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The Effects of the Omega-3 Fatty Acids Docosahexaenoic Acid on INM1 Gene Expression in Saccharomyces Cerevisiae

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Abstract

Bipolar disorder is a mental disorder associated with abnormal inositol levels. Preliminary studies show omega-3 fatty acids alter intracellular inositol levels; however, the cellular mechanism behind this process is unknown. Therefore, the focus of this study is to determine the effects of the omega-3 fatty acid docosahexaenoic acid (DHA) on the expression of *INM1* which encodes inositol monophosphatase. Using yeast as our model system, cells were grown in varying concentrations DHA and the expression on *INM1* was determined by RT-qPCR. The results show *INM1* expression increased with increasing concentrations of DHA; thus, the effects of DHA on inositol levels may be exerted via its impact on *INM1* expression.

Introduction

Bipolar disorder is a psychiatric illness that includes episodes of mania and depression. This illness is defined in two categories: bipolar I disorder (BP-1) and bipolar II disorder (BP-2). BP-1 includes symptoms of increased manic episodes, while BP- 2 includes depressive stages with a less intense form of mania called hypomania (Vieta *et al.* 1997). Current well used treatments for bipolar disorder include lithium and valproate (VPA). These drugs both decrease the intensity and frequency of the manic stage, with lithium being slightly more effective compared to valproate (Freeman *et al.* 1992). However, both treatments come with negative side effects.

A potential alternative treatment for bipolar disorder is omega-3 fatty acids (Shakeri *et al.* 2016). They have been studied extensively because omega-3 fatty acids are recognized for their health benefits (Simopoulos 1991). In using them as alternative treatments for bipolar disorder, side effects that could threaten the health and safety of patients are avoided.

Silverstone and colleagues (2005) discovered that patients suffering from bipolar disorder had abnormal intracellular inositol levels. Inositol is a chemical compound that plays an important role in secondary messenger signaling in the brain. Preliminary work in Dr. Marlene Murray's lab indicate that intracellular inositol levels are influences by omega-3 fatty acids (M. Murray, personal communication). To study the mechanism by which omega-3 fatty acids influence inositol levels, yeast cells were used as a model system for deciphering this mechanism. The yeast gene *INM1*, which encodes inositol monophosphatase, is essential in the biosynthesis of inositol. Although omega-3 fatty acids have been shown to influence the amounts of inositol produced, the mechanisms behind how omega-3 fatty acids work on a cellular level in this process have not been confirmed (Murray and Greenberg, 2000). Therefore, the focus of my study was determining the effects of the omega-3 fatty acid docosahexaenoic acid (DHA) on the expression of the *INM1* gene, a key component in inositol biosynthesis.

Background

Lithium and VPA are known to have various negative side effects, including nausea, tremors, and skin diseases such as psoriasis when treating bipolar disorder (Deandrea 1982). They are also primarily used to treat symptoms of mania since they are relatively ineffective in treating the depressive symptoms in bipolar disorder. As a result, omega-3 fatty acids are studied as an alternative treatment because they do not cause negative side effects and are likely to decrease symptoms of depression in bipolar disorder (Shakeri *et al.* 2016).

Stoll *et al.* (1999) showed in their study that omega-3 fatty acid ethyl esters (derivatives of omega-3) inhibit signal transduction pathways in neurons. They found that using these derivatives stabilized mood swings in bipolar disorder patients by stopping excessive cell signaling. This study concluded that patients treated with omega-3 fatty acids showed reduced depressive symptoms and therefore possible antidepressant effects could come from using omega-3 fatty acids to treat bipolar disorder. Similarly, Su *et al.* (2003) studied the effects of omega-3 polyunsaturated fatty acids in patients with depressive disorder. The results showed a greater decrease on the Hamilton Rating Scale for Depression (a qualitative survey to determine severity of depression) measured in patients after they took the omega-3 pills than the patients who took placebo (olive oil) pills. This indicated that the patients who took omega-3 pills showed less symptoms of depression.

One possible molecular pathway that can be studied to test if omega-3 fatty acids could be used as a treatment for bipolar disorder is the biosynthetic pathway of inositol, which is used as part of an important secondary messenger system in the brain (Levine 1997). In patients suffering through the manic stage of bipolar disorder, a higher level of inositol than should be present is found in the brain, while in the depressive stage, a lower level of inositol is present (Barkai *et al.* 1978). Lithium and VPA decrease inositol concentrations through inhibition of important steps in inositol biosynthesis (Shaltiel *et al.* 2004). Lithium decreases inositol levels in the brain by inhibiting

several enzymes, which accounts for lithium's benefits in treating the manic stage in bipolar disorder (Hirvonen *et al.* 1991). Tokuoka *et al.* (2008) also found VPA to decrease inositol levels through causing a defect in the signaling pathway of inositol. Similarly, Vaden *et al.* (2001) found a decrease in inositol mass found in yeast cells when lithium and VPA were introduced.

