continue research in Biochemistry.

**Chris Yates '12** is a chemistry major from Iowa City, IA. He is a member of the varsity men's tennis team and the president of the Alpha Tau Omega Fraternity. He participates in the Rhodes Student Associate for the sports information department and the voice of lynx varsity athletics. In his free time, Chris is a Teaching Assistant for organic chemistry, volunteers at The MED, enjoys reading, and watching movies. He has conducted research with the Department of Opthalmology at the University of Iowa and took part in the University of Illinois summer enrichment program. Animal Behavior was his favorite course. After graduation, he plans on attending medical school and earning his M.D.

# Docosahexaenoic Acid has Beneficial Effects on the Aging Brain that may Improve the Symptoms and Reduce the Risk of Alzheimer's Disease

Jessica Fawer
Rhodes College

*Docosahexaenoic acid (DHA) is a major component of omega-3 fatty acids, important nutrients necessary for normal brain development and functioning. Multiple studies have shown a negative correlation between levels of DHA in the diet and risk of dementia. These studies have also shown that reduction of DHA in the brain may contribute to neurodegeneration by augmenting neuroinflammatory responses and apoptosis, which can both lead to debilitated brain functioning. For this reason, researchers have dedicated much of their efforts to developing Alzheimer's disease treatments that increase levels of DHA. Although clinical trials have been conducted to examine the effects of DHA treatments on Alzheimer's disease, most of the trials were unsuccessful; however, the results may indicate that the success of the treatment is actually dependent upon the amount of degeneration that has already occurred in the brains of the Alzheimer's patients. Understanding the mechanism by which DHA may prevent the onset or reduce symptoms of Alzheimer's disease may lead to more effective treatments for this ubiquitous disease.*

**Introduction and Discussion**

Alzheimer's disease (AD) has become one of the most common causes of death in individuals over 60 and its prevalence is expected to significantly increase in the coming years; therefore, exploring medications for the treatment and prevention of the disease is now a central focus of research (Fantini and Yahi, 2010). Millions of people in the U.S. are currently affected by AD and the anticipated escalation in the number of cases may create a dilemma for the health care industry (Jicha and Markesbery, 2010). Multiple studies have found a negative correlation between AD and docosahexaenoic acid (DHA), an essential nutrient found in Omega-3 fatty acids (Lukiw and Bazan, 2008). With the increase in Alzheimer's research, more investigators are devoting their time to discovering a clinical treatment for the disease using this important fatty acid.

Docosahexaenoic acid is crucial in maintaining membrane integrity of neurons, as well as the formation and retention of memory (Jicha and Carr, 2010). DHA is believed to ameliorate symptoms of AD via several mechanisms. Amyloid-beta (Aß) plaques and tau tangles are the causes of cognitive decline in AD, and research has demonstrated that DHA can decrease the production of these proteins (Lukiw and Bazan, 2010). External Aβ plaques are formed from toxic peptides produced as a result of abnormal cleavage of amyloid precursor protein (APP), which causes a disruption in neuronal communication (Lukiw and Bazan, 2010). Hyperphosphorylation of tau protein, which is located in microtubules of cellular skeletons with the primary function of stabilizing the cell structure, leads to dissociation of tau that accumulates within the cell (Lukiw and Bazan, 2010). Also, oxidative stress contributes to the formation of plaques and tangles which DHA and its derivative neuroprotectin D1 (NPD1) have been shown to reduce. The evidence of DHA's involvement in neurodegeneration continues to support its potentially beneficial clinical use for treating and preventing AD, although human treatment using DHA has generally been unsuccessful.

***Alzheimer's Disease***

Alzheimer's disease is a fatal, incurable neurodegenerative disorder characterized by progressive cognitive decline: memory loss, problems completing mundane tasks, confusion, declines in communication, and mood adjustments (Fernandez et al., 2010). According to Williams et al (2010), the slow progression of neurodegeneration occurring in individuals with Alzheimer's disease usually does not reveal any of these cognitive symptoms until more than half of the degeneration has already occurred. The most apparent symptom is the inability to remember recently acquired information (Williams et al., 2010). People with AD may begin to repeatedly ask for information for which they have already heard the answer

(Williams et al., 2010). Also, problems completing familiar tasks is a telling sign. For example, a task that individuals should be capable of completing independently, such as balancing a check book, may be impaired (Fernandez et al., 2010). The accumulation of these exacerbating deficits eventually interferes with daily functioning, which may lead to the necessary care for individuals with AD by their family or placement in assisted living facilities.

The increasing costs of health care for Alzheimer's disease patients, as well as the need for assistance from family members, have contributed to the urgent need to develop preventative methods for this irreversible disease. Currently five million individuals in the U.S. are affected by AD (Jicha and Carr, 2010). The occurrence, however, is expected to multiply fourfold by 2050 (Jicha and Carr, 2010). One in five individuals over the age of 85 will be diagnosed with AD, and the number will rise with the increase in life expectancy due to health care improvements (Ramesh et al., 2010). Approximately 25 million individuals worldwide are presently diagnosed with AD, with the addition of 4.6 million new AD cases annually (Søgaard et al. 2009). A 2008 study of AD patients in the United States found that costs increased significantly with each worsening stage of the disease. For people receiving informal and formal care, the annual cost increases from approximately $20,000 at the earlier stages to $43,000 in the fourth year of diagnosis (Zhu et al., 2008). Not only would an understanding of the cause of AD and an effective treatment reduce the death rate of individuals over 60 years of age due to dementia (abnormal cognitive decline), it would also diminish the need for familial caregivers who receive no payments for their full-time assistance, as well as the high healthcare costs.

Research has shown that the degeneration associated with Alzheimer's disease is largely the result of irregular functioning of APP, but the occurrence of this defect in individuals without genetic mutations is a mystery. While the normal function of APP is thought to contribute to neuronal growth, repair, and survival, it is apparent that this membrane glycoprotein plays a definitive role in the progression of AD physiopathology (reviewed in Bobba et al., 2010). APP usually undergoes alteration by α- and ß-secretases (Stockley and O'Neill, 2007). In AD, an additional γ-secretase abnormally cleaves APP via proteolysis in the presence of ß-secretase (but not α-secretase) at the transmembrane segment, producing the beta-amyloid (Aß) peptide of 38-42 amino acids (Massoud and Gathier, 2010). These peptide fragments are then deposited outside of the cell to form the toxic Aß or senile plaques (Green et al., 2007). Neuronal communication is inhibited by the senile plaques and causes some of the neuronal loss that is characteristic of AD (Jicha and Carr, 2010). As demonstrated in figure 1, the apoptosis that occurs as a result of the Aß plaques can lead to other contributing factors of neurodegeneration such as neurofibrillary tangles, oxidative stress and excitotoxicity (Massoud and Gathier, 2010).

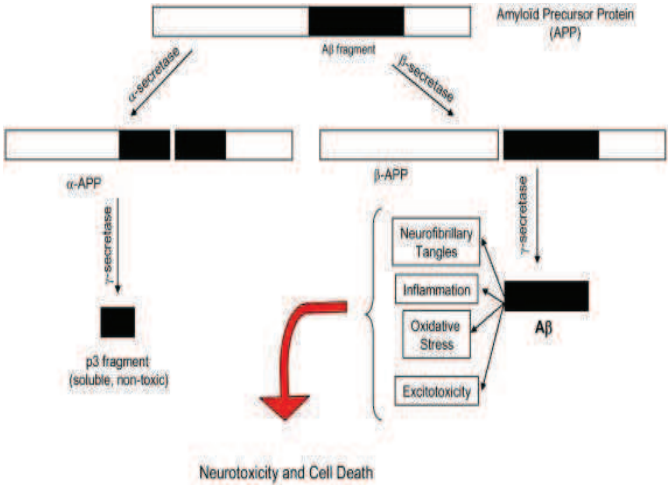


Figure 1: Pathophysiology of Alzheimer's Disease. In combination, ß-secretase and γ-secretase cleave amyloid precursor protein into the Aß peptide, leading to neurotoxicity and cell death. Alpha-secretase and y-secretase, in the absence of ß-secretase, cleave amyloid precursor protein into a non-toxic peptide fragment (Massoud and Gathier, 2010).

Secondary to Aß plaques in the pathology of Alzheimer's disease are tau protein tangles (Jaworski et al., 2010). The usual function of tau involves the stabilization of microtubules in the cellular cytoskeleton (Massoud and Gathier, 2010). Hyperphosphorylation of tau in Alzheimer's disease produces paired helical filaments (PHF) that ultimately cause neurofibrillary tangles (NFT) (Massoud and Gathier, 2010). Since microtubules are the means of transportation within a cell, an interruption in the cell's ability to transport signals due to NFTs eventually leads to neuronal death (Jaworski et al., 2010). Recent research suggests Aß plaques are responsible for the formation of tau tangles, though tau tangles may exist in the absence of Aß plaques and vice versa (Jaworski et al., 2010); thus, the protein dysfunction causality in AD remains unknown.

***Alzheimer's Disease Risk Factors***

Alzheimer's disease is associated with many different environmental and social factors that may contribute to the risk of developing this neurodegenerative disorder. The most apparent risk factor for the development of AD is age (Williams et al., 2010). A problem that arises when diagnosing AD is distinguishing cognitive decline due to aging and cognitive decline due to dementia. The symptoms for each may appear very similar in the early stages of AD, but implementing the use of mini mental state examinations (MMSE) during testing has led to greater accuracy (Laks et al. 2010). According to Kalaria et al., women have a consistently higher prevalence of AD, due to their higher life expectancies in comparison to men (2010). Education level has also been found to influence the risk of dementia. Individuals with little educational achievement or poor literacy may have a greater risk of developing AD than individuals who are intellectually stimulated in their careers and everyday lives. The reason for this correlation is unknown but researchers infer that individuals with higher educational achievement, and

therefore increased intellectual stimulation, develop increased cognitive reserve (the mind's elasticity and ability to fight neurodegeneration) and synapse formation. In addition, lower levels of education are usually associated with lower social class and inferior health.

An additional correlation has been found between cardiovascular health and AD; the risk factors for cardiovascular disease, which include high blood pressure, high cholesterol and poor diabetes care, may put an individual at risk for AD (Kalaria et al., 2010). An individual who suffers from the overproduction of amyloid ß-peptide may be genetically predisposed to this dysfunction of the APP, but there is a possibility that at least one environmental risk factor is necessary to allow the expression of AD genes (Calon et al., 2004).

Though less prevalent than environmental factors, some Alzheimer's patients have acquired a gene mutation responsible for the defective proteins. As Bi (2010) explains, this type of mutation is referred to as early onset Alzheimer's disease because symptoms emerge before age 40. Approximately 5% of all AD cases are familial early onset and can be attributed to mutations in three identified genes. Presenilin 1 (PSEN1) and presenilin 2 (PSEN2) are two of the genes that code for APP and mutations in both contribute to approximately 50% of familial Alzheimer's disease cases. Recent research has found that mutations in PSEN1 and PSEN2 demonstrate γ-secretase activity, which is a known contributor in the formation of Aß plaques (Bi, 2010). Both familial and sporadic Alzheimer's disease include Aß plaques and tau tangles. For this reason, the goal of Alzheimer's research is to reduce the occurrence of these dysfunctional proteins to decrease the amount of neuronal cell death in all cases, perhaps via DHA treatment.

***Docosahexaenoic Acid***

Docosahexaenoic acid is a long chain omega-3 (n-3) polyunsaturated fatty acid (PUFA) that exists in high concentrations within the nervous system (Akbar et al., 2005). Fish oils are enriched with this essential fatty acid, with low levels being linked to prenatal and early developmental defects (Lukiw and Bazan, 2008). Age-related cognitive decline and neurodegenerative diseases have also been heavily studied in association with DHA levels (Lukiw and Bazan, 2008). Eicosapentaenoic acid (EPA), another essential PUFA, undergoes desaturation via beta oxidation to form DHA. After consumption EPA and DHA are absorbed by the liver where the conversion takes place. DHA then travels through the blood stream to its intended locations where DHA is required for proper functioning, such as the brain and photoreceptors in the retina (Mukherjee et al., 2004).

The consumption of DHA is necessary to central nervous system development and preservation of neuronal functioning; however, neurons lack the capacity to produce DHA and must therefore be introduced exogenously (Lukiw and Bazan, 2008). Glial and endothelial cells, on the other hand, have some ability to synthesize DHA, but it remains questionable whether this considerably alters the total levels in the brain (Lukiw and Bazan, 2008). A DHA transporter brings DHA to the brain in blood plasma where binding lipoproteins specific to fatty-acids are located (Lukiw and Bazan, 2008). The highest concentrations of DHA appear in central nervous system synapses, myelin sheaths and photoreceptors; DHA makes up 60% of neuronal cell membranes (Lukiw and Bazan, 2008; Green et al., 2007). Interestingly, the occurrence of AD is not only positively correlated to life expectancy in Western countries, but also parallels the ratio of omega-6 to omega-3 PUFAs in the diet; higher n-6 to n-3 ratios can result in an inflammatory response (Yehuda et al., 2005; Lim et al., 2005). Likewise, AD patients have reduced brain DHA in comparison to healthy subjects of the same age (Green et al., 2007). Studies such as these involving DHA suggest that diet may be another risk factor for AD.

Polyunsaturated fatty acids provide neurons with essential membrane elements required for normal functioning (Jicha and Carr, 2010). As Tully et al. (2003) explains, these membrane lipids support the structure and function of proteins within the membrane and protein complexes. The addition of PUFA in the brain causes cell membranes to be more resilient as a result of the bent configuration (figure 2), and thus preserves the membrane structure. The PUFA insertion in the membrane also lowers the cholesterol fraction, contributing to the more flexible membrane. The ability of DHA to support a more efficient structure is perhaps responsible for the positive effects observed with an increase in DHA: lowering the risk of heart disease, improving memory, and influencing early development of the brain and retina (Tully et al., 2003).

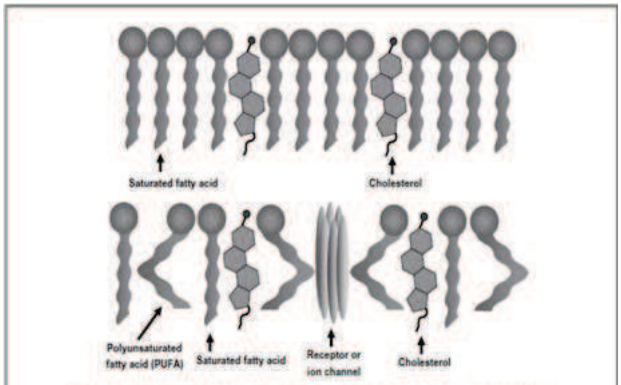


Figure 2. Cell Membrane. Polyunsaturated fatty acids have a bent structure and therefore provide the membrane with more flexibility. This also allows more receptors to be inserted in the membrane (Jicha and Carr, 2010).

This increased membrane fluidity allows for the insertion of more receptors in the synapse, which can enhance neuronal communication. PUFAs, specifically DHA, also maintain membrane structure by their conversion into phospholipids and second messengers that are involved with preventing neuronal death via inflammation and oxidative stress. The production of PUFAs entails crosslinking of their saturated fatty acid precursors, and this configuration promotes protein lipid relations that other saturated fatty acids do not allow (Tully et al., 2003). Therefore, PUFAs have been denoted crucial for membrane formation and stability, making intake of DHA essential for brain function and prevention of neuronal death.

The cell death associated with depletion of DHA in the brain has been linked to cognitive decline (Lim et al., 2005). Studies investigating the effect of low levels of DHA in rodent diets have shown links to inadequate cognitive learning abilities (Hiratsuka et al., 2009; Hashimoto et al.,, 2005). Altering the diets to increase DHA intake improved learning aptitude in the animals and thus suggests that DHA may also help improve memory in humans suffering from neurodegenerative diseases (Ikemoto et al., 2001). For example, according to Chung et al. (2008), rats fed diets high in DHA performed significantly better on a Morris water maze task than the rats on a DHA deficient diet. Hiratsuka et al. (2009) revealed a possible mechanism by which DHA may improve learning. In their study, DHA connecting phospholipid diets had higher plasmalogen levels, a group of phospholipids found in platelets that are associated with DHA, as well as higher learning abilities. A reduction in blood plasmalogen levels, which have been shown to have antioxidant effects, has been linked to aging and dementia, specifically within plasma phospholipids in AD patients. In addition, lower antioxidant levels may contribute to deficient performance on memory tasks. Though plasmalogen is not well understood, it is known to reduce the oxidation of cholesterol in the cell membrane, which contributes to cell survival; therefore, a possible explanation for the improvement of memory after raising levels of DHA may be that DHA increases plasmologen levels to provide antioxidants that inhibit or slow the progression of neuronal death (Hiratsuka et al, 2009; Maeba and Ueta, 2003). These studies on DHA and memory may help to elucidate our understanding of DHA's relationship to aging and cell survival; however, it does not fully explain its involvement in Alzheimer's disease.

Related to its ability to maintain cell membranes and promote cell survival, DHA has been shown to reverse the effects of degeneration in photoreceptors of the retina (Mukherjee et al., 2004). Age-related macular degeneration results in vision loss caused by choroidal neovascularization, the growth of new blood vessels from the choroid into a subretinal area of the eye (Mukherjee et al., 2004). In a mouse model of macular degeneration used by Sheets et al (2010), intraperitoneal injections of the DHA derivative neuroprotectin D1 (NPD1) attenuated the damage caused by the growth of new vessels that resulted from laser-induced lesions. Surprisingly, NPD1 treatment was only given for one week after the lesions were produced but the improvement of the damaged retina continued well after the treatment ceased. The mechanism by which NPD1 reverses the deleterious effects of neovascularization remains unknown. Possible pathways include decreasing the expression of interleukin-1β and tumor necrosis factor-α, two inflammatory cytokines. Also, NF-kB, a transcription factor involved with inflammation and the immune system, when activated promotes vascular endothelial growth factor (VEGF) to induce neovascularization; inhibition of NF-kB may be another possible mechanism by which NPD1 reduces choroidal neovascularization (Sheets et al., 2010). The oxidative stress induced during macular degeneration which causes the release of these toxic cytokines may be a factor in the development of AD as well. More specifically, the treatment of age-related macular degeneration using NPD1 to support the survival of neurons within the eye may have similar benefits when preventing or treating AD.

**DHA and Alzheimer's disease**

Through the recent investigation of DHA's association with AD, multiple researchers have discovered that individuals suffering from AD tend to have lower levels of DHA, supporting the hypothesis that treatments aiming to increase DHA levels may slow the disease progress (Tully et al., 2003). Although animal models of AD have shown improvement following DHA treatment, a successful human trial has not been observed (Devoree et al., 2009); however, evidence that a lower risk of developing AD is present with a diet rich in fish oil or other foods containing DHA strengthens researchers' confidence its ability to have a positive effect in humans as well (Schaefer et al., 2006).

The research into the beneficial effects of DHA on Alzheimer's disease can be attributed to the association of the disease to low levels of the essential fatty acid. Tulley et al. (2003) investigated DHA levels using a valid indicator (serum cholesteryl ester-fatty acid composition) because results of previous studies suffered due to rough approximations of DHA-containing food consumption. The comparison of AD patients to healthy age-matched controls showed AD patients had significantly less DHA levels in their plasma serum. The results, nonetheless, merely demonstrate a correlation between the variables. The DHA levels may be another sign of the disease, as low levels of DHA could indicate the disease progression, or a potential risk factor (Tulley et al., 2003). Since the discovery of this negative correlation researchers have begun to investigate the effects of adjusting the amount of DHA consumption has on the risk of developing AD, as well as the disease progress.

Since Aß- plaques and tau tangles are known causes of AD, the association of DHA and this disease may be due to its ability to reduce the occurrence of these toxic proteins. Lim et al. (2005) used a mouse model of AD to measure Aß levels in the perirhinal cortex, parietal cortex, and hippocampus, brain regions which are all in danger of developing Aβ plaques that cause AD. Mice were placed in three different diet conditions: control DHA, low DHA, or high DHA. Results (figure 3) indicated that the high DHA group experienced a decrease in Aß42, one of the two detrimental proteins formed from abnormal cleavage of APP and the one most associated with synaptic degradation. In addition, general plaque burden, calculated as the percentage of Aβ-positive cells within the total area measured, was evaluated by immunolabeling for an antibody against Aß1-13 and showed a significant decline in the high DHA diet (Lim et al., 2005). Therefore, dietary intake of DHA has the potential to reduce symptoms and slow development of AD by decreasing one of the causal factors, Aß plaques, through a mechanism that Lim et al. (2010) also discovered in this study.

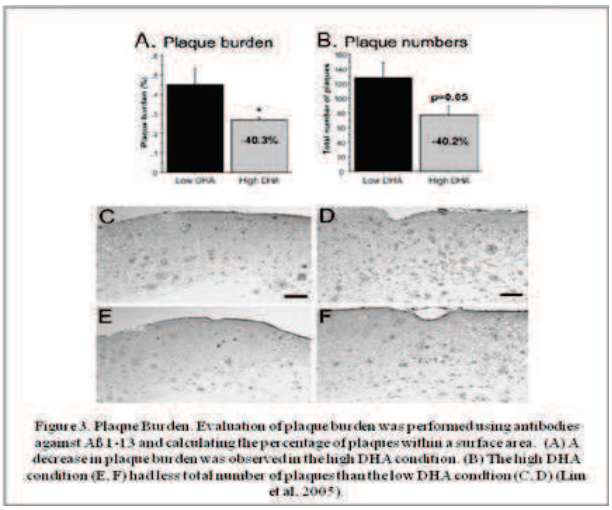


Figure 3. Plaque Burden. Evaluation of plaque burden was performed using antibodies against Aß 1-13 and calculating the percentage of plaques within a surface area. (A) A decrease in plaque burden was observed in the high DHA condition. (B) The high DHA condition (E, F) had less total number of plaques than the low DHA condition (C, D) (Lim et al. 2005).

To examine the reduction of Aβ plaques with the addition of more DHA in the diet, Lim et al. (2010) measured APP C-terminal fragments, in response to the three diet conditions, using western blot analysis. Results indicated a significant decrease in cytosolic APP compared to the control and low DHA groups, but an increase in membrane bound APP compared to both control and low DHA (figure 4).

