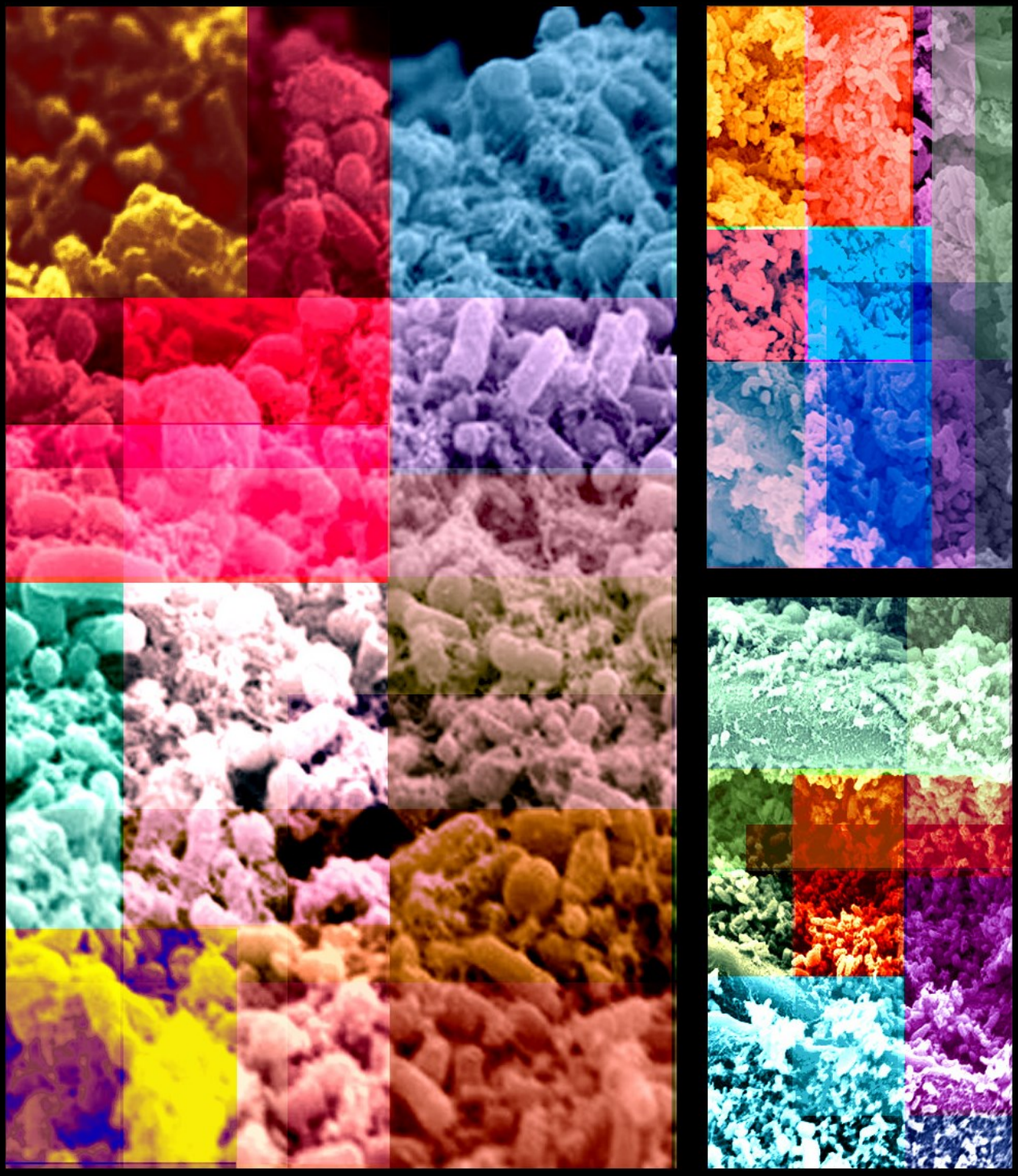
# JOSHUA

The Journal of Science and Health at the University of Alabama



May 2014 Volume 11

# From The Editor

Dear Readers,

Although this is my final editor's note and I have to say goodbye to both my Alma Mater and this publication, I am confident that I am leaving my readers in extremely capable hands. As I look forward to the next step in my academic journey, I know that I have been privileged to serve both my readership and this university's community.

*The Journal of Science and Health at the University of Alabama* is a culmination of talents emanating from some of the brightest young minds at our university, and I have certainly been privileged to introduce their ideas to our readers. I know that these new researchers will be the future leaders in their respective fields and I urge you to continue to follow both their progress and future publications. If as editor, my opinion carries any weight, I wish to encourage these bold young men and women to keep to their path. Research can be daunting, but researchers are fearless. They are not afraid to fail, because they know that in science, success is built upon both failures and successes. I count myself as one amongst them and I hope that I have communicated our communal passion for research and discovery. To each contributor, I extend my sincerest thanks for your commitment to science and I strongly encourage you to continue your research. Your *JOSHUA* articles stand as a testament to both your hard work and dedication.

Throughout my tenure at *JOSHUA*, I have had the opportunity to work with an unparalleled mentor, Dr. Caldwell, and I want to thank him for his support and faith. I have also enjoyed the support of a dedicated staff, and I want to thank them for their cooperative spirit and work ethic. I am confident that they will continue the fine tradition of publishing a highly ranked scientific journal.

As I step forward, I will occasionally glance over my shoulder to see the amazing things that I know will be happening at our university. And, as always, I sincerely hope that we have provided you with a most intriguing and educational read.

Sincerely,

*Jonathan R. Belanich*

Editor in Chief

About the Cover:

The scanning electronic microscope (SEM) images, taken by Kim Lackey using the Electron Microscope Facility in the Science and Engineering Complex building at the UA campus, show the immobilized *Clostridium tyrobutyricum* cells inside the matrix of fibrous-bed bioreactor. The images give the size of bacterial cells and the cotton fiber as well as an idea of cell immobilization. The images show that Clostridial cells can be attached, entrapped, or both on cotton fiber to reach high densities of cells (up to 100 g/L cell dry weight) due to the large surface area and void volume of the highly porous fibrous matrix.

Image credit: Kim Lackey





Questions should be directed to joshua.alabama@gmail.com, and more information and all archived volumes are available at bama.ua.edu/~JOSHUA.

# Table of Contents

# Production of Butyric Acid by Metabolically Engineered *Clostridium tyrobutyricum*

Robert Lind[1], George (Hayden) Gunn[1], Chao Ma[1], Margaret Liu[1]

[1]*Department of Chemical and Biological Engineering, University of Alabama, Tuscaloosa, Alabama 35487 USA*
*Corresponding author: Margaret Liu, (phone) 205 348-0868, (email) mliu@eng.ua.edu*

*Clostridium tyrobutyricum ATCC 25755 is an anaerobic acidogenic microorganism producing butyric acid, acetic acid, hydrogen, and carbon dioxide as its main fermentation products. Butyric acid is an important chemical with wide application in the chemical, food, and pharmaceutical industries. In this study, an effective butyric acid production process was developed using a novel metabolically engineered mutant C. tyrobutyricum ACKKO with the ack (encoding acetate kinase) gene deleted. Both free-cell and immobilized-cell fermentations were developed to produce butyric acid in a 2-L stirred tank bioreactor at pH 6.0, Temp 37 °C, and agitation speed 150 rpm. In free-cell fermentation, the engineered mutant ACKKO showed a slower cell growth rate than wild type (0.16 $h^{-1}$ vs 0.23 $h^{-1}$), but significantly higher butyric acid (38.7 g/L vs 23 g/L) and productivity (0.38 g/L/h vs 0.23 g/L/h) from glucose. The immobilized-cell fermentation of ACKKO boosted butyric acid titer to 45 g/L and productivity to 0.49 g/L/h after one round of cell adaptation, demonstrating the potential to further increase butyric acid production by gradual adaptation. These results suggested that the high-titer and high-productivity butyric acid production could be achieved by the metabolically engineered C. tyrobutyricum ACKKO mutant using immobilized-cell fermentation.*

## Introduction

Butyric acid is an important C4 fatty acid that is widely used in the chemical, food, pharmaceutical, and perfume industries [1,2] and has been produced using different processes. The currently dominant route for butyric acid production is through petroleum-based chemical synthesis, but it has received negative attention because of the public concerns on the environmental pollution caused by the petrochemical industry [3]. In addition, the consumer's preference to bio-based natural ingredients for foods, cosmetics, and pharmaceuticals has made the biochemicals produced from renewable resources become an increasingly attractive alternative. *C. tyrobutyricum* has the capability of yielding a higher titer of butyric acid while reducing the toxic chemical derivatives released from petroleum-based production.

*C. tyrobutyricum* ATCC 25755 is a gram-positive, rod-shaped, spore-forming, and obligate anaerobic bacterium that produces butyric acid as the main short-chain acid product from various carbohydrates [4]. Intense research has been performed to develop new technologies to improve butyric acid production, such as metabolic engineering and fermentation. Metabolic engineering is a powerful tool to construct a high butyrate-producing strain via gene manipulation. One engineered mutant, *C. tyrobutyricum* ACKKO, has been constructed by knocking out the acetate kinase (*ack*) gene in the byproduct (i.e., acetic acid) formation pathway [5]. Fermentation has been widely used in biotechnology industries, and its process development is essential to achieve high-level bioproduction. The major process parameters affecting butyric acid production include fermentation mode, agitation, temperature, pH, nutrient feeding strategy, etc.

The main objective of this study was to achieve a high butyric acid production (i.e., high titer and high productivity) by investigating a metabolically engineered *C. tyrobutyricum* ACKKO. Both free-cell and immobilized-cell fermentations were performed to develop an efficient butyric acid production process by integrating genetic engineering and process development.

## Methods and Materials

*Bacterial Strains and Media*

The purified engineered mutant was maintained on RCM plates in an anaerobic chamber (95% $N_2$, 5% $H_2$). The seed trains of both wild type (control) and engineered *C. tyrobutyricum* ATCC 25755 strains were maintained in liquid culture at 37°C in Reinforced Clostridial Medium (RCM; Difco, Kansa City, MO). All liquid fermentation cultures were grown at 37 °C in a previously described synthetic medium (CGM) [5]. The short-term stock cultures were stored

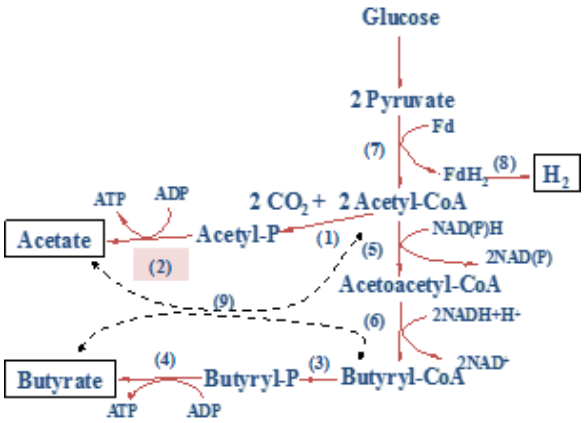



**Figure 1.** Metabolic pathway in *Clostridium tyrobutyricum*. Enzyme involved in acids' production: (1) phosphotransacetylase (*pta*); (2) acetate kinase (*ack*); (3) phosphotransbutyrylase (*ptb*); and (4) butyrate kinase (*buk*).

in serum bottles containing 50-mL CGM at 4 °C and long-term stock cultures were stored in CGM containing 15% glycerol at -80 °C.

*Fermentation Kinetics*

In free-cell fermentation, the activated fresh seed culture of ACKKO mutant was used to inoculate the 2-L CGM medium without antibiotics in a 3-L stirred tank bioreactor (FS-01-A; Major science, Saratoga, CA). The fermentation diagram is presented in Figure 2. The 400 g/L of concentrated glucose was used as carbon source to feed the bioreactor to avoid carbon depletion. Anaerobiosis was reached by sparging the initial medium with nitrogen. All fermentations were carried out at a fed-batch mode in duplicate, agitated at 150 rpm, and controlled at 37 °C and pH 6.0 by adding 5 N NaOH. Samples were taken daily for cell growth and bioproduction analysis.

**Figure 2.** Butyric acid fermentation setup.

In immobilized-cell fermentation, ACKKO cells were immobilized in a fibrous-bed bioreactor (FBB), which was made of a glass column packed with a spiral-wound cotton towel [6,7]. The FBB with a working volume of 750 mL was connected to the stirred tank bioreactor containing 2-L media in circulation. Both the fermentor and the FBB were sparged with nitrogen to achieve anaerobiosis before inoculation.

*Analytical Methods*

Clostridial cell growth was analyzed by measuring the optical density of cell suspension at 600 nm ($OD_{600}$) with a spectrophotometer (Biomate3; Thermo Fisher, Hudson, NH). One unit of $OD_{600}$ corresponded to 0.68 g/L (cell dry weight). High performance liquid chromatography (HPLC, Shimadzu, Atlanta, GA) was used to analyze the organic compounds, including glucose, butyrate, and acetate in fermentation broth. The HPLC system consisted of a refractive index detector (RID-10A), UFLC automatic injector (SIL-20A), a pump (LC-20AT), a column oven at 78 °C (RID-10A), and an analytical column (Rezex RHM, Phenomenex, Torrance, CA). The eluent was distilled water at a flow rate of 0.6 mL/min.

**Results**

The kinetics profiles of both free-cell and immobilized-cell fermentations using the metabolically engineered *C. tyrobutyricum* ACKKO are described in Figure 3. The initial glucose concentration of about 80 g/L was added to basal medium, and concentrated glucose was fed to the fermentation culture as needed (so-called fed-batch).

*Cell Growth*

It was found that the ACKKO cells grew exponentially in the first batch and entered into stationary phase with maximum $OD_{600}$ of about 10.5 in both fermentations. The cell growth rate in immobilized-cell fermentation (0.12 $h^{-1}$) was slightly lower than that in free-cell fermentation (0.16 $h^{-1}$) because most cells were immobilized inside the matrix of the FBB. As previously published, fermentation using wild type *C. tyrobutyricum* had higher cell growth of 0.23 $h^{-1}$ [6,7],

*Free-cell Fermentation*

In free-cell fermentation by ACKKO mutant, butyric acid production started after inoculation without obvious delay and continued to increase until fermentation completely stopped at a maximum concentration of 38.7 g/L due to product inhibition. It was

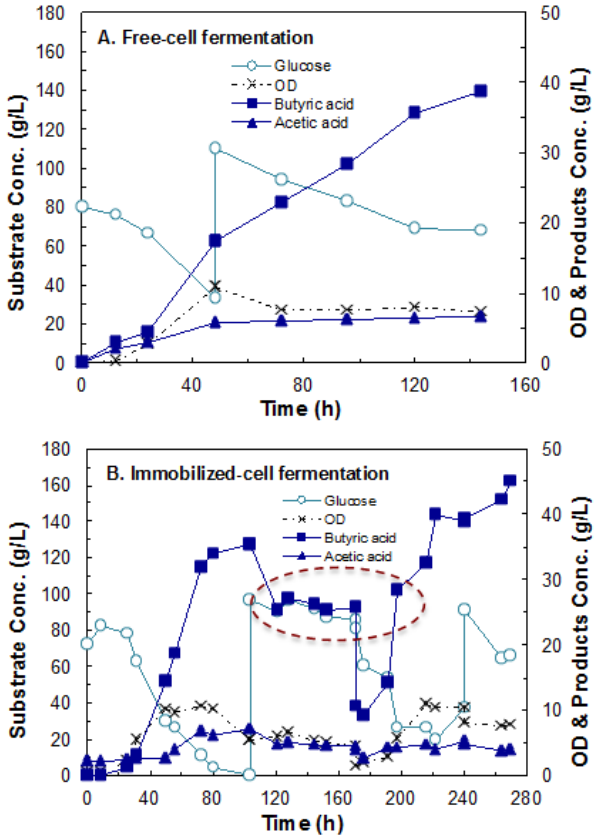



**Figure 3.** Fermentation kinetics of metabolically engineered *C. tyrobutyricum* ACKKO in free-cell fer-

reported that the wild type produced 23 g/L of butyric acid under the same fermentation condition. The productivity of butyric acid was 0.38 g/L/h, which was higher than that of wild type (0.23 g/L/h) [6]. The final concentration of byproduct, acetic acid, was about 6.7 g/L. The ratio of butyric acid/acetic acid (C4/C2) was 5.8 by ACKKO and 4.5 by wild type.

*Immobilized-cell Fermentation*

In immobilized-cell fermentation, the production of butyric acid was started at around 20 hrs, and reached 35.2 g/L in the end of the first batch. The butyric acid production dropped to 25.7 g/L caused by the pH shift from 6.0 to 5.5 (indicated by the circle in Figure 3). The fermentation medium was exchanged at 171 hrs, and butyric acid concentration dropped to 9.2 g/L at the beginning of the new fed-batch. The final butyric acid concentration in the second fed-batch reached 45.0 g/L, which was higher than that produced in free-cell fermentation (38 g/L). The butyric acid productivity was increased to 0.49 g/L/h in immobilized-cell fermentation from 0.38 g/L/h in free-cell fermentation. The immobilized-cell fermentation produced a final acetic acid concentration of 3.9 g/L and demonstrated a higher ratio of C4/C2 than that in free-cell fermentation, 11.5 vs 5.8.

**Discussion**

*Effect of Metabolic Engineering*

As compared to wild type, the cell growth rate of the metabolically engineered mutant was decreased by 30%, but butyric acid concentration and productivity were significantly increased by 35% and 65%, respectively. Moreover, the C4/C2 ratio was also increased in ACKKO, indicating higher butyric acid selectivity. The increased butyric acid production and tolerance in ACKKO can be attributed to the higher carbon flux through the butyrate formation pathway caused by the down regulation of the acetate pathway. The enzymes in the butyrate formation pathway have stronger tolerance than those in the acetate formation pathway [7], so the ACKKO cells showed higher butyrate tolerance. As described in Figure 1, higher butyrate production accumulated less ATP, which caused the slower cell growth (an ATP consuming process) in the ACKKO fermentation. The results obtained in this study demonstrated that metabolic engineering (i.e., knockout of *ack* gene) reduced cell growth rate, redistributed carbon flux, increased end-product tolerance, and improved butyric acid production.

*Effect of Cell Adaptation*

The immobilized-cell fermentation by the ACKKO mutant produced higher concentration of butyric acid, lower concentration of acetic acid, and thereby higher selectivity of butyric acid than the free-cell fermentation. The previously developed FBB has been used for cell immobilization and production of various biofuels, chemicals, and other industrial products, such as acetic, propionic, and butyric acids [7,8,9]. The highly porous fibrous matrix provides large surface area and void volume to allow high densities of cells immobilized. As described above, high butyrate concentration (45 g/L) and productivity (0.49 g/L/h) after one fed-batch of cell adaptation was achieved in immobilized-cell fermentation due to all of these advantages. The higher butyric acid concentration could significantly reduce the product recovery

and purification cost, which usually accounts for >50% of the total production cost. Moreover, the FBB can facilitate cell adaptation to acquire a high tolerance to toxic fermentation substrates and metabolites.

**Conclusion**

In this study, a novel engineered *C. tyrobutyricum* mutant ACKKO was applied in fermentation to achieve high production of butyric acid. The knockout of acetic acid formation pathway in ACKKO reduced cell growth rate but increased the concentration and productivity of butyric acid. The ratio of butyric acid/acetic acid (C4/C2) was decreased due to the carbon rebalance by gene manipulation. The cell adaptation in immobilized-cell fermentation using fibrous-bed bioreactor, in comparison to free-cell fermentation, improved the cell growth of metabolically engineered mutant in addition to improving butyric acid production. This study demonstrated that the integration of metabolic engineering and environmental adaptation via cell immobilization is a powerful tool to reach high butyric acid production using *C. tyrobutyricum*.

**References**

[1]. Zhang C, Yang H, Yang F & Ma Y. (2009). Current progress on butyric acid production by fermentation. Curr. Microbiol. 59: 656–663.
[2]. Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ & Brummer RJ. (2008). Review article: the role of butyrate on colonic function Aliment. Pharmacol. Ther. 27: 104–119.
[3]. Huang J, Cai J, Wang J, Zhu X, Huang L, Yang ST & Xua Z. (2011). Efficient production of butyric acid from Jerusalem artichoke by immobilized *Clostridium tyrobutyricum* in a fibrous-bed bioreactor. Bioresour. Tech. 102: 3923–3926.
[4]. Liu X & Yang ST. (2006). Kinetics of butyric acid fermentation of glucose and xylose by *Clostridium tyrobutyricum* wild type and mutant. Process. Biochem. 41: 801-808.
[5]. Liu X, Zhu Y & Yang ST. (2006). Construction and Characterization of *ack* Deleted Mutant of *Clostridium tyrobutyricum* for Enhanced Butyric Acid and Hydrogen Production. Biotechnol. Prog. 22: 1265-1275.
[6]. Huang YL, Mann K, Novak JM & Yang ST. (1998). Acetic acid production from fructose by *Clostridium formicoaceticum* immobilized in a fibrous-bed bioreactor. Biotechnol. Prog. 14: 800-806.
[7]. Zhu Y & Yang ST. (2003). Adaptation of *Clostridium tyrobutyricum* for enhanced tolerance to butyric acid in a fibrous-bed bioreactor. Biotechnol. Prog. 19: 365-372.
[8]. Lewis VP & Yang ST. (1992). Continuous propionic acid fermentation by immobilized *Propionibacterium acidipropionici* in a novel packed-bed bioreactor. Biotechnol. Bioeng. 40: 465-74.
[9]. Liang ZX, Li L, Li S, Cai YH, Yang ST & Wang JF. (2012). Enhanced propionic acid production from Jerusalem artichoke hydrolysate by immobilized *Propionibacterium acidipropionici* in a fibrous-bed bioreactor. Bioprocess. Biosyst. Eng. 35: 915-21.

**Acknowledgements**

This work was supported by the grant from the Department of Chemical and Biological Engineering, The University of Alabama and funding by the National Science Foundation (BRIGE 24512).

**About the Author**

Originally from Macon, Georgia, Robert Lind is a junior in the Department of Chemical and Biological Engineering at the University of Alabama and a member of American Institute of Chemical Engineers (AIChE). He has been researching in Dr. X. Margaret Liu's lab in the Department of Chemical and Biological Engineering for one and half year now, and in March 2014 he presented his research at the AIChE Southeastern Regional Conference in San Juan, Puerto Rico.

# High-Level Expression of Targeted Anti-Cancer Biopharmaceuticals Using CHO Cell

Hannah Bowers[1], Kimberly Triplett[1], Garrett Lim[1], Ningning Xu[1], Chao Ma[1], Margaret Liu[1]

[1]*Department of Chemical and Biological Engineering, The University of Alabama, Tuscaloosa, AL, 35487*

*Chinese Hamster Ovary (CHO) cell, the most popular mammalian cell used in the biopharmaceutical industry, was used to develop a production cell line to express the targeted anti-cancer therapeutic protein. Lonza GS vector system was applied to construct the over-expression plasmid of monoclonal antibody IgG1 for high-producing CHO K1/IgG cell line construction. The methionine sulfoximine (MSX) amplification and glutamine-free selection were performed to select and amplify IgG production. Multiple single clones were obtained using limiting dilution cloning, producing about 350-800 mg/L of IgG. The glycosylation of produced protein will be analyzed to define the lead clone for the process development of anti-cancer biopharmaceutical production.*

## Introduction

About one of every four deaths in the United States can be attributed to cancer, second only to heart disease in total deaths [1]. It is projected that approximately 600,000 Americans will die of cancer in 2014. Globally, over 13 million new cancer cases are diagnosed per year. Common cancer treatment strategies currently include radiation, chemotherapy, and surgery, but these methods can be potentially harmful to healthy cells. Scientists and clinicians have been dedicated to providing the highest life quality for patients diagnosed with cancer by improving cancer prevention, diagnosis, and survivorship. The development of innovative biopharmaceuticals and the improvement of protein quality and clinical efficiency of various therapeutic proteins represent an efficient course in cancer therapy.