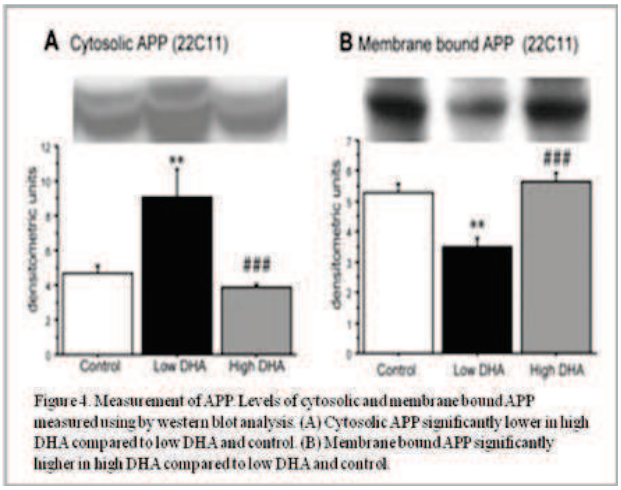


Figure 4. Measurement of APP. Levels of cytosolic and membrane bound APP measured using by western blot analysis. (A) Cytosolic APP significantly lower in high DHA compared to low DHA and control. (B) Membrane bound APP significantly higher in high DHA compared to low DHA and control.

In AD, membrane bound APP produces Aβ peptides after abnormal cleavage; thus, an increase in the protein could imply that less membrane bound APP is being abnormally cleaved to form the Aβ plaques. This finding was further elucidated with measurement of the specific products of APP cleaved by α and β-secretases. Both α and β C-terminal fragments were significantly reduced in the high DHA conditions compared to the low DHA condition (figure 5).

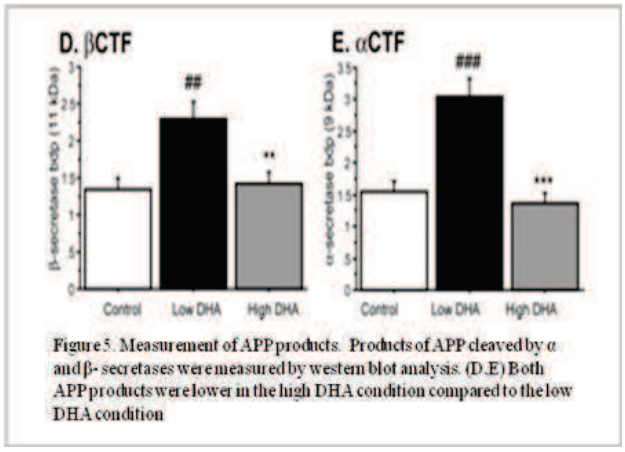


Figure 5. Measurement of APP products. Products of APP cleaved by α and β- secretases were measured by western blot analysis. (D,E) Both APP products were lower in the high DHA condition compared to the low DHA condition

Lim and his colleagues interpreted the data collected for APP as being the result of DHA's involvement in modifying the amount of α and β-secretase contact with membrane bound APP. They claim that DHA decreases the availability of α and β-secretases, decreasing the amount of membrane bound APP cleaved by the two enzymes. Though the results of this study confirm that a modification of secretase activity causes the reduction of Aβ plaques via DHA-rich diets in a mouse model, human trials are necessary.

In addition to altering the levels of α and β-secretases to partially inhibit the production of Aβ plaques, Green et al. (2007) found another mechanism by which DHA decreases the risk of developing AD. DHA levels were again manipulated in the food consumed by a transgenic AD mouse model but with different conditions: control, DHA, DHA and docosapentaenoic acid at 1:1 (DPA), DHA and arachidonic acid at 1:1 (ARA). DPA and ARA are both omega-6 fatty acids; maintaining a healthy ratio of omega-6 inflammation-promoting fatty acids to omega-3 inflammation-reducing fatty acids is essential for proper cell functioning. Presenilin 1 and nicastrin levels, proteins that help comprise the γ-secretase complex responsible for cleaving APP into Aβ peptides, were then analyzed using a western blot technique. Results indicated that all experimental conditions when measuring presinilen 1 levels were significantly lower than the control (figure 6). No changes in the nicastrin levels were observed.

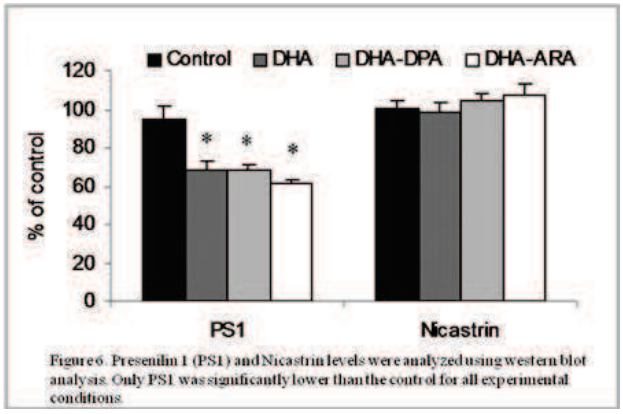


Figure 6. Presenilin 1 (PS1) and Nicastrin levels were analyzed using western blot analysis. Only PS1 was significantly lower than the control for all experimental conditions.

DHA, therefore, decreases levels of γ-secretase by specifically reducing presenilin levels, contributing to the decline in Aβ peptide production that forms the toxic plaques (Green et al., 2007). In the DHA/omega-6 conditions, however, the effects ceased after 9 months. In addition, this research found that the DHA in combination with DPA drastically reduced tau tangles by preventing phosphorylation. This discovery is not due to any changes in the pathology of Aβ plaques because the occurrence of tau modification continued into the period when DHA/omega-6 no longer had an effect on Aβ peptide production. The tau-reducing mechanism that DHA applies to ameliorate AD pathology remains unknown (Green et al., 2007). Like the experiment examining the effects of DHA on APP C-terminal fragments, the animal model of AD may not translate to the same reductions in factors contributing to AD development in humans. These studies demonstrate DHA's mechanisms for reducing Aβ plaques, while other DHA researchers focus on its effect on neurons within brain regions devoted to memory.

Another approach to studying DHA's involvement in AD is by examining neuronal activity in the memory centers especially targeted in the pathology, such as the hippocampus (Lim et al., 2005). Of particular interest is the dentate gyrus of the hippocampus, where neurogenesis has been found to continue into adulthood and is a known site of normal cognitive functioning (He et al., 2009). To investigate the effects of DHA on cognition, He et al. (2009) used the fat-1 transgenic mouse model that is capable of producing excess DHA by conversion of omega-6 fatty acids. Immunohistochemical staining with an antibody against bromodeoxyuridine (BrdU) to assess cell proliferation revealed a significant increase in BrdU-positive cells within the subgranular zone and the granule cell layer of the dentate gyrus as a result of more DHA production in the fat-1 mice (figure 7).

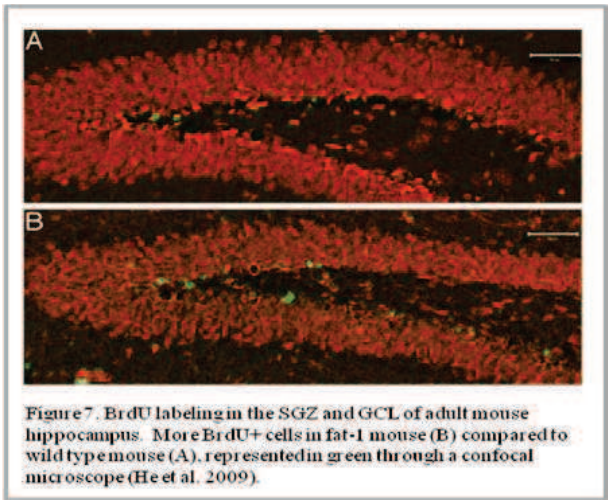

Figure 7. BrdU labeling in the SGZ and GCL of adult mouse hippocampus. More BrdU+ cells in fat-1 mouse (B) compared to wild type mouse (A), represented in green through a confocal microscope (He et al. 2009).

Therefore, DHA promotes neurogenesis in the adult hippocampus. He and his colleagues also observed an increase in synaptic spine density in the fat-1 mice as measured by Golgi-Cox staining of the hippocampus. Both sets of data in this study are consistent with the finding that DHA enhances performance on memory tasks (Lim et al., 2009, Hiratsuka et al., 2009; Hashimoto et al., 2005). The use of the fat-1 transgenic mouse model provided a more accurate method of manipulating DHA levels in this study because it eliminates most confounds that altering the levels of DHA in the diet might induce (He et al., 2009). A DHA treatment that is proven to raise DHA brain levels would thus be expected to have a similar effect on neurogenesis and spine density within the human hippocampus, which could explain the augmented cognition observed as a result of higher DHA levels.

**Conclusion**

Alzheimer's disease is an increasingly prevalent, fatal disease that requires necessary attention by researchers to focus on cures, treatments, and preventative therapies. Current treatments include cholinesterase inhibitors and N-methyl-D-aspartate (NMDA) receptor agonists, the goal of each being to improve the cognitive symptoms; unfortunately, neither provides alleviation of symptoms for more than one year (Bi, 2010). The studies presented provide evidence for the potential of DHA treatments to ameliorate symptoms as well as reduce the risk of AD by modifying the production of Aβ peptides via a reduction in secretases that cleave APP. While much research has been devoted to developing a way to manage the number of AD cases and deaths, AD etiology is not yet entirely understood which causes a problem for researchers to translate data from animal studies to human trials.

Conducting human research that involves measuring the Aβ plaques and its constituents with the same methodology as the previous studies is not feasible. Studies investigating the effects of DHA on the risk of AD or its ability to ameliorate the symptoms may only be measured externally via epidemiological studies that use MMSEs or similar tests to diagnose and classify dementias. Though limited epidemiological studies have shown a reduced risk for developing AD in humans with an adequate amount of DHA in the diet, causal factors have not been established.

Not all observational studies have found significant positive results for a greater DHA intake, which challenges DHA's involvement in AD while also presenting more evidence for the drawbacks of studying AD in humans. Devoree et al. (2009) and Kröger et al. (2009), using similar methodologies, found no correlation between risk of AD and levels of DHA in the diet. All observational studies, including the one conducted by Tully et al. (2003) that measured serum cholesteryl fatty acid levels in humans and found a significant negative correlation, must consider environmental factors since studies are usually conducted over a long time period. Devoree et al. (2009), for instance, examined individuals within a Dutch community that usually consume moderate amounts of fish each year. Much of the fish, however, is fried, which could counteract the beneficial effects of DHA. Also, the subjects in these studies are usually approaching the ages found to be most at risk for developing AD within the following decade; research has shown that diet earlier in life is more crucial when predicting risk of developing AD (Devoree et al, 2009). After considering the limitations in the study of DHA and AD in humans, it appears as though using transgenic mouse models of AD provide greater insight into the cellular

pathology of amyloid accumulation and its response to DHA. Again, problems arise with this method as well. The animal models of AD lack tau tangles, suggesting that differences between human and animal models may cause disparity in the results of experiments that manipulate pathology (Duff, 2002). Also, the pathology and symptoms of the animal model occur at a more rapid pace than the steady progression of the disease in humans (Duff, 2002). Even with these hindrances, transgenic mice continue to be the most helpful tool in AD studies.

The evidence to support the potential therapeutic qualities of DHA remains strong even with the lack of successful human trials. If DHA can reduce Aβ plaques in mice with the same constituents and synthesis as in human AD, it is reasonable to assume that DHA may have the same effects in human AD pathology. Perhaps the delivery method of DHA may need adjustment to compensate for the accelerated speed of breakdown that occurs in humans at the ages studied. Despite the lack of established effectiveness in human trials, researchers anticipate success in future studies. The benefit of establishing the role of DHA may play if proven effective include reducing the risk of AD, ameliorating symptoms, and alleviating the financial burden the disease forces upon the government as well as individual families. Even if DHA is found to only have preventative effects, the cost and number of AD cases would be significantly diminished.

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# The Boat Ride to Death

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*Apoptosis is the cell's response to unrepaired DNA damage, endoplasmic reticulum stress, or extracellular stimuli. The apoptotic signaling pathway can occur through two means: the extrinsic and intrinsic pathways. The former pathway relies on the permeabilization of the mitochondrial outer membrane, known as mitochondrial outer-membrane permeability (MOMP) the primary subject of this review. Regardless, both the intrinsic and extrinsic pathways have a relationship with MOMP and eventually converge at the point of executioner caspase activation; this results in the cleavage of regulatory and structural molecules and causes apoptosis. The balance of pro and anti- apoptotic regulators has a strong influence on cancerous cells and well as neurodegenerative disorders, and is a prime area for therapeutic intervention.*

**Introduction**

The dynamic between cell proliferation and death maintains an organism's equilibrium of its building blocks, cells. Cell death occurs through one of three independent pathways, type I (apoptosis), type II (autophagic), and type III (necrosis). By and large, the most common pathway is type 1 cell death (Lemasters *et al.,* 1998). This method of cell death results in the loss of cell function, upon which the cell is removed, most often replaced with a new cell. Apoptosis is the safest method of removal for organisms, as necrosis leads to the release of Hmgb1 protein, which promotes inflammation and the demise of neighboring cells; apoptosis, on the other hand, causes the death of specific cells through heightened specificity (Paola Scaffidi TM, Marco Bianchi 2002).

Apoptosis is the cell's response to unrepaired DNA damage, endoplasmic reticulum stress, or extracellular stimuli (Tait SW and Green DR 2010). The apoptotic signaling pathway can occur through two means: the extrinsic and intrinsic pathways. The former pathways relies on the permeabilization of the mitochondrial outer membrane, known as mitochondrial outer-membrane permeability (MOMP) (Fulda S and Debatin KM 2006), the primary subject of this review. The extrinsic pathway begins with ligation to a death receptor, Fas, TNF $\alpha$, or tumor necrosis related apoptosis inducing ligand (TRAIL), on the cell surface and can become associated in the MOMP pathway, but predominantly follows an independent path to cell death (Zapata JM *et al.* 2001). Regardless, both the intrinsic and extrinsic pathways have a relationship with MOMP and eventually converge at the point of executioner caspase activation; this results in the cleavage of regulatory and structural molecules and causes apoptosis (Figure 1) (Ghobrial IM *et al.,* 2005; Tait SW and Green DR 2010).

Abnormalities in the pathway that result in the failure of the signaling pathway to initiate apoptosis have detrimental effects on the viability of organisms. The over-expression of apoptotic inhibition can lead to proliferative, unchecked cell growth, resulting in cancer. Abnormalities can also result in cancer treatment failure, as many chemotherapeutic agents rely on the apoptotic pathway to exert their cell death effects. A prominent mechanism of pathway alteration and inhibition is cellular resistance to MOMP (Tait SW *et al.* 2010).

Alternatively, the detrimental effects of modified MOMP can be seen in acquired as well as genetic diseases, such as neurodegenerative diseases (Suzuki Y *et al.* 2001). There is evidence that hepatitis B and C encoded proteins, as well as hepatotoxins like ethanol, initiate MOMP(Green DR and Kroemer G 2004). The balance between pro and anti regulators of MOMP become of particular importance in neurodegenerative diseases. This balance of regulators, known as the apostat, is observably altered in the favor of apoptosis with diseases like fronto-temporal dementia, Alzheimer's, and Huntington's diseases (Bredesen DE 2008).

MOMP induced apoptosis plays a paramount role in maintaining the body's homeostasis and function. Understanding the pathway of MOMP mediated apoptosis is critical to a greater understanding of cancer mechanisms. Consequently, what are some of the current and potential therapeutic approaches, which act through this pathway for cell that undergo elevated and diminished MOMP?

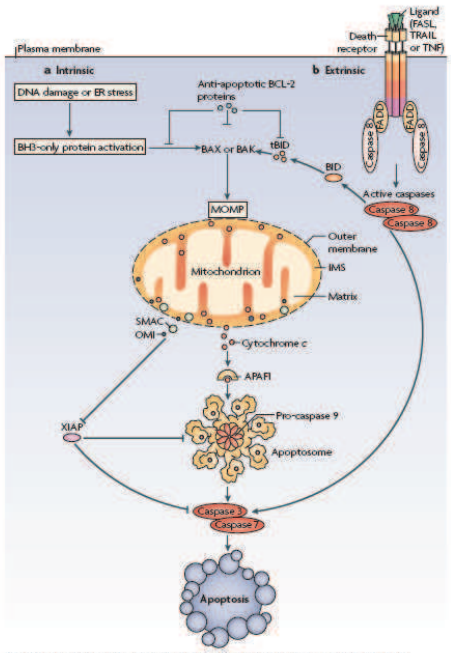


Figure 1 | Intrinsic and extrinsic pathways of apoptosis. a | Intrinsic apoptotic stimuli, such as DNA damage or endoplasmic reticulum (ER) stress, activate B cell lymphoma 2 (BCL-2) homology 3 (BH3)-only proteins leading to BCL-2-associated X protein (BAX) and BCL-2 antagonist or killer (BAK) activation and mitochondrial outer membrane permeabilization (MOMP). Anti-apoptotic BCL-2 proteins prevent MOMP by binding BH3-only proteins and activated BAX or BAK. Following MOMP, release of various proteins from the mitochondrial intermembrane space (IMS) promotes caspase activation and apoptosis. Cytochrome c binds apoptotic protease-activating factor 1 (APAF1), inducing its oligomerization and thereby forming a structure termed the apoptosome that recruits and activates an initiator caspase, caspase 9. Caspase 9 cleaves and activates executioner caspases, caspase 3 and caspase 7, leading to apoptosis. Mitochondrial release of second mitochondria-derived activator of caspase (SMAC; also known as DIABLO) and OMI (also known as HTRA2) neutralizes the caspase inhibitory function of X-linked inhibitor of apoptosis protein (XIAP). b | The extrinsic apoptotic pathway is initiated by the ligation of death receptors with their cognate ligands, leading to the recruitment of adaptor molecules such as FAS-associated death domain protein (FADD) and then caspase 8. This results in the dimerization and activation of caspase 8, which can then directly cleave and activate caspase 3 and caspase 7, leading to apoptosis. Crosstalk between the extrinsic and intrinsic pathways occurs through caspase 8 cleavage and activation of the BH3-only protein BH3-interacting domain death agonist (BID), the product of which (truncated BID; tBID) is required in some cell types for death receptor-induced apoptosis. FASL, FAS ligand; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand.

Figure 1 (Tait and Green, 2010)

***Extrinsically meditated death***

The first pathway for apoptosis is through external, ligand mediated cell death. Upon the binding of a death ligand (Fas , TNF α, or TRAIL) to their respective cell surface death receptor, the formation of the death signaling pathway complex begins. Within this complex are the adaptor proteins Fas-associated death domain protein (FADD) and caspases 8. The Fas-associated death domain protein (FADD) recruits caspase 8 via domain interactions and in turn leads to the dimerization of caspase-8 monomers by induced proximity, resulting in their activation (Boatright KM *et al.* 2003). This can lead to the death of the cell.

Caspase-8 can directly lead to the activation of executioner caspases-3 and -7. Upon cleavage by caspase-8, these inactive dimers are cut into active monomers (Chai J *et al.* 2001). These active monomers can carry out cell death through the cleavage of regulatory and structural molecules (Tait SW and Green DR 2010). However, active caspase-8 can potentially cause crosstalk between the intrinsic and extrinsic pathway, and therefore makes the extrinsic pathway worth mentioning in this review. It can influence the intrinsic pathway via the cleavage of BID to tBID, resulting in the activation of BAX and BAK (Tait SW and Green DR 2010).

The activation of BAK and BAX leads to mitochondrial permeability, by the activation of caspases-3, and -7 (Suzuki Y *et al.* 2001).

***Intrinsically mediated death***

The Bcl-2 family of proteins, which includes BAX and BAK, play a large role in mediating apoptosis and serves as the entryway into the intrinsically mediated death of a cell. These proteins regulate the permeability of the outer mitochondrial membrane, or MOMP (Kuwana ea 2006). We can think of MOMP mediated cell death through a simple analogy; the passage of souls across the River Styx. Upon a cell reaching MOMP, it has reached a point of no return, and is forced to a destiny of death. MOMP facilitates the release of cytochrome C and pro-apoptotic caspases from the mitochondrial intermembrane space to the cytosol (Suzuki Y *et al.* 2001). Cytochrome C interacts with Apaf-1, which catalyzes activation of caspase-9. This caspase in turn activates executioner caspases-3 and -7 and causes cell death.

*Paying Charon to ride the boat, how do we push the boat into the water?*

The balance between pro and anti-apoptotic proteins that mediate the permeability of mitochondria catalyzes intrinsic cell death. The anti-apoptotic BCL-2 proteins are usually integrated in the outer mitochondrial membrane (OMM) and contain four BH1-4 domains; these proteins are the BCL-2-related gene A1, BCL-2, BCL-2-related gene, long isoform (BCL-xL), BCL-w, and myeloid cell leukemia 1 (MCL-1). They function by inhibiting of pro-apoptotic proteins (Chipuk JE *et al.* 2010).

Pro-apoptotic proteins can be divided into either BH3-only or effector proteins. BH3 only proteins are known as “sensitizer” and/or “depressor” proteins. These proteins bind and inhibit the anti-apoptotic proteins. The proteins in this pro-apoptotic, BH3 only family, are: Bim, Puma, BMF, Bad, Bik, Hrk, Bid, and Noxa. Their inhibitory effect on the anti-apoptotic proteins occurs through domain interaction. The hydrophobic BH3 domain interacts with the BH1, BH2, and BH3 domains of the anti-apoptotic proteins, which leads to apoptosis inhibition (Petros N, Wang, olenjniczak, Meadows, Mack, Swift, Matayoshi 2000).

BID and BIM are unique BH3 only proteins and display the capability to inhibit the anti-apoptotic proteins as described. In addition, they also show the ability to mediate the activation of pro-apoptotic effector proteins. These pro-apoptotic proteins are in the BCL-2 protein family and are known as BAK (BCL-2 antagonist killer 1) and BAX (BCL-2 associated x protein) (Chipuk JE *et al.* 2010).