An anti-cancer biopharmaceutical is defined by the US Food and Drug Administration (FDA) as "a protein derived from living cells or tissues" used to treat or cure cancer. As a biopharmaceutical, monoclonal antibody (mAb) can specifically bind the antigen or receptor of the target cancer tissue tightly, interact with the immune system, and treat tumor cells through antibody-dependent cell-mediated cytotoxicity [3]. Chinese hamster ovary (CHO) cells have been widely utilized to produce anti-cancer therapeutic proteins, including mAbs and recombinant proteins. CHO cells can host a wild range of heterogeneous genes and regulate post-translational modifications (e.g. glycosylation, sialylation, and fucosylation) to improve the bioactivity, extend shelf life, and reduce the immunological response of the expressed therapeutic protein. It is important to develop an efficient bioprocess (i.e., high protein production, high product quality, and a short timeline) to meet the requirement of high dosage of therapeutic proteins and the growing cancer patient population.

Several CHO host cells have been applied widely in biopharmaceutical production, such as CHO K1, CHO DG44, CHO DXB-11 and CHO S. The parental CHO K1 is derived from the first ancestral CHO cell but contains less genome DNA. CHO DG44 is a metabolically engineered host cell derived from CHO-pro3-strain, with deleted dihydrofolate reductase gene (*dhfr*). The CHO DG44 cell line can be metabolically selected with proper heterogonous gene expression but its cell growth rate is slower than CHO K1. CHO DXB-11 has a *dhfr* deleted allele and a missense mutated allele. CHO S is originally derived from the ancestral CHO cell via cell adaptation, showing the fastest cell growth as compared to CHO K1 and CHO DG44.

The development of a high-yield protein-producing cell line is the first key step in therapeutic protein production, which relies on the efficiency of expression vectors. The optimal expression vectors can achieve high protein productivity and stable protein expression and can shorten the timeline of production cell line development. For example, the optimal expression vectors typically contain a stable gene expression cassette with various key elements including mRNA transcription promoter/enhancer, translation enhancing sequences, and a selection marker gene (e.g., *dhfr* or GS mini gene). The application of chemicals to selection marker gene, such as methotrexate (MTX) or methionine sulfoximine (MSX), can select and amplify gene copy number and thus significantly increase the expression level of the target gene. The protein expression level can also be greatly improved by increasing the transcription and translation efficiencies of relevant genes using novel elements (e.g. Selexic Genetic Elements) in expression vector [4]. Also, the ubiquitous chromatin opening element (UCOE) can open up chromatin to generate stable

expression irrespective of chromosomal integration site. Other vector elements, including special promoter, enhancer element, intron, chromatin modifier and insulator, have also been used to develop cell line platforms with improved protein expression level.

The overall objective of this study is to develop an efficient platform to produce therapeutic proteins with high quality and better productivity. We used the CHO K1 host cell to produce recombinant monoclonal antibody IgG. The DNA sequence was optimized and inserted into Lonza GS vector to achieve high protein expression in the CHO K1 cells. The L-glutamine selection and MSX amplification were applied to amplify IgG production. Finally, the IgG production was evaluated in order to understand the clone variation.

**Materials and Methods**

*Plasmid Construction*

Lonza Biologics' Glutamine Synthetase (GS) expression system was used as a cloning vector to construct the single expression vector pEE-IgG containing both heavy chain (HC) and light chain (LC). The expression of HC and LC were controlled by separate hCMV-MIE promoters. The mini GS gene was particularly used as a dominant selectable marker when the transfected CHO K1/IgG grew in L-glutamine-free medium, and the untransfected CHO K1 cells were killed by MSX selection reagent. The expression level of IgG was subsequently amplified using elevated levels of MSX (up to 25 μM).

The constructed plasmid DNA was amplified in *E. coli* DH5α and purified using the OMEGA Endo -free Plasmid DNA Maxi Kit (OMEGA BioTek, Norcross, GA). The concentration of plasmid DNA was determined by measuring the $OD_{260}$ and the purity was determined by the $OD_{260}/OD_{280}$ ratio. The plasmid DNA was concentrated, sterilized, and sequenced before transfection.

*Cell Culture*

The adherent CHO K1 parental cells were obtained from Fisher Scientific (Fisher, Waltham, MA). After adaptation into suspended and serum-free culture, the CHO K1 cells were cultured in CDM4 medium (Fisher, Waltham, MA). The cells were cultivated in 125-mL shaker flask in incubator at 37 ℃, 5% $CO_2$, and 110 rpm. The CHO K1 cells were regularly passaged twice a week to maintain healthy seed train. The viable and total cell counts were determined by hemocytometer using trypan blue.

*Cell Transfection*

Healthy CHO K1 cells with viability >95% were transected by linearized plasmid pEE-IgG facilitated with FreeStyle MAX reagent. The transfection was performed following the standard transfection procedure developed by manufacture (Life Technologies, Inc.). The viable cell density pre-transfection was optimized and $1.5x10^7$ c/mL was used in the pEE-IgG transfection. The transfected CHO K1 cell cultures were incubated in $CO_2$ incubator immediately.

*Selection and Amplification*

The L-glutamine free CDM4 medium supplemented with 1-25 μM of MSX was applied to select the successfully transfected CHO K1. Genomic amplification was achieved by adding MSX, a folic acid antagonist actively transported into cells by the folate transporter. MSX stock solution was prepared at 1 mM in NaOH solution. The selected or amplified cells were passaged into fresh selection or amplification medium at seeding density of $0.5x10^6$ c/mL twice a week. Once the viability reached >90%, the recovered cell pool was frozen and stored in liquid nitrogen tank.

*Single Cell Cloning and Evaluation*

Limiting dilution cloning was performed in 96-well plates to pick single clones. Low seeding density of 0.3 c/well was used to assure a single clone in each well. The top clone was defined using enzyme-linked immunosorbent assay (ELISA) following the procedure described in the literature [5]. In IgG productivity analysis, glucose was analyzed daily using YSI Select Biochemistry Analyzer (YSI Incorporated, Yellow Springs, Ohio), and fed to the shaker flask culture as needed to maintain glucose concentration between 2-6 g/L. The production of top clones was analyzed using High Performance Liquid Chromatography (HPLC, Agilent, Santa Clara, CA) following the procedure provided by the manufacturer.

**Results and Discussion**

*Plasmid Construction*

As shown in Figure 1, the plasmid PEE-lgG encoding an IgG was successfully constructed using the Glutamine Synthetase (GS) gene expression system. Both heavy chain (HC) gene and light chain (LC) genes were cloned into the polylinker downstream of two hCMV-MIE promoters. The hCMV-MIE promoter was derived from human cytomegalovirus stain AD169 and used to enhance the mRNA levels of the genes of interest. The GS cDNA expressed glutamine synthetase that enabled the metabolite selection of the successfully transfected IgG-producing cell line by

removing L-glutamine from cell culture medium. The constructed single plasmid pEE-IG contained both HC gene and LC gene, which enabled one-step transfection and simplifies cell pool selection.

The pEE-IgG plasmid (total size of 11,495 bp) was first confirmed with gel electrophoresis after double digestion with restriction enzymes *not*I and *Sal*I. The expected restriction fragment sizes (i.e., 7,542 bp and 3,953 bp) were determined from the restriction map of pEE-IgG plasmid (Figure 1A). As shown in the DNA gel (Figure 2B), two bands with sizes of about 7.5 kb and 4.0 kb were observed, demonstrating the right construction of pEE-IgG plasmid. The isolated pEE-IgG was further sequenced and confirmed that both LC and HC had been cloned into the GS vector properly.

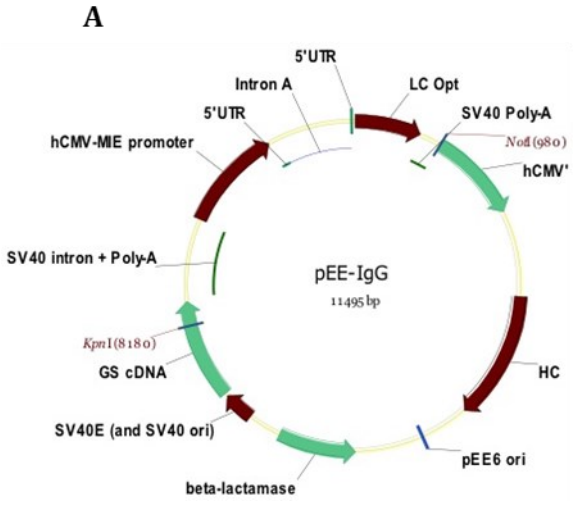
*Generation of Stable CHO K1/IgG Cell Line*

The stable CHO K1-IgG cell line was developed by transfecting CHO K1 cells with the constructed plasmid pEE-IgG, followed by metabolite selection, MSX amplification and single cell cloning. The FreeStyle MAX, a novel cationic lipid-based reagent designed to transfect multiple mammalian cells with high efficiency, was used in the development of transfection cell line. In addition to FreeStle MAX,some transfection reagents, such ascalcium phosphate, DEAE-dextran, and cationic lipid, have also been evaluated. These transaction reagents coat the transfection DNA (negative charged), neutralize or even create positive charge to assist DNA/transfection reagent complex to cross the cell membrane. As shown in Figure 2, the transfected CHO K1-IgG cell pool using FreeStyle Max obtained high transfection efficiency, 30%~50%, which was estimated using control transfected CHO K1/GFP cell pool at 48 hrs post transfection. The transfected CHO K1 were cultured in glutamine-free medium for stable selection, and stepwise amplification was performed using MSX to reach high -level expression of therapeutic protein.

*Production of IgG*

In productivity analysis, the selected top CHO K1 cell line (i.e. homogenous single clone) was cultured in 125-mL shaker flask with a seeding density of $0.3\times10^6$ c/mL. The IgG production of the top 6 clones was presented in Figure 3, showing that up to 780 mg/L of IgG was produced from simple fed-batch (i.e. feeding glucose only). The fed-batch process can be developed to further improve IgG production by 2-5 folds by developing specific feeding nutrients and rational feeding strategy.

It was found that the clone to clone variation was obvious, with IgG titer distribution between 347 mg/L to 780 mg/L. The variation of IgG production among CHO K1/IgG single clones was mainly caused by the random integration of the gene of interest into the genome of CHO K1 host cell during transfection, or the gene amplification by MSX. The non-targeting gene insertion could result in the silence of gene expression or instable gene expression. This issue can be addressed by vector optimization, such as epigenetic regulatory elements to promote the homologous integration and prevent transgene silencing.



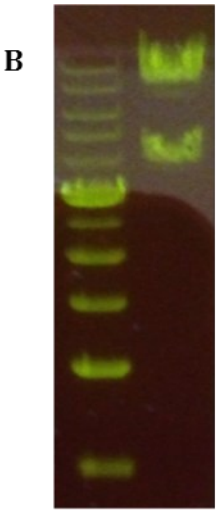




**Figure 1.** pEE-IgG plasmid construction. (A) Plasmid map. hCMV-MIE: human cytomegalovirus major immediate-early enhancer and promoter; SV40 ori: SV40 origin of replication; GS: glutamine synthetase. (B) plasmid analysis by DNA gel electrophoresis. Lane 1: DNA marker; Lane 2: pEE-IgG.

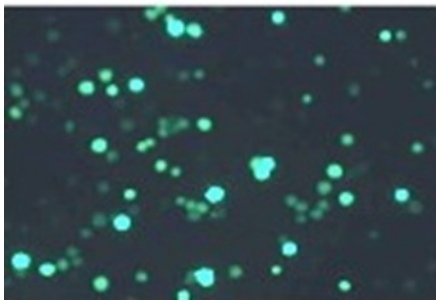


**Figure 2.** Transfection efficiency of CHO K1 cells at 48 hrs post transfection.

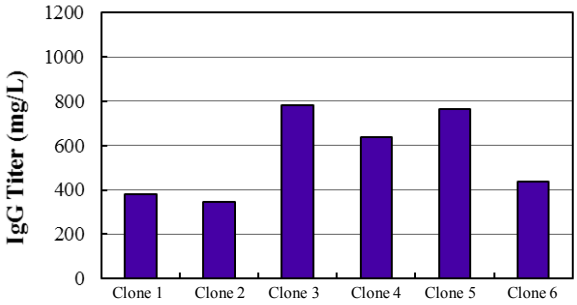



**Figure 3.** IgG Production of top 6 clones in shaker flask batch culture

## Conclusion

High IgG producing cell line could be developed by optimizing the codon of the interested gene, transfection optimization, gene amplification, and production process development. With the continuous increase in size of the market of mammalian-cell based biologics, it is of great interest to rationally develop a more efficient bioprocessing to achieve high product quality and productivity. The anti-cancer monoclonal antibody, IgG1, was overexpressed using the Lonza GS vector containing glutamine-free metabolite section element and strong transcriptional promoter. The stable CHOK1/IgG production cell line was construed and IgG expression was improved significantly by gene amplification. Both protein productivity and quality were used as the criteria in clone selection. In future, the advanced CHOnomics technologies will be used to develop a better understanding of cell metabolism and physiology in order to create an engineered host cell and further improve the bioprocessing efficiency of anti-cancer biopharmaceuticals.

## References

[1] Center M, Siegel R & Jemal A. (2008). Global Cancer Facts and Figures. American Cancer Society. 2: 1-2.

[2] Leone-Bay A. (2011). Next-Generation Protein Therapeutics Summit conference report. Therapeutic delivery. 2(10): 1233-1234.

[3] Leader B, Baca QJ & Golan DE. (2008). Protein therapeutics: a summary and pharmacological classification. Nature reviews. 7(1): 21-39.

[4] Omasa T, Onitsuka M & Kim WD. (2010). Cell engineering and cultivation of chinese hamster ovary (CHO) cells. Curr. Pharm. Biotechnol. 11(3): 233-240.

[5] Lequin R. (2005). Enzyme immunoassay (EIA)/ enzyme-linked immunosorbent assay (ELISA). Clin. Chem. 51(12): 2415-2418.

## Acknowledgements

This work was supported by the Research Grant Committee (RGC), the grant from the Department of Chemical and Biological Engineering, The University of Alabama, and funding by the National Science Foundation (BRIGE 24512).

## About the Author

Originally from Memphis, Tennessee, Hannah Bowers is currently a junior majoring in Chemical and Biological Engineering at the University of Alabama. She has been researching in Dr. X. Margaret Liu's lab in the Department of Chemical and Biological Engineering for one and a half years. In addition to Hannah's passion for research, she enjoys volunteering for an uninsured/low-income medical clinic in Memphis and hopes to attend medical school after graduation in 2015

Kimberly Triplett, a native of Florida, is a junior at the University of Alabama majoring in chemical engineering and minoring in vocal performance. She has worked in Dr. Xiaoguang Margaret Liu's biopharmaceuticals lab since January 2013. Outside of research, she serves as President of Alpha Epsilon Delta Pre-Health Honor Society and Executive Director for the STEM Alabama Education Initiative. Her honors include membership in the Anderson Society, Blue Key Honor Society, Mortar Board, and the 26th Order of The XXXI. She would like to thank Dr. Liu, Ningning Xu, and Chao Ma for their endless support, patience, and willingness to teach over the past three semesters.

# Magnetic Particles with a Polycaprolactone Coating and Preparation of Magnetic Micelles for Drug Delivery

Abigail L. Paulson[1], M. Adam Beg[1], Amanda L. Glover[1], Todd P. Davis[1], Jacqueline A. Nikles, Ph.D.[2], David E. Nikles, Ph.D.[1]

*[1]Department of Chemistry, University of Alabama, [2]Department of Chemistry, University of Alabama at Birmingham*

*Single crystal magnetite nanoparticles of varying sizes were synthesized by the thermal decomposition of iron oleate in high boiling organic solvents (benzyl ether or 1-octadecene). 3-aminopropyltrimethoxysilane was bound to the surface of the magnetite nanoparticles to give a surface of primary amine groups. A tin-catalyzed ring -opening polymerization of ε-caprolactone was initiated from the surface bound amines to give particles coated with covalently bound polycaprolactone. FT-IR and X-ray photoelectron spectra confirmed the presence of the polymer on the particle surface. The coated particles were incorporated into magnetic micelles made from poly (ethylene glycol-b-caprolactone) diblock copolymers. The particles were trapped in the semi-crystalline core of the micelles.*

## Introduction

Cancer is the second leading cause of death in the United States and the first in people ages 40-79. In 2009, 23% of deaths recorded, a total volume of 567,628 deaths, were caused by cancer. In 2013, a total of 1,660,290 new cancer cases and 580,350 cancer deaths are projected to have occurred [4]. Cancer is most commonly treated with surgery, chemotherapy, and radiation. Physicians consult patients on a case-by-case basis to determine the best course of treatment. These therapies, while usually effective, are harmful to the body. Both chemotherapy and radiation harm normal somatic cells and can cause damage to critical tissues. When the two are used in conjunction, the danger is even more apparent [3].

In order to prevent this damage, specific, targeting chemotherapeutic drugs are necessary. The objective of this project is to create a nanoscale, magnetically triggered vector for targeted cancer drug delivery. Synthesizing magnetic nanoparticles and modifying the surface chemistry of these particles allows the polymerization of the particle surface. This increases the ability to crystallize these particles with a chemotherapeutic agent inside a polymer micelle. Theoretically, the application of a magnetic pulse would allow for release of the drug at specific sites in the body.

The large-scale synthesis of monodisperse nanoparticles is possible with varying degrees of size control. The nanocrystals were synthesized through a thermal decomposition of a metal-oleate complex. Size can be influenced by solvent choice as well as reaction time [2]. The surfaces of these particles have been modified to adsorb to the surface of polyethylene glycol, as well as similar copolymers, to change the way the particles are treated within the body [1]. Polyethylene glycol (PEG) has been shown to delay and prevent opsonization and subsequent removal of nanoparticles and nanodevices by the body's immune response [5]. It was predicted that these properties could be used to create a magnetically loaded micelle for use in cancer drug delivery.

## Methods and Materials

*Iron Oleate Synthesis [2]*

The preparation of the metal-oleate complex was completed by reacting iron(III) chloride with sodium oleate. Iron(III) chloride (10.8 g) and sodium oleate (36.5 g) were added to a round-bottom flask and dissolved in a mixture of ethanol (80 mL), hexane (140 mL), and distilled water (60 mL). The solution was heated in an oil bath at 70 ˚C for a period of 4 hours. After being cooled to room temperature, the organic layer containing the iron-oleate complex was

washed three times with distilled water in a separatory funnel. The hexane was evaporated off, and the complex was allowed to dry in a crystallization disk.

*Magnetite Nanoparticle Synthesis [2]*

Nanoparticles were synthesized from the thermal decomposition of the iron-oleate complex in solution with oleic acid. The iron-oleate complex (27 g) was added into a three-neck round-bottom flask with oleic acid (5.4 g) and dissolved in octadecene or benzyl ether (200 g). The reaction was magnetically stirred and heated under nitrogen. The reaction was kept at reflux (320 ˚C) for a period of 30 minutes. The reaction was allowed to cool and was then precipitated with ethanol and centrifugation to give a viscous black solution.

*3-Aminopropyltrimethoxysilane Attachment*

An exchange of 3-aminopropyltrimethoxysilane (APTMS) for oleic acid on the surface of the particles occurred at room temperature in solution. The magnetite solution (20 g) was dispersed in toluene (100 mL). APTMS (20 mL) was added, and the solution was magnetically stirred under nitrogen in a round-bottom flask. After 72 hours, the solution was precipitated with acetone and centrifugation, then reconstituted in toluene and precipitated with acetone three more times.

*Polycaprolactone Surface Polymerization*

APTMS-coated nanoparticles (1 g) were added to a three-neck round bottom flask with oleylamine (36 µL), mesistylene (8 mL), distilled caprolactone (8 mL), and 6 drops of a $Sn(Oct)_2$ catalyst. The flask was sonicated for 15 minutes, then a stirbar was added, and the reaction mixture was heated to 110 ˚C while stirring under nitrogen. After 36 hours, the heat source was removed, and the reaction was allowed to cool. Dichloromethane was added to the black solution to reduce the viscosity. The solution was precipitated with ethanol and centrifugation.

*Creation of Magnetic Micelles*

Magnetic micelles were prepared by adding a solution of the diblock copolymer polyethylene glycol -b-caprolactone (10.0 mg) and iron oxide nanoparticles (1.0 mg) in tetrahydrofuran, THF, (filtered through 0.2 µm syringe filter) to ultrapure water dropwise with probe sonication. The THF was then allowed to evaporate overnight, and the resulting solution was filtered through a 0.45 µm syringe filter and diluted to a known final volume of 10 mL with ultrapure water.

**Results**

The iron-oleate complex produced between 26 and 28 g of product. The magnetite reaction was scaled down appropriately. Two different solvents were used to produce particles of differing sizes. The reactions each produced 500 mL of particles in solution. Transmission electron microscopy (TEM) was used to image these particles, showing mean sizes of around 24 nm and 16 nm (Fig. 1). The APTMS reaction gave a yield of 1.5 g of black particles; IR spectra comparison between the original magnetite and the APTMS nanoparticles confirmed the replacement (Fig. 2). Polymerization was quantified by atomic absorption showing 4% iron content. The presence of the polymer was also confirmed by IR spectroscopy (Fig. 2). The magnetite was loaded into the micelles, and dialysis calculations gave an average of 30% loading. TEM imaged the magnetite within the micelles (Fig. 3).