Currently, there are two models for BAK and BAX activation; the direct activator requirement model and the neutralization model (Tait SW and Green DR 2010). The direct activator model is thought to work through two possible routes rooted in the idea that BAK and BAX need to be directly activated by a BH3-only protein, like BID or BIM. In this route, anti-apoptotic BCL-2 proteins are in complex with a pro-apoptotic, BH3-only protein. This binding prevents the inhibition of BID and BIM proteins, which are subsequently capable of interacting with effector proteins (Figure 2A).

Alternatively, the direct activator model may function by BCL-2 initially binding to a pro-apoptotic BH3-only protein. However, in the presence of other pro-apoptotic BH3 proteins, competition may ensue between the two pro-apoptotic proteins which leads to release of MOMP catalysts BAX and BAD (Figure 2B). The neutralization model suggests that effector proteins are bound to BCL-2 proteins, and upon the presence of pro-apoptotic BH3 proteins, competition ensues and the effector protein is released and can induce MOMP (Figure 2C) (Chipuk JE *et al.* 2010). The controversy is ongoing. It appears that there is mounting evidence in favor of the direct activator model, although the idea that the neutralization model occurs simultaneously cannot be discarded (Chipuk JEG, D.R. 2008).

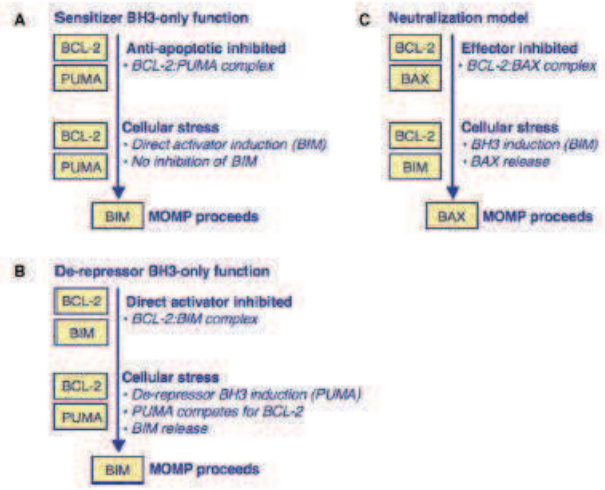


Figure 2
(Chipuk *et al.,* 2010)

Although different models are suggested for the activation and mechanistic function of BAX and BAK, it is known that BH3 proteins are essential in the process (Chipuk JEG, D.R. 2008). The two BH3 domain proteins, BID and BIM are particularly important in inducing BAK and BAX oligomerization through BH3 and BH4 rearrangements of these effector proteins (Chipuk JE *et al.* 2010). BAK interaction with BID's truncated form, tBID, or another BH3 protein, causes the exposure of its BH3 domain and insertion into the hydrophobic groove of an analogously activated BAK protein, creating a homodimer (Dewson G *et al.* 2008). The BH3 protein, BIM, also catalyzes activation, through a similar mechanism. However, BIM's activation of BAX is unique, binding to the side opposite the hydrophobic binding pocket (Moldoveanu T *et al.* 2006).

These effector protein homodimers can further oligomerize, forming higher order oligomers through dimer-dimer interactions (Figure 3) (Kuwana T *et al.* 2005). It has recently been found that higher order oligomerization occurs at the protein's alpha-6 helix; this study by Dewson, et al., reports oligomerization of BAK proteins (Dewson G *et al.* 2009). These oligomerized proteins can then form either pores or vesicles that allow for the release of inter-membrane proteins, like the caspases and cytochrome C (Antonsson B *et al.* 1997; Schendel SL *et al.* 1997). The quantity of monomers necessary for MOMP to occur is an area of active investigation, with some studies indicating as little as four and others reporting thousands (Saito M *et al.* 2000; Amotz Nechushtan CS, Itschak Lamensdorf, Soo-Han Yoon, Richard Youle 2001).

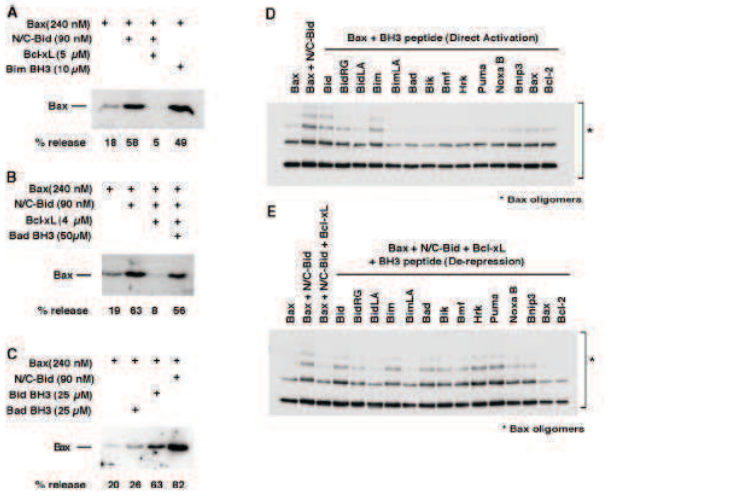


Figure 5. BH3 Peptides Induce Bax Association with Lipid Membranes and Increased Bax Crosslinking
The indicated recombinant proteins and BH3 peptides were incubated with liposomes as in Figure 2, and one part of the assay mix
subjected to sucrose float-up centrifugation to collect the membranes, which were then analyzed for Bax content by immunoblotting.
(A) Bim BH3 peptide induced Bax membrane association and membrane permeabilization to the same extent as N/C-Bid. Note that
association correlated with fluorescein-dextran release.
(B) Bad BH3 peptide, a derepressor, increased Bax association with the membrane as well as dextran release.
(C) The Bad peptide by itself did not stimulate Bax membrane association. The data shown are representatives of five (A), three (B), and
(C) experiments.
(D) Direct activators, Bim and Bid BH3 peptides, induce increased Bax oligomerization detected by crosslinking using BMH. The mu
peptides induced less oligomerization of Bax.
(E) Derepressors restored Bax oligomerization. Only higher molecular weight species (dimers and above) are visible because the antibod
relatively insensitive in recognizing monomeric Bax (data not shown). The data are representative of five (D) and two (E) experiments.

Figure 3
(Kuwana *et al.,* 2005)

By altering the ratio of pro-apoptotic and anti-apoptotic BCL-2 proteins, the fate of mitochondrial integrity can be managed. As a result, the BCL-2 family members can serve as possible therapeutic agents for diseases with unregulated apoptosis, such as cancer. Antisense BCL-2 therapy combined with chemotherapy has been shown to induce apoptosis in cancerous cell lines. Antisense BCL-2 (oblimersen sodium) is an 18bp phosphorothioate oligonucleotide that targets the first six codons of BCL-2 mRNA. This method of chemotherapy co-treatment has demonstrated decreased BCL-2 expression and enhanced mitochondria-dependent apoptosis (Gupta S *et al.* 2009).

Delivery of a modified BAX vector, via gene therapy, has also shown promise to solve unregulated cell proliferation. As mentioned, BAX forms dimers with anti-apoptotic proteins, which inhibits its function. By creating a truncated version of BAX that cannot be bound to the anti-apoptotic proteins, the cells will be more likely to undergo apoptosis and suppress tumor growth (Usui K *et al.* 2003).

The use of BH3 mimetic peptides and synthetic drugs is also under investigation as a means to induce cell death in cancerous lines. BH3 mimetic peptides, derived from the pro-apoptotic proteins, have been generated in order to replicate the effects of pro-apoptotic proteins. These mimics show greater success in vitro, because in vivo they are limited by poor cell permeability, solubility and stability (Moreau C *et al.* 2003). The most successful mimetic has been the synthetic

drug ABT-737. This is a cell permeating BH3 mimetic that targets the BH3-binding groove on BCLX-$_{L}$, resulting in its inactivation. Due to the prevalence of other anti-apoptotic genes some tumor tupes are not as affected, however, lymphomas, leukemia, and small cell lung cancer have shown susceptibility (Oltersdorf T *et al.* 2005). ABT-737 is insoluble in the human body, its substitute, ABT-263, an oral medication, shows similar effectiveness but is soluble (Tse C *et al.* 2008).

*Riding a one way river*

Upon the activation of BAK and BAX and activation of MOMP, a mitochondrion has started rowing down the river of death with no sight for salvation. The oligomerization of BAK and BAX essentially pushes the boat into the river through the release of further effector proteins. The exact mechanism for release is still unclear; it is either through the formation of proteinaceous channels or lipidic pores.

BCL-2 proteins show structural similarities to bacterial pore forming toxins, which suggests the executor proteins, BAX and BAK might form proteinaceous channels in the mitochondrial outer membrane which release downstream executioner proteins (Muchmore SW *et al.* 1996; Suzuki M *et al.* 2000). These BCL-2 proteins are shown to induce the formation of the mitochondrial apoptosis-induced channel, or MAC. The formation of this channel simultaneously correlates with the release of cytochrome C (Figure 4). The formation of this channel is due to the interactions of helices 5 and 6 of BAX or BAK, likely with around 10 monomers oligomerized in the final channel. It is proposed that as more monomers join the oligomer, the hole grows in size, accounting for the initially slow release of cytochrome C. Support for this model is seen in the deletion of helices 5 and 6, which show no release of cytochrome C (Martinez-Caballero S *et al.* 2009). The proteinaceous channel does not account for the release of large molecules, like AIF and endonuclease G, while the lipidic pore model does.

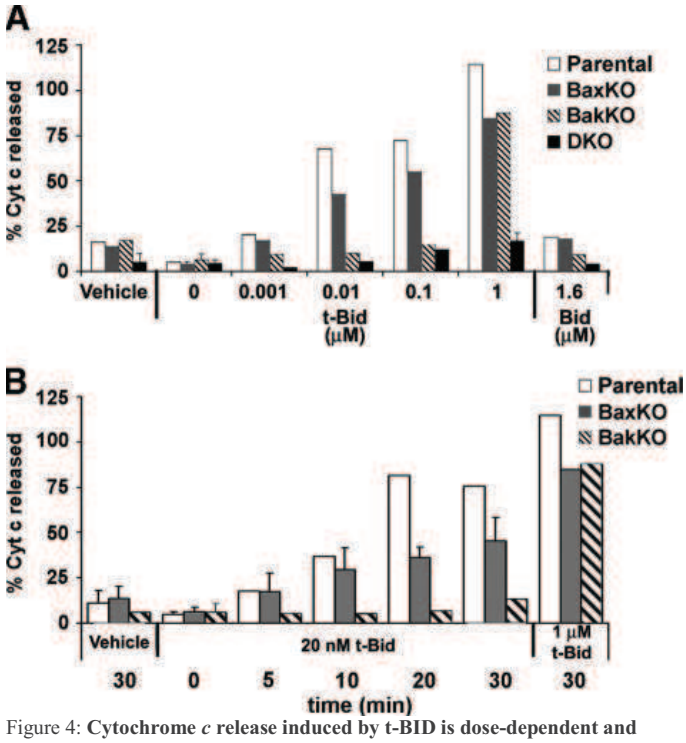


Figure 4: **Cytochrome *c* release induced by t-BID is dose-dependent and requires expression of Bax and/or Bak.** Mitochondria isolated from the indicated cell lines were incubated with varying concentrations of t-Bid or with Bid or vehicle, and cytochrome *c* (*Cyt c*) release was quantified by ELISA. *A, histograms* show the dose-dependent release of cytochrome *c* by t-Bid after 30 min, relative to release by 80 μg/ml alamethicin. The $EC_{50}$ of t-Bid for cytochrome *c* release for Parental and Bax KO cell lines was 7 and 17 nm with correlation coefficients of 0.92 and 0.98, respectively. $EC_{50}$ was estimated at 250 nm t-Bid for mitochondria from Bak KO cells. *B, histograms* show the time-dependent release of cytochrome *c* from isolated mitochondria after incubation with either 20 nm t-Bid at various times or 1 μm t-Bid after 30 min relative to alamethicin. Data are mean ± S.E.
(Mar*tinez-Caballero et al., 2009)*

Instead of channel formation, BAX and BAK oligomers can potentially bend and break the membrane resulting in the formation of lipid-composed pores. In a study by Basanez, et al, it was proposed that BAX molecules alter the properties of positively curved membranes (consistent with a mitochondrion), allowing for lipidic pore formation, which occurs through the bending of the lipid monolayer (Basanez G *et al.* 2002). Evidence for this model is seen through the effects of cardiolipin on membrane permeability. In vitro, cardiolipin containing liposomes demonstrated MOMP. Mitochondrial cardiolipin dependent permeabilization was seen in cells treated with Bid, Bim BH3 and oligomerized BAX. However, there was non-dependency witnessed when tBid was administered. This suggests that there may very well be dependency on cardiolipin to induce permeability; however, pathways activated by tBid are likely present and potentially undergo independent pathways for permeabilization. Cryo-electron microscopy of BAX-permeabilized liposomes displayed openings of 50-100nm in the mitochondria; this size hole would account for the release of large proteins (Schafer B *et al.* 2009).

As the membrane becomes permeable, the river current increases with the release of cytochrome C and other proteins, specifically SMAC, OMI, AIF, and endonuclease G

into the cytosol. Cytochrome C initiates the assembly of APAF-1 into a heptameric structure, this is known as the apoptosome (Tait SW *et al.* 2010). The apoptosome activates caspase-9 through dimerization (Boatright KM *et al.* 2003). Upon activation, caspase-9 is capable of cleaving the inter-chain linker, which blocks translocation that is necessary for its activation between the two respective monomers of executioner caspases-3 and-7 (Boatright KM *et al.* 2003).

Critical to apoptosome and caspase3/7 activity is the release of SMAC and OMI from the membrane. Upon the release of SMAC and OMI, their N termini bind to the third beta strand of the BIR3 domain in XIAP. XIAP is part of the IAP (inhibitor of apoptosis proteins) family and specifically inhibits the apoptosome and the executioner caspases. This inhibits XIAP's activity, allowing the apoptosome to form and the executioner caspase to act (Liu Z *et al.* 2000).

These executioner caspases cleave and inhibit critical members of the electron transport chain. This results in the gradual loss of membrane potential and ability of the cell to produce energy. A component of complex 1 of the electron transport chain, NDUFS1, shows particular susceptibility to proteolytic cleaving; the non-cleavable mutant displays resistance to immediate loss of membrane potential or ATP depletion (Ricci JE *et al.* 2004). Caspases-3 and-7 also cleave the inhibitor of the caspase-activated deoxyribonuclease, this activation leads to nuclear condensation (Ghobrial IM *et al.* 2005). These caspases also induce cleavage of protein kinases, cytoskeleton proteins, DNA repair proteins, cell cycle regulation, and signaling pathways that contribute to cell survival. These effects of caspases, in addition to reduced mitochondrial function because of MOMP, result in cell death (Ghobrial IM *et al.* 2005; Lakhani SA *et al.* 2006). This speaks to the importance of caspase activity in cell death.

In support of MOMP being a one way river is evidence showing that cells can undergo caspase independent cell death (CICD); that is the cell will still die if caspases are blocked. Upon MOMP, intermembrane space proteins are released into the cytosol that facilitate cell death, even if caspase activity is blocked. Release of AIF and endonuclease G contribute to nuclear chromatin condensation and DNA fragmentation(Saelens X *et al.* 2004). In addition, CICD is likely a result of the loss of mitochondrial function due to MOMP (Tait SW *et al.* 2010).

Interestingly, it appears that some cells display resistance to CICD assuming caspase inhibition (Tait SW and Green DR 2008). These cells, which display resistance to MOMP, are termed iMOMP cells. These iMOMP cells demonstrate sustained mitochondrial functionality and a maintained electropotential gradient over the membrane despite apoptotic stimuli (measured using potentiometric dye TMRE) (Figure 5).

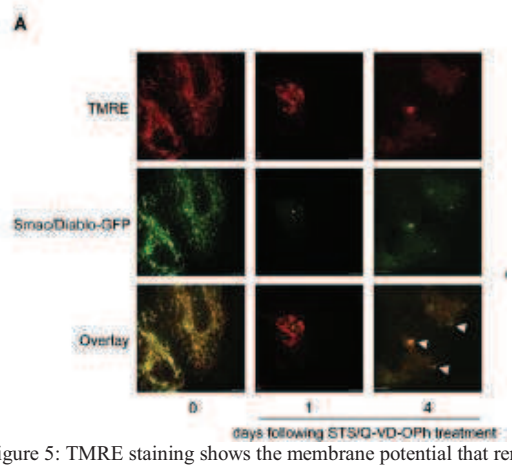


Figure 5: TMRE staining shows the membrane potential that remains after stimulation of MOMP. SMAC-GFP is used to show that remaining mitochondria are in fact the ones that display remaining membrane potential. (Tait *et al.* 2010)

Surprisingly, and of great importance with respect to cancer, is the repopulation of mitochondrial populations after MOMP induction in cells displaying iMOMP. Single cell clonogenic survival assays were used to investigate the relationship between survival after MOMP and iMOMP cells. It was found that cells which displayed higher levels of iMOMP had better clonogenic survival in response to apoptotic stimuli (provided caspases were blocked) and repopulated their mitochondria, leading to the idea that the iMOMP'd mitochondrion served as "seeds" for the proliferation of new mitochondria (Figure 6) (Tait SW *et al.* 2010).

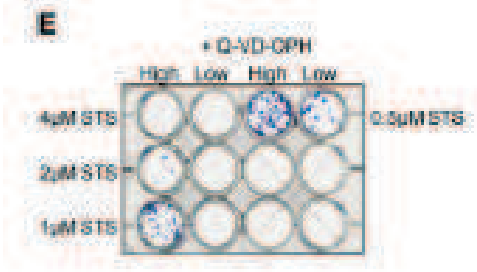


Figure 6: E) HeLa cells expressing GAPDH and Smac-GFP were treated for 16 hr with STS (0.5 mM-4 mM) plus Q-VD-OPh (20 mM) and sorted into 20% highest and lowest GFP-expressing populations. Then 2 3 105 cells from each population were plated down in the presence of Q-VD-OPh (20 mM) and examined for clonogenic outgrowth. As a control, 1 3 106 cells were treated with STS (0.5-4 mM) for 16 hr and examined for clonogenic outgrowth (middle panel). Colony number from three independent experiments; error bars represent SD. Scale bars represent 10 mm. Here we see that cells displaying clonogenic outgrowth contained mitochondria that had displayed resistance to MOMP.
(Tait *et al.* 2010)

Many cancers display caspase inhibition post MOMP. This, in conjunction with resistance to MOMP, creates a double-edged sword for cancer. If cancer cells are able to inhibit caspases following MOMP, and are able to withstand the effects of MOMP, there is great potential for chemotherapeutic resistant cancers. This fear is only heightened from the finding that cells displaying iMOMP are

able to repopulate their mitochondria populations and maintain survival and growth (Tait SW *et al.* 2010).

The hypothesis for how iMOMP occurs lies in the mechanisms for MOMP, which have already been discussed. Using fluorescently tagged BAX and OMI proteins, it appears that cells displaying iMOMP show no translocation of BAX to the outer mitochondrial membrane. Following from what has already been discussed, this would inhibit the release of caspase-dependent killers of the cell, as well as maintain the functionality of the mitochondria (Tait SW *et al.* 2010).

The failure of BAX and BAK translocation is likely a result of anti-apoptotic BCL-2 activity. Fluorescent tagging of BCL-2 proteins demonstrates that cells which have resistance to effector protein translocation display heightened levels of BCL-2 expression. This over-expression provides mitochondria with a protective shield. This finding was confirmed with the use of ABT-737, a BH3 mimic which inhibits BCL-2 function. When ABT-737 was administered, mitochondria that had previously displayed iMOMP soon underwent MOMP. Not surprisingly, ABT-737 has shown great potential as a chemotherapeutic drug (Tait SW *et al.* 2010).

iMOMP presents clear dangers when in combination with cancerous cell lines. However, iMOMP might not always need to be viewed as a threat to the body. iMOMP's heightened levels of BCL-2 proteins might provide knowledge on how to protect individuals who suffer from neurological diseases, specifically diseases that target mitochondria for death. Furthermore, in cells where mitochondria are targeted for death, if small populations of mitochondria can be salvaged, the cell might be spared.

*Rowing against the current: naturally and assisted*

There is always the possibility that a cell has accidentally been pushed into the pathway that leads to apoptosis. As a preventative measure to this, the cell has regulatory mechanisms meant to curtail MOMP and abort cell death. For the most part, these abort mechanisms are focused on controlling apoptosome formation and caspase 9 activity. These points of regulation also provide great entry points for chemotherapeutic treatment

By altering the activity of cytochrome C, the downstream effectors will never be activated, and the caspase activity will be diminished. It has been shown that oxidized cytochrome C promotes its agency, whereas its reduced form results in inhibited activity, a possible point for cellular regulation. Furthermore, there is evidence that permeabilization of the mitochondria not only releases cytochrome C, but also facilitates oxidation by cytochrome oxidases; the question must also be raised if there are cytochrome reductases as well. Pharmacologically, death can be stalled by using reductases, as seen with in vitro use of TMPD (Borutaite V and Brown GC 2007).

Apoptosome formation and activity can be down regulated by simple ions within the cell. Cells can inhibit apoptosome assembly through the presence of potassium; even at physiological levels of $K^+$, apoptosome formation is inhibited, suggesting this is a method of regulation over accidental release of cytochrome C (Kelvin Cain CL, Xiao-Ming Sun, David Brown, Gerald Cohen 2001) . Physiological intracellular levels of calcium have been found to inhibit apoptosome activity through the inhibition of the dimerization step that forms the heptameric ring, supporting yet another method for prevention of accidental death (Bao Q *et al.* 2007).

Regulation of cell death can also occur by modulating caspase 9 activation. Phosphorylation of caspase-9 at the inhibitory site, Thr125, is an intervention point for apoptosis. This site is phosphorylated by CDK1/cyclin B1 during mitosis and by drugs which inhibit mitosis; nonphosphorylatable mutants have shown resistance to this phosphorylation and subsequently are not inhibited (Allan LA and Clarke PR 2007).

Many chemotherapeutic drugs can disrupt the integrity of mitochondrion and induce cell death through the opening of the MAC pore. Synthetic ligands of the peripheral-type benzodiazepine receptor have been shown to induce apoptosis by these means. Specifically, benzodazepines, isoquinoline carboxamides, idoleacetamides, phenoxyphenyl-acetamides, and pyrazolopyrimides can cause a drop in mitochondrial transmembrane potential and increase the release of cytochrome c and SMAC proteins (James ML *et al.* 2006). Importantly, these small molecule ligands overcome the cytoprotective effects of anti-apoptotic BCL-2 proteins. The sensitivity of cells to these molecules provides great candidates for chemotherapy (Decaudin D *et al.* 2002).

Lamellarins are a family of marine alkaloids sequestered from ascidians and sponges that hold anticancer potential. The most recognized member of the family, lamellarin D, has been shown to promote MAC dependent release of cytochrome C and AIF from mitochondria (Kluza J *et al.* 2006). Synthetic analogs of lamellarin D have been used as preclinical drug candidates and show acceptable absorption, distribution, and metabolism qualities. The most successful synthetic analog is amino derived PM031379 which shows little toxicity to non tumor cells and potent anticancer activity in human tumor xenografts (Kluza J *et al.* 2006).

**Conclusion**

The death of cells is a highly regulated, precise process. When the pathway for apoptosis of cells is altered, detrimental effects can occur, putting an organism at risk. This can be seen on both sides of the equation, with heightened apoptosis resulting neurodegenerative diseases, and decreased apoptosis causing unchecked cell growth.

Facilitation of apoptosis occurs through extrinsic and intrinsic pathways. Extrinsically, death ligands, like Fas cause the activation of caspase-8. Caspase-8 can directly activate caspases -3 and-7 or can cause cross talk with the intrinsically mediated pathway, via the BID protein. BCL-2 proteins are the primary mediators for apoptosis through MOMP. BCL-2 proteins can be divided into pro-apoptotic and anti-apoptotic proteins. Currently, there are two proposed models for pro-apoptotic, BAX and BAK, activation: the neutralization model and the direct activator model; until more information is acquired, it appears that the direct activator model will provide the best explanation for activation. Regardless, the BH3 domain proteins are essential to the process through their role in the oligomerization of BAX and BAK. At the root BAK

and BAX activation is the ratio of BCL-2 proteins. When the ratio of pro-apoptotic to anti-apoptotic proteins becomes large enough, apoptosis occurs. This provides potential entry points for chemotherapeutics. So far, we have seen therapies such as antisense BCL-2 nucleotides, delivery of BAX vectors, and the use of BH3 mimetic peptides that drastically reduce cancer cell's resistance to apoptosis.

After BCL-2 activation and MOMP, the mitochondrion releases proteins that facilitate death. The means for this release is currently disputed. On one side are the advocates for the formation of proteinaceous channels from BAK and BAX oligomerization. Contenders argue that BAX and BAK oligomers cause bending of the membrane, which results in the formation of lipid pores. Recent research displays a dependency on cardiolipin, suggesting that the lipid pore model is gaining momentum. Regardless, a deeper understanding of which method is used might provide new methods for cancer regulation.

Recent research by Tait, et al, shows that some cells remain resistant to caspase independent cell death. These cells display resistance to MOMP, and potentially create oncogenic, chemotherapeutic resistant lines. Their resistance to MOMP, or iMOMP, likely rests with mitochondrial over expression of anti-apoptotic proteins that prevent BAX and BAK permeabilization. Furthermore, upon MOMP, these mitochondria that display iMOMP can serve as seeds for the oncogenic growth of new mitochondria within the cell. Further understanding of this phenomenon might provide insight on combating chemotherapeutic resistant cancers. Furthermore, applying these principles of death resistance to diseases in which mitochondria are targeted by the body for death could solve certain neurodegenerative and autoimmune diseases.

Drugs which target post MOMP effector proteins also show potential as chemotherapeutic agents. Altering the redox status of cytochrome C, apoptosome formation, and caspase 9 activity are all means through which the cell mediates the progression of cell death. These serve as targets for pharmacological intervention. Altering the permeability of the mitochondrial membrane also displays potential for inducing death in cancer cells. Some of these include synthetic ligands, which bind to the peripheral-type benzodiazepine receptor, as well as lamellarin D.

The pathway for MOMP is extensive, with many potential locations for modulation, either naturally or artificially. The largest hurtle in combating cancer and apoptotic-inducing diseases is sifting through the various hypothesis for how MOMP occurs. With a deeper understanding of MOMP and MOMP recovery, cell death can be better regulated.

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# High levels of microRNA-210 in rheumatoid arthritis

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*Objective: Previous studies have established that a range of microRNAs (miRNA) are induced by hypoxia in a number of cell types. Despite the prevalence of hypoxia in rheumatoid arthritis (RA) synovia, no studies have examined the effect of hypoxia on miRNAs in this disease as we do in this study.*
*Methods: RNA was isolated from synovial fibroblasts (SF; n=5 each) exposed to hypoxic conditions (1% $O_2$ for 2, 6 and 20 h) by MirVana kit and from synovial tissues (RA: n=8 and OA n=7) using Trizol reagent. The expression of miR-106b, -203 and 210 and selected predicted targets for miR-210 were analyzed by Real-time-PCR. Results: Expression of microRNA-210 (miR-210) is increased after 20 hours in hypoxia in RASF (fold change: 2.1±0.1, p = 0.021) and in OASF (1.6±0.2, p=0.025). There was no difference in the basal expression of miR-210 in RA- and OASF under normoxic or hypoxic conditions. In addition, the expression of miR-210 was significantly higher in RA (Ct: 4.75±0.66) versus OA (6.62±0.43, p=0.037) synovial tissues.*
*In silico analysis of genes modulated by hypoxia in RASF identified RASSF5, SUMO3, IL-12A as possible miR-210 target genes. Accordingly, RASSF5 was downregulated 40% (0.6±0.01 fold, p=0.003), SUMO3 40% (0.6±0.1, p=0.022) and IL-12A 60% (0.4±0.1, p=0.017) after 20 h under hypoxic conditions in RASF. Moreover, these potential target genes were also found to be less expressed in whole synovial tissue biopsies from RA (n=5) patients compared to OA (n=4) (RASSF5: dCt:19.2±0.5 vs 17.6±0.5, p=0.065; SUMO3: 13.9±0.6 vs 12.1±0.5, p=0.049, IL-12A: 19.0±0.5 vs 17.3±0.5, p=0.042).*
*Conclusions: The increased expression of miR-210 under hypoxic conditions may have implications for the activated and apoptosis-resistant phenotype of RASFs, due to the likely relationship to RASSF5, SUMO3, and IL-12A. The down regulation of these genes via miR-210 may help RASF to adapt to the hypoxic and inflammatory environment in RA joints.*

**Introduction**

Although the discovery of microRNAs (miRNAs) is fairly recent, their importance in controlling a variety of processes involved in disease initiation and progression was quickly established. Our understanding of the mechanisms that regulate miRNA expression, however, is still incomplete. Recently, hypoxia has received much attention as a possible factor that regulates miRNA expression.

Hypoxia is involved in a number of diseases, including many kinds of cancer (Huang X et al., 2009). Hypoxia is also known to play a role in rheumatoid arthritis (RA) tissues (Del Rey et al., 2010). The abnormally high density of synovial fibroblasts (SFs) is responsible for creating hypoxic conditions in this disease (Paleolog 2004). Hypoxia contributes to angiogenesis, which in turn perpetuates inflammation. A better understanding of the changes that SFs undergo in hypoxia may reveal novel therapeutic targets that could limit angiogenesis. Additionally, how hypoxia might contribute to the active phenotype of invasive rheumatoid arthritis synovial fibroblasts (RASFs) is not yet fully understood.

Nevertheless, a number of cellular changes that occur in response to hypoxia are known and may serve as a starting point for investigating the effects of hypoxia on the active phenotype of RASFs. One known change, for example, is that hypoxia inducible factor (HIF-1α) is normally degraded under normoxic conditions, but is stable and active during hypoxia. HIF-1α acts as a transcription factor that promotes the transcription of a wide range of genes, including those involved in the cell cycle, apoptosis, and respiration (Chan and Loscalzo, 2010). Furthermore, a number of studies have demonstrated increased expression of a number of miRNAs in a variety of cell types after exposure to hypoxic conditions (Kulshreshtha et al., 2008; Kulshreshtha et al., 2007). Of note, miR-210 is the most cited miRNA that appears to be induced by hypoxia (Chen et al., 2010; Crosby et al., 2009). miRNAs that are induced by hypoxia may help cells adapt to low oxygen levels by regulating mRNA translation.

Considering the large number of other tissues in which hypoxia has been shown to induce miRNA expression, it is likely that hypoxia also induces miRNA expression in RASFs; however, no studies have yet investigated the effect of hypoxia on miRNA expression in RASFs. We therefore decided to examine miRNA expression in RASFs after

exposure to hypoxia and study some of the downstream effects of such changes in miRNA expression.

## Materials and Methods

### *Patients and fibroblast cultures*

Synovial fibroblasts (SF) were derived from tissues extracted during surgery from either rheumatoid arthritis (RA) or osteoarthritis (OA) patients. SFs from passages 3-7 were used in the experiments. All patients signed a consent form approved by the local Institutional Review Boards.

### *Culture conditions and hypoxia*

SFs were grown in Dulbecco's modified Eagle's medium (Life Technologies, Basel, Switzerland) containing 10% fetal calf serum (FCS), fungicide, penicillin, and streptomycin. For exposure to hypoxia, SFs were transferred to an incubator (Forma Scientific, Illkirch, France) at 37°C and exposed to a humidified atmosphere containing 5% $CO_2$ and 1% $O_2$ volume/volume for 2, 6, or 20 hours. Controls were cultured under the same conditions but with 20% $O_2$ v/v (normoxia). Approximately 150,000 cells were seeded per well on 6-well plates and used once they had reached 50-90% confluence. Medium was changed immediately prior to exposure to hypoxia. Real-time PCR (see below) was used to analyze expression of vascular endothelial growth factor (VEGF) mRNA to insure that hypoxia had, indeed, been induced.

### *Quantitative real-time PCR*

Total RNA was isolated according to the manufacturer's protocol using the RNeasy Mini Kit from Qiagen (Düsseldorf, Germany). For isolation of RNA from flash-frozen tissue biopsies, Trizol reagent was used according to protocol (Invitrogen, Basel, Switzerland). Reverse transcription was performed using random hexamers from Qiagen and the BioRad C1000 Thermal Cycler (Hercules, CA, USA). Quantification of mRNA was performed using SYBR Green real-time PCR on the Applied Biosystems 7500 Real Time PCR System (Applied Biosystems) using self-designed primers from Microsynth (Balgach, Switzerland). Primer sequences are listed in Supplemental Table A.

MicroRNA was isolated according to the manufacturer's protocol using the *mir*Vana miRNA Isolation Kit from Applied Biosystems/Ambion (Rotkreuz, Switzerland). Reverse transcription was performed using primers from Applied Biosystems/Ambion and the Applied Biosystems Thermal Cycler, Model 9700. Quantification of miRNA was performed using TaqMan real-time PCR on the Applied Biosystems 7500 Real Time PCR System (Applied Biosystems) using primers from Applied Biosystems/Ambion.

To exclude genomic DNA, DNAse purification was performed during the total RNA extraction process. Furthermore, samples without reverse transcriptase were used as a control. 18S was used as an endogenous control for the analysis of mRNA expression. In the analysis of miRNA, let-7a was used as an endogenous control. All measurements were performed in duplicate. The threshold cycle ($C_t$) and comparative $C_t$ method were used to calculate differences. Data were analyzed using a one sample, two-tailed t-test to determine statistical significance on GraphPad Prism 5 (GraphPad Software, La Jolla, California).

### *Immunohistochemistry and in situ hybridization*

Cuts of paraffin-embedded RA tissues were prepared as slides, deparaffinized, and treated with peroxidase. Immunohistochemical detection of HIF-1α was performed using the HIF-1α antibody MA1-516 available from Affinity BioReagents (Rockford, IL) and biotynilized goat-anti-mouse secondary antibody from Dako (Baar, Switzerland). Mouse IgG1 was used as a negative control. Slides were developed with diaminobenzidine (DAB) and counterstained with Hämalaun.

## Results

### *Effect of hypoxia on miRNA expression*

Due to the restrictions of this project, a microarray analysis of the expression of many miRNAs in hypoxic RASFs was not possible. Instead, we decided to focus on miR-106b, miR-203, and miR-210. Several considerations guided the selection of these miRNAs for study. First of all, expression of miR-210 and miR-106 has been shown to increase in other cell types during hypoxia (Kulshreshtha et al., 2008; Chen et al., 2010; Crosby et al., 2009). A study by Distler and others found that expression of miR-203 is elevated in RASFs (2007). Additionally, TargetScan (Whitehead Institute for Biomedical Research) and Microcosm (European Bioinformatics Institute) predict that both miR-106b and miR-203 may regulate mRNAs that code for genes that play important roles in the pathogenesis of RA such as VEGF, Id-2, MMP-3, and CXCL12.

TaqMan real-time PCR revealed that RASFs exposed to hypoxic conditions with 1% $O_2$ for 2, 6, and 20 hours did not show altered expression of either miR-106b or miR-203 in comparison to RASFs cultured in normoxia (Figure 1A and 1B). Although the expression of miR-210 was not altered in RASFs in hypoxia for 2 or 6 hours, its expression was significantly up regulated after 20 hours, with a mean x-fold change ± SEM of 2.1 ± 0.3 ($p = 0.021$; Figure 1C). Future experiments, therefore, focused solely on miR-210.

### *Comparison of miR-210 expression in OA and RA*

OASFs appear to react similarly to RASFs in hypoxia. Expression of miR-210 in OASFs ($n = 5$) and RASFs ($n = 5$) also remained similar to each other after 20 hours in hypoxia with mean x-fold changes of 1.6 ± 0.2 and 2.1 ± 0.3, respectively (Figure 2B). Likewise, there was no significant difference between the baseline expression of miR-210 in OASFs and RASFs. In unstimulated cultures, OASFs had a mean delta $C_t$ value of 2.5 ± 0.3, while RASFs had a mean delta $C_t$ value of 3.2 ± 0.3 (Figure 2A).

On the other hand, however, RA tissues ($n = 8$; $dC_t$ 4.75±0.66) have greater expression of miR-210 than OA tissues ($n = 7$; $dC_t$ 6.62±0.43; $p=0.037$; Figure 3).

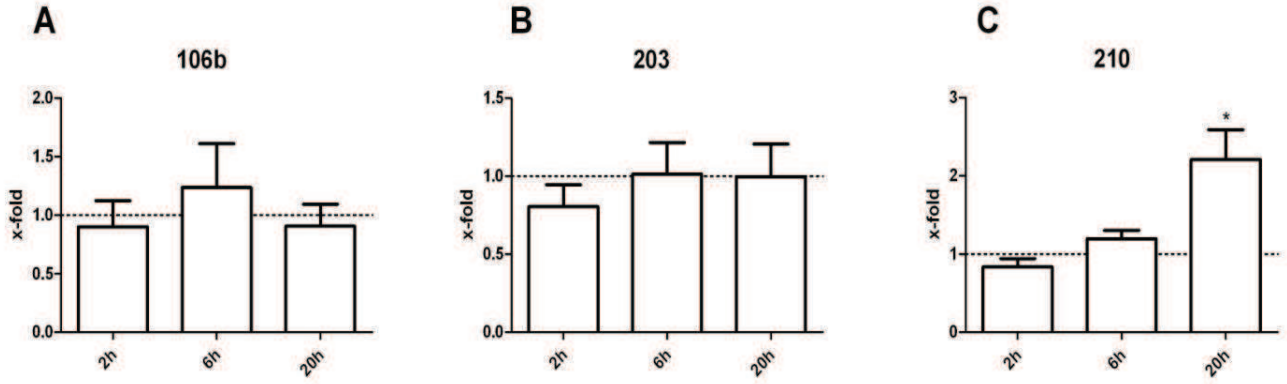


Figure 1: X-fold change in the expression of selected miRNAs after 2, 6, and 20 hours in hypoxia. miR-106b (A) and miR-203 (B) showed no alteration in expression at any time point in comparison to normoxic controls (defined as a value of 1). Expression of miR-210 increased only after 20 hours in hypoxic conditions. Values are the mean x-fold change for RASFs (n = 4; n = 5 for miR-210 at 20 hours) under hypoxic conditions (1% $O_2$) compared to the same RASFs under normoxic conditions (20% $O_2$). Error bars show standard error of the mean (SEM). * indicates $P < 0.05$.

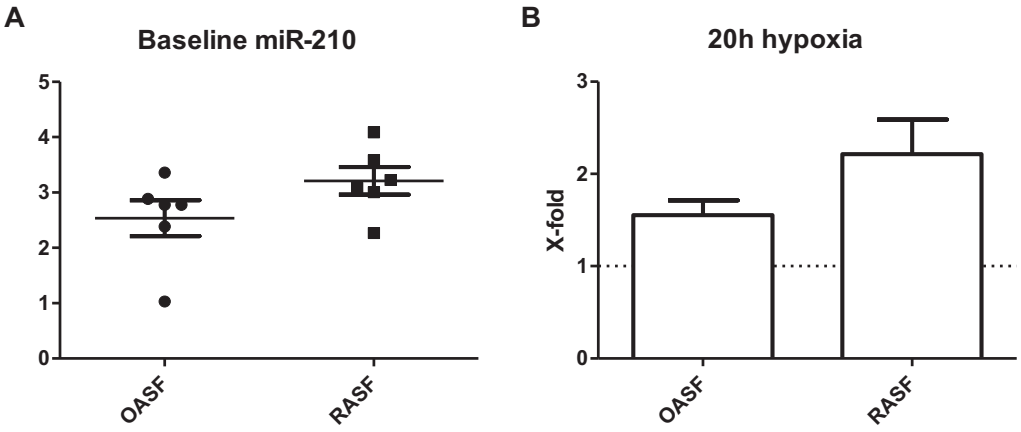


Figure 2: Comparison of miR-210 expression in OASFs and RASFs. Baseline expression of miR-210 is similar between OASFs (n = 6) and RASFs (n = 6), as indicated by the $\Delta C_t$ values in unstimulated cells (A). After 20 hours in hypoxic conditions (1% $O_2$), miR-210 expression remains similar between OASFs (n = 5) and RASFs (n = 5), as indicated by the mean x-fold change compared to unstimulated cells (B). Error bars show SEM.

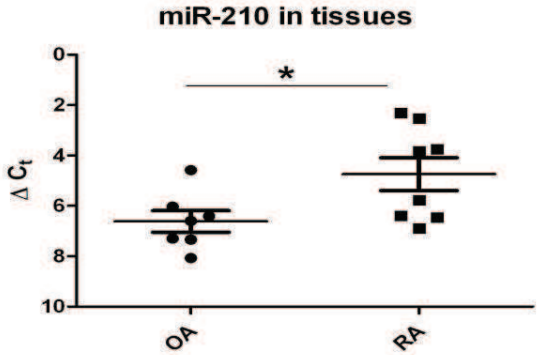


Figure 3: Comparison of miR-210 expression in OA and RA tissues. Values shown are $\Delta C_t$ values for RNA isolated from OA (n = 7) and RA (n = 8) tissue biopsies. Error bars show SEM. * indicates $P < 0.05$.

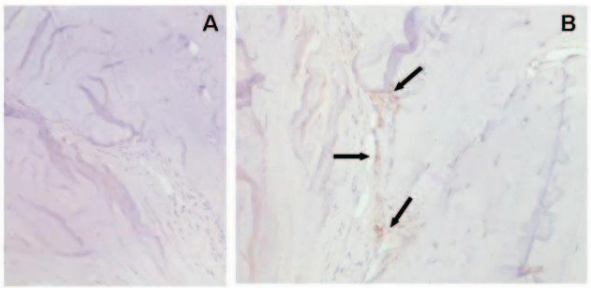


Figure 4: Immunohistochemistry of HIF-1α in RA synovial tissue (B) with negative control staining for comparison (A). Hypoxic SFs, shown by arrows, are seen at sites of invasion into cartilage.

***Localization of hypoxia in RA tissues***

Immunohistochemistry of HIF-1α in RA synovial tissues revealed hypoxic SFs at sites of invasion into cartilage (Figure 4).

*Targets of miR-210*

To study the targets of miR-210, we began by analyzing mRNA expression of a select number of genes in both RASFs and OASFs exposed to hypoxia. The genes were selected by comparing the miR-210 targets predicted by algorithms such as Microcosm, TargetScan, and PicTar (New York University) with genes that are known to have decreased expression in RASFs in hypoxia (Del Rey and others 2010). We ultimately decided to examine chemokine ligand 2 (CXCL2), neuronal pentraxin 1 (NPTX1), interleukin-12A (IL-12A), Ras association domain family member 5 (RASSF5), and SMT3 suppressor of mif two 3 homolog 3 (SUMO3).

Real-time PCR performed on mRNA samples from OASFs and RASFs in both normoxic and hypoxic conditions revealed that these cells express little to no CXCL2 or NPTX1. Conversely, we found that RASSF5 expression in RASFs is down regulated 0.6 ± 0.1-fold after 20 hours in hypoxia (p=0.003). Similarly, SUMO3 expression is down regulated 0.6 ± 0.1-fold (p=0.022). Expression of IL-12A is likewise down regulated by 0.4 ± 0.1-fold (p = 0.017; Figure 5A). Nevertheless, in OASFs the changes in RASSF5, SUMO3, and IL-12A expression were not significantly altered (Figure 5B).

On the tissue level, these same genes had lower expression in RA tissues than in OA tissues. For RASSF5 the delta $C_t$ value in RA was 19.2±0.5 compared to 17.6±0.5 in OA (p=0.065; Figure 6A). The delta $C_t$ value for SUMO3 in RA was 13.9±0.6 compared to 12.1±0.5 in OA (p=0.049; Figure 6B). IL-12A had a delta $C_t$ value of 19.0±0.5 in RA compared to 17.3±0.5 in OA (p=, p=0.042; Figure 6C).

**Discussion**

Although miR-106b has been demonstrated to be up regulated by hypoxia in other cell types, our results do not suggest that it is up regulated by hypoxia in RASFs (Kulshreshtha et al., 2008). Similarly, despite the known increased expression of miR-203 in RASFs as compared to OASFs, our results do not suggest that this increase in expression is caused by hypoxia.