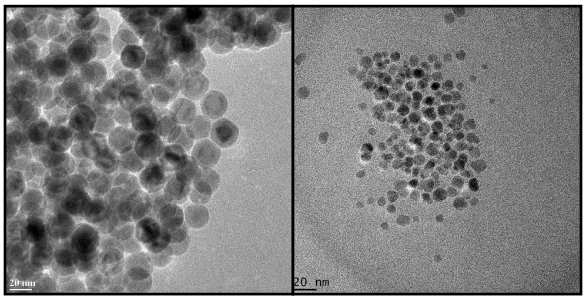



**Figure 1**. TEM of the magnetite particles from both

**Discussion**

The reaction to create magnetite is both reproducible and adjustable. Size control is achieved with the use of different solvents and heat times. The reduction of the aliphatic carbon-hydrogen bonding in the IR spectra as well as the disappearance of the oxygen indicates that the APTMS did replace oleic acid at the particle surface. Polycaprolactone can be polymerized from the particle surface, and these particles can

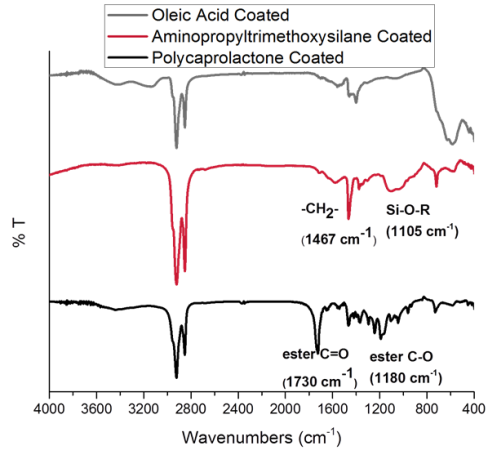



**Figure 2.** Infrared spectra of the magnetite particles with three different surfactants.

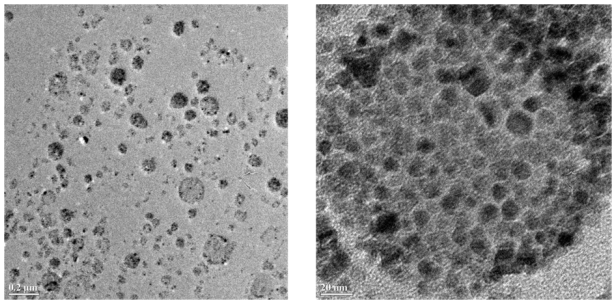


**Figure 3.** TEM of the magnetic micelles show more than one micelle in a variety of sizes and a close up of one micelle showing multiple nanoparticles within its core.

be readily dispersed into copolymer micelles. Current experiments demonstrating drug loading and magnetic heating will show the viability of this concept for cancer treatment. A successful cancer treatment system such as this would have high drug loading with little to no drug release until induced by magnetic heating. In the pursuit of this goal, many variables must be adjusted to provide the ideal delivery vector.

A targeted cancer drug delivery system could have multiple uses in cancer treatment. In addition to a targeted treatment for tumors, the magnetic properties of such a system could be used to detect malignant cancers through magnetic resonance imaging (MRI). With the metastasis of cancerous tumors currently leading to a steep decline in survival chances for patients, a way to clearly detect the spread of tumors should be very beneficial to cancer treatment. In addition, this type of drug delivery would reduce the sickness associated with chemotherapy and the risks associated with surgery. Though cancer treatment research is far from the finish line, the progress is promising.

## References

[1] Owens III, D. E. and Peppas, N. A. 2006. Opsonization, biodistribution, and pharmacokinetics of polymericnanoparticles. International Journal of Pharmaceutics **307**: 93–102.

[2] Park, J., An, K., Hwang, Y., Park, J.G., Noh, H.J., Kim, J.Y., Park, J.H., Hwang, N.M. and Hyeon, T. 2004. Ultra-large scale syntheses of monodisperse nanocrystals. Nature Materials **3:** 891-895.

[3] Phillips, T. L. and Fu, K. K. 1976. Quantification of combined radiation therapy and chemotherapy effects on critical normal tissues. Cancer **37**: 1186–1200.

[4] Siegel, R., Naishadham, D. and Jemal, A. 2013. Cancer statistics, 2013. CA: A Cancer Journal for Clinicians **63**: 11–30.

[5] Soppimath, K. S., Aminabhavi, T. M., Kulkarni, A. R. and Rudzinski, W. E. 2001. Biodegradable polymeric nanoparticles as drug delivery devices. Journal of Controlled Release **70**: 1-20.

## Acknowledgments

The author would like to thank the University of Alabama and the generosity of Dr. Nikles for providing these research opportunities.

## About the Author

Abigail Paulson is a sophomore at the University of Alabama. She is originally from Raleigh, North Carolina and is currently pursuing a degree in Chemical and Biological Engineering. In addition to her work in the Nikles Lab at the University, she will be returning to the National Institutes of Health for the summer to continue a project on growth and development. Abigail is also a member of the University Fellows Experience.

# Signal-Modulated Alternative Splicing in Neurons: Implications for the Formation and Maintenance of Synaptic Networks

Dylan Marchione

*Although the phenomenon of alternative splicing was discovered nearly four decades ago, the mechanisms that control it are poorly understood. A growing body of evidence suggests that, rather than being a stochastic process, alternative splicing is tightly regulated. The topic of signal-modulated alternative splicing is an exciting new area of research with tremendous fundamental implications. If the process is, as it appears, ubiquitous throughout nature, it represents a grossly underappreciated mechanism for cellular adaptation in response to a changing environment. Here, the topic of signal-modulated alternative splicing is presented and a fascinating example involving the $Ca^{2+}$/CaM-mediated alternative splicing of neurexin-1 (Nrxn1) is described.*

**Pre-mRNA Processing: 5' Capping, Polyadenylation, and Splicing**

After a gene is transcribed, the RNA transcript is subject to a series of modifications collectively referred to as pre-mRNA processing. The first modification to occur is 5' end capping, in which a methylated guanine nucleotide is attached to the 5' end of the pre-mRNA via a triphosphate linkage [1]. The primary function of this "5' cap" is to allow for proper delivery of the mRNA molecule to the ribosome via interaction with the eukaryotic translation initiation factor 4E (eIF4E) [2]. At the 3' end of the pre-mRNA, a mechanistically distinct yet functionally related process occurs. Following transcription, the 3' end of the pre-mRNA is cleaved, and a chain of adenosine monophosphates is added [1]. This polyadenylation stabilizes the mRNA molecule and helps to promote translation initiation [2]. Whereas these two mechanisms are generally well understood, the third process, splicing, is somewhat enigmatic.

Most simply, splicing can be defined as the process by which introns are excised from the pre-mRNA transcript [3]. The process is carried out by a multisubunit complex called the *spliceosome* [4]. The core components of the spliceosome are the small nuclear ribonucleoproteins (snRNPs) U1, U2, U4, U5, and U6, although a number of accessory proteins are also required for splicing to occur. Spliceosome assembly begins when the U1 snRNP binds to a 5' exon/intron junction on the pre-mRNA, referred to as the 5' splice site. At the opposite end of the same intron is the 3' splice site. The 3' splice site region is defined by three distinct sequence elements: the branch point, the polypyrimidine tract, and the terminal AG. The branch point is bound by SF1, the polypyrimidine tract is bound by U2AF 65, and the AG dinucleotide is

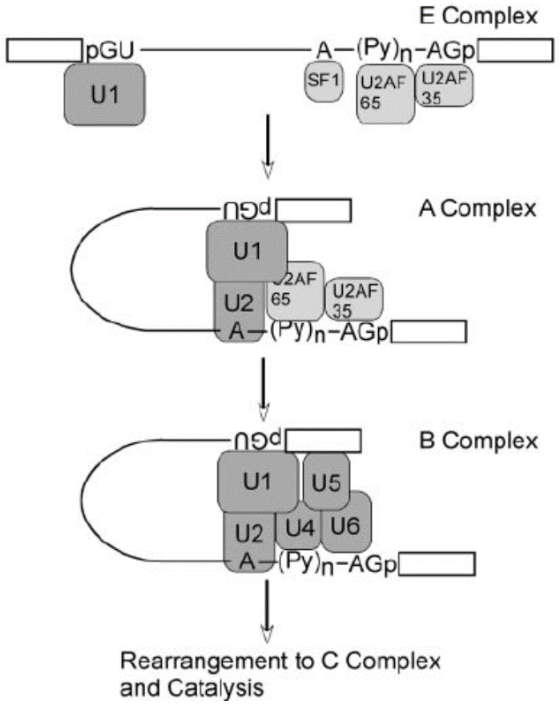



**Figure 1:** Mechanism of spliceosome assembly [Black 2003].

bound by U2AF 35. Collectively, these proteins comprise the E (early) complex [3].

After formation of the E complex, the U2 snRNP binds SF1 at the branch point and simultaneously binds U1, causing folding of the pre-mRNA and forming the A complex. Following this step, the U4/U5/U6 tri-snRNP binds to the A complex, resulting in

formation of the B complex. After B complex formation, a complex rearrangement occurs resulting in the formation of the catalytically active C complex [3]. Once the C complex is formed, two transesterification reactions occur. The first results in the formation of a branched RNA intermediate referred to as the intron lariat. In the second, the intron lariat is excised and the exons are ligated [5].

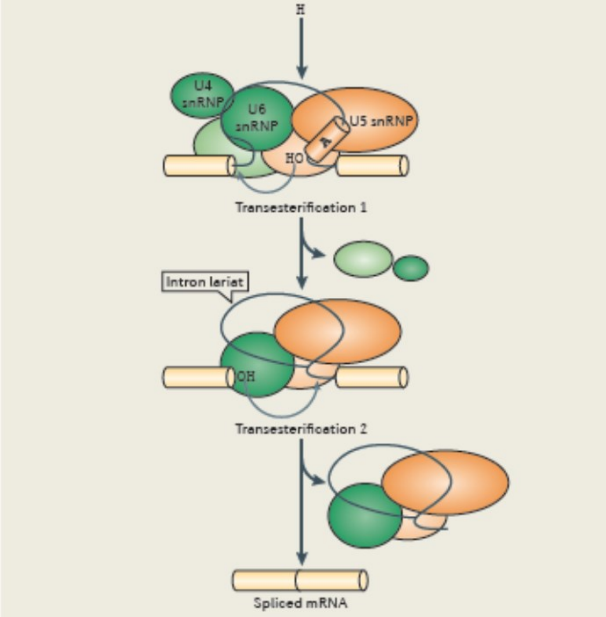



**Figure 2:** The splicing reaction [5].

**Alternative Splicing**

Rather than being a simple, deterministic process, splicing is in fact highly variable and entails an appreciable amount of complexity. Any changes in the assembly of the spliceosome can result in different splice site choices, which will in turn result in the production of different mRNA molecules [3]. Such a phenomenon, in which a single pre-mRNA can be processed to produce different mRNA molecules, is referred to as alternative splicing. Evidence suggests that alternative splicing affects a staggering 95% of mammalian genes [5]. Depending on the number of introns within a given gene, alternative splicing can allow for the generation of a wide variety of proteins. The canonical example of this is the *Drosophila melanogaster* Down Syndrome Cell Adhesion Molecule (DSCAM) gene, which has been shown to produce an astonishing 38,016 different variants as a result of alternative splicing [6]. Several modes of alternative

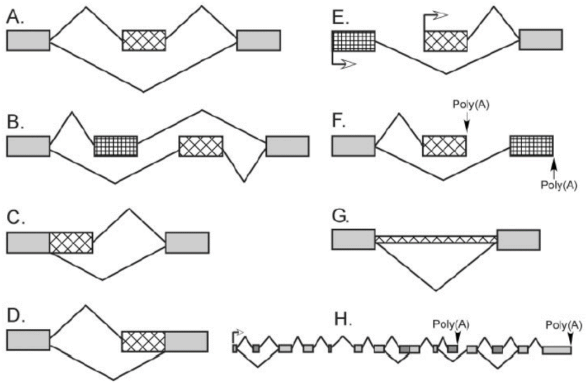



**Figure 3:** Patterns of alternative splicing. (A) Variable inclusion of an exon. (B) Mutually exclusive exons. (C, D) Alternative 5' or 3' splice sites. (E, F) Alternative promoters and or alternative poly(A) sites. (G) Variable inclusion of an intron. (H) Combinatorial alternative splicing. [Black 2003].

splicing are outlined in Figure 3.

**Factors Influencing Alternative Splicing**

In order for splicing to occur, the spliceosome must physically interact with the pre-mRNA. The interaction between components of the spliceosome can be influenced by a number of regulatory proteins. The best studied splicing regulators are members of the Ser/Arg-rich protein family (SR proteins) and heterogeneous nuclear ribonucleoproteins (hnRNPs), which function antagonistically with respect to alternative splicing [7].

SR proteins activate splicing by strongly promoting U1 snRNP binding to exons [8]. It has been shown that SR protein activity is significantly influenced by phosphorylation [9]. Concordantly, SR proteins are known substrates of a number of kinases, including SRPK1 and 2, Clk, Topoisomerase I, and Akt, as well as the phosphatases PP1 and PP2A [9]. While hnRNPs also readily bind pre-mRNA, this interaction actually inhibits assembly of the splicing machinery [10]. Nonetheless, the activity of hnRNPs is similarly controlled by phosphorylation: PKA, Casein Kinase II, and Mnk1/2 have all been shown to phosphorylate members of the hnRNP protein family [9].

The understanding that the activity of both SR proteins and hnRNPs can be influenced by phosphorylation immediately suggests that cells might

possess regulated signal transduction networks to modulate splicing in response to cellular conditions. While this does, in fact, appear to be the case, care must be taken to avoid oversimplifying the nature of such an interaction. Not only are the activities of many splicing-associated proteins influenced by phosphorylation, they have also been shown to be affected by other post-translational modifications including methylation, SUMOylation, and acetylation [9].

Further complicating the issue is the fact that the aforementioned post-translational modifications also influence subcellular localization as well as protein-protein interactions [9]. This picture becomes even more complicated when the effects of additional processes such as relative rates of transcription, core-splicing-machinery protein levels, intron size, and splice site competition are considered [7]. Nonetheless, multiple pathways have been identified that unequivocally implicate the existence of signal-modulated alternative splicing [9].

### Activity Dependent Alternative Splicing of the *Nrxn1* Gene

One intriguing example of signal-induced alternative splicing is the activity-dependent alternative splicing of neurexin-1. Briefly, in a mouse model it has been shown that neuronal stimulation activates calcium/calmodulin-dependent kinase IV(CaMKIV), leading to phosphorylation of the protein Sam68 at Ser20. This phosphorylation influences splicing so as to favor the exclusion of exon 20 from *Nrxn1*. The precise mechanism by which this occurs is not fully understood, but accumulating evidence suggests that it depends upon the interaction between Sam68 and the splicing factor U2AF65. This is very interesting because exon 20 falls within a critical region of neurexin. Referred to as alternatively spliced segment 4 (AS4, occasionally denoted SS4), the region is a 30 amino acid sequence which directly influences the interaction between neurexin and a number of ligands that mediate synaptogenesis, one key group of which are the neuroligins [11].

CaMKIV is a member of the calmodulin kinase (CaMK) signal transduction cascade. An overview for CaMK signaling is included in **Figure 4.** Enriched in nervous tissue, this pathway mediates the cellular response to calcium signaling. The pathway is initiated when an influx of calcium ions activates the messenger protein calmodulin (CaM). The $Ca^{2+}$/CaM complex binds to and activates a number of downstream targets, collectively referred to as the CaMKs. In this pathway, the $Ca^{2+}$/CaM complex both activates the kinase CaMKK and binds to the downstream kinase CaMKIV thereby exposing a critical phosphorylation site. Historically, CaMKIV is primarily regarded as a regulator of transcription, with the characteristic example being CaMKIV-dependent activation of the transcription factor CREB [12]. As such, the finding that CaMKIV influences translation as well distinguishes it as a core regulator of neuronal gene expression.

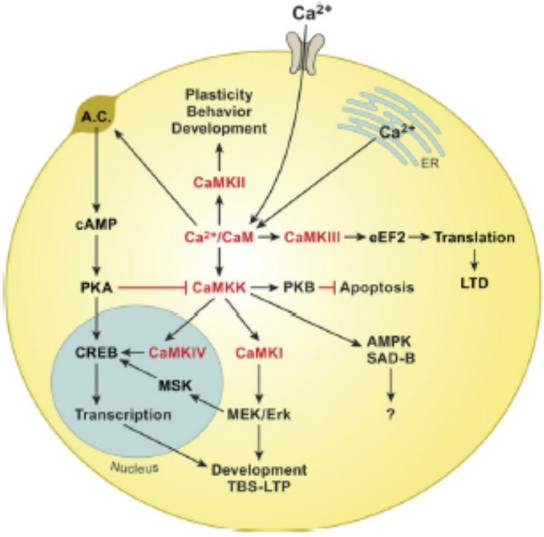



**Figure 4:** The CaMK pathway [12].

Sam68 (Src-associated in mitosis 68 kDa protein) was originally discovered as a substrate of Src that promoted cell-cycle progression [9]. However, the defining characteristic of the protein is not its role in the cell-cycle, but its interaction with RNA. Sam68 contains a 70-amino acid RNA binding domain, called a KH domain, that places it in the STAR (Signal-Transduction and RNA) family [11]. Intriguingly, this domain is also found in hnRNPs. As might be predicted from this structural homology, Sam68 has previously been shown to influence alternative splicing. In the best-defined pathway, in T-cells, activation of the Ras pathway leads to the Sam68-dependent inclusion of a variable exon in the

cell-surface glycoprotein *CD44* [13].

As previously mentioned, the mechanism by which Sam68 influences splicing of *Nrxn1* is not currently known. However, evidence suggests that it relies on the protein U2AF65 [11]. First, Sam68 shares sequence similarity with the branch-point-binding protein SF1 (refer to **Figure 1)**. Reminiscent of Sam68, SF1 has been shown to be phosphorylated on Ser20, and this phosphorylation controls its interaction with U2AF65 [14]. Supporting this hypothesis, it has also been shown that Sam68 interacts with U2AF65 *in vivo* in non-neuronal cells. In the study, it was proposed that Ras-driven phosphorylation causes Sam68 to dissociate from the pre-mRNA, allowing for spliceosome organization and subsequent exon splicing [15]. It is possible that a similar mechanism accounts for the signal-responsive alternative splicing of *Nrxn1*.

Neurexins were first identified in 1992 as a result of a screen for possible receptors for α-latrotoxin, a primary component of black widow venom that was known to cause massive neurotransmitter release [16]. Starting with peptide sequences derived from the purified α-latrotoxin receptor, the authors worked backwards to generate corresponding oligonucleotides and then conducted a cDNA library screen. The screen revealed two sets of overlapping cDNAs encoding neuron-specific cell surface proteins which the authors named neurexin I and II. Early sequence analysis of these proteins revealed homology to the extracellular matrix proteins agrin, laminin, and slit andit was therefore hypothesized that neurexins might play a role in shaping cell-cell interactions [16]. Indeed, subsequent analysis has revealed that neurexins do in fact mediate cell-cell interactions via their transsynaptic interaction with another family of transmembrane proteins called neuroligins [17].

**Neurexins and Neuroligins in Synaptic Function**

Both neurexins (NXs) and neuroligins (NLs) are neuronally-expressed transmembrane proteins. Interest in the function of NXs and NLs was piqued when it was demonstrated that their heterologous expression in non-neuronal cells induced the formation of synapse-like connections with co-cultured neurons [18]. Nearly a decade later, a comprehensive model was proposed that implicated NX-NL interactions as being important for the maturation, activity-dependent validation, and maintenance of synapses [17].

According to the current understanding, early in synaptic development, the NX-NL complex functions to stabilize the transient points of contact between axons and dendrites. After initial stabilization of these nascent synaptic sites, the interaction of NX and NL leads to a rapid accumulation of postsynaptic proteins, resulting in the rapid assembly of a functional postsynaptic complex. Similarly, NX-NL complexes have been shown to promote the assembly and maturation of the presynaptic machinery as well. Significantly, evidence suggests that various aspects of NX-NL interactions are activity-dependent [17].

Interestingly, both neurexins and neuroligins can undergo extensive alternative splicing, allowing for the generation of thousands of possible mRNA transcripts. When presented with both the extent of their splicing capacity and their role in synaptic development, it is difficult to resist suspecting that complex alternative splicing of neurexin and neuroligin might play a role in determining synapse identity and network connectivity during brain development [17].

The nature of the interaction between NX and NL seems to support such a role. A diagram showing NX-NL complex formation is included in **Figure 5.** The proteins interact via their extracellular domains, forming a heterotetramer. Intriguingly, both NXs and NLs undergo alternative splicing at multiple sites localized to the NX-NL interface. As would be

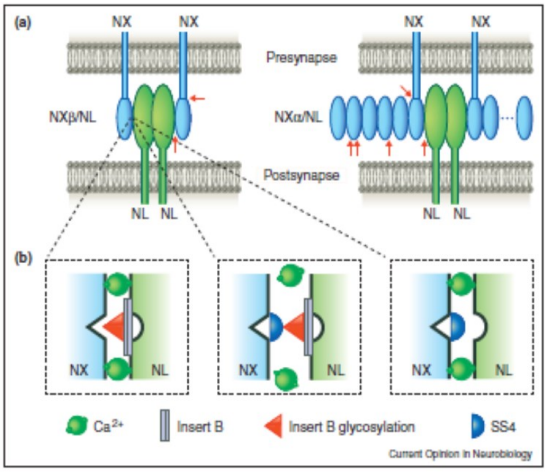



**Figure 5:** Complex formation between neurexin and neuroligin [17].

expected, differential inclusion of these potential splice sites can significantly influence NX-NL binding affinities [17].

**Neurexins and Neuroligins in Disease**

Dysfunction in neurexins and/or neuroligins have been implicated in a number of cognitive diseases, including schizophrenia, autism spectrum disorder (ASD), Tourette's syndrome, and generalized learning disability [19]. These observations are not surprising given the critical role for these proteins in the maintenance of neural circuits. What might not be so intuitively clear is the relative contribution of misregulated splicing to cognitive disease. Prior investigations seeking a correlation between NX/NL dysfunction and cognitive diseases have focused specifically on the role of mutations; however, given the recent findings regarding the apparent physiological relevance of regulated splicing, it would be interesting to determine how antagonism of this process would affect cognition. Such an investigation would provide greater insight into mechanisms governing synaptic network formation and potentially identify the alternative splicing machinery as a target for intervention in neurologic dysfunction.

**Conclusion**

Alternative splicing is a highly complex process that has the potential to substantially augment the protein-coding capacity of a genome without increasing its size. Recent insights into the regulation of alternative splicing have demonstrated that cells have a much greater capacity than previously appreciated to dynamically alter protein expression in response to a changing environment. The poorly studied phenomenon of signal-modulated alternative splicing has been implicated in processes as complex as the formation and maintenance of synapses. This result alone warrants significant additional investigation. However, irrespective of the role of alternative splicing in influencing synaptic integrity, there is still a critical need for a greater investment of resources into studying the mechanisms governing alternative splicing. Cumulatively, the recent evidence suggests that dynamic regulation of alternative splicing is not merely a curiosity but may instead be a massively neglected fundamental biological process.