In contrast, miR-210 is clearly up regulated by hypoxia in RASFs, although the change in expression is somewhat delayed, not appearing until 20 hours after exposure to hypoxia. This finding is in line with previous studies that have found that hypoxia induces increased miR-210 expression in a number of cell types (Crosby et al., 2009; Huang et al., 2009).

RASFs and OASFs appear to express miR-210 in similar amounts under control conditions. Additionally, OASFs react to hypoxia in a manner similar to RASFs, as their production of miR-210 increased to levels comparable to RASFs after 20 hours in hypoxia. The relationship between hypoxia and miR-210 is therefore not specific to RASFs. Again, this finding supports the results of other studies that have found miR-210 to be up regulated by hypoxia in different cell types.

A difference between RA and OA is, however, noticeable on the tissue level. RA tissues express greater levels of miR-210 than OA tissues. This finding is logical in light of the more extensive hyperplasia, and therefore more numerous hypoxic regions, found in RA joints than in OA joints. A few of the RA tissues analyzed expressed similar levels of miR-210 as in OA tissues, but these tissue samples likely did not contain extensive hypoxic regions.

We found that hypoxic RASFs expressed less RASSF5, SUMO3, and IL-12A mRNA. The likely relationship between miR-210 and these genes is further enhanced by the finding that these genes also have lower expression in RA tissues than in OA tissues, since RA tissues express more miR-210 than OA tissues. Whether these decreases are, in fact, due to direct regulation by miR-210 has yet to be determined. Transfection with pre- and anti-miR-210 will reveal whether these genes are, in fact, direct targets of miR-210.

If these genes are truly direct targets of miR-210, their down regulation in hypoxic RASFs would have implications for the activated and apoptosis-resistant phenotype observed in RASFs. RASSF5 activates Mst1, which induces apoptosis. With decreased levels of RASSF5, RASFs would become less apoptotic. Down regulation of SUMO3 would also help the RASFs to adapt to hypoxic conditions, because lower levels of SUMO3 would decrease the amount of sumoylated (and active) PARP1, which inhibits coactivation of HIF-1α. Lastly, IL-12A is involved in immune cell activation, so its down regulation would also have repercussions for the inflammatory nature of RA.

Apoptosis assays on RASFs transfected with pre- and anti-miR-210 also have the potential to provide important information about the effect of miR-210 on the reduced apoptosis rates observed in activated RASFs.

Additionally, in situ hybridization will be a useful tool for visualizing the expression of miR-210 in RA tissues. These stainings can be compared to our stainings for HIF-1α to determine whether there is a correlation between locations of hypoxia and the localization of miR-210.

Lastly, it will be important to repeat all of these experiments to increase the number of data points available for statistical analysis.

In light of the many studies that have established an increase in miR-210 expression in hypoxia in other cell types, our finding that this miRNA is also up regulated by hypoxia in RASFs is no surprise. Nevertheless, this discovery opens the door to a new realm of investigation in RASFs. MiR-210 likely targets a number of genes that, when down regulated, contribute to the activated phenotype of RASFs. A better understanding of precisely which genes miR-210 targets will allow for greater knowledge of what makes RASFs so destructive. More effective treatments can then be developed.

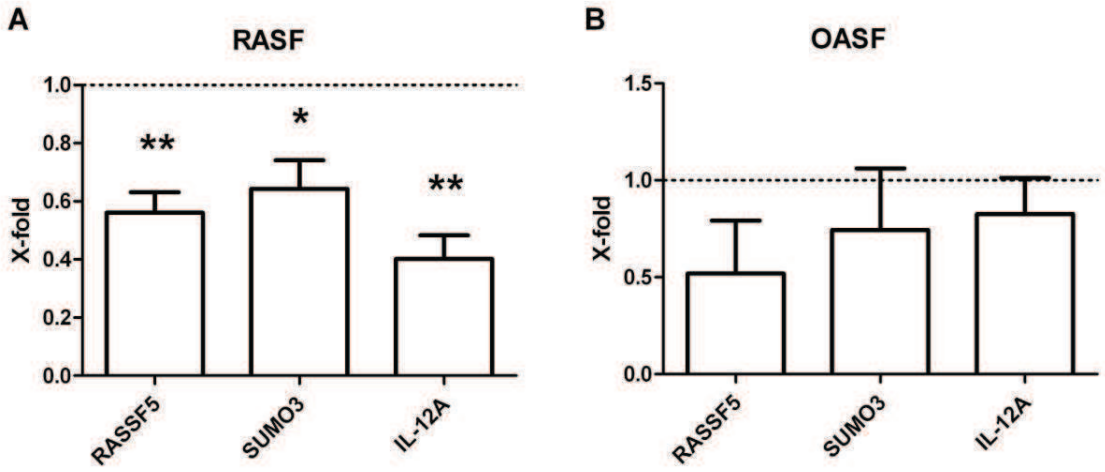


Figure 5: X-fold change in mRNA expression in RASFs (n = 5) and OASFs (n = 4) after 20 hours in hypoxic conditions (1% $O_2$) compared to the same cells in normoxic conditions (20% $O_2$; defined as 1). Values shown are mean x-fold change. Error bars indicate SEM. * indicates $P < 0.05$, ** indicates $P < 0.01$.

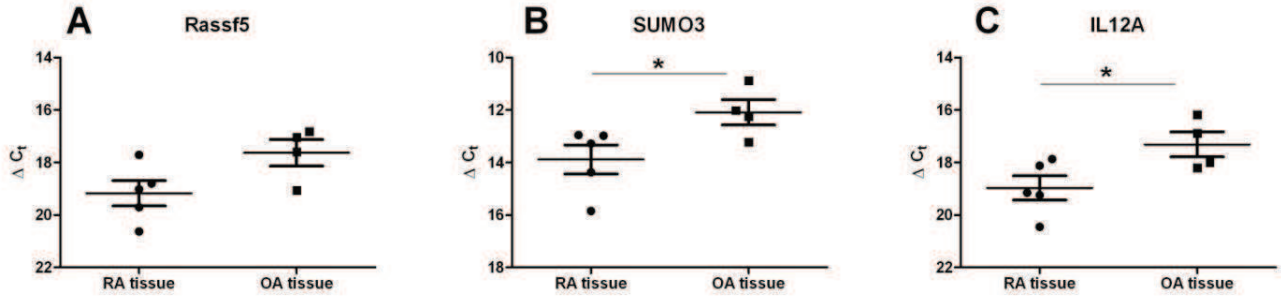


Figure 6: Delta $C_t$ values for mRNA expression in RA tissues (n = 5) and OA tissues (n = 4). Error bars indicate SEM. * indicates $P < 0.05$.

**Acknowledgements**

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# Defining a Role for the Anterior Cingulate Cortex in Insight

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*The insight process is characterized by four elements: 1) mental impasse, an inability to solve a presented problem, 2) restructuring of the problem to break impasse, 3) a deeper understanding after the solution, and 4) a suddenness with which the solution arrives. Insight has been well-studied using electroencephalography (EEG) and event-related functional magnetic resonance imaging (fMRI) techniques, which implicate the involvement of several brain regions, such as the prefrontal cortex and the right temporal lobe. To a lesser degree, activation of the anterior cingulate cortex (ACC) has been observed during insight and related tip of the tongue (TOT) moments. Already known to be responsible for monitoring cognitive conflict during stimulus processing and transitioning between attentional sets, the ACC's functions may also extend to playing a major role in breaking mental impasse through restructuring of possible responses. Here, the missing links between insight process and execution are provided, thereby defining a control-exerting, conflict-resolving model for the ACC during insight. This model helps to demonstrate the importance of learning processes and their relationship to attention and cognition in light of the prevalence of attentional deficits.*

**Insight: Introduction and Importance**

Many famous discoveries have been the result of insightful ("Aha!") moments. According to legend, Archimedes shouted "Eureka!" after realizing how to calculate density from water displacement (Jung-Beeman et al., 2004). Insight occurs when one suddenly is able to solve a task or answer a question that previously seemed unanswerable due to mental constraints, such as misunderstanding information.

While insight problems are commonly thought to be verbal or riddle problems, insight can be used to solve a wide variety of tasks and should be primarily viewed as a process rather than according to problem type. For example, insight may provide understanding of figurative speech, the abrupt ability to predict a book's ending, or the sudden realization of the solution to a critical thinking problem (Kounios & Beeman, 2009). It may even be seen as a creative skill since it is the bridging of two mentally distant ideas to form a solution (Friedman & Forster, 2005).

Many brain regions exhibit increased activity specifically during insight problems, but the connection in activity between these regions is poorly understood. Many medial frontal and temporal areas show such enhanced activity (Kounios et al., 2006). The left dorsolateral prefrontal cortex (DLPFC, BA 9) and anterior cingulate cortex (ACC; BA 24, 32, and 33) exhibit increased activation specifically during insight tasks (according to fMRI and EEG) (Tian et al., 2010; Qiu et al., 2009; Luo et al., 2004). Luo et al. (2004) had participants solve riddles while being observed through fMRI, while Qiu et al. (2009) took electrophysiological recordings during mental preparation for problems. The variance in problem type leading to common activation pattern suggests that the brain might solve these queries using a common network independent of problem type (Tian et al., 2010).

This paper attempts to clearly define a role for the ACC by juxtaposing insight literature with studies on cognitive decision-making, attention, conflict monitoring, and stimulus response processes during which the ACC is known to be activated and that are purportedly important during insight. Thus, the overlapping yet vaguely connected functions and processes are integrated in this brain region.

Positing a role for the ACC in insight poses this region as an integral part of the learning process. The ACC is an important mediator of attentional control, which is crucial during the learning process; without directed attention, it becomes difficult to distinguish important stimuli from unimportant stimuli. It is not surprising then that individuals with attention-deficit hyperactivity disorder (ADHD) demonstrate bilaterally reduced (interhemispheric difference was 2%) ACC volume, independent of medical treatment (Makris et al., 2010). Moreover, individuals with obsessive-compulsive disorder have shown decreased errors on highly conflicting tasks due to an increased ability of the ACC to learn from errors (Hammer et al., 2009). Thus, delineating the ACC's role in insight more broadly applies to learning and attention, of which a greater understanding could lead to more effective therapies for learning and attention deficits.

ACC involvement in insight provides a specific context in which to connect attentional processes to learning and cognitive understanding. A thorough examination of the characteristics of insight and then a consideration of theories of ACC activity will converge on implications for the two's relationship.

## II. Insight

"Insight" refers to a specific set of conditions that occur during problem solving attempts in which one is unable to produce the correct answer because of a mental blockade that prevents accurate understanding of the problem or query. Four salient elements distinguish insight from typical problem solving: mental impasse, restructuring, deeper understanding, and suddenness.

*Mental Impasse*

Mental impasse occurs when, after attempting to produce a correct response to a query (verbal or procedural), one simply quits after being unable to make any further progress. Several studies suggest that a focus on irrelevant or insignificant cues leads to impasse by redirecting attention away from items relevant to generating a correct response (Lockhart et al., 1988; Isaak & Just, 1995). Mental impasse can also originate from an inability to retrieve information necessary to complete the query. This cause of impasse is present in tip of the tongue (TOT) states, when individuals are confident they know an answer to a question but are unable to produce it (Maril et al., 2001). Thus, working memory's (WM) ability to process and/or retrieve long-term memories for interpretation becomes exhausted (Sandkuhler & Bhattacharya, 2008). Attention-monitoring processes steer the relevance of WM items. Since WM items in impasse are inadequately chosen or processed, inhibiting forward progress, it seems that attention-monitoring deficits are involved in forming mental impasse (Awh, Vogel, & Oh, 2006). Thus, upon reaching mental impasse, the brain has depleted its bank of possible responses without finding an adequate one.

*Restructuring*

The second step in insight involves breaking impasse through reevaluating available information in a novel way (restructuring) that better fits the query, precipitating a sensible response. The individual transitions from an inability to solve a problem to a state of knowing how to reach the solution (Siegler, 2010). Two competing theories exist that attempt to explain the nature of the restructuring process: the conscious, controlled theory and the subconscious recombination theory.

Proponents of the conscious, controlled theory argue that people search for and utilize *invariants* (properties that do not change across problems or when the problem is reevaluated) to break through mental impasse (Davidson, 1995; Kaplan & Simon, 1990). Kaplan & Simon (1990) argue that insight may occur when constraints are placed on 'search' problems that mandate the use of invariants, creating a better representation of the problem. They extend the theory of 'search' problem solving, which is deliberate and analytical, to insight situations, thus suggesting that insight is highly conscious. However, other studies (Aziz-Zadeh, Kaplan, & Iacoboni, 2009; Polson & Jeffreis, 1982 as cited in Novick & Sherman, 2003) refute this similarity, arguing that insight and noninsight (which is often conscious) problem solving are qualitatively different processes though they may use common brain regions. Specifically, the search strategy yields thoughts available to WM as well as progressively strengthening understanding; insight is not a consciously accessible process, is unavailable to conscious working memory, and causes sudden (Davidson, 1995, p. 128) and unpredictable (Metcalfe, 1986) understanding.

Those in support of the subconscious recombination theory suggest that automatic semantic network activation causes a break in impasse. Ash and Wiley (2006) demonstrated that problems utilizing the restructuring phase of insight are not dependent on the ability to *consciously* control one's attention. Participants were given insight problems with either many ("MMA group," meant to give them conscious attentional control) or few possible trails of faulty thinking ("FMA" group, meant to induce restructuring).

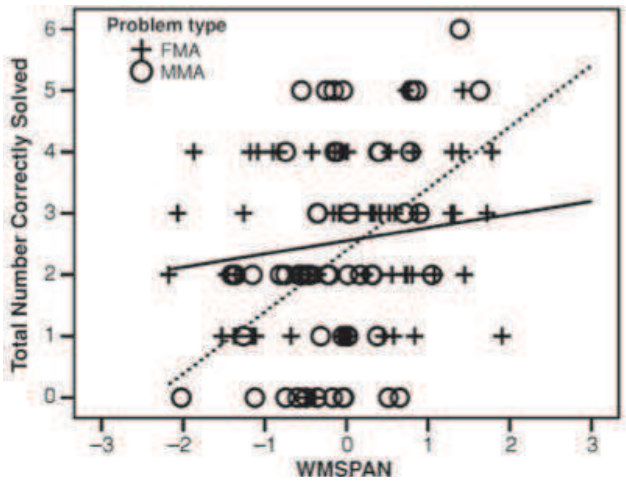



**Figure 1: Scatterplot relating working memory span scores and number of problems correctly solved**. The dashed line represents the regression line for the many moves problems (MMA), and the solid line represents the regression line for the few moves available (FMA) problems. Working memory predicted solution success on MMA problems but not on FMA problems (adapted from Ash and Wiley 2006).

A significantly higher positive correlation was found between solution success and WM span with the "many" group. In contrast, the FMA group—meant to represent individuals using restructuring—did not show solution predictability from memory span (Figure 1). The results support the automatic, subconscious recombination theory by showing that memory capacity does not predict restructuring (the FMA group) success or solution time. Moreover, the FMA group showed a relatively consistent solving time independent of working memory span. Interestingly, however, the MMA group did show a significant improvement in solution time as WM span increased (Figure 2). Since the MMA group was designed to replicate the overall insight process as opposed to only restructuring, this finding suggests that in some instances insight may depend on attentional control (Ash & Wiley, 2006). The conflict monitoring and selection-for-action theories of the ACC, discussed later, may explain these results in the context of the ACC.

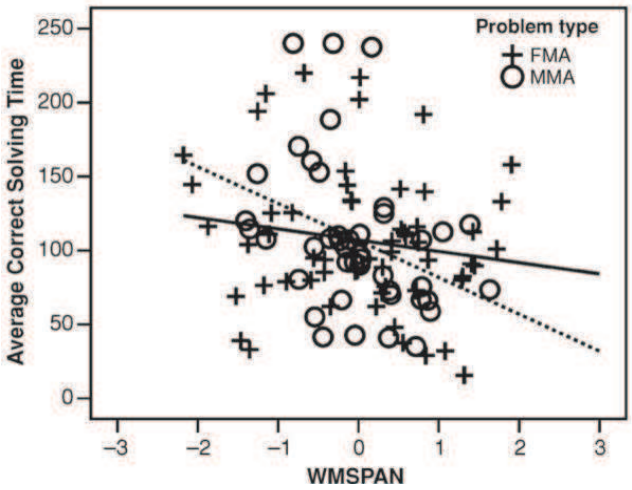



**Figure 2: Scatterplot relating working memory span scores and correct solving time.** The dashed and solid lines represent the regression line for many moves available (MMA) and few moves available (FMA) problems, respectively (adapted from Ash and Wiley, 2006).

Metcalfe (1986) found that participants could not predict their metacognitions for problem solving, although they were fairly successful at predicting memory performance. Thus, she argues that insight is a subconscious process, characterized by sudden moments of illumination that cannot be predicted and are thus subconscious.

Siegler (2000) builds on the subconscious recombination theory. He proposes that insight strategy begins unconsciously, and then arises into consciousness due to increasing use. He asked 31 German students to complete inversion tasks (e.g., A+B-B, 18+24-24). The insightful strategy for this task is simply to say the first number ("A," or "18), while the noninsightful way is to actually compute the answer (a slower route). Temporal measurement of task completion revealed whether students used this shortcut, and verbal reports indicated whether or not the student was *aware* of using the shortcut. He found (Figure 3) that students began by using computation, but after discovering the unconscious shortcut, more and more were able to verbally account for the drastic decrease in solution time (conscious shortcut).

Metcalfe's (1986) and Siegler's (2000) results together suggest that insight problem solving 1) does not involve *conscious* long-term memory retrieval in the same way as do typical problem solving attempts, and 2) while insight may, at first attempt, be a primarily subconscious route of problem solving, it does have the ability to traverse into consciousness with use.

*Deeper Understanding*

True insight leads to a correct solution, which creates a better understanding of the previously unsolvable problem (Sandkuhler & Bhattacharya, 2008). This component of insight is the "Aha!" phenomenon resulting from restructuring, and will therefore not be discussed in detail.

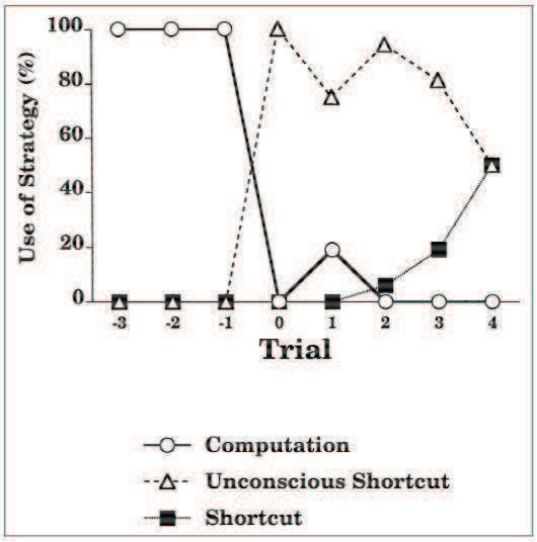



**Figure 3: Percentage use of computation, unconscious shortcut, and shortcut strategies in an inversion task**. Once students discovered the shortcut, they abandoned the computational method but were not immediately able to verbalize the difference in solution time. They progressively switched to exclusively using the conscious shortcut. Pretrial numbers indicate trials immediately before the first use of the unconscious shortcut (adapted from Siegler 2000).

*Suddenness*

The three-process theory of insight delineates three salient aspects of insight that help to explain its suddenness: selective encoding, selective combination, and selective comparison.

Selective encoding occurs when new features of previous stimuli become apparent; this process involves the restructuring of mental representations to include information that was previously thought to have been irrelevant, as well as the exclusion of so-thought relevant information. Thus, selective encoding occurs also during the restructuring phase of insight (Davidson, 1995, p. 128).

Selective combination builds upon selective encoding by utilizing the newly reorganized information in a new way.

The suddenness that one experiences during insight, however, is most aptly described by selective comparison, which is the establishment of a non-obvious mental relationship between new and known information (Davidson, 1995, p. 128).

These three processes are not unique to insight; only when these processes are not immediate (and thus require restructuring and reorganization of information) can a problem-solving method be termed insightful (Davidson, 1995, p. 129).

Of course, all of these processes seem spontaneous to the person, occurring without detection and without providing predictability of a correct answer (as shown by Metcalfe, 1986). The suddenness occurs when these process have generated a novel way of understanding information.

**II. The Anterior Cingulate Cortex (ACC)**

*ACC Connections*

Understanding of the ACC's neural connections will help elucidate its potential role in insight. Interestingly, the ACC is a crossroads of many neural systems, including the limbic, motor, and cognitive systems. In the limbic system, the ACC connects directly to the anterior thalamic nucleus and to the brainstem nuclei mediating autonomic control (Vogt et al., 2004; Wang et al., 2005). The ACC communicates with the motor and cognitive systems through connections to the primary motor and prefrontal cortices, respectively (Paus, 2001).

The ACC, and cingulated cortices in general, share strong neuronal linkages with several prefrontal cortical regions; the most commonly paired region with the ACC (in the literature) is the dorsolateral prefrontal cortex (DLPFC) (MacDonald et al., 2000). Several studies (Aziz-Zadeh, Kaplan, & Iacoboni, 2009; MacDonald et al., 2000) implicate a partnership between the PFC and ACC in controlling attention. The PFC inputs onto the cingulated motor areas within the cingulated sulcus, which also receives input from primary motor cortex and premotor cortex (Morecraft & Van Hoesen, 1993). The ACC also directly projects to the motor cortex and spinal cord (Wang et al., 2005; Dum and Strick, 1993).

Given the ACC's many anatamo-functional roles, the fact that it is involved in many tasks and processes, including reading, WM, attention, and emotion, is not surprising. Wang et al. (2005) suggest that ACC activation is related not to a certain task type, but rather to the amount of possible responses that a certain process could produce.

***The ACC in Attention & Cognitive Conflict***

Cognitive conflict is important in the context of insight. Conflicting memory sets or pieces of information are thought to create mental impasse and must be reworked through the restructuring phase to lead to the "Aha!" moment of insight.