**References**

[1] Moore MJ and Proudfoot NJ. Pre-mRNA processing reaches back to transcription and ahead to translation. Cell. 2009 Feb 20;136[4]:688-700. doi: 10.1016/j.cell.2009.02.001.
[2] Kapp LD and Lorsch JR. The molecular mechanics of eukaryotic translation. Annu Rev Biochem. 2004;73:657-704.
[3] Black DL. Mechanisms of alternative pre-messenger RNA splicing. Annu Rev Biochem. 2003;72:291-336. Epub 2003 Feb 27.
[4] Brody E and Abelson J. The "spliceosome": yeast pre-messenger RNA associates with a 40S complex in a splicing-dependent reaction. Science. 1985 May 24;228[4702]:963-7.
[5] Kornblihtt AR, Schor IE, Alló M, Dujardin G, Petrillo E, and Muñoz MJ. Alternative splicing: a pivotal step between eukaryotic transcription and translation. Nat Rev Mol Cell Biol. 2013 Mar;14[3]:153-65. doi: 10.1038/nrm3525. Epub 2013 Feb 6.
[6] Schmucker D, Clemens JC, Shu H, Worby CA, Xiao J, Muda M, Dixon JE and Zipursky SL. Drosophila Dscam is an axon guidance receptor exhibiting extraordinary molecular diversity. Cell. 2000 Jun 9;101[6]:671-84
[7] Nilsen TW and Graveley BR. Expansion of the eukaryotic proteome by alternative splicing. Nature. 2010 Jan 28;463[7280]:457-63. doi: 10.1038/nature08909.
[8] Staknis D, and Reed R. SR proteins promote the first specific recognition of Pre-mRNA and are present together with the U1 small nuclear ribonucleoprotein particle in a general splicing enhancer complex. Mol Cell Biol. 1994 Nov;14[11]:7670-82.
[9] Lynch KW. Regulation of alternative splicing by signal transduction pathways. Adv Exp Med Biol. 2007;623:161-74.
[10] Bennett M, Piñol-Roma S, Staknis D, Dreyfuss G and Reed R. Differential binding of heterogeneous nuclear ribonucleoproteins to mRNA precursors prior to spliceosome assembly in vitro. Mol Cell Biol. 1992 Jul;12[7]:3165-75.
[11] Iijima T, Wu K, Witte H, Hanno-Iijima Y, Glatter T, Richard S, and Scheiffele P. SAM68 regulates neuronal activity-dependent alternative splicing of neurexin-1. Cell. 2011 Dec 23;147[7]:1601-14. doi: 10.1016/j.cell.2011.11.028.
[12] Wayman GA, Lee YS, Tokumitsu H, Silva AJ, and Soderling TR. Calmodulin-kinases: modulators of

neuronal development and plasticity. Neuron. 2008 Sep 25;59[6]:914-31. doi: 10.1016/j.neuron.2008.08.021.
[13] Matter N, Herrlich P and König H. Signal-dependent regulation of splicing via phosphorylation of Sam68. Nature. 2002 Dec 12;420[6916]:691-5.
[14] Wang X, Bruderer S, Rafi Z, Xue J, Milburn PJ, Kramer A and Robinson PJ. Phosphorylation of splicing factor SF1 on Ser20 by cGMP-dependent protein kinase regulates spliceosome assembly. EMBO J. 1999; 18:4549–4559.
[15] Tisserant A and König H. Signal-regulated Pre-mRNA occupancy by the general splicing factor U2AF. PLoS One. 2008 Jan 9;3[1]:e1418. doi: 10.1371/journal.pone.0001418.
[16] Ushkaryov YA, Petrenko AG, Geppert M and Südhof TC. Neurexins: synaptic cell surface proteins related to the alpha-latrotoxin receptor and laminin. Science. 1992 Jul 3;257[5066]:50-6.
[17] Krueger DD, Tuffy LP, Papadopoulos T, and Brose N. The role of neurexins and neuroligins in the formation, maturation, and function of vertebrate synapses. Curr Opin Neurobiol. 2012 Jun;22[3]:412-22. doi: 10.1016/j.conb.2012.02.012. Epub 2012 Mar 15.
[18] Scheiffele P, Fan J, Choih J, Fetter R and Serafini T. Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. Cell. 2000 Jun 9;101[6]:657-69.
[19] Südhof TC. Neuroligins and neurexins link synaptic function to cognitive disease. Nature. 2008 Oct 16;455[7215]:903-11. doi: 10.1038/nature07456.

**About the Author**

Dylan Marchione is from Philadelphia, Pennsylvania. He is a senior University Scholar's student in Biological Sciences. He conducted his undergraduate and graduate research with Dr. Stevan Marcus. Next year he is entering a PhD program in Pharmacology at the University of Pennsylvania.

# Microglial Activation in Inflammation-Mediated Neurodegeneration

Anna Moyer

*Sustained microglial activation contributes to uncontrolled inflammation and tissue pathology in many neurodegenerative disorders. Microglia recognize danger signals released by necrotic cells and respond via signal transduction pathways that regulate transcription factors. The subsequent production of pro-inflammatory cytokines, enzymes, and reactive oxygen species results in feed-forward amplification of neuroinflammation. If uncontrolled, this response can lead to collateral tissue damage and enhance the pathogenesis of neurodegenerative disorders. Specifically, the activity of the enzymes iNOS and PHOX creates reactive oxygen and nitrogen species, which have deleterious effects on neuronal survival. Primary phagocytosis of neurons is also potentially responsible for the loss of viable neurons. As a result, drugs that block the progression of neuroinflammation by curtailing microglial activation offer potential therapies for lessening tissue damage, and understanding their mechanisms of action can provide greater insight into microglial activation and signaling. Potential therapeutics include natural bioactive compounds and new derivatives of natural products developed to block specific components of microglial activation.*

## Introduction

Chronic neuroinflammation plays a key role in the neuronal pathology and disease progression of a variety of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis [25]. Although these disorders have diverse etiologies and intricacies, sustained microglial activation contributes to uncontrolled inflammation and tissue pathology in many diseases with a neurodegenerative component [12]. Microglia are the resident mononuclear phagocytes of the nervous system, and as the primary immune cells in the brain, they are responsible for surveying the microenvironment for and responding to components released by damaged or stressed host cells [15]. Upon recognizing danger signals from necrotic cells, microglia switch from a resting state to an activated phenotype and promote inflammation by producing pro-inflammatory cytokines, enzymes, and reactive oxygen species [25]. Although microglial activation is a function of the innate immune system that serves to eliminate pathogens and to aid repair, detrimental side effects may result in collateral damage to the nervous system [12]. Specifically, if feed-forward amplification of inflammation remains unchecked, chronic inflammation may potentiate the neurotoxicity common to neurodegenerative disorders [25]. As a result, developing therapeutics that alter the behavior of microglia after activation is a major target for treating neuroinflammation, but designing these therapies requires identifying specific microglial signaling cascades and enzymes and their roles in the progression of inflammation [43,49].

Microglial activation involves a number of inflammatory mediators and effectors. Upon engagement of "danger" receptors, microglia become more mobile, activate phagocytic NADPH oxidase (PHOX) to create superoxide, and produce pro-inflammatory cytokines that activate more microglia and astrocytes [25]. Downstream transcription factors ultimately regulate hundreds of genes, and proteins such as inducible nitric oxide synthase (iNOS) and cytochrome c oxidase (COX-2) are upregulated [12]. Together, iNOS and PHOX potentiate neuronal death by a "dual key" mechanism of neuronal killing in which the enzyme products nitric oxide and superoxide synergize to create the more damaging peroxynitrite [37]. Microglia can also phagocytose damaged neurons and may scavenge neurons that are stressed yet viable [24]. This combination of reactive species and phagocytosis ultimately perpetuates neuronal mitochondrial damage and cell death, which likely contributes to the pathogenicity of neurodegenerative disorders [25].

## Microglial Sensing of Tissue Injury

Microglia present in the uninjured central nervous system are highly ramified and have motile

processes that survey the environment for damage [7]. In this state, microglia express only low levels of immune receptors, but when receptors engage with endogenous or pathogen-associated molecules, these cells undergo dramatic changes resulting in an amoeboid morphology and activated state [7, 63]. The receptors implicated in microglial activation can respond to a variety of molecules, including lipopolysaccharide (LPS), cytokines, adenosine triphosphate (ATP), glutamate, and protein aggregates [28]. Sensing of infection occurs via pathogen recognition receptors, such as the Toll-like receptor (TLR) family, which respond to components of bacteria and viruses but are also implicated in several chronic inflammatory diseases [25, 28].

Sensing of disturbances in tissues occurs by damage receptors, which sense components released by stressed host cells [12]. There are a variety of molecules that interact with microglial receptors in neurodegenerative disorders. Damaged neurons release extracellular nucleotides, and ATP induces microglial chemotaxis via ionotropic P2X and metabotropic P2Y purinergic receptors [40]. The adenosine A1 receptor is present on microglia in limited amounts under normal conditions but is upregulated significantly in response to ATP [34]. Selective stimulation of this receptor inhibits microglial activation, perhaps through modulation of calcium influx, but its role in neurodegeneration is not well characterized. Toll-like receptor 4 (TLR4) was initially identified as the microglial receptor that responds to LPS from Gram-negative bacteria; however, it is now recognized that TLR4 has numerous endogenous ligands and is implicated in traumatic brain injury, aging, and neurodegenerative diseases [57].

In Alzheimer's disease, the accumulation of β amyloid (Aβ) results in extracellular senile plaques. Microglia sense Aβ through TLR4, which activates phagocytosis of extracellular Aβ [25]. In a mouse model of Alzheimer's disease, the loss of TLR4 results in greater accumulation of Aβ [51]. Similarly, mice with a loss-of-function mutation in TLR4 display decreased microglial activation and aggravated cognitive deficits [47]. These results suggest that at some stages in the progression of Alzheimer's disease, microglial activation may confer neuroprotection by clearing Aβ plaques. Intracellular inclusions of α-synuclein form in the brains of Parkinson's patients, and the creation of oligomeric α-synuclein intermediates is an initial cause of neuronal death [25, 51]. Dying neurons may release components that activate microglia, including ATP and α-synuclein [25]. TLR4 is required for α-synuclein dependent microglial activation in primary murine cells, and treatment of microglia with α-synuclein results in the release of pro-inflammatory cytokines, creation of reactive oxygen species, and phagocytosis of extracellular α-synuclein [22]. In both Parkinson's disease and Alzheimer's disease, the beneficial and injurious effects of microglial activation are difficult to distinguish: Microglial cells not only phagocytose detrimental extracellular proteins via stimulation of TLR4, but also release reactive oxygen species and other components that have the potential to damage neurons [22, 25].

**Transduction and Effectors of Neuroinflammation**

TLR signal transduction is complex and ultimately regulates hundreds of genes. Upon activation, TLRs recruit adaptor proteins such as MyD88 and TRIF, which results in transforming growth factor β activated kinase 1 (TAK1) activation [41, 58]. TAK1 is a central regulator of downstream pathways and activates the transcription factor nuclear factor kappa B (NF-κB) and members of the MAPK family, including ERK, JNK, and p38 [21]. These pathways effect expression of pro-inflammatory cytokines, the enzymes iNOS and COX-2, and a variety of other proteins [12]. This induction of gene expression may take several hours to initiate changes in protein levels, but receptor ligation also rapidly triggers protein kinase C (PKC) to activate PHOX. Together, these responses lead to the creation of reactive oxygen species, activation of other microglia and astrocytes, and phagocytosis of extracellular molecules and cellular debris [25].

**Phagocytic NADPH Oxidase (PHOX)**

The NADPH oxidase system is a multi-unit enzyme that is associated with membranes and is triggered by microglial activation [11]. NADPH oxidases contain six transmembrane domains and binding sites for NADPH and FAD. There are at least six isoforms of the catalytic subunit of NADPH oxidase, and mi-

croglia primarily express phagocytic NADPH oxidase, which contains the catalytic subunit Nox2 [16]. PHOX is composed of $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, rac, $p22^{phox}$, and the catalytic subunit Nox2 (Fig. 1). In addition to expressing PHOX, microglia also express non-phagocytic NADPH oxidase with the catalytic subunit Nox1.

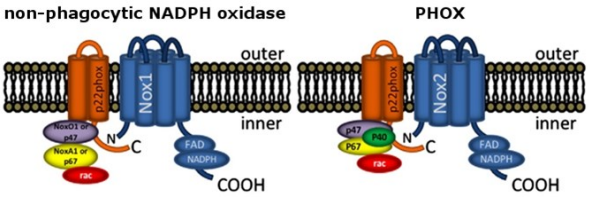



**Figure 1:** Structures of non-phagocytic NADPH oxidase and PHOX. To generate superoxide, NADPH binds to Nox and transfers electrons to heme groups and to oxygen on the outer membrane [11].

Nox2 was the first subunit of NADPH oxidase described, and as a result, the function of PHOX is the most well-defined of the NADPH oxidases [11]. To activate this enzyme, PKC phosphorylates $p47^{phox}$, which exposes a binding site that interacts with $p22^{phox}$. $p67^{phox}$ subsequently associates with $p47^{phox}$ and assembles the functional enzyme by recruiting activated rac. PHOX produces superoxide ($O2^{\cdot}$-) by passing an electron through NADPH to FAD, from FAD to heme groups associated with Nox2, and across the membrane to oxygen. Superoxide is extremely unstable and rapidly dismutates to hydrogen peroxide or reacts with other radicals to form oxidants [12]. Hydrogen peroxide is further reduced to water and the hydroxyl radical (-OH) via the Fenton reaction [50]. These molecules are antimicrobial and can function in redox signaling to promote cell proliferation, angiogenesis, and wound healing [14]. However, activated PHOX can produce up to 10 nmol of superoxide per minute per $10^6$ cells, and in the context of chronic inflammation, these molecules can damage neurons and other cells.

Dysfunction of PHOX is associated with both immune system abnormalities and neurodegenerative diseases [11]. Mutations in Nox2 cause chronic granulomatous disease, which is a rare primary immunodeficiency syndrome [48]. Patients with this disorder display heightened susceptibility to infections because their immune cells cannot create adequate levels of superoxide in response to bacterial and fungal invasion. Early mechanistic studies of chronic granulomatous disease led to the basic understanding of PHOX and its role in immunity, but the functions of PHOX and other NADPH oxidases during neuroinflammation are complex. Extracellular superoxide produced by PHOX is toxic to neurons, while intracellular superoxide signals the production of pro-inflammatory cytokines [8]. Nox2 is activated in Alzheimer's disease, Parkinson's disease, multiple sclerosis, HIV-associated dementia, neuropathic pain, and many other central nervous system disorders [50]. Deletion of Nox2 in mouse models of Parkinson's disease and Alzheimer's disease results in neuroprotection, and pharmacological inhibition of Nox2 in patients with these disorders has the potential to reduce the damage caused by PHOX activation. However, most available drugs target PHOX indirectly and may not cross the blood blain barrier, decreasing the utility of blocking Nox2 in human patients.

**Inducible Nitric Oxide Synthase (iNOS)**

Inducible nitric oxide synthase is an enzyme that is not expressed in healthy central nervous system tissues but is upregulated in microglia and astrocytes in response to pro-inflammatory cytokines [12]. There are three isoforms of NOS: neuronal NOS1, inducible NOS2 (iNOS), and endothelial NOS3 [9]. iNOS produces L-citrulline and nitric oxide from L-arginine by

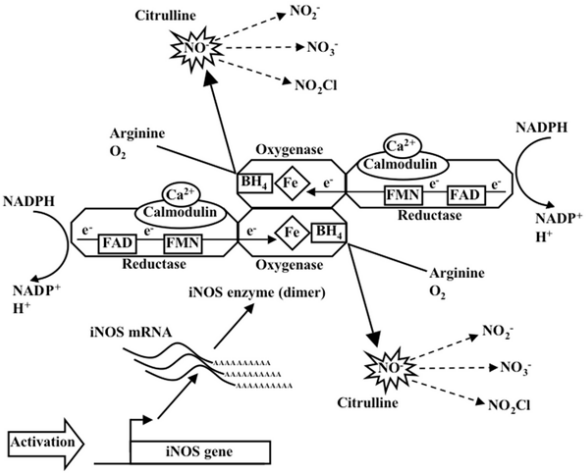



**Figure 2:** Structures and catalytic activity of iNOS and its cofactors. Electrons pass from NADPH through FAD, FMN, Fe, and BH4 to produce L-citrulline and nitric oxide [26].

transferring an electron from NADPH to oxygen through a variety of cofactors [32]. NOS is a large enzyme composed of one polypeptide with two functional domains. Its domain architecture resembles that of cytochrome p450 BM3, but NOS must dimerize at an interface that contains tetrahydrobiopterin ($BH_4$) [1, 32].

Upon microglial activation, iNOS expression is upregulated and active iNOS forms (Fig. 2 ). Calmodulin and calcium associate with the iNOS dimer to stabilize the structure, and the catalytically active enzyme produces nitric oxide [26]. In this multi-step reaction, one electron from NADPH passes from the flavin reductase to the oxygenase domain, which reduces Fe(III) to Fe(II) [32]. Next, the heme group binds molecular oxygen and is reduced by one electron from BH4 . Two protons initiate the cleavage of the $O_2$ bond, generate Fe(III)-O, and release one molecule of water. Fe(III)-O inserts itself into a terminal N -H bond in L-arginine, which ultimately forms L-citrulline and nitric oxide and resets the enzyme cofactors. Because this reaction is complex, the multiple cofactors and substrates necessary for producing nitric oxide may not be present in the cell [31]. In certain conditions, such as L-arginine or $BH_4$ depletion, iNOS may create superoxide instead of nitric oxide via an "uncoupling" mechanism.

Low levels of nitric oxide produced by iNOS are used in cellular signaling and may have protective effects [9, 12]. In primary cell culture, the addition of nitric oxide increases the motility of microglia after activation with lipopolysaccharide [46]. Nitric oxide can increase cyclic GMP levels by interacting with guanyl cyclase, and activation of downstream signaling pathways may be responsible for this heightened mobility. However, activated iNOS can produce very high levels of nitric oxide, which leads to inhibition of mitochondrial cytochrome oxidase in neurons and subsequent depolarization and glutamate release [4]. Accordingly, selective inhibition of iNOS in a rat model of Parkinson's disease results in decreased activation of microglia and increased survival of dopaminergic neurons [10]. The beneficial effects of inhibiting iNOS occur at a narrow range of drug treatment, which suggests that iNOS has both beneficial and neurotoxic properties that must be balanced to maximize neuronal survival.

**Synergism of PHOX and iNOS**

PHOX generates superoxide, iNOS generates nitric oxide, and these two radicals combine to create peroxynitrite (ONOO-) in a reaction that is very rapid [23]. Because peroxynitrite is extremely reactive and has a half-life of less than 10 milliseconds, its presence is difficult to detect directly *in vivo*. However, peroxynitrite reacts with tyrosine to form stable 3-nitrotyrosine, and these residues can be assayed as a proxy for peroxynitrite levels [9]. Under basal conditions, the maximum rate of peroxynitrite formation is about 0.3 μM per second, but in inflammatory states when PHOX and iNOS are highly active, peroxynitrite can form two to three times more quickly [23]. Although peroxynitrite is antimicrobial, if formed at its maximum rate, this compound would cause the death of the cell expressing PHOX and iNOS in less than an hour.

A "dual key" mechanism of neuronal killing by PHOX and iNOS suggests that both superoxide and nitric oxide are necessary for stimulating neuron death [37]. In mixed cultures of neurons and glia, the stimulation of PHOX alone or iNOS alone does not result in the death of neurons. When both PHOX and iNOS are activated, more than half of the cultured neurons die within 48 hours. This decrease in survival is abrogated by either PHOX or iNOS inhibitors and by peroxynitrite scavengers. Moreover, once initiated, the interaction between nitric oxide and superoxide could proceed via positive-feedback amplification [33]. N9 microglial cells continue to produce nitric oxide after treatment and subsequent removal of lipopolysaccharide, and this production is halted by treatment with a PHOX inhibitor. The production of nitric oxide by iNOS is also limited by directing siRNA against the catalytic subunit of PHOX, and the release of nitric oxide from exogenous sodium nitroprusside increases the activity of PHOX. Together, these results suggest that the creation of reactive species could create the self-propelling cycle of inflammation implicated in the progression of neurodegenerative disorders and that treatment inhibiting either iNOS or PHOX could confer neuroprotection by blocking the production of peroxynitrite.

**Hypoxia**

The central nervous system is sensitive to changes in available oxygen, and hypoxia promotes excitotoxicity by releasing glutamate and by promoting immoderate N-methyl-D-aspartate receptor (NMDAR) activation [61]. Blocking synaptic NMDARs decreases the detrimental effects of hypoxia, but reducing enzymatic degradation of glutamate does not alter neuronal survival. Microglia can alter hypoxic damage of neurons via multiple mechanisms. Nitric oxide produced by iNOS can stimulate the release of calcium from astrocytes, which leads to increased glutamate release [12]. Additionally, although nitric oxide alone may not be neurotoxic, concomitant hypoxia can potentiate neuronal death [36]. The mitochondrial enzyme cytochrome oxidase typically has a $K_m$ for oxygen of less than 1 μM, which means that it is unaffected by moderate hypoxic conditions. However, nitric oxide is an inhibitor of cytochrome oxidase and competes with oxygen, resulting in an increase in the observed $K_m$ of oxygen to cytochrome oxidase. Consequently, low levels of nitric oxide in hypoxic conditions can synergize to inhibit cytochrome oxidase, which ultimately results in neuronal death.

**Phagocytosis**

As macrophages, microglia phagocytose necrotic cellular debris and apoptotic neurons [24]. Microglia take up extracellular Aβ in Alzheimer's disease, α-synuclein in Parkinson's disease, and other protein aggregates implicated in various neurodegenerative diseases [25, 51]. Dying neurons display "eat-me" signals on their cell membranes that interact with microglial receptors to initiate phagocytosis [24]. A common "eat-me" signal is phosphatidylserine, but proteins such as calreticulin are also involved in signaling that a cell should be phagocytosed [38]. Phosphatidylserine is typically located exclusively on the inner membrane leaflet of cells because the enzyme aminophospholipid translocase removes phosphatidylserine from the outer leaflet. ATP depletion, necrosis, apoptosis, and oxidative stress can all effect phosphatidylserine display by inactivating aminophospholipid translocase or by activating the phospholipid scramblase. Phosphatidylserine display activates microglial chemotaxis and phagocytosis of the neuron [24]. Although phosphatidylserine display typically initiates phagocytosis of an apoptotic neuron, this change in membrane composition is reversible and may not trigger phagocytosis if phosphatidylserine is returned to the inner leaflet [38]. Additionally, non-toxic levels of oxidative stress and glutamate may initiate phosphatidylserine display in otherwise viable neurons [13]. This killing of live neurons by microglia is termed primary phagocytosis because the primary cause of neuronal death is phagocytosis. Low doses of Aβ can induce phosphatidylserine display and primary microglial phagocytosis of neurons in a co-culture of rat cells, which suggests that primary phagocytosis could play a role in neuronal death in Alzheimer's disease and other neurodegenerative diseases.

**Inhibition by Natural Bioactive Compounds**

Drug treatment for neurodegenerative diseases has traditionally focused on compensating for the detrimental effects of the disorders rather than conferring neuroprotection [52]. However, because the species created by iNOS and PHOX are potentially neurotoxic and primary microglial phagocytosis can damage viable neurons, abrogating microglial activation has the potential to confer neuroprotection [17]. Natural bioactive compounds, existing approved CNS drugs, and new synthetic molecules are all potential candidates for minimizing microglial activity [17, 49]. Natural-based inhibitors of inflammation have been employed as traditional medicines for decades, but only recently have the bioactive components of herbal plants been more extensively researched. Although diverse botanicals show general anti-oxidative and anti-inflammatory properties, some compounds have distinct and specific mechanisms for blocking the activation of microglia during neuroinflammation [60]. Compounds investigated for their ability to mitigate neuroinflammation by preventing microglial activation include curcuminoids, flavonoids, gastrodin, ginsenosides, green tea isolates, inflexin, kavalactones, obovatol, resveratrol, squamosamides, and various other derivatives of natural products. For anti-inflammatory therapies to be effectual, they must gain access to the central nervous system, target specific

pathways of microglial generation, and act at advanced stages of the disease [17].

**Curcuminoids**

Curcuminoids are isolated from turmeric (*Curcuma longa*) and include curcumin, demethoxycurcumin, and bisdemethoxycurcumin [53]. Curcumin interacts both directly and indirectly with many molecular targets, including COX-2 and TLR4 [2]. In BV2 microglial cells treated with LPS, curcumin prevents the nuclear translocation of NF-κB, which suppresses the transcription of pro-inflammatory cytokine genes [27]. Consequently, treatment with curcumin significantly inhibits the expression of iNOS and COX -2 and prevents the production of pro-inflammatory cytokines. Demethoxycurcumin is a natural derivative of curcumin and shows more potent suppression of pro-inflammatory cytokines than curcumin in rat primary N9 microglia activated with LPS [66]. Demethoxycurcumin attenuates the LPS-induced phosphorylation of MAPKs, including p38, JNK, and ERK, and it blocks the nuclear translocation of NF-κB. Demethoxycurcumin may also decrease the activity of PHOX by suppressing the expression of one of its catalytic subunits. In addition to suppressing microglial activation, curcumin may also act directly on nigrostriatal dopaminergic neurons in a mouse model of Parkinson's disease [56]. In these mice, treatment with curcumin increased the survival of tyrosine hydroxylase positive neurons, diminished microglial activation, and preserved levels of the antioxidant enzyme superoxide dismutase (SOD1) in response to 6-hydroxydopamine insult.

Due to its pleiotropic effects, curcumin has been found to treat a wide variety of neurodegenerative diseases, disorders with inflammatory components, and cancers, and it is safe at doses as high as 15 g/day for 3 months [2]. However, curcumin displays poor bioavailability, and many of its metabolites are biologically inactive [3]. As a result, creating synthetic curcumin analogs that are effective at modulating inflammation but are more bioavailable than curcumin is a current area of research. Of 46 synthesized curcumin-like diarylpentanoids, three inhibited iNOS production in LPS-activated macrophage RAW 264.7 cells [29]. In HAPI microglial cells, two structural analogs of curcumin also reduced nitric oxide production in response to LPS [53]. Because curcurmin regulates multiple signaling pathways that are involved in inflammatory diseases, synthetic curcuminoids have the therapeutic potential to treat a variety of human disorders, including neurodegenerative diseases.

**Ginsenosides**

Ginsenosides, or ginseng saponins, are isolated from *Panax ginseng*, which contains a heterogeneous mixture of 30 saponins [17, 30]. Ginseng has been used as a general tonic for over 5000 years, but the particular pharmacological targets of its individual components are an active area of research [42]. Ginseng and ginsenosides have been demonstrated to act upon the central nervous system, cardiovascular system, and immune system, and are divided into protopanaxadiols and protopanaxatriols [6, 30].

Ginsenosides inhibit the phosphorylation of JNK and ERK in N9 microglia activated with LPS, reduce the production of nitric oxide and the pro-inflammatory cytokine TNFα, and block the nuclear translocation of NF- κB [62]. The protopanaxatriol Re inhibits LPS-stimulated inflammation in BV2 microglial cells by reducing the phosphorylation of the MAPK p38, which leads to a reduction in iNOS and COX-2, but does not affect JNK activation [30]. The ocotillol-type ginsenoside pseudoginsenoside-F11 inhibits inflammation in N9 microglial cells treated with LPS, potentially by disrupting the association of TLR4 with its adaptor MyD88, which leads to a decrease in TAK1, MAPK, and protein kinase B signaling and inhibits expression of iNOS, COX-2, and PHOX [60]. Moreover, the ginsenoside Rg1 may suppress dopaminergic neuron loss in an *in vivo* model of Parkinson's disease by modulating p38 phosphorylation [59]. The ginsenoside Rb(1) is reported to cross the blood brain barrier after intravenous infusion in a model of cerebral stroke and to prevent neuronal apoptosis [64]. However, despite the large number of studies reporting the activities of ginsenosides, the specific molecular targets of each component of ginseng should be elucidated before these compounds can be considered for treatment of neuroinflammation [42].

**Obovatol**

Obovatol is one of 250 compounds isolated from the herb *Magnolia obovata* [19]. In mouse macrophage-like RAW 264.7 cells, obovatol inhibited LPS-induced nitric oxide production and expression of iNOS and COX-2 [20]. Simultaneous treatment of these cells with LPS and obovatol reduced NF-κB nuclear translocation and inhibited phosphorylation of the MAP kinases ERK and JNK. Obovatol also protects neurons from microglial activation in microglia-neuron co-culture, and microarray analysis of these microglial cells showed that 108 of the 257 genes expressed in response to LPS returned to normal levels when pretreated with obovatol [39].

Affinity purification suggests that peroxiredoxin 2 (Prx2) is the molecular target of obovatol. Prx2 is a peroxidase, and the addition of obovatol significantly increased the ability of Prx2 to reduce hydrogen peroxide levels *in vitro*. Pretreatment of mice with obovatol before LPS injection improved memory function and reduced amyloid β levels in the brains of these mice [18]. Long-term treatment of mice expressing mutant human amyloid precursor protein with obovatol also improved cognitive function by inhibiting amyloid β fibrillation. Further analysis of the safety and efficacy of obovatol is necessary before its use as a therapeutic can be considered, but these studies suggest that modulating the activity of Prx2 offers an additional approach for treating neuroinflammation.

**Squamosamides**

Compounds extracted from *Annona glabra* are used in traditional Chinese medicines and are thought to have anti-cancer and anti-apoptotic features [65]. A cyclic analogue of a squamosamide extracted from this tree was synthesized to exhibit stronger antioxidant properties than its natural correlate. This compound, named FLZ, can cross the blood-brain barrier and ameliorates dopaminergic neuron loss in a rat model of Parkinson's disease [5]. When challenged with 6-hydroxydopamine, MN9D dopaminergic neurons treated with FLZ showed increased survival and decreased expression of proteins associated with Parkinson's disease, and these changes are perhaps the result of increased protein kinase B signaling. FLZ may inhibit microglial activation by suppressing Src tyrosine kinase signaling: Treatment of BV2 microglial cells with FLZ resulted in a decrease in Src tyrosine kinase phosphorylation and a reduction in PHOX activity [52]. Because activation of Src signaling activates PHOX, the inhibition of Src by FLZ could repress microglial activation. However, FLZ is also reported to act by inducing the expression of heat shock proteins, and because FLZ is relatively new, further inspection of the mechanisms of neuroprotection by this compound and its *in vivo* effects is crucial [35].

**Conclusions**

Despite the damaging actions of microglia, their activation may also confer neuroprotection, and understanding how microglia equipoise the beneficial and injurious results of inflammation is important when constructing therapies to ameliorate only the detrimental facets of neuroinflammation [12]. Developing therapeutics that block specific aspects of microglial signaling and activation has two potential benefits: Identifying novel drug targets could lead to a greater understanding of these signaling cascades, and drug candidates with anti-inflammatory properties could offer improved treatments for a variety of neurodegenerative diseases [49]. For instance, inhibiting either iNOS or PHOX blocks the feed-forward, "dual key" production of damaging reactive species [37]. A variety of natural compounds merit further study for their ability to curtail microgliosis, but microglial activation is complex, and its roles in neuroinflammation are not fully elucidated. In the future, an increased understanding of how microglia effect neuronal death could lead to better treatments for those with neurodegenerative disorders.

**References**

[1] Alderton W, Cooper CE & Knowles RG. (2001). Nitric oxide synthases: structure, function and inhibition. Biochem J, 357:593-615.

[2] Aggarwal BB & Sung B. (2008). Pharmacological basis for the role of curcumin in chronic diseases: an age-old spice with modern targets. Trends Pharmacol Sci, 30:85-94.

[3] Anand P, Kunnumakkara AB, Newman RA & Aggarwal BB. (2007). Bioavailability of curcumin:

problems and promises. Mol Pharm, 4:807-18.
[4] Bal-Price A & Brown GC. (2001). Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. J Neurosci, 21:6480-91.
[5] Bao XQ, Kong XC, Kong LB, Wu LY, Sun H, & Zhang D. (2014). Squamosamide derivative FLZ protected dopaminergic neuron by activating Akt signaling pathway in 6-OHDA-induced in vivo and in vitro Parkinson's disease models. Brain Res, 1547:49-57.
[6] Beamer CA & Shepherd DM. (2012). Inhibition of TLR ligand- and interferon gamma-induced murine microglial activation by Panax notoginseng. J Neuroimmune Pharmacol, 7:465-76.
[7] Benarroch EE. (2013). Microglia: Multiple roles in surveillance, circuit shaping, and response to injury. Neurology, 81:1079-88.
[8] Block ML. (2008). NADPH oxidase as a therapeutic target in Alzheimer's disease. BMC Neurosci, 9 Suppl 2:S8.
[9] Bonilla IM, Sridhar A, Gyorke S, Carounel AJ, & Carnes CA. (2012). Nitric oxide synthases and atrial fibrillation. Front Physiol, 3:105.
[10] Broom L, Marinova-Mutafchieva L, Sadeghian M, Davis JB, Medhurst AD, & Dexter DT. (2011). Neuroprotection by the selective iNOS inhibitor GW274150 in a model of Parkinson's disease. Free Radic Biol Med, 50:633-40.
[11] Brown D & Griendling KK. (2009). Nox proteins in signal transduction. Free Radic Biol Med, 47:1239-53.
[12] Brown GC & Neher JJ. (2010). Inflammatory neurodegeneration and mechanisms of microglial killing of neurons. Mol Neurobiol, 41:242-7.
[13] Brown GC & Neher JJ. (2014). Microglial phagocytosis of live neurons. Nat Rev Neurosci, 15:209-16.
[14] Chan EC, Jiang F, Peshavariya HM, Dusting GJ. (2009). Regulation of cell proliferation by NADPH oxidase-mediated signaling: potential roles in tissue repair, regenerative medicine and tissue engineering. Pharmacol Ther, 122:97-108.
[15] Chan WY, Kohsaka S, & Rezaie P. (2007). The origin and cell lineage of microglia: new concepts. Brain Res Rev, 53:344-54.
[16] Cheret C, Gervais A, Lelli A, Colin C, Amar L, Ravassard P, Mallet J, Cumano A, Krause KH, & Mallat M. (2008). Neurotoxic activation of microglia is promoted by a nox1-dependent NADPH oxidase. J Neurosci, 28:12039-51.
[17] Choi DK, Koppula S, & Suk K. (2011). Inhibitors of Microglial Neurotoxicity: Focus on Natural Products. Molecules, 16:1021-1043.
[18] Choi DY, Lee JW, Lin G, Lee YK, Lee YH, Choi IS, Han SB, Jung JK, Kim YH, Kim KH, Oh KW, Hong JT & Lee MS. (2011). Obovatol attenuates LPS-induced memory impairments in mice via inhibition of NF-κB signaling pathway. Neurochem Int, 60:68-77.
[19] Choi DY, Lee JW, Peng J, Lee YJ, Han JY, Lee YH, Choi IS, Han SB, Jung JK, Lee WS, Lee SH, Kwon BM, Oh KW & Hong JT. (2012). Obovatol improves cognitive functions in animal models for Alzheimer's disease. J Neurochem, 120:1048-59.
[20] Choi MS, Lee SH, Cho HS, Kim Y, Yun YP, Jung HY, Jung JK, Lee BC, Pyo HB, & Hong JT. (2007). Inhibitory effect of obovatol on nitric oxide production and activation of NF-kappaB/MAP kinases in liposaccharide-treated RAW 264.7 cells. Eur J Pharmacol, 556:181-9.
[21] Dai L, Aye Thu C, Liu XY, Xi J, & Cheung PC. (2012). TAK1, more than just innate immunity. IUBMB Life, 64:825-34.
[22] Fellner L, Irschick R, Schanda K, Reindl M, Klimaschewski L, Poewe W, Wenning GK, & Stefanova N. (2013). Toll-like receptor 4 is required for α-synuclein dependent activation of microglia and astroglia. Glia, 61:349-60.
[23] Ferrer-Sueta G & Radi R. (2009). Chemical biology of peroxynitrite: kinetics, diffusion, and radicals. ACS Chem Biol, 4:161-77.
[24] Fricker M, Oliva-Martin MJ, & Brown GC. (2012). Primary phagocytosis of viable neurons by microglia activated with LPS or Aβ is dependent on calreticulin/LRP phagocytic signalling. J Neuroinflammation, 9:196.
[25] Glass CK, Saijo K, Winner B, Marchetto MC, & Gage FH. (2010). Mechanisms underlying inflammation in neurodegeneration. Cell, 140:918-34.
[26] Grayfer L & Belosevic M. (2012). Cytokine Regulation of Teleost Inflammatory Responses, New Advances and Contributions to Fish Biology, Prof. Hakan Turker (Ed.), ISBN: 978953-51-0909-9, InTech, DOI: 10.5772/53505.

[27] Jin CY, Lee JD, Park C, Choi YH, & Kim GY. (2007). Curcumin attenuates the release of pro-inflammatory cytokines in lipopolysaccharide-stimulated BV2 microglia. Acta Pharmacol Sin, 28:1645-51.
[28] Kettenmann H, Hanisch UK, Noda M, & Verkhratsky A. (2011). Physiology of microglia. Physiol Rev, 91:461-553.
[29] Lee KH, Ab-Aziz FH, Syahida A, Abas F, Shaari K, Israf DA, & Lajis NH. (2009). Synthesis and biological evaluation of curcumin-like diarylpentanoid analogues for anti-inflammatory, antioxidant and anti-tryosinase activities. Eur J Med Chem, 44:3195-200.
[30] Lee KW, Jung SY, Choi SM, & Yang EJ. (2012). Effects of ginsenoside Re on LPS-induced inflammatory mediators in BV2 microglial cells. BMC Complement Altern Med, 12:196.
[31] Lei H, Luo S, Quin H, & Xia Y. (2013). Molecular mechanisms of endothelial NO synthase uncoupling. Curr Pharm Des, Epub ahead of print.
[32] Li H & Poulous TL. (2005). Structure-function studies on nitric oxide synthases. J Inorg Biochem, 99:293-305.
[33] Lijia Z, Zhao S, Wang X, Wu C, & Yang J. (2012). A self-propelling cycle mediated by reactive oxide species and nitric oxide exists in LPS-activated microglia. Neurochem Int, 61:1220-30.
[34] Luongo L, Guida F, Imperatore R, Napolitano F, Gatta L, Cristino L, Giordano C, Siniscalco D, Di Marzo V, Bellini G, Petrelli R, Cappellacci L, Usiello A, de Novellis V, Rossi F, & Maione S. (2014). The A1 adenosine receptor as a new player in microglia physiology. Glia, 62:122-32.
[35] Ma B, Li M, Ma T, Liu GT, & Zhang J. (2014) Neuroprotective effects of compound FLZ in an ischemic model mediated by improving cerebral blood flow and enhancing Hsp27 expression. Brain Res. Epub ahead of print.
[36] Mander P, Borutaite V, Moncada S, & Brown GC. (2005). Nitric oxide from inflammatory activated glia synergizes with hypoxia to induce neuronal death. J Neurosci Res, 79:208-15.
[37] Mander P & Brown GC. (2005). Activation of microglial NADPH oxidase is synergistic with glial iNOS expression in inducing neuronal death: a dual-key mechanism of inflammatory neurodegeneration. J Neuroinflammation, 12:2-20.
[38] Neher JJ, Neniskyte U, & GC Brown. (2012). Primary phagocytosis of neurons by inflamed microglia: potential roles in neurodegeneration. Front Pharmacol, 3:27.
[39] Ock J, Han HS, Hong SH, Lee SY, Han YM, Kwon BM, & Suk K. (2010). Obovatol attenuates microglia-mediated neuroinflammation by modulating redox regulation. Br J Pharmacol, 159:1646-62.
[40] Ohsawa K, Irino Y, Sanagi T, Nakamura Y, Suzuki E, Inoue K, & Kohsaka S. (2010). P2Y12 receptor-mediated integrin-beta1 activation regulates microglial process extension induced by ATP. Glia, 58:790-801.
[41] Okun E, Griffioen KJ, Lathia JD, Tang SC, Mattson MP, & Arumugam TV. (2009). Toll-like receptors in neurodegeneration. Brain Res Rev, 59:278-92.
[42] Radad K, Moldzio R, & Rausch WD. (2011) Ginsenosides and their CNS targets. CNS Neurosci Ther, 17:761-8.
[43] Ransohoff RM & Cardona AE. (2010). The myeloid cells of the central nervous system parenchyma. Nature, 468:253-62.
[46] Scheiblich H, Roloff F, Singh V, Stangel M, Stern M, & Bicker G. (2014). Nitric oxide / cyclic GMP signaling regulates motility of a microglial cell line and primary microglia in vitro. Brain Res, Epub ahead of print.
[47] Song M, Jin J, Lim JE, Kou J, Pattanayak A, Rehman JA, Kim HD, Tahara K, Lalonde R, & Fukuchi K. (2011). TLR4 mutation reduces microglial activation, increases Aβ deposits and exacerbates cognitive deficits in a mouse model of Alzheimer's disease. J Neuroinflammation, 8:92.
[48] Stasia MJ & Li XJ. (2008). Genetics and immunopathology of chronic granulomatous disease. Semin Immunopathol, 30:209-35.
[49] Suk K & Ock J. (2012). Chemical genetics of neuroinflammation: natural and synthetic compounds as microglial inhibitors. Inflammopharmacology, 20:151-8.
[50] Surace MJ & Block ML. (2012). Targeting microglia-mediated neurotoxicity: the potential of NOX2 inhibitors. Cell Mol Life Sci, 69:2409-27.
[51] Tahara K, Kim HD, Jin JJ, Maxwell JA, Li L, & Fukuchi K. (2006). Role of toll-like receptor signaling in Abeta uptake and clearance. Brain, 129:3006-19.

[52] Tai W, Ye X, Bao X, Zhao B, Wang X, & Zhang D. (2013). Inhibition of Src tyrosine kinase activity by squamosamide derivative FLZ attenuates neuroinflammation in both in vivo and in vitro Parkinson's disease models. Neuropharmacology, 75:201-12.
[53] Tocharus J, Jamsuwan S, Tocharus C, Changtam C, & Suksamrarn A. (2011). Curcuminoid analogs inhibit nitric oxide production from LPS-activated microglial cells. J Nat Med, 66:400-5.
[56] Tripanichkul W & Jaroensuppaperch EO. (2013). Ameliorating effects of curcumin on 6-OHDA-induced dopaminergic denervation, glial response, and SOD1 reduction in the striatum of hemiparkinsonian mice. Eur Rev Med Pharmacol Sci, 17:1360-8.
[57] Trotta T, Porro C, Calvello R, & Panaro MA. (2014). Biological role of Toll-like receptor-4 in the brain. J Neuroimmunol, 268:1-12.
[58] Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, & Chen ZJ. (2001). TAK1 is a ubiquitin dependent kinase of MKK and IKK. Nature, 412:346-51.
[59] Wang Q, Zheng H, Zhang ZF, & Zhang YX. (2008). Ginsenoside Rg1 modulates COX-2 expression in the substantia nigra of mice with MPTP-induced Parkinson disease through the P38 signaling pathway. Nan Fang Yi Ke Da Xue Xue Bao, 28:1594-1598.
[60] Wang X, Wang C, Wang J, Zhao S, Zhang K, Wang J, Zhang W, Wu C, & Yang J. (2014) Pseudoginsenoside-F11 (PF11) exerts anti-neuroinflammatory effects on LPS-activated microglial cells by inhibiting TLR4-mediated TAK1/IKK/NF-κB, MAPKs and Akt signaling pathways. Neuropharmacology, 79:642-656.
[61] Wroge CM, Hogins J, Eisenman L, & Mennerick S. (2012). Synaptic NMDA receptors mediate hypoxic excitotoxic death. J Neurosci, 32:6732-42.
[62] Wu CF, Bi XL, Yang JY, Zhan JY, Dong YX, Wang JH, Wang JM, Zhang R, & Li X. (2007) Differential effects of ginsengosides on NO and TNF-alpha production by LPS-activated N9 microglia. Int Immunopharmacol, 7:313-20.
[63] Yan K, Zhang R, Sun C, Chen L, Li P, Liu Y, Peng L, Sun H, Qin K, Chen F, Huang W, Chen Y, Lv B, Du M, Zou Y, Cai Y, Qin L, Tang Y, & Jiang X. (2013). Bone marrow-derived mesenchymal stem cells maintain the resting phenotype of microglia and inhibit microglial activation. PLoS One, 8: e84116.
[64] Zhang B, Hata R, Zhu P, Sato K, Wen TC, Yang L, Futija H, Mitsuda N, Tanaka J, Samukawa K, Maeda N, & Sakanaka M. (2006). Prevention of ischemic neuronal death by intravenous infusion of a ginseng saponin, ginsenoside Rb(1), that upregulates Bcl-x(L) expression. J Cereb Blood Flow Metab, 26:708-21.
[65] Zhang D, Hu X, Wei SJ, Liu J, Gao H, Qian L, Wilson B, Liu G, & Hong JS. (2008). Squamosamide derivative FLZ protects dopaminergic neurons against inflammation-mediated neurodegeneration through the inhibition of NADPH oxidase activity. J Neuroinflammation, 5:21.
[66] Zhang L, Wu C, Zhao S, Yuan D, Lian G, Wang X, Wang L, & Yang J. (2010). Demethoxycurcumin, a natural derivative of curcumin attenuates LPS-induced pro-inflammatory responses through down-regulation of intracellular ROS-related MAPK/NF-kappaB signaling pathways in N9 microglia induced by lipopolysaccharide. Int Immunopharmacol, 10:331-8.

**About the Author**

Anna Moyer is a senior from Boalsburg, Pennsylvania majoring in biology at the University of Alabama. Working with Dr. Janis O'Donnell, she studies neuroinflammation in a fruit fly model of Parkinson's disease. Anna plans to graduate with bachelor's and master's degrees before pursuing a doctorate in molecular genetics. Her long-term goal is to explore fundamental questions in human genetics and disease mechanisms via model organisms, in hope that understanding details about cellular processes will one day lead to more effective therapies for disorders with genetic components. Anna is a member of the Phi Beta Kappa Honor Society and has received an Arts and Sciences Outstanding Sophomore Award, the Grantland and Louise Rice Achievement Award, and a scholarship from Sigma Tau Delta International English Honor Society.

# A Review of Literature on False Memory Phenomena and Anosognosia in Dementia Patients: Insight into the Development of Targeted Interventions

Samuel Creden

*Though false memory phenomena (FMP) and anosognosia are two distinct phenomena frequently recorded in dementia patients, little research addressing potential treatments or interventions for either phenomenon exists. Despite this, this literature review proposes the development of such treatment models is both possible and necessary. FMP, which include false recognitions and recollections, "déjà" phenomena, and memory-related delusions and confabulations, are collectively examined in pursuit of a more thorough clinical understanding of the phenomena. Additionally, a causal link between anosognosia and FMP is proposed, thereby expanding both clinical understanding of FMP in dementia and the importance of further research.*

## Introduction

This paper will address false memory phenomena (FMP) as a holistic issue, including confabulation, déjà vu, déjà vécu, delusions, false recollection, and false recognition. In prior studies, phenomena of this type have been collectively labeled "memory distortions" [1, 10]. While the current consensus is that memory distortions are causally linked to flawed encoding and recall processes within the episodic memory, these studies have shown that individuals with Alzheimer's disease or its prodromal condition, mild cognitive impairment, are particularly vulnerable to the expression of false memories for events which they have never experienced [23]. No matter the details of their manifestation, false memory phenomena in dementia patients are both clinically important and wanting for attention. Unfortunately, current research on FMP is relatively scant, and there is even less available on the diagnosis of and response to FMP in dementia patients. Furthermore, an overwhelming majority of the current body of research comprises cross-sectional, correlational, or otherwise observational studies. The main purpose of this literature review, therefore, is twofold: to examine such manifestations and risk factors of FMP, and to explore the applied implications of this body of literature to ascertain whether the experimental development of a clinical, multidisciplinary intervention for FMP in dementia patients is possible.