The ACC plays a major role in attentional control (Luks et al., 2002; Carter et al., 1998; D'Esposito et al., 1995) and cognitive conflict monitoring (MacDonald et al., 2000; Carter et al., 1998). Whether the ACC actually initiates strategic functions in executing these processes as opposed to merely monitoring them is the matter of an intense debate (Weissman et al., 2005; Carter et al., 1998). Proponents of the *conflict-monitoring theory* posit a monitoring function for the ACC, while those arguing for ACC-implemented control subscribe to the *selection-for-action theory*.

*Conflict-Monitoring Theory Studies*

These studies argue against the ACC exerting any top-down attentional control, even at the response level, and instead favor a *modulatory* function for the ACC (Morishima et al., 2010; Milham et al., 2003; MacDonald et al., 2000; Carter et al., 1998; Botvinick et al., 1999). Other studies have further demonstrated the ACC's modulatory function to be response-level limited (Milham et al., 2003).

Carter et al. (1998) found increased ACC activity during incorrect responses (of which participants were aware) to Continuous Performance Tests, which measure attention (Figure 4; Riccio et al., 2002). Interestingly, this study also found that ACC activity increases when correct responses requiring high competition are produced (Figure 4; Carter et al., 1998).

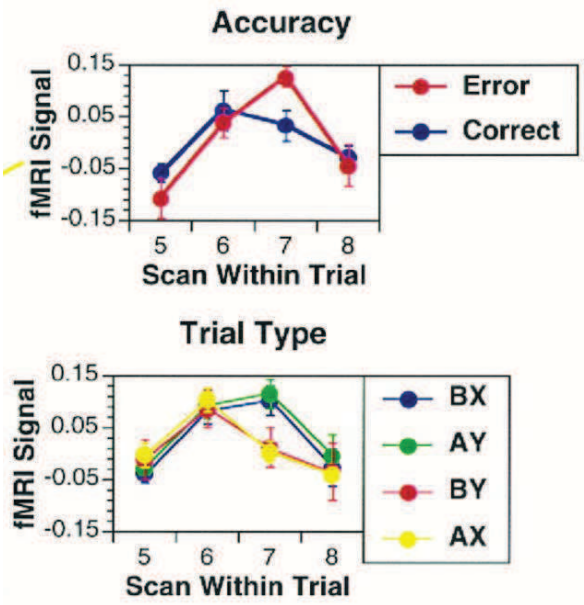



**Figure 4: ACC fMRI activity during Accuracy and Trial Type studies**. The plots for error and trial-type effect changes over time as percent difference from baseline MRI signal. In the Accuracy plot, an error effect was observed temporarily when incorrect response activity exceeded correct response activity. In the Trial Type plot, a transient increase in ACC activity was observed for trials AY and BX, the high response competition trials (adapted from Carter et al., 1998).

The more recent studies supporting the conflict-monitoring theory have extended the control of attention to include the PFC. Carter et al. (2000) emphasize the ACC's evaluative function that requires another area (the dorsolateral prefrontal cortex; DLPFC) to exert cognitive control. Paus, in his review (2001), points out that this argument would predict equal activation at the response and sensory levels, with further, downstream modulation specified later by the DLPFC.

However, Milham et al. (2001) found that the ACC is exclusively involved in response-level conflict. Their Stroop task event-related fMRI study compared groups presented incongruent-eligible tasks and incongruent-ineligible tasks to neutral task sets (congruent-ineligible and congruent-eligible). Incongruent means that the word and ink color were different colors, while eligible and ineligible refer to whether the color word was within the defined response group of words. They found that the ACC was significantly more active only for incongruent-eligible tasks, while the L PFC demonstrated increases in activity in both incongruent-eligible and incongruent-ineligible trials (Milham et al., 2001; Supplementary Figure 1). According to Milham et al. (2001), incongruent-eligible tasks create cognitive conflict at response and non-response levels, while incongruent-ineligible tasks may only cause non-response level conflict. Thus, one would expect increased activation during both incongruent tasks *if*

that brain region is involved in non-response level conflict; they conclude that the ACC, which did not produce those results, is only involved in response level conflict (Milham et al., 2001). Furthermore, Fleck et al. (2006) found that ACC activity decreased with confidence in decisions and increased with response time, suggesting that the ACC indeed is involved in cognitive conflict at the response-level (Fleck et al., 2006).

MacDonald et al. (2000) conducted event-related fMRI studies and a version of the Stroop task, finding a significant increase in left DLPFC fMRI activity when participants were asked to read the color as opposed to the word (Figure 5, bottom left). Additionally, activity in the ACC was significantly higher for incongruent (word and font color are different colors) Stroop tasks, a condition representing increased cognitive conflict (Figure 5, top right). MacDonald et al. (2000) concluded that the left DLPFC likely implements control, while the ACC simply monitors for conflict and is more active during the response (the later stages of processing, denoted by scans 6-10 in Figure 5).

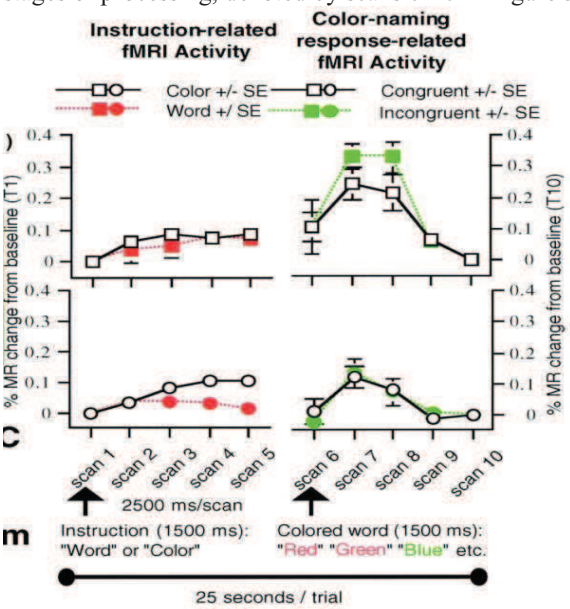



**Figure 5: Stroop task-related fMRI activity across time in the L DLPFC and the ACC.** Significant differences were found for the L DLPFC (bottom left quadrant) and ACC (top right quadrant). The top left quadrant indicates an insignificant difference in ACC activity between "word" and "color" recall, while the bottom right quadrant shows an insignificant difference in L DLPFC activity between congruent and incongruent color-naming responses (MacDonald et al., 2000).

Carter et al. (2000) provide evidence that the ACC, along with serving an evaluative role, *signals* that different or novel strategic processes should be evoked in cases when the ACC has detected cognitive conflict.

Participants underwent an event-related fMRI monitoring of the Stroop task, in two task sets: "high-expectancy" for congruent (word is printed in the same color) and for incongruent (word is printed in another color) stimuli. ACC activity was differentially heightened during incongruent tasks in which participants were presented predominantly (80%) congruent trials (Figure 6A), and only a minority of incongruent tasks (20%; meant to "surprise" the brain by presenting more conflicting conditions that require strategic processes to present a correct answer).

| Trial-type | Mean Response Time (ms) |
|---|---|
| Mostly incongruent | 44 |
| Mostly congruent | 154 |

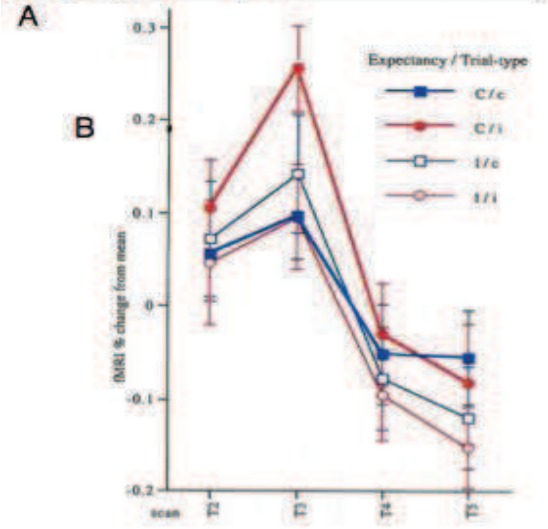



**Figure 6:**
**B. fMRI differential ACC activations from the mean for various Stroop task groups**. ACC activity was transiently the greatest in the congruent-expectant incongruent trial type. While ACC activity is not modulated by trial type, the congruent conditions seem to be (as seen by the significant difference between C/c and C/i) (adapted from Carter et al., 2000).
**A. ANOVA-revealed expectancy-trial-type interaction** demonstrates a significantly quicker response for mostly incongruent trials compared to mostly congruent trials (adapted from Carter et al., 2000).

Moreover, the study also demonstrated that when participants expecting congruent trials were shown an incongruent task, a significantly longer response time was observed, suggesting that strategic processes were less active, though cognitive conflict was high. In mostly incongruent trials, in which one would have high expectancy of these more demanding tasks, the short response time suggests that cognitive conflict was minimal and strategic processes were in place (Carter et al., 2000). The results argue that the ACC might not implement strategic processes itself. If this were true, one would have expected differential (between congruent and incongruent trials) activation of the ACC during the "mostly incongruent" set (Carter et al., 2000).

Thus, ACC's role in attention is thought to be exclusively to monitor (MacDonald et al., 2000; Botvinick et al., 1999; Carter et al., 1998), while the DLPFC, proximal and connected to the ACC, has been suggested to implement attentional selection and conflict resolution via strategic processes (MacDonald et al., 2000; Carter et al., 1998). However, the abundance of selection-for-action theory studies

that respond directly to these studies deserves consideration, particularly since many argue for a combined theory that distinguishes between anatamo-functional areas of the ACC. Moreover, both theories could likely converge onto the current understanding of insight, which will become apparent later.

*Selection-for-Action Theory Studies*

The selection-for-action theory argues that the ACC, along with other brain regions, actively chooses relevant information to be further acted upon (Posner et al., 1988). One early study (Posner et al., 1988) found that PET activation of the ACC was higher when participants were asked to pick from a long (up to 25 items) list of dangerous animals compared to a short (down to 1 item) list, suggesting ACC involvement in selection for action.

Another more recent study (Gruber et al., 2010) delineates the salient tenet of selection-for-action studies. It observed fMRI activation on three trials in which the frequency of an unexpected color congruency task varied. A negative correlation appeared between *intra*-individual ACC activity levels and response times to the atypical trials. Thus, faster response times were associated with higher ACC activation between multiple trials for the same individual. This association suggests that the ACC is actively involved in action selection; highly active ACC causes short response time because the ACC is actively choosing action items and is thus providing an expedited solution. When the ACC is relatively inactive, the longer response time occurs because the ACC is not exerting this selection-for-action function (Gruber et al., 2010).

Neither exclusively in favor of the conflict monitoring of selection-for-action theory, Luks et al. (2002) combine the ACC's monitoring role with selection-for-action's contexts (attentional set shifting). Their study (Supplementary Figure 2) agrees that the ACC monitors for cognitive conflict or competition at the stimulus and response levels, but generalizes this monitoring function to include all instances involving inhibition of goal-directed activation. An event-related fMRI experiment studied whether ACC is active exclusively during response selection, or if it also monitors task-related attention shifting; they had several interesting finds related to the ACC.

They found that the ACC was significantly more activated only after informative cues, during which the establishment and maintenance of attention is needed (Luks et al., 2002). They suggested the ACC monitors conflict related to attentional set maintenance, an explanation further supported by their finding that ACC activity was insignificant after neutral cues. Such would be expected because no attentional set had been created that needed observation (Luks et al., 2002). They conclude that the ACC thus monitors the distribution of attention among competing "attentional sets" (Luks et al., 2002). They noted that informative cues rely on WM in addition to attentional control, an important connection since WM and attentional control influence one another (Burgess et al., 2010; Courtney et al., 1997 as cited by Luks et al., 2002). Interestingly, a deficiency in task set maintenance in ADHD individuals has been associated with decreased DFPLC volume and less functional WM (Burgess et al., 2010), suggesting interplay between attention, WM, and the DFPLC.

Weissman et al. (2005) make some critiques of MacDonald et al. (2000), specifically that their 12.5 s cue-target interval was too long to accurately measure attention; they were not convinced that attention was directed during cue presentation, and chose to use shorter response intervals (1.25s). They demonstrated that ACC activity actually increases when interference from important tasks increases, indicating that the ACC likely does focus attention toward relevant stimuli. Later, it will become clear that this capability is important in overcoming mental impasse by refocusing on appropriate and helpful information.

*Reconciling the Selection-for-Action and Conflict Monitoring Theories*

Given the large bodies of literature advocating both theories, it seems undeniable that elements of both are likely correct. As Botvinick et al. (1999) state, the conflict monitoring theory by definition does not exclude the selection-for-action theory; it simply argues that the ACC does play a role in overseeing and signaling cognitive conflict. Neither does the selection-for-action theory exclude the possibility of the conflict monitoring theory. An appropriate model for ACC function might take into account the most supported and central findings of each, which are the ACC's conflict-detecting function as well as its ability to steer attention toward pertinent stimuli. Thus, both theories are useful in explaining the role of the ACC in the context of insight, a process dependent on attentional allocation and the monitoring of and control of cognitive conflict.

**III. Anterior Cingulate Cortex and Insight**

The ACC may be involved in the insight process during the mental impasse and restructuring phases. Previously, Sandkuhler and Bhattacharya (2008) suggested that areas involved in attentional control could contribute to solving insight problems. While they were interested in the posterior parietal lobe, also involved in attention, the same implications are true for the ACC: attentional control is important for maintaining and diverting attention to relevant stimuli; mental impasse is thought to occur when irrelevant or less helpful information and stimuli are made central in attention (Lockhart et al., 1988). The ACC also shows promising connections during the restructuring phase. The following chronological descriptions of the ACC through each step in insight will help to demonstrate a role from beginning to end.

*The ACC and the Immediate Pre-Insight State*

Kounios et al. (2006) show evidence suggesting that the activity pattern of the ACC immediately prior to attempting solution of a problem may determine whether one will use insight or noninsight processes. They conducted an fMRI study while asking participants to solve visually presented word-association insight problems (e.g., given the words *pine, crab, sauce*; the solution is *apple*). Interestingly, they found that the ACC was significantly more active during pre-insight strategies than for pre-noninsight strategies (Figure 7; Kounios et al., 2006).

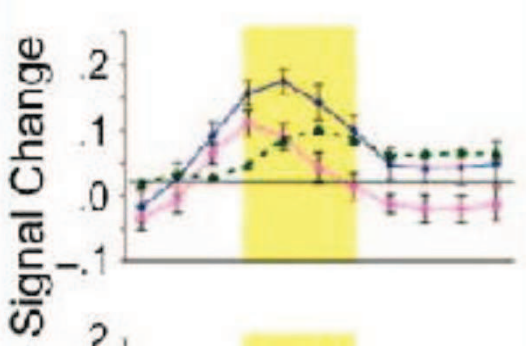



**Figure 7: ACC fMRI signal change among insight vs. noninsight strategies immediately preceding solution**. ACC was more activated pre-solution when insight was used (blue) than when noninsight was used (pink). The yellow shaded portion indicates the preparation phase. The green line shows (blue-pink) (from Kounios et al., 2006).

These results suggest the ACC may play a role in the processes of insight even before mental impasse. Notably, during the preparation phase, no cognitive response conflict existed (Kounios et al., 2006), suggesting that the ACC is *not* limited to monitoring or responding to such conflict. The authors posit that the ACC may have created a "clean slate" for problem solving by inhibiting irrelevant thoughts, perhaps to the previously attempted trial (Kounios et al., 2006). While this may seem relevant to mental impasse, these effects could be occurring before that stage if the ACC somehow attempts to diminish the remnant attentional set or cognitive conflict from the previous task, similar to switching attentional subgroups or signaling the PFC to switch/clear attentional subgroups (Luks et al., 2001; Banich et al., 2000). (While not expanded upon in the study, the fMRI did seem to yield a small area of activity within the PFC. See Figure 3 of Kounios et al., 2006). Other studies have also posited that ACC activity is associated with helpful preparation for insight problem solving (Tian et al., 2010).

Thus, it is possible that when the ACC becomes active preceding insight, it serves to prepare and alert its conflict and attentional monitoring and controlling capabilities, leading to a successful insight solution (obviously, only successful solutions are solved via insight). Moreover, this finding implies that increased ACC activity likely serves to promote successful insight solution as opposed to inhibiting it. These results do not limit ACC activity to pre-impasse stages.

*The ACC and Mental Impasse*

The ACC has shown selective activation during tip of the tongue (TOT) instances, which are similar to insight situations. Maril et al. (2001) found that when participants were asked to recall questions, the ACC was significantly more activated during TOT experiences (which involve cognitive conflict) than when the answer was known (no TOT) or not known. Tip of the Tongue states may be viewed as similar to mental impasse in that cognitive conflict may be preventing the correct response or attention to the right stimuli that would give the correct response. Maril et al. (2001) pointed out that the activation was not due to increased retrieval effort, but was more likely attributable to "impending success." Importantly, neither tip of the tongue solutions nor insight solutions are predictable, suggesting that they both rely on subconscious processes (Metcalfe, 1986), which likely includes cognitive conflict monitoring processes to choose the correct response. In fact, TOT states may simply be the interference of attentional control steering away from the correct answer. Similarly, mental impasse may be caused by a focus on the wrong information. Since the ACC may both monitor conflict and mediate attentional control, it could be responsible for both helping lead to insight solution, but also could help to *cause* mental impasse if it directs attention toward the wrong set of stimuli.

A handful of studies have suggested direct ACC involvement in mental impasse (Tian et al., 2010; Anderson et al., 2009; Maril et al., 2005; Luo, Niki, & Phillips, 2004). Luo, Niki, & Phillips (2004) suggest the ACC may mediate conflict between sensible resolutions and overpowering incorrect solutions. They presented confusing, insight sentences followed by a short blank screen, and then the solution. After leaving the MRI machine, participants answered a survey on whether they understood the sentence and/or its solution. When subjects used insight (defined as when revealing the solution created sudden comprehension), the ACC was significantly activated, as was the left PFC. Participants reported that they were at first unable to solve the problem because they were thinking about the problem in the wrong way; this obstacle causes mental impasse. It may be that when the PFC fails to implement strategic control processes, irrelevant information processing (as Carter et al., 2000 demonstrated); resultantly, the ACC is required to signal conflict to various areas of the brain including the PFC (Luo, Niki, & Phillips, 2004). While MacDonald et al. (2000) proposed a response-associated monitoring function of the ACC, the fact that ACC was equally activated for insight and difficult solutions but less so for non-insight solutions suggests that in insight, the ACC activity may not be limited to response-level processing (Luo, Niki, & Phillips, 2004).

*ACC and Restructuring*

In fact, the ACC may also play a direct role in response-level processing in insight (Anderson et al., 2009). This study found that fMRI ACC activity increased immediately preceding but especially after responses were requested for word-association insight cues. The authors contextualize their results using the ACT-R (Adaptive Control of Thought-Rational) theory, a product of the Anderson lab that argues ACC activity is associated with subgoal changing and number. Subgoals are intended to help find solutions to problems. The PFC, specifically the lateral inferior PFC (LIPFC), is said to execute retrieval processes, and the ACC thus becomes active once a solution has been found by the LIPFC and requires processing (Anderson et al., 2009). Importantly, the LIPFC is essentially the same brain region as the DLPFC.

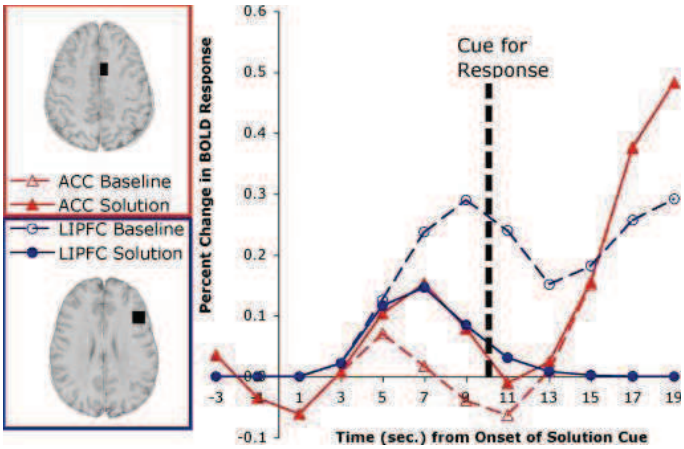



**Figure 8: ACC and LIPFC fMRI signal percent change from norm for baseline and solution trials.** ACC activity increased with time preceding the response as well as at post-response cue, while LIPFC was significantly active concomitant with the ACC pre-response cue period. However, a significant difference between LIPFC and ACC activity only occurred post-response cue. Importantly, this particular figure is a model generated from previous figures (adapted from Anderson et al., 2009).

While other studies have focused on what Anderson et al. (2009) would identify as the pre-response cue activation of the ACC, Anderson et al. focus on the post-response *cue* (which, importantly, is not post-response) activity. The ACC at that point may be 'scrambling' to produce a response out of retrieved solution by creating subgoals (Anderson et al., 2009). Such a process contributes to the restructuring phase of insight in that it involves active rearrangement of retrieved information. Thus the ACC may be incorrectly delegating subgoals in cases of incorrect responses or when problems take a significant amount of time to solve; such a mistake may be seen as mental impasse or even preliminary, "rough drafts" of restructuring. Anderson et al. (2009) identify the LIPFC (synonymous to the DFPLC) as initiating and completing "retrieval processes." Thus, DLPFC activity is proposed to precede ACC activity in insight, and the ACC becomes active only once a solution has been found and needs processing. These results, while showing some dissimilarity to MacDonald et al. (2000), also share in common the chronology of these areas' activity and to some degree, the amount of direct control the ACC versus the DLPFC has (both suggest the DLPFC is more involved in direct query-solving activity).

Another fMRI study (Aziz-Zadeh et al., 2009) demonstrated significant differences in brain activity levels between insight and normal problem-solving techniques. Among others, the right PFC, pons, angular gyri, right insula, and ACC were significantly more active when insight problem solving was used (Figure 9). The authors suggest that the ACC must inevitably be in partnership with these other brain areas because of the myriad regions stimulated during insight. Interestingly, the right PFC activity increased close to the same time interval as the ACC (Figure 9). These results show a difference in activation pattern from what Anderson et al. (2009) found.