Particular insight into false memory production comes with an examination of research concerning anosognosia, the phenomenon in which a patient is not aware they are suffering from a particular symptom or disability. Anosognosia is cited as a common symptom in several types of dementia, particularly Alzheimer's disease, in which anosognosia becomes more prevalent as the disease progresses [22, 29]. Though no presently published research exists that causally links false memory production with anosognosia, several researchers have noted a relationship between the two [9, 17]. Furthermore, research concerning each phenomenon is being actively published. In the pursuit of the primary purpose of this literature review, a secondary purpose becomes evident: explore the interplay between anosognosia and FMP to ascertain whether research intended to establish a causal relationship between them might be warranted.

## Methods

This literature review considers "false memory phenomena" to be an umbrella term comprising the phenomena of recollective confabulation, déjà vu, déjà vécu, delusions, false recollection, and false recognition.

Searches were conducted primarily within the PsycINFO, PsycARTICLES, and PubMed databases, with additional attention given to The University of Alabama's database aggregate search tool Scout. Search criteria were contrived as follows: key terms entered were "dementia" and "anosognosia", with sub-key terms including "false memory", "recollective confabulation", "déjà vu", "déjà vécu", "false recognition", "false recollection", and "delusion". Each search was constructed by grouping at least one of the

key terms with at least one of the sub-key terms (e.g. "dementia AND recollective confabulation AND delusion"). Without any additional inclusion or exclusion criteria, these searches collectively produced roughly 28,000 results. Ninety-nine candidate works were chosen based on relevance and convergence across different searches.

Search results were selected for inclusion within this study if they included a sufficient association of key and sub-key terms; evaluation of a proposed or existing treatment; or identifiable significance, relevancy, or urgency placed upon the study of false memory production in dementia. A sufficient association of key and sub-key terms entails an identifiable association or correlation between anosognosia and false memory, a causal relationship found between anosognosia and another variable, or a causal relationship found between false memory production and another variable (i.e. insight into risk factors or underlying cognitive mechanisms of either phenomenon). Studies not explicitly related to individuals with dementia or their caregivers were excluded. Finally, dated (at least ten years old) and obsolete studies were excluded unless they could be considered sufficiently seminal to the field. Following this additional criteria, thirty-one works were chosen for inclusion within this literature review.

**Findings**

*Justification of the Simultaneous Study of all FMP*

As the proposed concept of FMP comprises such an expansive and varied collection of phenomena, its study might seem at worst excessively broad and at best worryingly ambitious. However, an examination of the clinical definitions of these phenomena dispels such concerns and instead demonstrates that the study of any one particular phenomenon is nearly impossible without the study of the rest (see Table 1). Despite already having well-formed definitions of each phenomenon, both the distinguishing nuances and interrelated similarities deserve particular attention. Notable among these are the direct and indirect acknowledgements of the similarity of all these concepts.

Where false recognition entails the simple remembrance of a stimulus not previous experienced, false recollection entails the additional remembrance of specific details. This fits with a previously proposed distinguishing model for recollective confabulation vs. familiarity [18], with false recognition paralleling familiarity and false recollection paralleling (or, perhaps, equating to) recollective confabulation. Additionally, the two phenomena fit elegantly with the paradigm of item-recognition memory (i.e. distinguishing dissimilar stimuli, such as a key and a microphone) and detail-recognition memory (i.e. distinguishing similar stimuli, such as a silver key and a bronze key) proposed by Abe et al. [1]. By calculating indices for both types of memory, the researchers found that patients with Alzheimer's disease demonstrate an impaired ability to use item-specific recognition and proposed their impaired detail-recognition memory as a potential factor. Here, false recognition parallels to item-recognition memory, and false recollection parallels to detail-recognition memory. This comparison is particularly poignant, as both false recollection and detail-recognition memory can be redefined as more specific versions of their counterparts.

Parallels are drawn by two separate studies between false recognitions/recollections and déjà vu/vécu, respectively [18, 19]. Déjà vu, defined as a sense of familiarity not founded in reality, parallels false recognition so closely that the two might be considered nearly synonymous. Déjà vécu, an iteration of déjà vu more sensitive to contextual details, demonstrates the same quality when compared to false recollection. The principle difference between false recognitions and recollections and déjà phenomena, the researchers state, is that déjà phenomena are delusion-like in their tenacity.

[1] It should be noted that, within this context, déjà vu is discussed as a pathological experience. While healthy persons can experience nonpathological instances of déjà vu [19], such phenomena are beyond the scope of this review.

[2] Additionally, déjà vu has been used as an umbrella term, encompassing related phenomena such as déjà senti and déjà visité [19],Though the study of such phenomena may be relevant at some point in the near future, they are beyond the scope of the current review.

[3] It should be noted that, within this context, delusions are discussed as contextually comprehensible. Where delusions of sheer incomprehensibility are possible (e.g. bizarre delusions), such phenomena are beyond the scope of this review. For further clarification, see [13].

[4] This definition applies particularly to what Moulin calls "recollective confabulation" [17].

*Clinical definitions of false memory phenomena*

| Specific Phenomenon | Definition |
|---|---|
| False Recognition | The process whereby people erroneously claim to have experienced a stimulus which they have not previously encountered [1]<br>Labeled as approximately synonymous with déjà vu [18, 19] |
| False Recollection | The mental restoration of a stimulus never actually experienced during which details are recalled [1]<br>Labeled as approximately synonymous with déjà vécu [18,19] |
| Déjà vu[1,2] | A delusion-like phenomenon in which a patient demonstrates familiarity with unfamiliar materials [17] |
| Déjà vécu | A delusion-like phenomenon in which erroneous contextual information is recalled [19] |
| Delusion[3] | A fixed, false belief held even when confronted with contradictory evidence [16]<br>A belief that is adopted and maintained uncritically despite implausibility in light of general knowledge or presented evidence [13] |
| Confabulation | A fleeting, mistaken belief that is more isolated, transient, and variable than a delusion or delusion-like phenomenon [16]<br>An erroneous memory that is either true but inappropriately retrieved or outright false [4]<br>The reproduction of false information from a remembered, non-existent episode that is used to justify false recognition[4] [17] |

**Table 1**

Such tenacity is also what distinguishes delusions and confabulations. Between the fixedness and adherence to most psychological models demonstrated by delusions and the transience and marked variability of confabulations, the two are often viewed as discrete though closely related phenomena [16]. However, this consensus is not unanimous, with some explaining that the phenomena are distinct but potentially inclusive: spontaneous confabulations are delusional by nature, and confabulations provoked by direct questioning are less persistent than their spontaneous counterparts [13]. Regardless of whether delusions and confabulations overlap conceptually or actually, the merit of their combined study is clear, as is that of the inclusion of delusion-like déjà phenomena and confabulatory false recall phenomena. Thus, this literature review refers to any instances of false recognition, false recollection, déjà vu, déjà vécu, or appropriately memory-related delusion and confabulation collectively as "false memory phenomena", except where the differences discussed become critically relevant.

*Justification of the Included Study of Anosognosia*

Presently, anosognosia and FMP are not linked through direct experimentation or observation. However, based on review of the current body of literature, this study proposes that research directly tailored to establishing a causal link (or the lack thereof) between anosognosia and FMP is both important and necessary. Despite no such research existing currently, several studies propose implications and venture post-hoc hypotheses, that anosognosia and the onset of FMP may be casually linked. Gallo et al. indicate that the relationship between anosognosia and memory accuracy (that is, whether a particular memory is true) is modulated by retrieval monitoring through both the demands of the task and the capacity of the patient [9]. In addition to retrieval monitoring, anosognosia has been linked with memory error monitoring [24]; reduplicative paramnesia, a type of FMP-related delusion [17]; and disinhibition in patients demonstrating mild cognitive impairment in multiple domains [6, 27]. This review will further explore retrieval monitoring, error monitoring, and inhibition as underlying cognitive mechanisms related to the suppression of FMP, thereby making the current, tangential relationship between anosognosia and FMP more robust.

*Importance of the Study of False Memory Phenomena and Anosognosia*

Several factors contribute to the importance of this line of study, most notable among them the prevalence of FMP and anosognosia. Conclusions drawn on the prevalence of anosognosia are varied. Where some reports indicate anosognosia is present in anywhere ranging from 20% to 80% of dementia patients [6], others indicate 47% of Alzheimer's disease patients experience the phenomenon [27]. Nevertheless, even a phenomenon recorded in 1 patient in 5 merits attention. Concurrent phenomena such as subjective quality of life and negative affect are examined later in this literature review, and a consideration of the potential results of future study follow.

*Correlates with False Memory Phenomena*

Chief among any diagnostic model is a series of identified correlates, including risk factors, comorbidities and, especially within the field of clinical psychology, underlying cognitive mechanisms. As a major component of intervention is diagnosis, an examination of such correlates is certainly warranted.

The first type of correlate to be examined is risk factors (see Table 2.1). It should be noted that this table is meant not to provide a comprehensive compilation of all risk factors linked with FMP, but to provide a primer for such a compilation and a framework for future research. For this purpose, risk factors are any noncognitive quality a patient may demonstrate that

could indicate a vulnerability to the onset of FMP. The potential use of this compilation to a diagnostic model is straightforward. With knowledge of risk factors, a clinician may develop a working predictive model for the onset of FMP. Of course, to effectively apply such a model, one must develop or otherwise discern methods that test, measure, or otherwise quantify the presence of these risk factors. Fortunately, the appropriate psychological inventories and diagnostic tests for each of these risk factors all already exist.

Of particular note among these listed risk factors is the surprising fact that as veridical memory improves, susceptibility to FMP actually increases. This notion contradicts what may appear true on an intuitive basis. Generally, FMP production is expected to manifest alongside deficits in memory. However, the identification of this risk factor grants valuable insight into the necessities of a carefully constructed, targeted intervention. An intervention that addresses deficits in veridical memory (e.g. amnesia) but neglects the subsequent vulnerability to FMP potentially

*Identified risk factors of anosognosia and the production of false memory phenomena*

| Risk Factor | Details |
|---|---|
| Availability of veridical memories | When controlled for other variables like frontal-lobe dysfunction, amnesics demonstrate reduced rates of false recognition [3, 4] |
| Dysfunction of ventromedial prefrontal cortex | The authors propose that among cases of behavioral variant frontotemporal dementia, disturbances of the activity of the ventromedial prefrontal cortex produce confabulations [16]. |
| Frontotemporal dysfunction | Several authors identify that dysfunction in the frontal and temporal lobes, as well as the circuits linking them, positively correlates with false recognition, confabulation, and related phenomena [8, 14, 17, 18, 24]. |
| Negative emotional valence | The authors propose that difficulty with or aversion to reliving past events brought about by negative emotional valence surrounding them is involved in the genesis of anosognosia [12, 24]. |
| Prevalence of amyloid-β (1-42) | Prevalence of false recognitions positively correlates with concentration of amyloid-beta (1-42) in cerebrospinal fluid [10]. |
| Stage of disease progression | Severity of the dementia positively correlates with the prevalence of anosognosia [31]. |
| Type of dementia | A cross-sectional study found that patients with Alzheimer's disease experienced FMP more often than patients with fronto-subcortical dementia, normal pressure hydrocephalus, and vascular dementia [11]. |

**Table 2.1**

invites the onset of FMP. Though this effect has not been demonstrated within individuals, cross-sectional studies have indicated that patients with increased capacities for veridical memory also experienced more frequent instances of FMP [3, 4]. The implication of these studies' results is that the correlation between veridical memory and FMP also applies not only between individuals, but within individuals.

The second type of correlate to be examined is behavioral and psychological effects (see Table 2.2). As before, this table is meant to serve not as a comprehensive list of behavioral and psychological effects of FMP and anosognosia but as a primer for one. Many of these entries could be considered non-cognitive neuropsychiatric symptoms, also called behavioral and psychological symptoms, of dementia. However, this concept is applied only to cases of

*Identified behavioral and psychological effects of false memory phenomena and anosognosia*

| Effect | Details |
|---|---|
| Increased caregiver burden | Patients' anosognosia as measured by the Experimenter Rating Scale (a clinical judgment) accounts for 14.7% of the total variance of self-reported caregiver burden [30]. |
| Reduced reliability of patients' self-reported quality of life | Caregivers' perceptions of their patients' quality of life as measured by the Quality of Life (QoL)—Alzheimer's Disease scale decreased with the onset of anosognosia, where patients' self-reported scores increased [6].<br>Discrepancies between the QoL-proxy (report by caregivers) and QoL-dementia (self-report by patients) inventories increased with patients' anosognosia as measured by the Clinical Insight Rating [2]. |
| Agitation and Irritability<br>Aberrant Motor Behaviors<br>Apathy | Anosognosic patients demonstrate increased levels of these phenomena when compared to non-anosognosic patients [27].<br>Notably, apathy correlated only among patients with mild dementia [6]. |

**Table 2.2**

dementia in which anosognosia and/or FMP are present, and it is additionally applied to caregivers of patients present with anosognosia and/or FMP. Entries in this table are listed as such because they are noticeably concurrent with FMP or anosognosia but related neither to their onset (as risk factors are) nor their underlying cognitive mechanisms. Such comorbidities further reveal the importance of this line of study, as the entries are overwhelmingly negative from a clinical viewpoint.

*Underlying cognitive mechanisms related to false memory phenomena and anosognosia*

| Mechanism | Details |
|---|---|
| Deficits in source monitoring | The authors propose that the impaired ability to identify and evaluate the source of experiential content is involved in the genesis of confabulation [16, 17, 18, 20, 24]. |
| Deficits in inhibition[5] | The authors propose that the impaired ability to inhibit the translation of experiential content into beliefs is involved in the genesis of confabulation [4, 7, 11, 13]. |
| Deficits in retrieval monitoring and encoding | The authors propose that the conflation of memory encoding with memory retrieval leads patients to believe a recently encoded memory was actually just retrieved [4].<br>This conflation is amplified with unique (i.e. not routine) situations such as death [19]. |
| Disruptions to temporal coding mechanism | The temporal coding mechanism describes neural activity downstream of the hippocampus that signals recollection [19]. |

**Table 2.3**

The final type of correlate to be examined is underlying cognitive mechanisms related to FMP, either through suppression or genesis (see Table 2.3). As before, this table is meant not to provide a

[5] In this context, "inhibition" is substituted for "memory inhibition", applicably defined as the capacity to discriminate between memories of experienced information and information not actually experienced [11].

comprehensive list of underlying cognitive mechanisms of FMP and anosognosia but a primer for one.

Notable among these results is the aforementioned relationship between retrieval monitoring, inhibition, and anosognosia. Furthermore, the anosognosic phenomenon of cryptomnesia—in which a forgotten memory returns to a person who recognizes it not as a memory but a novel experience, conceptually links anosognosia and source monitoring. The relationships between anosognosia and cognitive mechanisms underlying FMP discussed both within this study and in the current body of literature provide support for the conceptual, causal relationship between anosognosia and FMP itself. Several of these cognitive mechanisms, like anosognosia, are understandably difficult to measure and observe diagnostically. Options for measuring anosognosia include a dual questionnaire administered to both the patient and their caregiver, clinical judgment on the part of the presiding professional, and the comparison of patients' self-assessments with examination results [24]. However, Rosen notes that each of these methods bears a particular shortcoming. The first option is labor-intensive and requires the presence of a caregiver, the second option has no standardized scale and is thus potentially inaccurate, and the final option requires a substantial investment of time. Inventories, questionnaires, or clinical examinations designed to measure the cognitive mechanisms at hand with both sensitivity and specificity were not found.

**Discussion**

Moving forward from this survey of literature to a model of intervention requires considerable critical thought. Risk factors and metrics, including the presence of anosognosia, have been provided to aid in the identification of both the predisposition for false memory production and the actual onset of FMP. Some underlying cognitive mechanisms of FMP have been identified to provide contextual understanding of particular instances. Subsequent steps include incorporating these risk factors and cognitive mechanisms into both preventative and responsive treatment models. These models, however, must be abstract and procedural. As with most clinical applications of knowledge, textbook cases are rare. Factors related to neuropsychological function, affective functioning, sociodemographic characteristics, caregiver well-being, and the nature of the relationship between the patient and caregiver have all been identified as factors contributing to the severity of anosognosia [5]. Though the present literature review is a suitable primer for developing a case-specific, applied model of intervention, particular care must be taken to consider the contextual factors of the patient's anosognosia (if any) when doing so.

Underscoring the caution with which intervention must be approached are the delicate balances that must be maintained in designing a plan, among which are those between routine and repetition. As discussed above, unique stimuli can produce an experience of déjà vécu, likely due to errors involving the conflation of memory encoding and retrieval [4, 19]. This suggests a potential trend of routine across the context-sensitive treatments. In order to mitigate the frequency of unique stimuli, caregivers might design, introduce, and adhere to a particular routine with their patient. However, researchers have noted an increase in the frequency of false recognition with repeated, similar stimuli [1]. This in turn suggests that if a caregiver implemented a routine as an intervention against FMP, the rigidity with which they would have to adhere to that routine (in order to ensure the patient experiences no stimuli that are not identical to those of the routine) might render such a routine impractical.

A potential solution lies in the recognition that semantically-related FMP are infrequent. With "gist-based" memory rarely left intact, patients are left unable to synthesize relationships across stimuli presented to them [21, 26]. In response, a caregiver may contrive a "routine" with varied stimuli with the knowledge that once every stimulus bears unique qualities, no stimulus will be truly unique. However, the detriments of such a complex intervention are at this point plain. With both routines with absolute rigidity and with absolute variety demonstrating such problems, the intuitive conclusion is that an optimal intermediate must exist.

A similar balance presents itself when we examine factors that correlate with the progression of dementia. Anosognosia, with which this study proposes concurrence with FMP, also correlates positively with the progression of the disease. However, veridi-

cal memory impairment correlates negatively with FMP and positively with the progression of the disease. From these established trends, the intuitive conclusion that anosognosia and veridical memory impairment correlate positively can be drawn (by comparing the relationships of each with disease progression), as can the conclusion that they correlate negatively (by comparing the relationships of each with FMP)—notably, the relationship must be modulated by a spurious variable in either case. Research has previously proposed cognitive reserve as that spurious variable, reporting that low levels of cognitive reserve correlate with earlier onset of anosognosia and supporting the former conclusion [28]. Thus, records of FMP within both early and late stages of the disease exist; in early stages of the disease veridical memory availability presents a risk factor for FMP, and in late stages of the disease anosognosia does the same, despite a recorded decline in FMP among patients with more severe dementia [4, 14]. FMP related to anosognosia in late stages of dementia would offset this decline. Though veridical memory availability is certainly more desirable than anosognosia as a quality, clearly treatment design must take into consideration both the level of anosognosia and the level of veridical memory availability to be optimal.

Within the current body of literature are a number of studies that propose and review potential treatments. Martin proposes that a treatment targeting the reduction of FMP and simultaneous preservation of FMP is possible, offering proactive education and warning of patients about particularly sensitive phenomena as a candidate [15]. Martin's research is corroborated by Gallate et al., who propose inhibition of left anterior temporal lobe activity through magnetic pulse stimulation as a mechanism of reducing semantically related FMP (the left anterior temporal lobe is largely responsible for semantic memory) without impacting the availability of veridical memory [8]. Gallo et al. propose that pairing words (i.e. phonological stimuli with pictures (i.e. visuospatial stimuli), thereby involving more neural pathways, could potentially reduce the frequency of FMP occurrence [9], though a cohort study by Pierce et al. providing contrary evidence must be noted [20]. Ross, Spencer, Blatz, & Restorick propose collaboration between couples as a way to introduce conscious error-monitoring into the memory retrieval process [25]; however, this method is reactive to FMP, not proactive prevention. Finally, Romberg et al. propose memantine as a pharmacological intervention with demonstrable results in mice [23]. Though whether the FMP-related effects of memantine translate to human subjects is unclear, memantine is already an approved medication for moderate-to-severe Alzheimer's disease and Lewy body dementia.

**Conclusion**

Ultimately, the initial purposes of this literature review have been fulfilled. Several risk factors for FMP have been examined and summarized. Based on the findings outlined above and the conclusions drawn from them, one may conclude that the experimental development of a clinical, multidisciplinary intervention for FMP in dementia patients is both possible and necessary. Of particular note among the risk factors for FMP is anosognosia, which is likely to be causally linked with FMP but has yet to be studied with the purpose of establishing such a link.

Despite its limited size, the present body of literature reveals significant insight into the development of a model of diagnosis and customizable intervention for FMP and anosognosia in dementia. The several propositions for treatments and interventions that already exist corroborate this review's initial proposal that such a model is possible. The prevalence of related phenomena and nature of clinical correlates lend importance to the study of FMP and anosognosia. Through established relationships with its underlying cognitive mechanisms, anosognosia is related—potentially causally—with FMP. Finally, the available information has been compiled and analyzed to create a suitable primer for further experimental study of such models of diagnosis and customizable intervention. Directions for further research into the design of targeted interventions for patients experiencing FMP or risk factors associated with FMP have been outlined through the examination of potentially troublesome variables (rigidity of the intervening routine, stage of disease progression, and availability of veridical memory and cognitive reserve) as well as through the summarization of existing prototypical interventions.

**References**

[1] Abe N, Fujii T, Nishio Y, Iizuka O, Kanno S, Kikuchi H, Takagi M, Hiraoka K, Yamasaki H, Choi H, Hirayama K, Shinohara M, & Mori E. (2011). False item recognition in patients with Alzheimer's disease. Neuropsychologia: 49(7), 1897–902.
[2] Berwig M, Leicht H, & Gertz HJ. (2009). Critical evaluation of self-rated quality of life in mild cognitive impairment and Alzheimer's disease--further evidence for the impact of anosognosia and global cognitive impairment. The Journal of Nutrition, Health & Aging: 13 (3), 226–30.
[3] Budson AE, Sullivan AL, Daffner KR, & Schacter DL. (2003). Semantic versus phonological false recognition in aging and Alzheimer's disease. Brain and Cognition: 51(3), 251–61.
[4] Ciaramelli E, Ghetti S, Frattarelli M, & Làdavas E. (2006). When true memory availability promotes false memory: evidence from confabulating patients. Neuropsychologia: 44(10), 1866–77.
[5] Clare L, Nelis SM, Martyr A, Roberts J, Whitaker CJ, Markova IS, Roth I, Woods RT, & Morris, RG. (2012). The influence of psychological, social and contextual factors on the expression and measurement of awareness in early□stage dementia: Testing a biopsychosocial model. International Journal of Geriatric Psychiatry: 27(2), 167–177.
[6] Conde-Sala JL, Reñé-Ramírez R, Turró-Garriga O, Gascón-Bayarri J, Juncadella-Puig M, Moreno-Cordón L, Viñas-Diez V, & Garre-Olmo J. (2013). Clinical differences in patients with Alzheimer's disease according to the presence or absence of anosognosia: Implications for perceived Quality of Life. Journal of Alzheimer's Disease: 33(4), 1105–1116.
[7] de Boysson C, Belleville S, Phillips NA, Johns EK, Goupil D, Souchay C, Bouchard R, & Chertkow H. (2011). False recognition in Lewy-body disease and frontotemporal dementia. Brain and Cognition: 75(2), 111–8.
[8] Gallate J, Chi R, Ellwood S, & Snyder A. (2009). Reducing false memories by magnetic pulse stimulation. Neuroscience Letters: 449(3), 151–4.
[9] Gallo, DA, Chen JM, Wiseman AL, Schacter DL, & Budson AE. (2007). Retrieval monitoring and anosognosia in Alzheimer's disease. Neuropsychology: 21 (5), 559–68.
[10] Hildebrandt H, Haldenwanger A, & Eling P. (2009). False recognition correlates with amyloid-beta (1-42) but not with total tau in cerebrospinal fluid of patients with dementia and mild cognitive impairment. Journal of Alzheimer's Disease: 16(1), 157–65.
[11] Hildebrandt H, Haldenwanger A, & Eling P. (2009). False recognition helps to distinguish patients with Alzheimer's disease and amnestic MCI from patients with other kinds of dementia. Dementia and Geriatric Cognitive Disorders: 28(2), 159–67.
[12] Kalenzaga S, & Clarys D. (2013). [Relationship between memory disorders and self-consciousness in Alzheimer's disease]. Gériatrie et Psychologie Neuropsychiatrie du Vieillissement: 11(2), 187–96.
[13] Langdon R, & Bayne T. (2010). Delusion and confabulation: mistakes of perceiving, remembering and believing. Cognitive Neuropsychiatry: 15(1), 319–45.
[14] Lavoie DJ, Willoughby L, & Faulkner K. (2006). Frontal lobe dysfunction and false memory susceptibility in older adults. Experimental Aging Research: 32 (1), 1–21.
[15] Martin, DB (2008). The effect of warning and instruction on the suppression of false memories in normal aging, mild cognitive impairment, and dementia of the Alzheimer's type. Dissertation Abstracts International: Section B: The Sciences and Engineering: 68.
[16] Mendez MF, Fras IA, Kremen SA, & Tsai PH. (2011). False reports from patients with frontotemporal dementia: delusions or confabulations? Behavioural Neurology: 24(3), 237–44.
[17] Moulin C. (2013). Disordered recognition memory: recollective confabulation. Cortex: A Journal Devoted to the Study of the Nervous System and Behavior: 49(6), 1541–52.
[18] Moulin C, Conway MA, Thompson RG, James N, & Jones RW. (2005). Disordered memory awareness: recollective confabulation in two cases of persistent déjà vecu. Neuropsychologia: 43(9), 1362–78.
[19] O'Connor AR, Lever C, & Moulin C. (2010). Novel insights into false recollection: A model of déjà vécu. Cognitive Neuropsychiatry: 15(1-3), 118–144.
[20] Pierce BH, Waring JD, Schacter DL, & Budson AE. (2008). Effects of distinctive encoding on source-based false recognition: further examination of recall-to-reject processes in aging and Alzheimer disease. Cognitive and Behavioral Neurology : Official Journal of the Society for Behavioral and Cognitive Neurology:

21(3), 179–86.
[21] Plancher G, Guyard A, Nicolas S, & Piolino P. (2009). Mechanisms underlying the production of false memories for famous people's names in aging and Alzheimer's disease. Neuropsychologia: 47(12), 2527–36.
[22] Rabins PV, Lyketsos CG, & Steele CD. (2006). Practical dementia care. (2nd ed.). New York: Oxford University Press.
[23] Romberg C, McTighe SM, Heath CJ, Whitcomb DJ, Cho K, Bussey TJ, & Saksida LM. (2012). False recognition in a mouse model of Alzheimer's disease: rescue with sensory restriction and memantine. Brain : A Journal of Neurology: 135(Pt 7), 2103–14.
[24] Rosen HJ. (2011). Anosognosia in neurodegenerative disease. Neurocase: 17(3), 231–241.
[25] Ross M, Spencer SJ, Blatz CW, & Restorick E. (2008). Collaboration reduces the frequency of false memories in older and younger adults. Psychology and Aging: 23(1), 85–92.
[26] Sommers MS, & Huff LM. (2003). The Effects of Age and Dementia of the Alzheimer's Type on Phonological False Memories. Psychology and Aging: 18(4), 791–806.
[27] Spalletta G, Girardi P, Caltagirone C, & Orfei MD. (2012). Anosognosia and neuropsychiatric symptoms and disorders mild Alzheimer disease and mild cognitive impairment. Journal of Alzheimer's Disease: 29(4), 761–772.
[28] Spitznagel MB, & Tremont G. (2005). Cognitive reserve and anosognosia in questionable and mild dementia. Archives of Clinical Neuropsychology: 20 (4), 505–515.
[29] Turró-Garriga O, Conde-Sala J, Reñe-Ramírez R, López-Pousa S, Gascón-Bayarri J, & Garre-Olmo J. (2013). [Prevalence of anosognosia in Alzheimer's disease]. Medicina Clinica: 13, 353-9.
[30] Turró-Garriga O, Garre-Olmo J, Vilalta-Franch J, Conde-Sala JL, de Gracia Blanco M, & López-Pousa S. (2013). Burden associated with the presence of anosognosia in Alzheimer's disease. International Journal of Geriatric Psychiatry: 28(3), 291–7.
[31] Turró-Garriga O, López-Pousa S, Vilalta-Franch J, & Garre-Olmo J. (2012). [Evaluation of anosognosia in Alzheimer's disease]. Revista de Neurologia: 54 (4), 193–8.

**About the Author**

Sam Creden is a junior at The University of Alabama from Chicago, Illinois majoring in Biology and Psychology with a minor in Blount liberal arts. He currently works in Dr. Ryan Earley's Integrative Animal Behavior laboratory, where he examines the physiological effects of stress on mangrove rivulus. Previously, he has worked in Dr. Will Hart's Social Cognition laboratory. Sam's research interests lie in bringing several disciplines—among them endocrinology, toxicology, oncology, and psychiatry—together, and hesincerely hopes to someday study as a physician scientist. Outside of school, he enjoys working as a resident assistant in Paty Hall, volunteering wherever he can, and spending his free time playing board games.

# Denisovans: From a Pinky to a People

Trever Chidester

*Every so often, the paleoanthropological world is surprised with a finding that rewrites the books. With new advances in DNA analysis, researchers are finally able to make the most out of the smallest finds. Deep in a Siberia Cave, a new discovery has caused a huge uproar in what was thought to be the hominid community of the paleolithic world. Here, the discovery, identification, and analysis of a new hominid species is explained in its entirety.*

Three hundred years before a remote, Siberian cave would make headlines in the scientific world, the nearby village told stories of a banished hermit named Denis, who took shelter in the large, three-chamber grotto. It was a cozy home, just up the mountain from a flowing river and fully equipped with a naturally made chimney. Little did Denis know that he had not been the first resident of the cave that would eventually bear his name. In fact, over the last 100,000 years, he would be one of three hominid species to ever take refuge in the perfect prehistoric home. One of those hominid species had never before been seen, but they would eventually take the name of Denisovans. The recent discovery of this relatively young hominid has blown up in the paleoanthropological world. With new advances in DNA analysis, researchers have been able to reconstruct entire genomes from just a pinky bone. Although the genome has opened up many doors for interpreting the species, traditional methods of researching the specimen are very limited due to the small amount of fossilized remains discovered. Nevertheless, researchers have already imaged an entirely new paleolithic world with the information present. Possibly one of the most important discoveries in recent paleoanthropology, a finger bone and millions of base pairs decoded from ancient DNA support the uncovering of a new hominid species known as Denisovans that are claimed to have interacted with *Homo neanderthalensis* and *Homo sapiens* across the Eurasian continent.

Since the beginning of the search for human origins, each discovery has had an "underdog" story of how a new species came to be known. Finding a small sliver of a bone in the Altai Mountains of southern Siberia is nothing to celebrate for an archaeologist wanting to make history. Ironically, the tiny fossil dug up by Alexander Tsybankov in 2008 would be the biggest find of his life [8]. The young, Russian archaeologist decided to take a shot in the dark by sending in the piece of bone to be identified. Based on the small grooves, researchers concluded that it must have once served as the fingertip to a primate. Because of what they knew about the fossil record and geographic distribution of primates, the fossil had to be from a hominid [8]. After DNA analysis, it was discovered that this small finger bone belonged to a previously unknown hominid species. This little pinky bone was so well preserved that analysts were able to extract seventy percent of the organism's DNA. Most fossilized bones only contain about five percent of the ancient DNA because of degradation [5]. Remarkably, the organism's entire genome was able to be sequenced from the small remains.

Of course, a discovery such as this would trigger a frenzy of archaeological digs in the Denisova cave, all with intensions of finding more specimens. If the site were not already remarkable enough, a small toe bone and two teeth were discovered and all assumed to be Denisovan. To the surprise of the most highlighted ancient DNA geneticists, Svante Pääbo, the small toe bone had actually belonged to a distant cousin, known as *Homo neanderthalensis* [4]. Just when they thought the gold mine at Denisova could not get any better, several tools and a beautifully crafted bracelet were discovered in the same level as the bones, indicating that early humans once occupied the cave as well [4]. Pääbo described the Denisova cave as "the one spot on Earth that we know of where Neanderthals, Denisovans, and modern humans all lived" [8]. Right now, this cave is the only evidence we have of the Denisovans' existence. However, with the information that was drawn from population genetics and DNA comparisons, there is a lot to be said about this ancient hominid species.

Paleoanthropology has come an extremely long way to be able to tell so much about an unknown

specimen from just the pinky bone. Because of the DNA, geneticists can tell that the finger bone belonged to a Stone Age girl who lived around 75,000 years ago. The growth plate on the finger was not entirely fused, which indicated that the girl was around six or seven years old when she died of some unknown cause [1]. Although it is difficult to know what exactly the species may have looked like, some of the alleles present in the genome suggest that the girl probably had dark skin, as well as brown hair and eyes [2]. Looking at the surrounding environment of the Altai Mountains, it can be assumed that the girl's "tribe" likely hunted bear, lynx, and wild boar. The Denisova cave was most likely a winter home during the months of harsh cold and was perfect for fires as indicated by the large hole in the top of the cave [4].

Other than small indications of the individual's life, the genome can also give a broad view of different characteristics of the species as a whole. Through a thorough analysis of Denisovan and modern human's genomes, researchers found that a set of genes that is involved with brain function in hominids is present in both genomes. While the Denisovans do not possess all of the complex genes for brain functioning that *Homo sapiens* do, the new hominid group does prove to have a specific gene that is involved with speech. It is hard to tell if the Denisovans had a primitive type of language, but there is no doubt that they had the ability to have one [1]. The genome also indicates that the species had relatively low genetic diversity. By splitting the DNA of the young girl to generate each of her parent's DNA, geneticists can tell how genetically different her parents were. Denisovans had a significant one-third less variation within their species compared to *Homo sapiens*. While some might suggest that inbreeding was the cause for the low variation, geneticists were able to localize where the similarities occurred. Because the matching sequences occurred scattered across the strands of DNA, inbreeding was not a factor [8]. This suggests that the Denisovans were most likely a low populated group. Ancestors of the Denisovans split with what would become modern humans around 500,000 years ago in Africa [8]. It is now believed that a group of *Homo heidelbergensis* left Africa, spread around Eurasia, and evolved into the Neanderthals and Denisovans. *Homo heidelbergensis* is said to be the common ancestor of these three hominids [7].

By looking at the three genomes and comparing them with each species' number of mutations using a chimpanzee genome as the control, geneticists can estimate the time a species may have diverged. This is made possible by looking at the rate of mutation and applying it to the genomes [5]. Humans have less genetic similarity with chimpanzees because there has been more time for *Homo sapiens* to accumulate mutations. These new technologies in DNA analysis are what generated the dates to when Denisovans may have become a new species. With that being said, the little girl would have never been able to be distinguished as her own species with the use of traditional comparative methods. The Denisovans are the first hominid species to be classified based on DNA analysis [5].

Without significant advances in these technologies, generating an entire genome from a pinky would have never been possible. When the Neanderthal project first started in 1997, only 360 base pairs were able to be extracted from the fossilized tissue. With the new discovery in 2010, over 5.5 billion base pairs were able to be uncovered. This is due to both an advancement in sequencing the DNA and the ability to draw a genome using a smaller sample size. Similarly, cost efficiency has also played a role in the genome project. In 2005, decoding one base pair cost $1,000. This would have made sequencing the Denisovan genome indubitably impossible to afford. However, in a matter of just five years, the Denisovan's base pairs were able to be decoded at just ten cents a pair [6]. This monumental decrease in cost is what has made the entire identification of this new species possible.

A site like the Denisova cave that is known to have housed three different hominid species begs two very important questions: Did the hominids live in the cave at the same time, and did they interbreed with one another? While dating techniques are not advanced enough to tell if the hominids occupied the cave at the exact same time, DNA analysis can tell a great deal about introgressed DNA, the small amounts of DNA that are originally from one species but also found in another [3]. It is extremely difficult to dispute the fact that *Homo sapiens* once bred with their

distant hominid cousins, the Neanderthals and the Denisovans. DNA of modern humans outside of Subsaharan Africa contain up to four percent Neanderthal DNA. Similarly, populations in Oceania contain up to six percent Denisovan DNA [6]. The presence of these early hominids' DNA in our genome is all the evidence needed to show that *Homo sapiens* are a product of more than just "Out of Africa" evolution. Instead, the site at Denisovas highly supports the gradual and complex evolution of *Homo erectus* to *Homo sapiens* known as multiregionalism [4]. While this has been the minority view in the paleoanthropology world for a long time, this new evidence might support the hypothesis that our origins are a mosaic of different ancient hominids [1].

When looking at the relatively small amount of DNA that modern humans could have inherited from Neanderthals and Denisovans, many would wonder why this number is at all significant. On the contrary, these foreign genes have been shown to be very advantageous for the human race. As mentioned earlier, DNA comparisons show that Denisovans share up to "eight human-specific genes that code for particular brain functions" that have led to the advancement of the hominids [1]. Similarly, some genetic variations from these prehistoric peoples are linked to immunity, which would have been essential to the global spread of *Homo sapiens* [6]. According to the evolutionary theory, genetic mutations are what gives rise to new adaptations. It is very possible that these specific, shared mutations would have been inherited from these early hominids.

While Denisovans were not quite as wide spread as the species of hominids that covers the planet today, their range played a key role in determining who they bred with and where they bred with them. The DNA evidence suggests, without a doubt, that the early hominids were quite friendly with each other in the sense of reproducing. One paleoanthropologists suggests that the paleolithic Eurasia is "looking at a Lord of the Rings-type world — that there were many hominid populations" [2]. This implies that a great deal of interaction occurred, and by looking at the genomes, the locations of those interactions can be identified. Genomes of the native Australian Aborigines and Melanesians explicitly show the presence of Denisovan DNA. But, to many geneticists' surprise, mainland Asians including Chinese and Mongolians show no trace of Denisovan DNA [1]. Since the little girl was discovered in a cave in southern Siberia, the information does not seem to line up. However, just like anything else in human evolution, the Denisovans and their dispersal is a puzzle that invites a number of hypotheses.

The biggest controversy in dealing with the populations genetics of Denisovans is whether or not this hominid species was able to cross the Wallace line. One of the largest biogeographical disjunctions in the world, the Wallace line runs right through southeast Asia and the Oceania islands. This strong ocean current has prevented all placental mammals from crossing into Australia except for modern humans and rodents. Playing a significant role in the evolution and competition of the region, the line has kept the east dominated by marsupials while the west is inhabited by the placental mammals [3]. This is extremely important to consider when the only Denisovan fossils are west of the line while the only genetic evidence of their existence is east of the line. Some suggest that Denisovans had to have crossed the Wallace line because of the lack of their introgression with mainland, modern humans. Even the genome from an ancient Chinese *Homo sapiens* supports the fact that Denisovans did not interbreed with the group that would populate China and the rest of Asia [3]. Others have believed that the recently discovered Hobbit man or *Homo floresiensis* could have been a "regional representative" of the Denisovans. However, this hypothesis is highly unlikely due to the two species genetic divergence [3]. Another analysis gives a contradicting theory that claims that the Denisovans were not able to cross the Wallace line. Instead it suggest that a primary group of *Homo sapiens* spread throughout Asia and interbred with Denisovans on the mainland. Then, when another exodus of *Homo sapiens* out of Africa swept through Europe and Asia, it forced the previous "hybrids" to move south to Australia and the surrounding island. This implies that modern humans carried the Denisovans' genes across the Wallace line without the hominid group ever making the trip themselves [5]. Of course, these hypotheses are extremely premature and will only become more accurate as more fossil evidence is uncovered.

The future of paleoanthropology looks extremely promising in the search to solve the puzzle of human origins. Thanks to the advances in technologies and the increase in cost efficiency, more and more specimens that are uncovered can be analyzed and added to the massive hominid genome project. Some paleogeneticists like Pääbo believe that "there will be more" and early hominid fossil hunting will shift to the east to uncover more about modern humans [4]. While it is obvious that most of the future in paleogenetics still has yet to be dug up, there is still much to be done with what is already available. By sequencing more living African populations, who were unaffected by introgression, the traits that are inherited from African ancestry and traits from inbreeding in Eurasia can be more easily distinguished [6]. Nevertheless, there is much to be done in the field and in the lab so that it may be understood what made *Homo sapiens* different.

From just the fingertip of a young girl to the billions of base pairs of a once unknown hominid species, paleoanthropology is even closer to discovering the mystery of the complex lineages that are human evolution. It is remarkable that the technology today is close to reconstructing, from just her pinky, a little girl that once roamed the earth 75,000 years ago. While looking at the hominid interactions thousands of years ago may seem irrelevant today, the introgression that once occurred can tell a great deal more about *Homo sapiens* than was ever thought imaginable. Understanding the small differences in these genomes can give insight into why Neanderthals do not roam the world today and why modern humans did not go extinct. With Denisovans and their genome, paleoanthropology is one step closer to finding out what makes *Homo sapiens* the species that lived.

**References**

[1] Bower, Bruce. 2012. "Denisovan DNA reveals history of enigmatic group." Science News 182
[2] Callaway, Ewen. 2013. "Mystery Humans Spice Up Ancients' Sex Life". Nature.
[3] Cooper, A. and Stringer, C. B. 2013. "Did the Denisovans Cross Wallace's Line?" Science. 342 (6156), 321-323.
[4] Gibbons, Ann. 2011. "Who Were the Denisovans?". Science. Vol. 333 no. 6046 pp. 1084-108
[5] Harmon, Katherine. 2012. "New DNA Analysis Shows Ancient Humans Interbreed With Denisovans". Scientific American.
[6] Hawks, John. 2013. "Significance of Neandertal and Denisovan Genomes in Human Evolution" Annual Review of Anthropology, Vol. 42: 433 -449
[7] National Geographic. 2013. "Why am I Denisovan" <https://genographic.nationalgeographic.com/denisovan/>
[8] Shreeve, Jamie. 2013. "The Case of the Missing Ancestor." National Geographic 224, #1

**About the Author**

Trever Chidester is a sophomore at the University of Alabama from Detriot, MI. He is studying Anthropology and History with a minor in Evolutionary Studies. Much of his time at Alabama is balancing research in Anthropology and History. In the coming fall, he will be the Vice President of the Evolutionary Studies Club. He hopes to continue his studies and one day receive a joint PhD in the two fields.

# The Grey Whale (*Eschrichtius robustus*) and Nutrient Cycling - the Effect of the "Ocean's Bulldozers" on their Habitat

Anna P. Hawkins[1]

[1]*Department of Biological Sciences, Box 870344, University of Alabama, Tuscaloosa, AL, 35487*

*The grey whale (Eschrichtius robustus) is a bottom-feeding species of whale in the suborder Mysticeti that feeds by scraping its baleen plates along the seafloor. This feeding practice of the grey whale is unique to the species and releases very large amounts of marine sediment into the water column. Because of this, it is possible that grey whales have a significant impact on nutrient cycling in their habitats. The Eastern population of grey whales feeds in the Bering Sea and is thought to be recovered from exploitation (~20,000 individuals). In the present study, this population of whales has been found to release approximately 3.85 billion cubic meters of sediment into the water column every year. The Western population of grey whales is still considered severely depleted (~100 individuals) and feeds along the coasts of Russia, China, Korea, and Japan, and was found to release about* $1.93 \times 10^7$ *cubic meters of sediment per year. In comparison, abyssal storms only release about 14,000 kg of sediment per year. The sediments that grey whales release in the Bering Sea are especially nutrient-rich. They are enriched in silicon dioxide, iron, phosphorus, organic carbon, and nitrogen. It is therefore reasonable to conclude that grey whales have a positive localized effect on nutrient cycling and productivity (particularly in benthic communities) in their two feeding grounds.*

## Introduction

Grey whales (*Eschrichtius robustus*) feed primarily on bottom-dwelling amphipods and other benthic infauna by scraping their baleen plates along the sea floor [9]. This process dislodges edible and inedible materials alike, and leaves depressions along the otherwise flat bottom. These depressions are elliptical in shape (Fig. 1) and are quickly colonized by species that are regularly observed as early colonists of disturbed areas of seafloor [1, 8] As the grey whales feed, they release large volumes of sediment into the water column. In 1983, Nerini and Oliver published a study on grey whales and their effect on the structure of the Bering Sea floor, claiming that grey whales annually turn over 9-27% of Bering Sea sediment, and in 1984, Johnson and Nelson stated that "the volume of sediment injected into the water column [by the population of whales in the Bering Sea] is at least 1.2 billion cubic meters per year," which is over twice the yearly sediment load of the Yukon River.

Because the grey whale population in the Bering Sea releases such large amounts of sediment into the water column every year (Fig. 2), some scientists believe that grey whales have a significant impact on nutrient cycling and overall productivity in their

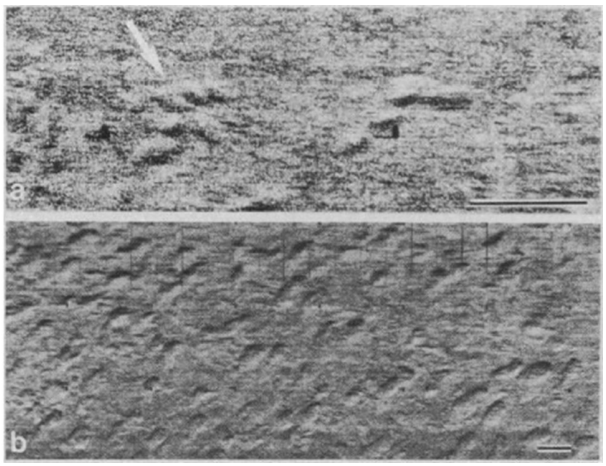



**Figure 1:** Side-scan sonar image of grey whale feeding troughs. (a) shows fresh feeding troughs and (b) shows troughs that are quite a bit older and have been enlarged by the Bering Sea currents. Scale bars represent 10 meters. Taken from Johnson and Nelson (1984).

environment. Johnson and Nelson (1984) state that "grey whale feeding causes recycling of nutrients that would otherwise be trapped in sediment" and that the whales are most likely significant contributors to the high productivity of the Bering Sea benthos [5]. Anderson and Lovvorn (2008) agree, stating that grey whale feeding may release enough nutrients to allow

the populations of benthic invertebrates to increase over long periods of time, which increases the ecosystem's productivity throughout the food web [1]. The objective of the present study is to more exactly determine the extent to which grey whales affect nutrient cycling and overall ecosystem productivity. It is hypothesized that grey whales release a significant amount of nutrients into the water column and have an important positive impact on the overall productivity and health of their environment.