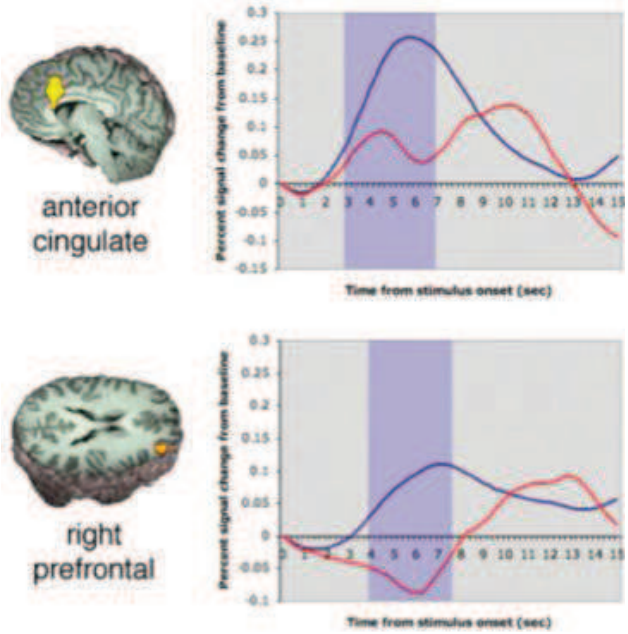



**Figure 9: fMRI activation pattern for insight (blue) and noninsight 'search' (red) problem solving.** In the ACC, insight activity was increased above the baseline as well as above noninsight problem solving during the 3-7 sec interval after stimulus presentation. For the PFC, activity was increased above baseline only for insight and from 4-7.5 sec (Aziz-Zadeh, Kaplan, & Iacoboni 2009).

A difference in problem type could have produced these different activation patterns; while Anderson et al. (2009) utilized word association, solvable only by insight, Aziz-Zadeh, Kaplan, and Iacoboni (2009) used anagrams (scrambled words), which can be solved using insight or noninsight skills. The separated activation patterns for 'search' (noninsight) and insight problems were markedly different. In fact, the noninsight trial showed two distinct moments of activation (Figure 9; maxima approximately 4 sec and 11.5 sec), a pattern not unlike Anderson et al.'s findings (Figure 8). Aziz-Zadeh, Kaplan, and Iacoboni (2009) may have incorrectly classified some insight problems as noninsight, or Anderson et al.'s trial was in fact solvable using noninsight strategies. Future research should specifically examine the pre- and post-response cue activation differences for *both* noninsight as well as insight strategies. The ACC function proposed by Anderson et al. (2009) however does not preclude the conflict monitoring theory's role. Possible ways of integrating these views into a unified role of the ACC in insight will be discussed later.

The conflict monitoring theory has not been widely used to describe the ACC's role in insight. Since it situates the ACC as contributor to attention allocation by monitoring for cognitive conflict, the theory provides a promising model for understanding restructuring, which is largely thought to involve overcoming a focus on irrelevant information. MacDonald et al. (2000), Milham et al. (2001), and Carter et al. (2000) provide fundamental evidence for the conflict

monitoring theory. These studies found that the ACC was activated during both correct and incorrect responses, particularly for those involving high cognitive conflict (such as incongruent trials in the Stroop task). Moreover, they identify the PFC as an executive partner of the ACC. For insight, it may be that in this theory the ACC monitors for conflict—which is likely high during mental impasse—and signals this conflict to the PFC (and other brain regions, which Aziz-Zadeh, Kaplan, and Iacoboni (2009) may have identified), which exerts direct control to 'restructure' or reevaluate to what items attention is being allocated. This process could continue until the ACC no longer signals conflict presence upon a satisfactory solution being found. Discussed later, this monitoring model may be further explained by the ACT-R theory.

The selection-for-action theory argues that the ACC does not just monitor, but directly focuses attention toward relevant stimuli (Weissman et al., 2005; Luks et al., 2004). Certainly, such a model allows for an important role for the ACC in insight, arguing for its firsthand executive control of attention allocation. If this is the case, then the ACC might monitor for conflict and then select certain items or responses over others (Posner et al., 1988), thereby contributing directly and actively to rearrangement of information into new ways of thinking (restructuring). Longer responses to insight problems, including instances in which a solution suddenly arises after significant temporal delay (Darsaud et al., 2010), could be due to failure of a relatively inactive ACC to aid in solution selection (Gruber et al., 2010).

*ACC and Suddenness*

The suddenness from insight is in many ways an effect of the nature of restructuring, which was previously shown to be predominantly subconscious (Metcalfe, 1986). The involvement of the ACC in creating a feeling of suddenness thereby originates in its role in restructuring. Previously described, suddenness in insight originates from selective comparison: when a non-obvious mental relationship quickly forms between old and novel information (Davidson 128). Thus, the seemingly instantaneous presence of a correct solution evokes an emotionally sudden sensation. The ACC's role in conflict monitoring to aid in the attention to relevant information and then to a conscious solution may assist in this feeling (Aziz-Zadeh et al., 2009; Siegler, 2000).

*ACC and Mental Impasse, Restructuring, & Suddenness: Putting it all together*

In some ways, the ACT-R theory of the ACC (Anderson et al., 2009) and the conflict monitoring theory pose quite different functions for the ACC in insight; in the former, the ACC has specific executive functionality and may exercise control over attention, while in the latter, the ACC simply watches for cognitive conflict and relates it to other cortical areas. The conflict-monitoring theory provides for a more temporally 'upstream' function in that the ACC would contribute to the generation of a successful response by indirectly inhibiting irrelevant information. However, the ACT-R theory would place the ACC more downstream, responsible for bridging retrieved information to a response through active subgoal generation. The selection-for-action theory may help to provide a perspective that reconciles these roles.

Anderson et al. (2009) contrast the ACT-R theory with the conflict-monitoring and error detection theories. The error detection theory of the ACC is quite outdated; several sources refute this theory (MacDonald et al., 2000; Carter et al., 1998) and it borders on a straw man argument used to contrast the ACT-R theory. Anderson et al.'s (2009) results do not exclude the conflict-monitoring theory given the significant increase in ACC activity pre-response as well, during which conflict monitoring between competing processes could be occurring. Thus, the ACT-R may describe just one of the functions of the ACC, especially during the restructuring phase of insight, in that the ACC would have control over information processing through its way of breaking retrieved information into subgroups.

Moreover, an integrated approach of the selection-for-action theory combined with the ACT-R and conflict monitoring theories may provide a more descriptive and global way of characterizing the ACC's role in insight. Upon detecting cognitive conflict, subgoals (the product of selection-for-action theory's action) may be partitioned by the ACC to reorganize and process conflicting solutions or information. Of course, the ACC may relay conflict signals to other areas, such as the PFC, that assist in exerting top-down control of attention allocation, helping the ACC to package subgoals to produce a response out of a solution.

In insight, solutions are often thought to be inhibited by a focus on irrelevant information, creating mental impasse that is eventually overcome by reorganizing the way of understanding given information (Sandkuhler & Bhattacharya, 2008). This integrated model maps onto this process. The selection-for-action theory accounts for the selective action necessary to rearrange items, and conflict monitoring may signal the initiation of this action. Then, according to the ACT-R theory, the ACC creates subgoals—which may be the product of restructuring—used to group solutions in preparation for response.

A consideration of the connection between WM and attention helps to further explain this process, since attention has been likened to a "gatekeeper" of WM (Awh, Vogel, & Oh, 2006). WM is comprised of the active items being attended to at any given time. However, WM is quite small; visual WM can only hold up to four items at a time (Vogel et al., 2001 as cited by Awh Vogel, & Oh, 2006). Thus, competition for WM space is high, particularly when multiple stimuli are present or there is high cognitive conflict (Awh, Vogel, & Oh, 2006). Working memory items may be controlled by the ACC; as explained by the conflict-monitoring and selection-for-action theories, the ACC may monitor for conflict within WM and may exert choice over what items are included in WM (MacDonald et al., 2000). In the context of insight, it could be possible that irrelevant or incorrect solutions outcompete important stimuli for working memory space (and therefore attention as well), leading to mental impasse and an inability to produce the correct solution (Awh, Vogel, & Oh, 2006).

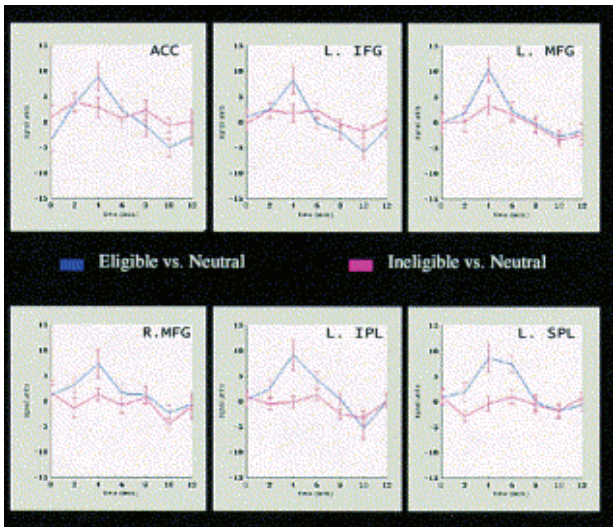
## IV. Conclusions & Implications

The involvement of the ACC in insight may be multifaceted, as explained by several theories of the ACC. The ACC might play a role as a monitor of cognitive conflict (MacDonald et al., 2000), an executor of attention control (Gruber et al., 2010), and a partitioner of solution subgroups (Anderson et al., 2009); all of these processes are exemplified in the context of insight, which involves breaking mental impasse using restructuring, a process reliant on regrouping of information in novel ways. The selection-for-action and conflict monitoring theories pose different roles for the ACC, but these roles are not necessarily conflicting. Moreover, the ACT-R theory (Anderson et al., 2009) provides a third perspective, and an integrated model of ACC function provides a temporally comprehensive account for ACC involvement in insight processes.

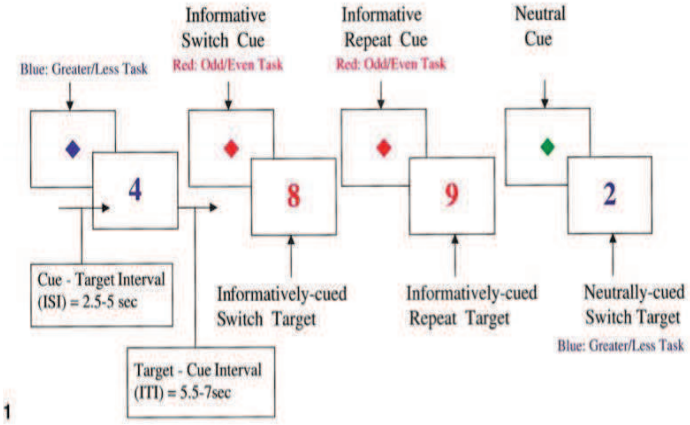
The ACC's involvement in learning processes poses it as an important area to understand in light of the 21.5% increase in ADHD prevalence in the past four years (Visser et al., 2010). Individuals with ADHD have reduced working memory ability as well as underactive attentional control and response selection mechanisms, including decreased left DLPFC activity and possibly also ACC activity (Burgess et al., 2010). Moreover, schizophrenic patients may be sensitive to heightened cognitive demands manifested by high activity in the ACC (Wilmsmeier et al., 2010). Obsessive-compulsive individuals demonstrate reduced error rates for high-conflict tasks, which utilize the ACC (Hammer et al., 2009). Thus when ACC function deviates from normal, serious attention and learning deficit or hyperactivity may result.

These results pose a challenge for a better understanding of the cognitive and neurocomputational processes of normal and disordered learning, but may serve as a substrate for studies to utilize to investigate how certain alterations in the ACC may affect the response times and success rate of the insight process. Perhaps the knowledge from future insight studies on these described deficits could prove helpful to better understanding the exact cognitive abnormalities, leading to more detailed and more successful therapies.

## Supplementary Materials




**Supplementary Figure 1: fMRI data from Milham et al., 2001.** This figure presents fMRI activity relative to baseline levels for different brain regions. Eligible and ineligible refer to whether the color word was within the defined response group of words.




**Supplementary Figure 2: Explanation of Luks et al. 2001 experimental task setup.**
Blue numbers required participants to respond whether it was greater or less than 5, while red numbers asked them to say whether the number was odd or even. The red and blue diamonds informed participants that a task of corresponding color was next. Green cues could be followed by either task. Thus on green cues, participants did not know the goal of the task until presented with the actual blue or red number.

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# Ontogeny of Nut-Cracking Behavior in the Capuchin Monkey (Cebus sp.)

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*Research studies have attributed the ontogeny of nut-cracking behavior exhibited by capuchin monkeys to a wide range of factors, but the topic is still debated among primatologists. Capuchins are a New World primate often researched both in captivity and in the wild because their development is widely related to the development of early hominids. In this paper, three theories are reviewed as to how tool use and nut-cracking emerged and what factors led to their development. The Socioecological Evolution Theory suggests that manipulative behavior and tool use have evolved as successful adaptions to a varying habitat. The Perception-Action Theory proposes that species-typical forms of exploratory action support the ontogeny of such goal-directed actions. The Independent Emergence Perspective argues that capuchin's exploratory behavior is not linked to cognitive abilities or increased intelligence. All three perspectives addressed specific variables they felt most important in the evolution, but none were able to find meaningful correlations. It is possible then, that tool use developed because of a wide variety of confounding factors.*

**Introduction**

The capuchin monkey (Cebus sp.) is often studied because of the high level of intelligence among New World primates and for it's unique extractive foraging behavior (Fragaszy et al., 2004). There is evidence from studies and observations of tool use in capuchins, which provide possible insight on the evolution of primate cognition (Ottoni & Mannu, 2001). Capuchins typically inhabit tropical or subtropical forests such as the Amazon Rainforest of Brazil and Venezuela (Myers et al., 2008). In addition, species of capuchin have been observed in fragments of woodland during the dry season (Fragaszy et al., 2004). These monkeys are typically tree dwellers, but will descend to the canopy floor to forage . Capuchins are omnivorous foragers feeding on a variety of insects, seeds, pith, eggs. Though a large portion of their diet consists of fruit (Myers et al., 2008). While foraging they have been observed tearing up vegetation and pounding seeds against branches in an effort to extract embedded foods (cited by Myers et al., 2008). Terborgh, 1983, ,argued that capuchins are described as destructive extractive foragers because capuchins deal with the environment in a vigorous and persistent way with he purpose of exploiting potential food (cited by Jalles-Filho, 2008). The most recognizable behavior performed by capuchins while foraging is the use of a hammer and anvil to crack open nuts and seeds.

There is evidence that stone tools were first produced by Hominids up to 2.5 million years ago (Schick and Toth, 1993 cited by Westergaard, 1995). There are reports of spontaneous tool use of capuchins by Gonzalo Fernandez de Oviedo as early as 1526 (cited by Ottoni and Mannu, 2001). There is evidence of tufted capuchin monkeys using stones in a percussive manner to cut objects, which requires the sequential combination of two stones (Westergaard and Suomi, 1994). Using a stone (hammer) to pound apart nuts placed on a hard surface (anvil) is the most complex form of tool use by a nonhuman species because it involves producing two spatial relations in sequence (Fragaszy et al., 2004). The nut-cracking behavior observed in species of capuchin monkey involves four sequential steps: (1) Find and pretreat a nut or seed; (2) Find the appropriate stone to use as a hammer; (3) Place the food on the hard surface; (4) Successfully crack the nut open. The foraging process of nut-cracking is unique and impressive because it involves a complex sequence of planning, finding the correct tool, and using more than one tool working together., The appreciation of an object's affordance and planning is the foundation of human technology and regards stone pounding as a precursor to tool use (Visalberghi et al., 2009).

In order to better understand how and why capuchins perform the complex adaptive behavior of nut-cracking, the confounding factors that influenced the emergence of this behavior are reviewed. There are three hypotheses that aim to explain capuchins' ability to intelligently use tools. The first theory examines the Socioecological Evolution Theory, which attributes tool use to an increase in reproductive fitness through adaptation to environmental and social pressures. The second theory explores the Perception-Action Theory, which postulates that tool use is grounded in perceptual learning arising from an action directed towards an object (Resende et al., 2008). Other scholars disagree with the Perception-Action Theory and form a third hypothesis to explain nut-cracking behavior. Thus, the third section of my review discusses how

manipulation does not correlate with effective tool use or cognitive abilities, but relies upon many other factors.

Socioecological Evolution Theory

Besides chimpanzees, capuchins are the only primates to routinely use stones for nut-cracking in natural settings (Resende et al., 2008). Many primatologists and ethologists believe the emergence of this behavior exemplifies increased fitness within certain species of capuchin that make it an advantage for survival and reproduction. The explanation behind this theory of evolution is that the more intelligent individuals have the most adaptive traits and will be most successful at obtaining nutrients and successfully reproducing.

In primates, evolution of a behavior such as learning can most often be attributed to social pressures (Jolly, 1972). One of the most prevalent social factors considered in the evolution of behavior is dominance. Capuchin groups are structured with one dominant male who gets the first choice in food along with a priority group of younger males chosen by the leader Myers et al., 2008). This social structure provides pressure on subordinate individuals to adapt to a wider variety of foods because those individuals often have limited choices. Subordination especially occurs between the dominant male and the females of the group. The ability to crack nuts is less important for females because, according to social structure, the dominant male usually collects food for the females of the group (Pannozzo, 2003). It has been reported that adult females performed the fewest nut-cracking episodes out of each sex-age class (Ottoni and Mannu, 2001). Adult males averaged fourteen nut-cracking episodes per individual whereas juveniles averaged over forty episodes each. From the data presented by Ottoni and Mannu, it is possible that juveniles are the most active because they are restricted access to more desirable resources, or because they have not been able to combine the actions into the appropriate sequence for successful nut-cracking (2001)., Other primatologists argue that social factors play a role due to the fact that females use tools less frequently than males (Jalles-Filho, 2008). There were also observations of anxiety of subordinate individuals when manipulating the testing apparatus or near it. Observations that were similar to those of Ottoni and Mannu. The social pressures of dominance, age, and sex contribute to the need for the capuchins to have a large variety in diet, as well as adaptive techniques for obtaining food. The individuals that are more intelligent are able to learn to forage more effectively and obtain more food for energy and will therefore be more fit.

Social structure is only a portion of the explanation for how nut-cracking behavior became an advantageous trait according to the Socioecological Evolution Theory. There are many factors involving habitat and resource adaptation that play a strong role in the evolution of nut-cracking. In a woodland habitat, capuchins have been observed to exhibit nut-cracking behavior most frequently in the dry season thought they forage year round (Fragaszy et al., 2004). A seasonal shift in habitat from the tropical rainforest to dry woodland, which includes a shift in available food resources, was very important in contributing to the need for an adaptive foraging behavior. The greater the intelligence of an individual, the better that individual could learn to exploit changing ecological circumstances. This line of thought follows the idea that species that are able to obtain resources from different areas seasonally, like capuchins, will be more intelligent because this type of foraging promotes better memory, more knowledge of environment, and the ability to plan ahead. Westergaard and Suomi hypothesized that under the right circumstances, capuchins use stone tools to cut and percuss in a way that could be similar to early hominids, but within their New World habitat (1994). Increased intelligence can also be attributed to the analysis needed to perform the complex tasks an extractive forager uses to obtain food.

The Socioecological Evolution Theory proposes that nut-cracking is an adaptive trait that has evolved from social influences and environmental pressures causing a need for an adaptive foraging behavior. Although it is possible that nut-cracking evolved, it does not explain the process of learning necessary for a complicated action such as tool use. In order to evolve, there would have to be a line of adaptive traits that led up to nut-cracking, but nut-cracking is a specific sequence that is not successful without preceding steps.

Perception-action Theory

Many scholars do not consider nut-cracking behavior to have evolved, but argue that it has emerged from learning spatial relationships during innate manipulation of objects. Capuchin monkeys have had success in learning to effectively use tools because they spontaneously manipulate objects in ways that develop spatial relations between objects and surfaces, and because they spontaneously use objects as tools (Fragaszy & Cummins-Sebree, 2005). The Perception-Action Theory postulates that skills are acquired through linked action and perceptual learning (Resende et al., 2008). Learning about spatial relations and how to produce a desired goal is what lead to the emergence of tool use. It has been suggested that the actions which support learning are actions commonly evident in the species-typical repertoire of exploratory behaviors (Resende et al., 2008). Learning the mental and physical capacity of tool use reflects experimental discovery through actions, perceptual learning, and practice in a particular context.

ElisabettaVisalberghi, purposed that there is a global developmental pattern in capuchins to crack nuts with a percussive tool (cited by Resende et al., 2008). First, there was a simultaneous emersion of direct percussive actions and manipulation of stones or nuts. Second, the monkeys placed the nut on the anvil and struck it with a stone, but were not effective at opening the nut. Last, the monkey eventually became effective at opening nuts by choosing the correct substrate. Since there are variables in substrate and nut or seed toughness, the effectiveness of nut-cracking requires practice. Observations that not all individuals are able to learn proficient nut-cracking and that some sub-adults have the basic actions required, but not the appropriate sequence, supports the analysis that nut-cracking takes practice (Ottoni and Mannu, 2001).

From the Perception-Action Theory it was predicted that direct actions with an object or surface will come before actions that combine an object with a surface (Fragaszy, 2007). It was predicted that capuchins would spontaneously bang different objects against different substrates in a playful

way before performing the goal-directed action of using an object to crack open a nut (Resende et al., 2008). It was found the first step in capuchins learning to crack nuts to be similar to the first step in the global development pattern and prediction of the Perception-Action Theory (Ottoni and Mannu, 2001). It was observed that the manipulation of stones and nuts occurred simultaneously to direct percussion of the nut against the substrate. The next step was non-effective nut-cracking once the monkeys began to release the nut and continue to direct action toward it (Resende et al., 2008). It appeared that the observed learning pattern fit that of the global pattern, but a study of effective tool selection by wild bearded capuchin monkeys found that the monkeys were almost always able to determine the best substrate on the first trial (Visalberghi et al., 2009). These results come from the observation that capuchins gain information about a tool by moving, lifting, or tapping the object. If information on correct tool choices comes during manipulation and exploration of an object, then there should be not be a step in which tool use is ineffective unless there is another factor to be considered in the pattern of learning.

The reason nut-cracking is difficult, and why it is interesting that a nonhuman primate performs this behavior, is because it involves producing a sequential, static, permissive, direct relation between hammer stone and nut (Fragaszy, 2007). Resende et al. noted that placing the nut on the anvil and releasing it seemed to be the most difficult barrier to learning to crack nuts (2008). The act of releasing a nut onto another object indicates that capuchins have learned the principles of static and dynamic relations. It takes experience and learning to understand the correct process of nut-cracking. The Perception-Action Theory explains the difficulty of releasing a nut to behavior based upon its foraging ecology (Resende et al., 2008). Capuchins are primarily arboreal, use vigorous actions in foraging, and are cautious about releasing an object because of the risk of losing it.

The Perception-Action Theory clarifies the importance of species-typical exploratory activity in learning skilled action sequences, which is evident in spontaneous manipulative and exploratory actions with an object (Resende et al., 2008). This theory explores capuchin monkeys' pattern of learning, which supports the link between manipulative development and the emergence of tool use. Perception-Action Theory is not able to explain how capuchins are able to learn to release a nut in order to strike it with a hammer. Ottoni and Mannu offered the importance of social factors for this independent trait (2001). They reported that the majority of individuals who watched others perform nut-cracking behavior were infants and juveniles, which indicates that learning to release a nut could be, in some portion, dependent on the social group.

**Independent Emergence**

From the Perception-Action Theory has emerged opposition to it. Scholars, such as B.J. King, argue that extractive foraging has no correlation to the evolution of intelligence or complex cognitive skills (Jalles-Filho, 2008). King argues that feeding strategy is not linked to mental development, which is evident in primates and extractive foraging. This is observed by looking at the group of primates and foragers as a whole. Many species of extractive foragers do not have high levels of cognitive abilities, so the development of nut-cracking among primates is unique to capuchins and chimpanzees (Resende et al., 2008).

The Perception-Action prediction that capuchin's exploratory behavior was addressed by Jalles-Filho, who studied manipulative propensity and tool use. According to a report by Jalles-Filho, there was no correlation found between tool use and manipulative propensity in capuchin monkeys (1995). Jalles-Filho, performed another study that concluded that the overall manipulative propensity of the monkeys is not correlated to the disposition and efficiency to use tools or in any way could it be used to predict tool use (2008). In these two studies, all the subjects used the tools provided, but there was no guarantee of systematic expression to achieve a goal, such as obtain food. It cannot be argued that capuchins do not have the morphological and cognitive capabilities for tool use, but many studies have had little success in correlating manipulative propensities to goal-directed tool use (Jalles-Filho, 1994).

Conclusion

The capuchin monkey is able to crack open a nut using a hammer and anvil technique, which can be explained as a complex sequence of goal-directed actions involving the use of more than one tool. This article reviews three different lineages of thought that set out to explain how tool use and nut-cracking emerged in capuchin monkeys. The Socioecological Evolution Theory ties social pressures to the environmental pressures of adapting to a changing variety of habitats and resources. Evolution could explain the emergence of tool use, but is unlikely due to the intermediate adaptive traits necessary for nut-cracking to emerge. The Perception-Action Theory forms a pattern of development for capuchins and is based on the thought that species routinely perform specific actions and that specific types of tool use are species dependent. The Perception-Action Theory is widely accepted, but is incomplete in that it does not explain how the capuchins learned the static relations of setting a nut on an anvil and releasing it. The first two theories related extractive foraging to the propensity for tool use. Other primatologists argued that foraging has no correlation to cognitive abilities or high intelligence. This argument deserves discussion because of the fact that nut-cracking was developed uniquely among primates and that not all foragers are cognitively intelligent. It may be that the integration of tool use throughout the history of the capuchin monkey requires the coordination of many confounding variables.

An important issue that must be raised in relating many studies of capuchins to evolutionary or emergence implications is that sample size is always very small. Capuchins are quite active and it is difficult to obtain a lot of observational or quantitative data on wild species. Captive studies also must be careful because although it is possible to control many variables in the laboratory, implications relating to capuchins as a whole are difficult. Further research should study the complexity of object manipulation compared to the ability of capuchins to learn. Along with this study could test the developmental pattern in which capuchins could learn

various complex actions. Future research must take in to account the diversity of individuals as well as the species in order to discuss the emergence of an action.

A possible area of research that may help explain the development of nut-cracking is to observe and record how capuchins use tools for activities other than nut-cracking. Wild capuchins were observed using a stick to club a snake (Boinski, 1988 cited by Westergaard, 1995). There has been no evidence found of the limits of tool use in capuchins. Research should examine how a tool is used, such as a hammer or a weapon, and compare it to the goal that capuchins are attempting to achieve. Such research could explain the motivations that led to the emergence of tool use based upon a larger range of uses and conditions of use.

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# The Predatory Behavior of Spiders

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*All spider species have their own unique predatory behaviors that have allowed them to become successful predators. While certain species depend on their venom to capture prey, others can utilize both silk and venom to immobilize prey. Many methods of catching prey have also evolved. Some species discharge viscous spit to cripple prey, while others hunt spiders of different species for food. Spider predatory behavior is correlated with the morphological and physiological attributes of each species. Individuals of the same species can also exhibit separate predatory behaviors. Because spiders are one of the most diverse taxa, the complexity of spider predatory behavior is unfathomable, but more knowledge about these unique predators is discovered every day. Further research on spider predatory behaviors can show how spiders could regulate insect population growth, and by doing so, spiders could increase biodiversity and improve the earth's ecological health.*

**Introduction**

Predators are a vital part of ecology. From lions in African savannas to sharks in oceans, predators help maintain the earth's ecological balance (Stevens et al., 2000; Woodroffe and Frank, 2005). Large predators are known for their ability to stalk, attack, and kill prey, but small predators like spiders have evolutionarily developed different methods of capturing prey (Table 1). Spiders, in the order Araneae, depend on insects as their primary food source. Even though spiders evolved before insects, insects are believed to be a more diverse taxon due to their arms race between predators like spiders (Vollrath and Selden, 2007). Throughout the years, insect species diversity substantially increased, and spiders evolutionarily developed better techniques to capture their insect prey. It is also important to note that not all spider species pursue insects. Some of the largest species, spiders from the family Theraphosidae, can hunt larger animals like birds (Isbister et al., 2003). Bird-eating spiders are found in tropical areas of the world and have their own unique predatory behavior separate from most spiders (Vollrath and Selden, 2007; Isbister et al., 2003).

Whether spiders consume insects or birds for nutrition, all spiders have evolved to become successful predators because of their unique ability to produce silk (Vollrath and Selden, 2007). Different spider species produce silk that differ in viscosity, strength, and thickness (Vollrath and Selden, 2007). With multiple types of silk, many spiders can construct various forms of webs, and some spider species are known for their capacity to create intricate webs to capture prey and/or deter predators (Eberhard, 1982; Vollrath and Selden, 2007); however, silk production did not evolve in spiders just for web-building. Even though all spiders can generate silk, not all spiders can build webs (Malli et al., 1999; Wigger et al., 2002; Wullschleger and Nentwig, 2002). Many spider species compose and utilize silk for multiple tasks such as wrapping prey (Gilbert and Rayor, 1985; Robinson et al., 1969), transportation (Richman and Jackson, 1992), and protection for eggs from the elements and predators (Richman and Jackson, 1992; Vollrath and Selden, 2007).

Along with silk production, many spiders have glands that produce venom to immobilize and/or kill prey (Isbister et al., 2003; Malli et al., 1999; Robinson et al., 1969; Wigger et al., 2002; Wullschleger and Nentwig, 2002). Because species have dissimilar venom potencies, their predatory behaviors contrast as well. While some species can simultaneously utilize venom and web engineering to catch food, venom production is especially important for spider species that cannot construct webs (Malli et al., 1999; Wigger et al., 2002; Wullschleger and Nentwig, 2002). In addition to venom, certain species such as *Scytodes sp*. utilize their unique ability to produce glue-like spit to help immobilize their prey either before or after injecting venom and prey wrapping (Gilbert and Rayor, 1985; Miller, 2006). Spider species possess their own particular physiological and morphological attributes that correlate to their behavior (Vollrath and Selden, 2007). These factors have allowed spiders to become excellent predators.

Research in the predatory behaviors of spiders is relevant in understanding biodiversity and ecosystem sustainability. Since insects are the most abundant taxa in the world, spiders are an important part of the food chain because they regulate the population growth of insects. For example, the green rice leafhopper (*Nephotettix cincticeps*) is a common pest in southwest Japan, and spiders are the only predators that can efficiently prevent green rice leafhoppers from

overpopulating (Kiritani et al., 1972). If they are permitted to overpopulate, certain insect species can significantly harm other animal species as parasites, disease vectors, or by over-consuming vegetation. Spiders are natural pesticides that protect other animal species from the negative effects of insect overpopulation (Miliczky et al., 2000; Schmidt et al., 2005).

**Table 1. COMMON PREDATORY BEHAVIORS OF SPIDERS**

| Species or Family | Silk | Web-Building | Venom | Spitting | Wrapping | Jumping | Cooperative Prey Capture | Citation |
|---|---|---|---|---|---|---|---|---|
| *Nephila clavipes* | X | X | X | | X | | | (Robinson et al., 1969) |
| *Argiope argentata* | X | X | X | | X | | | (Robinson et al., 1969) |
| *Argiope savignyi* | X | X | X | | X | | | (Robinson et al., 1969) |
| *Argiope ftorida* | X | X | X | | X | | | Robinson et al., 1969 |
| *Cupiennius salei* | X | | X | | X | | | (Malli et al., 1999; Wigger et al., 2002; Wullschleger and Nentwig, 2002) |
| *Scytodes sp.* | X | X | X | X | X | | | (Gilbert and Rayor, 1985) |
| *Scytodes socialis* | X | X | X | X | X | | X | (Miller, 2006) |
| Salticidae | X | X | X | | X | X | | (Richman and Jackson, 1992) |

*Note:* The behaviors marked off could be found within some individuals of a species but not practiced by the species as a whole.

***The Correlations Between Silk, Venom, and Predatory Behavior***

Even though the usage of silk is not common in all arachnids, spider species that expend time and energy to fabricate webs for prey encounter obstacles promptly after prey have collided with the web. The prey's location must be pinpointed immediately before they have the opportunity to escape, and they must be ambushed in a manner that will not allow them to escape or harm the spiders (Robinson et al., 1969). Web-building spiders can properly detect the location of their prey from the vibrations diffused from the prey's struggle to escape (Richman and Jackson, 1992). After the prey has become immobilized by a venomous bite(s) and/or silk wrapping, the spiders must decide how to free the prey from the web and transport the food to a location for feeding or storage (Robinson et al., 1969). The entire process of catching and eating food is strenuous and time-consuming, but these behaviors have shown to be evolutionarily successful in spider species. The majority of web-building spiders wrap their prey. Wrapping is a mechanism that allows spiders to immobilize their prey immediately (Robinson et al., 1969), and it provides spiders with the opportunity to store their prey for later consumption (Gilbert and Rayor, 1985). Certain spiders may bite their prey before wrapping it or vice versa. Depending on the prey and spider species, the prey may die from the silk wrap, immediately after the injection of venom, or the combination of both (Gilbert and Rayor, 1985; Robinson et al., 1969). Many factors such as prey size and structure, venom potency or volume, and spider morphology determine how each species behaves aggressively toward its prey.

*Nephila clavipes*, an orb-web spider species, primarily bites its prey before wrapping, which develops under three circumstances. First, the prey can be wrapped after being transported from the capture site to the feeding location. Secondly, if the prey cannot be moved from the web, *Nephila clavipes* will wrap the prey at the capture site. Lastly, *Nephila clavipes* can free its prey from the web and wrap it at the capture site before moving it elsewhere (Robinson et al., 1969). In contrast, *Argiope argentata*, *Argiope savignyi*, and *Argiope ftorida* utilize silk as a weapon before biting their prey. Prey can be lifted off the web by the jaws and wrapped at the feeding site, or the spider can wrap its prey while transporting it elsewhere (Robinson et al., 1969). These wrapping techniques show how each species has evolutionarily adapted to obstacles in acquiring food.

***The Correlations between Venom and Predatory Behavior***

For spiders that do not build webs like the wandering spider (*Cupiennius salei*), venom is the most important tool in immobilizing prey (Malli et al., 1999; Wigger et al., 2002; Wullschleger and Nentwig, 2002). This species will inject

more venom into more difficult prey species to prevent escape, and in addition to venom concentration, these spiders will treat their prey differently depending on their preys' size and structure (Malli et al., 1999; Wigger et al., 2002; Wullschleger and Nentwig, 2002). In an experiment performed by Wigger et al. (2002), the wandering spiders utilized the lowest concentrations of venom on crickets (*Acheta domesticus*) and stick insects (*Carausius morosus*), slightly higher doses of venom on blowflies (*Protophormia sp.*), and the highest concentration of venom on ground beetles (*Poecilus cupreus*; Figure 1). Crickets and stick insects were easily caught, injected with little venom, and eaten. Despite the fact that blowflies can fly, the wandering spiders did not have difficulty in catching blowflies, but upon capture, blowflies would vibrate their flight muscles. These spasmodic movements caused the wandering spiders to inject more venom into blowflies. Ground beetles were the most problematic prey. After catching the beetles, the wandering spiders had to continuously spin the bodies around to find good injection sites. Upon discovering sufficient injection sites, more venom was used to immobilize ground beetles. Ground beetles are known to have a chemical defense against spider venom in their bodies, which could be another reason for changes in the wandering spiders' behavior. This type of spider predatory behavior lends support to the venom ptimization theory, which states that spiders know they must utilize their venom frugally (Wigger et al., 2002).

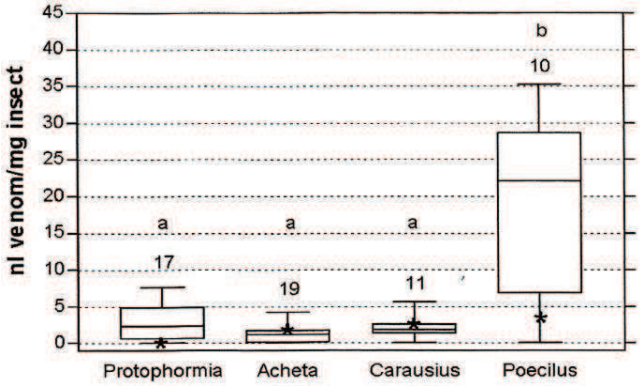



Figure 1. The concentration of venom from wandering spiders (*Cupiennius salei*) injected into four prey species. The crickets (*Acheta domesticus*), stick insects (*Carausius morosus*), and blowflies (*Protophormia sp.*), categorized by *a*, require lower doses of venom than the ground beetles (*Poecilus cupreus*), *b* (Wigger et al., 2002).

In another experiment, Wullschleger and Nentwig (2002) delved deeper into research by Wigger et al. (2002) to test if wandering spiders actually recognize the quantity of venom in their glands and if these spiders could select prey according to this awareness. Two different cockroach species, *Blatta orientalis* and *Nauphoeta cinerea*, were chosen as prey items. *Blatta orientalis* has significantly higher sensitivity to venom than *Nauphoeta cinerea*. Upon introduction of these two cockroaches, the spiders, with full venom glands, attacked both prey species equally. After electrically milking the venom glands or allowing the spiders to repeatedly bite crickets, the spiders preferred *Blatta orientalis* as prey over *Nauphoeta cinerea*. These results demonstrate that the predatory behavior in spiders is dependent on the volume of venom in their glands. Wullschleger and Nentwig (2002; Figure 2) found support for the venom optimization theory through their study of the predatory behavior of spiders that solely hunt with venom as their main weapon.

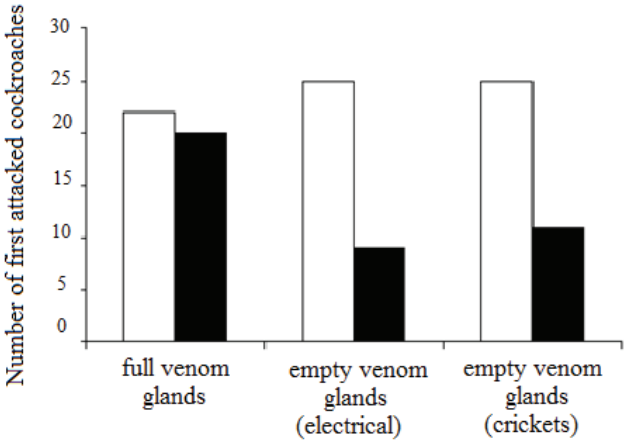
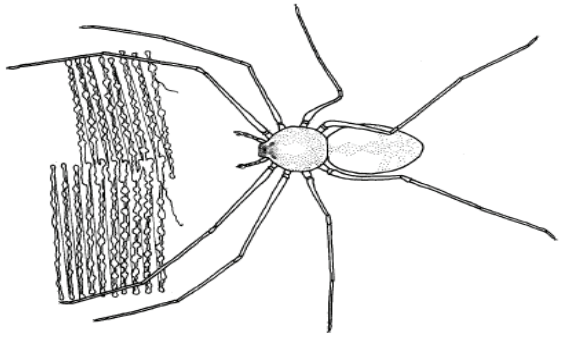



Figure 2. The cockroach prey-choice of wandering spiders (*Cupiennius salei*) dependent on empty or full venom glands. When wandering spiders had full venom glands (far-left bars), they preferred both *Blatta orientalis* (white bars) and *Nauphoeta cinerea* (black bars) equally. After getting their venom extracted via electrical milking (middle bars) and cricket exposure (far-right bars), wandering spiders preferred *Blatta orientalis* more than *Nauphoeta cinerea* (Wullschleger and Nentwig, 2002).

***Spitting Predatory Behavior***

Apart from venom injecting and wrapping, some species attack their prey via spitting. After the prey makes contact with its web, the spitting spider (*Scytodes sp.*) cautiously approaches the prey while randomly tapping it. When the spider grasps the prey with its forelegs, it ejects glue-like spit toward the prey (Figure 3). Immediately after spitting, *Scytodes sp.* uses its legs to rotate the prey in the drying spit. After the spit cements, the spider bites the prey, further immobilizing it. Once the spider realizes that its prey is secure, the spider bites into the shell of dry spit, pulls its victim out, and proceeds to wrap it with silk (Gilbert and Rayor, 1985).



***Spitting Spiders and Cooperative Prey Capture***

Malagasy spitting spiders (*Scytodes socialis*) from Eastern Madagascar's dry deciduous forests display similar

spitting behaviors as the spiders (*Scytodes sp*.) in the previous section, but Miller (2006) elaborates on their cooperative predatory behaviors. In order to cooperatively capture prey, multiple adult males, adult females, and juveniles share the same webs. With more predator participation, the spiders can capture larger prey than they could alone. Usually all adult males, adult females, and juveniles work together to catch large prey, but some individuals that preyed without assistance did not share with the group (Miller, 2006). Like many spider species, Malagasy spitting spiders utilize webs to trap their prey. Unlike the spitting spiders (*Scytodes sp*.), Malagasy spitting spiders bite their prey immediately to immobilize it and only spit on the most difficult prey items.

***Jumping Spider (Salticidae) Predatory Behavior***

In contrast to most spider species, jumping spiders, from the family Salticidae, have keen vision. Where most species rely on silk or venom to obtain prey, jumping spiders' main weapon is eye-sight. Their acute eye-sight has allowed them to be diurnal hunters that do not need webs in order to capture prey. Unlike other species, jumping spiders do not need to directly approach prey in a straight line. These spiders can find alternate routes to reach their food, and by doing so, a visual of the prey can temporarily be lost (Richman and Jackson, 1992). This confident predatory behavior resembles the behaviors of large mammals in the animal kingdom. Salticids are known for being active and aggressive predators. Upon discovering their prey, salticids will promptly leap on the prey without examining their surroundings first. While most spider species will carefully survey the environment before pursuing prey (Richman and Jackson, 1992), jumping spider behaviors indicate their lack of concern for predators in comparison to other species. In fact, some salticids are the predators of other spider species. Certain jumping spiders will invade the webs of web-building spiders such as orb-weavers. By advancing across the web or jumping directly through the web, salticids will steal insects from the web or snatch spiders that built the webs. Another salticid tactic to catch other spider species for food is to mimic the behavior of insects on another spider's web by maneuvering the silk to create vibrations. After the web-builder feels the vibrations executed by the jumping spider, it walks toward the direction of the vibrations expecting food, whereas the jumping spider attacks before the web-builder has a chance to escape (Richman and Jackson, 1992). The spiders in the Salticidae family have clearly evolved to become cunning predators that can manipulate the predatory behaviors of other spider species.

**Conclusion**

The predatory behaviors of spiders demonstrate the intricacies of the order Araneae. Even though spiders are assertive creatures, all of these behavioral circumstances show the agility and coordination skills of spiders. Every species analyzes their prey and almost instantly reacts in their own unique manner. From spitting to jumping and venom injecting to wrapping, spiders exercise their individual morphological and physiological characteristics to seize prey.

Further research in spider predatory behavior can be advantageous for ethologists who want to regulate insect populations in specific locations of the world. In the areas where insects are damaging crops, spreading deadly diseases, and disturbing habitats of other animals, spider predators could be less abundant due to other environmental factors (Miliczky et al., 2000; Schmidt et al., 2005). By studying spider behavior, researchers can increase spider population sizes to decrease insect populations.

Taxonomists could have an interest in spider predatory behaviors to classify new species. Currently, there are 41,719 species of spiders in the world, and it is possible that many other species have yet to be described (Platnick, 2010). While certain species may have similar appearances, they could have contrasting predatory behaviors that classify them into different species.

Overall, further research can expand the knowledge of these intriguing and valuable animals to the public. Many people may view spiders as pests and dangerous creatures because they do not know the ecological importance of spiders (Nyffeler and Sunderland, 2003). The grotesque physique and estranged behavior of spiders can cause people to fear them, but it is crucial for humans to appreciate spiders as predators that indirectly protect all animals.

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