**Figure 2:** Aerial view of grey whale feeding behavior. This image shows the plumes of sediment created in shallow water as the whale scrapes its baleen plates along the bottom. Image captured by Bruce G. Marcot and published online at http://www.taos-telecommunity.org/epow/EPOW-Archive/archive_2009/EPOW-091102.htm

Fossil records have shown that in the past there may have been a population of grey whales in both the Atlantic and the Pacific Oceans, but now, due in part to exploitation by humans, the Atlantic population is extinct and the Pacific population has been divided (Fig. 3) into Western and Eastern segments [11]. The Eastern population of grey whales consists of about 20,000 individuals and is said to be fully recovered from whaling [11]. This population feeds in the Bering Sea every summer and migrates along the West coast of the United States in winter [11]. The Western population of grey whales lives along the coasts of Russia, China, Korea, and Japan and is still considered to be critically depleted [11]. This population consists of only about 100 individuals [11]. The present study examines the amount of sediment released into the water column annually by both grey whale populations. It is hypothesized that the Eastern population releases significantly more sediment into the water column every year, simply because that population is so much larger than the Western population.

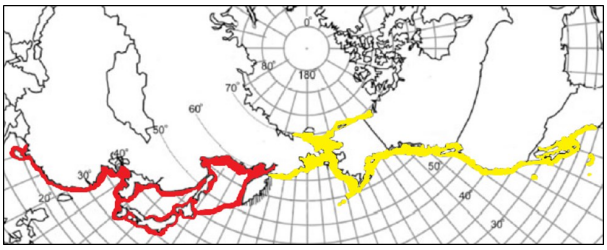


**Figure 2:** Aerial view of grey whale feeding behavior. This image shows the plumes of sediment created in shallow water as the whale scrapes its baleen plates along the bottom. Image captured by Bruce G. Marcot and published online at http://www.taos-telecommunity.org/epow/EPOW-Archive/archive_2009/EPOW-091102.htm

Additionally, the nutrient content of Bering Sea sediment was examined in order to determine whether the grey whales are significantly helping to increase productivity or whether their feeding practices have a negligible effect on productivity. According to März et al. (2013), silicon dioxide makes up about 60-80 weight percent of the sediment in the Bering Sea. Concentrations of other nutrients, such as iron, organic carbon, nitrogen, and phosphorus, were studied by Zhang et al. in 2009, and it was found that Bering Sea sediments are relatively rich in all of these nutrients [7, 12]. Because of the relative nutrient-richness of the Bering Sea, it is hypothesized that grey whales contribute significantly to the overall productivity of the area.

Depending on how much grey whales affect nutrient cycling in their feeding ranges, their disappearance from that area could have broad effects on the ecosystem as a whole. The overall purpose of the present study is to determine how much sediment grey whales release into the water column per year and their effect on the productivity of their environment.

**Materials and Methods**

The information and numerical data required for the present study were obtained from published sources released to online databases. The databases utilized for this study were Web of Science, SCOUT, and JSTOR. Published resources were collected using a methodical approach. Information obtained included resources about grey whales and their feeding behavior, nutrients and nutrient cycling in the Bering Sea, and the sediment released by abyssal storms. All of the sources were reviewed and relevant information extracted.

The different parts of the posed question were approached methodically. The information collected from the papers was used to aid in drawing conclusions about the questions posed at the start of this project. As very little previous research has been done on this subject specifically, some of the published resources were used to back up claims made in others. However, the most important part of this project was the calculations done based on the limited numerical data available.

Calculations were performed to determine the approximate volume of sediment released into the water column by grey whales each year. Average dimensions of the troughs made by the whales were obtained from Johnson and Nelson (1984) and were approximately equal to dimensions reported by Nerini and Oliver (1983) [5, 8]. In both papers, the troughs were reported to be elliptical in shape. Johnson and Nelson reported an approximate value for the amount of sediment released by grey whales per year (at least $1.2 \times 10^9$ $m^3$ $year^{-1}$, which they claimed to be over twice the yearly sediment load of the Yukon River), but they provided no methods for their calculations, and their estimate was based on an old estimate of grey whale population size (approximately 16,000 individuals) [5]. However, they did make reports of the mean number of days that grey whales spend feeding per year, the average concentration of edible infauna on the Bering Sea floor, and the mean daily food intake of the grey whale. Using these values, the approximate volume and mass of the sediment released by grey whales was calculated for the Bering Sea (Eastern) population of approximately 20,000 individuals [11]. The methods for these calculations could have differed from those used in Johnson and Nelson's study. The authors do not report whether they used the volume of a whole ellipse with the dimensions given, or if they used the volume of half an ellipse. For the purposes of this study, it was assumed that the seafloor was flat, which would mean that each feeding trough would represent a displaced volume of sediment equal to half of the volume contained in a 3D ellipse of equal size. Therefore, the volume of a half ellipse was used in the relevant calculations. The same calculations were performed for the Western population of about 100 individuals [11]. As there is very little data available on the Western population and the nature of its habitat, the same values used for the Bering Sea population had to be used. The only difference was the number of whales the calculation was scaled to. It can be assumed that the calculations are reasonably accurate approximations of the amount of sediment released into the water column per year by both populations of grey whales.

Other calculations that were performed include the approximate settling time for the sediment released, the approximate percentage of the grey whale feeding area that is disturbed every year, the approximate amount of silica released into the water column by grey whales, and how the approximate amount of sediment released by benthic storms compares to the amount of sediment released by grey whales.

**Calculation Methods**

These calculations were performed with the assumption that grey whales only feed in the summer months and do not feed at all while they're migrating. While there are some reports of grey whales feeding during migration, the prevailing scientific opinion is that grey whales only feed at their summer feeding grounds [9]. Also, in calculating the weight of the sediment released, the density of silica ($2.95$ g $mL^{-1}$) was used to approximate the density of the sediment as a whole, because the sediment in the Bering Sea is 60-80% silica [7].

*Approximate Amount of Sediment Disturbed by Grey Whales*

In order to calculate the amount of sediment

disturbed per year, equations one through eight (Table 1) were utilized in chronological order. The values shown in Table 2 for days spent feeding per year, food concentration on the Bering Sea floor, and daily food intake were also used. First, the approximate area of the troughs made by grey whales was calculated (Equation 1, Table 1) using the dimensions summarized in Table 4. That area was used to calculate the approximate amount of food obtained per trough using Equation 2 (Table 1). The average number of troughs made by one whale per day was calculated using the amount of food obtained per trough and Equation 3. This value was multiplied by the number of days spent feeding per year (Equation 4), and then multiplied by the number of individuals in the population (Equation 5). Then, the approximate volume of the troughs was calculated using Equation 6. This result was multiplied by the number of troughs made per year (Equation 7) in order to determine the approximate volume of sediment released per year. The average weight of this volume of sediment was determined using Equation 8.

*Approximate Percentage of the Grey Whale Feeding Range Disturbed*

This calculation was completed using the approximate area of the grey whale feeding range as reported by Johnson and Nelson in 1984 (Table 2) and Equations 9 and 10 (Table 1) [5].

*Sediment Settling Time Using Stokes's Law*

This calculation was completed using Stokes's Law (Equation 11, Table 1) and the variables summarized in Table 3. Then, the minimum and maximum depths of grey whale feeding areas from Swartz et al. (2006) were divided by the rate determined by Stokes's Law in order to determine the maximum and minimum times that it would take for the sediment to settle [11].

*Approximate Amount of Silica Released by Grey Whales per Year*

The amount of sediment released per year was calculated using Equation 12 (Table 1). The value for the percentage of silica in the sediment is 70%, as reported by März et al. (2013) [7].

| Equation Number | Equation Details | Used to find the: |
|---|---|---|
| 1 | $A_{ellipse} = \pi(r_1)(r_2)$ | Area of an ellipse |
| 2 | (mean food concentration) × (mean trough area) | Average amount of food obtained per trough |
| 3 | (daily food intake) / (amount of food obtained per trough) | Average number of troughs per day |
| 4 | (average number of feeding days per year) × (number of troughs per day) | Average number of troughs per year |
| 5 | (troughs per year) × (population size) | Upscaling to reflect population size |
| 6 | $V_{half\ ellipse} = (1/2)(4/3)\pi(r_1)(r_2)(r_3)$ | Half of the volume of an ellipse |
| 7 | (average volume per trough) × (average troughs per year) | Average volume of sediment released per year |
| 8 | (volume of sediment) × (density of sediment) | Average weight of sediment released per year |
| 9 | (feeding area) × (average trough depth) | Maximum volume of sediment that can be released |
| 10 | (actual volume suspended) / (maximum volume) × 100 | Percent of feeding area sediment resuspended |
| 11 | $v_s = \frac{2}{9}\frac{(\rho_p - \rho_f)}{\mu}(g)(R^2)$ | Sediment settling time (Stokes's Law) |
| 12 | (total amount of sediment released) × (0.7) | Amount of silica released per year |
| 13 | (concentration of sediment release) × (volume of water) | Amount of sediment released per day by a benthic storm |

**Table 1:** All the equations used for calculations in this study, along with brief descriptions of their use. All equations are referred to in the text by their given number.

*Approximate Amount of Sediment Released by Benthic Storms*

There is not much information available on the amount of sediment stirred up by benthic storms, so these calculations were slightly more difficult to perform. In order to determine the amount of sediment released by these storms, Equation 13 was used (Table 1), along with the values for the concentration of sediment released in a benthic storm and the depth of benthic storms. First, the volume of water in a one-square-meter area of water over a 5,000 meter deep benthic storm was calculated. That result was then multiplied by the concentration of sediment released in a storm (Equation 13) to determine the amount of sediment released per day by a benthic storm. Since most ben-

| Value Name (all values are approximate) | Value and Units |
|---|---|
| Days spent feeding per year | 180 days |
| Concentration of food on the Bering Sea floor | 171 g $m^{-2}$ |
| Daily food intake | 1100 kg $day^{-1}$ |
| Area of grey whale feeding range | 22,000 $km^2$ = 2.2 × $10^{10}$ $m^2$ |
| Concentration of sediment released by a benthic storm | 750 µg $L^{-1}$ per day |
| Benthic storm depth | 5000 meters |

**Table 2:** Values used in calculating the amount of sediment dispersed per year by grey whales and by benthic storms. All variables are approximate. Values for grey whale sediment dispersal are from Johnson and Nelson (1984). Values for benthic storm sediment dispersal are from Richardson et al. (1993).

thic storms can last on average for about a week [10], that result was multiplied by seven days to determine approximately how much sediment is released by one benthic storm. This value was then compared with the amount of sediment released by grey whales, in order to determine how many storms are equivalent to one year of feeding by grey whales.

**Results**

*Amount of sediment released per year*

Bering Sea Population

For the Bering Sea population of 20,000 whales, the volume of sediment released per year is equal to 3.85 × $10^9$ $m^3$ $year^{-1}$, which is equal to 1.02 × $10^{13}$ kg $year^{-1}$ (using the density of silica as the density of the sediment). This is equivalent to 70% of the feeding area in the Bering Sea, and corresponds to a release of 2.7 × $10^9$ $m^3$ $year^{-1}$ of silica. In comparison, Johnson and Nelson estimated that grey whales suspended 1.2 x $10^9$ $m^3$ $year^{-1}$ with a population estimate of 16,000 whales [5]. Using this value in place of the current value and performing the same calculation, the whales would disturb only 21.82% of their feeding area per year.

| Variable Name | Value and Units |
|---|---|
| $\rho_p$ (particle density) | 2.65 g $mL^{-1}$ |
| $\rho_f$ (fluid density) | 1.025 g $mL^{-1}$ |
| g (gravitational acceleration) | 9.81 m $s^{-2}$ |
| R (particle radius) | 200 µm = 200 × $10^{-6}$ m |
| $\mu$ (kinematic viscosity of seawater) | 1.05 × $10^{-6}$ $m^2$ $s^{-1}$ |
| depth of feeding | 5-15 meters |

**Table 3:** Variables needed for successful calculation of the sediment settling time using Stokes's Law. The value of each variable is given in column two. Seawater density, gravitational acceleration, and seawater viscosity are scientifically accepted constants. For the particle density and particle radius, the density of silica was used and the radius of an average-sized diatom was used, respectively. These values were chosen because the sediment of the Bering Sea is so enriched in silica (März et al., 2013). The depth of the grey whale's feeding areas is from Swartz et al. (2006).

Western Population

For the Western population of about 100 whales, the volume of sediment released per year is equal to 1.93 × $10^7$ $m^3$ $year^{-1}$. This is equal to 5.11 ×$10^{10}$ kg $year^{-1}$.

*Sediment settling time - Stokes's Law*

Using Stokes's Law, the settling rate for silica-enriched sediment was determined to be 0.135 m $s^{-1}$. This rate was used to determine high and low estimates for the time it takes the particles to settle, using depths of 5 meters and 15 meters. It was determined that in 5 meters of water, it takes about 37 seconds for the sediment to settle, while in 15 meters of water it takes about 111 seconds for the sediment to settle. By averaging these values, it takes approximately 74 seconds for the particles in the Bering Sea to settle (Fig. 4).

| Dimension | Value and Units |
|---|---|
| Length | 2.5 meters |
| Width | 1.5 meters |
| Depth | 0.25 meters |

**Table 4:** The approximate dimensions of the elliptical grey whale feeding troughs as reported by Johnson and Nelson (1984). To calculate the area and volume of each trough, the dimensions were converted to radii by dividing each by two (except for depth, since the depth was already a radii because the seafloor is flat).

*Amount of sediment released by benthic storms*

The average benthic storm lasts for about one week. This means that each storm releases about 26.25 kg of sediment into the water column. In comparison with the amount of sediment released by grey whales, the mass of sediment released by Eastern grey whales is equal to $3.89 \times 10^{11}$ benthic storms, while

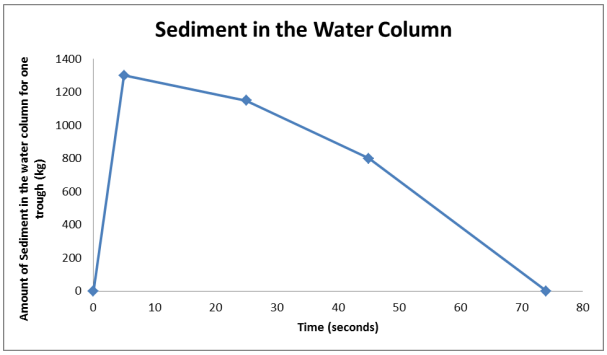



**Figure 4:** An approximate sediment-settling profile for one trough made by a grey whale. Time zero is at the start of the grey whale disturbance event. The sediment is released into the water column at once and settles back down to the bottom in an average of 74 seconds.

the mass of sediment released by Western grey whales is equal to $2.00 \times 10^{9}$ benthic storms.

**Discussion**

Based on the results presented in this paper, it is clear that grey whales release incredibly large amounts of sediment into the water column every year, and have some kind of effect on their environment. In the Bering Sea, the approximate calculated value for the amount of sediment injected into the water column ($1.02 \times 10^{13}$ kg year$^{-1}$) is equal to the mass of 31,875 Empire State Buildings; 159,375,000 blue whales; or 124,390,244 space shuttles. As a comparison, benthic storms only release about 26.25 kilograms of sediment per storm, which means that one year of grey whale feeding in the Bering Sea is equivalent to $3.89 \times 10^{11}$ benthic storms.

That huge amount of sediment is enriched with nutrients needed for survival by most organisms, and releasing those nutrients most likely has broad implications for the ecosystem as a whole. According to Nerini and Oliver (1983), grey whales may be important structuring agents in their communities that allow colonists dependent on their disturbances to flourish, and their effects may ripple far up the food chain [8]. The feeding activities of grey whales probably act a bit like human plowing - it prepares the benthos for the next year's "crop" of benthic amphipods.

The effect of grey whales is probably most easily seen in the large amount of amphipods and other benthic organisms in the Bering Sea - 171 g m$^{-2}$ [5]. However, they have been documented to have an effect on other organisms as well. Anderson and Lovvorn (2008) reported that benthic-feeding birds take advantage of grey whale feeding behavior to forage on prey that is otherwise unavailable to them [1]. Anderson and Lovvorn (2008) go on to assert that grey whale foraging helps populations of benthic invertebrates to increase over long periods of time [1]. This increase could also have an impact on the populations of amphipod-eating fish species. Since grey whales tend to feed in relatively shallow water (5-15 meters deep), it is very possible that they could have an effect on organisms higher in the water column in addition to those living in the benthos.

The Bering Sea sediments that grey whales disturb are very rich in many nutrients needed to support life. One reason for this high nutrient content could be the yearly phytoplankton blooms along the

receding ice sheet every summer. Cooper et al. (2012) state that, since populations of zooplankton are depressed at the beginning of summer from being under the ice sheet all winter, most of the organic matter from the phytoplankton bloom drifts to the bottom without being subject to zooplankton grazing [2]. Most of this organic material would remain locked in the benthic sediment and would be unusable for other organisms if it were not for grey whales arriving in the Bering Sea later in the summer and stirring up sediments.

Since the Bering Sea is relatively geographically isolated and protected from strong currents, it is safe to assume that the effect of grey whales is localized. However, their effect on nutrient cycling could quite possibly jump-start the whole food web in their feeding ranges by increasing the amount of benthic infauna that the environment can support. Without grey whales, it is possible that the productivity of the Bering Sea and the coasts of Russia, China, Japan, and Korea could decrease drastically, which would have far-reaching implications for human fishing and other activities in those regions.

**References**

[1] Anderson, E.M., Lovvorn, J.R. (2008). Gray whales may increase feeding opportunities for avian benthivores. *Marine Ecology Progress Series*, *360*, 291-296.
[2] Cooper, L.W., Janout, M.A., Frey, K.E., Pirtle-Levy, R., Guarinello, M.L., Grebmeier, J.M., Lovvorn, J.R. (2012). The relationship between sea ice break-up, water mass variation, chlorophyll biomass, and sedimentation in the northern Bering Sea. *Deep-Sea Research II*, *65-70*, 141-162.
[3] Encyclopedia Britannica. (2013). Bering Sea and Strait. *Encyclopedia Britannica Online Academic Edition*.
[4] Encyclopedia Brittanica. (2013). Stokes's Law. *Encyclopedia Brittanica Online Academic Edition.*
[5] Johnson, K.R., Nelson, C.H. (1984). Side-scan sonar assessment of gray whale feeding in the Bering Sea. *Science (New Series)*, *225*(4667), 1150-1152.
[6] Lovvorn, J.R., Cooper, L.W., Brooks, M.L., De Ruyck, C.C., Bump, J.K., Grebmeier, J.M. (2005). Organic matter pathways to zooplankton and benthos under pack ice in late winter and open water in late summer in the north-central Bering Sea. *Marine Ecology Progress Series*, *291*, 135-150.
[7] März, C., Schnetger, B., Brumsack, H.J. (2013). Nutrient leakage from the North Pacific to the Bering Sea (IODP Site U1341) following the onset of Northern Hemispheric Glaciation. *Paleoceanography*, *28*, 68-78.
[8] Nerini, M.K., Oliver, J.S. (1983). Gray whales and the structure of the Bering Sea benthos. *Oecologia*, *59* (2/3), 224-225.
[9] Rice, D.W., Wolman, A.A. (1971). The life history and ecology of the gray whale (*Eschrichtius robustus*). *American Society of Mammalogists*, 2-37 and 109-140.
[10] Richardson, M.J., Weatherly, G.L., Gardner, W.D. (1993). Benthic storms in the Argentine Basin. *Deep-Sea Research II*, *40*(4/5), 975-987.
[11] Swartz, S.L., Taylor, B.L., Rugh, D.J. (2006). Gray whale *Eschrichtius robustus* population and stock identity. *Mammal Review*, *36*(1), 66-84.
[12] Zhang, J., Guo, L., Fischer, C.J. (2010). Abundance and Chemical Speciation of Phosphorus in Sediments of the Mackenzie River Delta, the Chukchi Sea and the Bering Sea: Importance of Detrital Apatite. *Aquatic Geochemistry*, *16*, 353-371.

**Acknowledgements**

I would like to thank Dr. Robert Condon and Natalie Ortell for their help in refining my research question, and for their continued support of my efforts throughout the research process. I want to thank Dr. Jeffrey Krause for his advice on Bering Sea nutrients. I would also like to thank the Dauphin Island Sea Lab for providing the resources and venue to present my findings. I am grateful to the University of Alabama and Dr. Julie Olson for giving me this opportunity, and to Sarah White for her help in proofreading this paper.

**About the Author**

Anna Hawkins is a senior from Huntsville, Alabama studying Marine Science and Biology at the University of Alabama. In addition to her research, she also works for the Department of Biological sciences as a museum technician for the University's Herpetological

Collection. She is a member of the Computer Based Honors program and is the President of the University's Marine Science Club. After Anna completes her undergraduate degree, she hopes to work in conservation or as an aquarist and continue her education in Marine Biology in a Ph.D. program. Eventually, she hopes to have a career in aquarium management or studying cetaceans.

JOSHUA was made possible by the following organizations:

University of Alabama
Office of the President

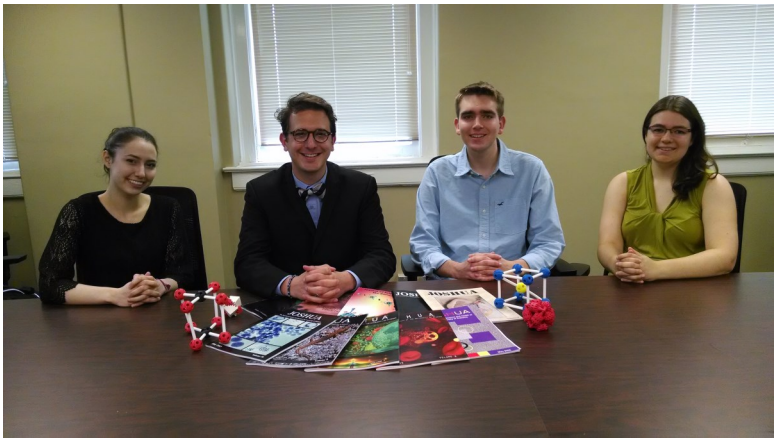
Tri-Beta National Honor Society
Kappa Beta Chapter

Howard Hughes Medical Institute

National Science Foundation

Computer Based Honors Program

And by the 2013-2014 JOSHUA Editing Staff



# 2015 Submission Guidelines

We accept articles from current undergraduate students at The University of Alabama (UA). If you are a graduate student or recent alumnus of UA, we will consider your article if the majority of your work was conducted while you were an undergraduate at UA. Undergraduate students from other institutions may submit; however, priority will be given to those who conducted their research at UA.

1. Your name, e-mail address, and phone number must be included.

2. Your submission must relate to science or health.

3. Your work must be sponsored by a faculty member.

4. The length of your submission must be between 2000 and 4500 words. We will accept longer submissions if the author can limit the submission to the required length for the publication, and any extra material is able to be published online.

5. Figures, charts, and graphs are allowed but not required. (Note: The color will be mostly black and white.)

6. Your paper must contain an abstract.

7. Your citations must follow the guidelines listed on our website at BAMA.UA.EDU/~JOSHUA.

8. The deadline for submission is February 13, 2015.

9. E-mail submissions to joshua.alabama@gmail.com


SHELBY HALL


THE UNIVERSITY OF ALABAMA
1831