On the contrary, in an ongoing unpublished study conducted by Dr. Marlene Murray, omega-3 fatty acids seem to increase inositol levels in yeast cells (M. Murray, personal communication); however, this is a preliminary study and the mechanisms behind how the omega-3 fatty acids increase inositol concentrations are unknown. Therefore, Dr. Murray's lab uses yeast a model system to understand the mechanism behind omega-3 fatty acid effects on inositol production. The inositol production pathway in yeast is already known, and is one possible cellular pathway that could explain the mechanism behind how omega-3 fatty acids influence inositol levels. This pathway, which is similar in humans but more complex, is an important secondary messenger system in the brain. Figure 1 shows the inositol production pathway, where glucose-6-phosphate is converted into inositol-1-phosphate through the *INO1* gene, and inositol-1-phosphate is then converted into inositol through the *INM1* gene. Vaden and colleagues found that valproate decreased inositol levels in yeast cells while VPA increased expression of *INO1*, signifying a feedback loop (2001). One explanation for this result could be that VPA seems to increase *INO1* expression in order to produce more inositol-1-phosphate, and through this effect, produce more inositol, that would make up for the decrease in inositol levels that it caused. However, while the *INO1* gene has been studied, not much research has been conducted on *INM1*. Thus, the aim of my study is to test what effect the omega-3 fatty acid, docosahexaenoic acid (DHA), has on the expression of the *INM1* gene.

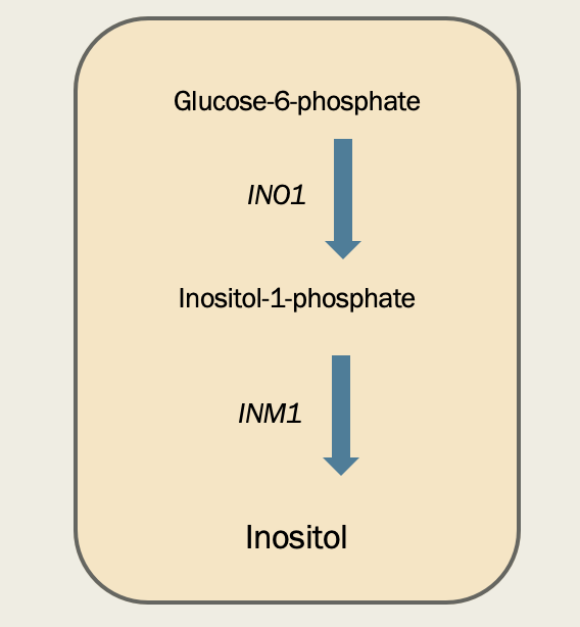

Fig. 1 The inositol production pathway in Saccharomyces cerevisiae which shows the genes INO1 and INM1 and their roles in producing inositol.

Methods

In order to measure the expression of *INM1*, several general steps were taken. First, yeast cultures were developed and cells grown in various media, followed by extraction of yeast cell mRNA. Then, RT-qPCR was performed, and gene expression was calculated. A melt curve analysis was then conducted to test for contamination and accuracy of data. Essentially, yeast cell DNA was extracted, purified, and then amplified to see the differences between treatment (DHA) groups and the control group.

Yeast Culture Development. In the first step, yeast cultures were developed in petri dishes with growth media, which contain nutrients that support the development of yeast. The yeast cells were then inoculated in various experimental conditions, as listed in the following: a control sample without treatment, a positive control sample of yeast cells grown in a VPA solution, and three experimental samples containing 0.2mM DHA, 0.4 mM DHA, and 0.6mM DHA treatments, respectively. A positive control was used because the effects of VPA were already known and would

provide a comparison to the DHA treatment samples. Using various concentrations allowed for a comparison of the *INM1* gene expression quantity across dosages of DHA.

Spectrophotometry was then used to determine the cell density of the yeast grown in this medium. This method determined the sample size of yeast (in mL) to be used for each sample by measuring the quantity of yeast cells grown in the various treatments. After placing the measured amount of yeast cells for each sample, they were allowed to grow overnight in a shaking incubator. Spectrophotometry was performed again to determine yeast cell density in each sample after growth in the incubator. This gave an indication of cellular density, so that the quantity of cell growth could be measured for each of the variable and the control groups.

Extraction of mRNA. After the yeast cells were grown, the mRNA (messenger RNA) from these cells were extracted. By measuring the cellular density of each test tube in the previous step, the amount of mRNA extracted from the cells were measured, creating equal sample sizes of mRNA. This allowed for the yeast mRNA counts to start at the same level in order to accurately obtain the amount of INM-1 expressed later in the experiment. After obtaining the determined volume of cells to be used, mRNA was extracted using the Bio-Rad spin protocol (Bio-Rad instruction manual 2014). This procedure uses centrifugation as the main method of extraction. Centrifugation separates cellular material based on their densities, so the yeast samples will separate larger cellular matter like mRNA to the bottom of the sample tube in a solid cell pellet, while leaving smaller materials in a liquid supernatant in the top layer. From the cell pellet, mRNA was extracted.

RT-qPCR. Following mRNA extraction, RT-qPCR was performed. This step can be further divided into two steps of RT and qPCR. First, mRNA was converted into cDNA, and qPCR was then performed to quantify gene expression levels.

Conversion to cDNA. The mRNA samples were converted to cDNA (complementary DNA) using reverse transcriptase (RT), an enzyme that converts DNA into RNA. To quantify the amount

of cDNA converted from mRNA for each sample, the five samples were divided into halves. One half of each sample was used as a control by not adding any RT. The second half of each sample was used as the treatment sample by adding RT. The absence of RT would result in the inability for mRNA to be converted into cDNA, allowing for a measurement of cDNA by comparing this control sample to the RT-introduced sample. This resulted in a total of 10 samples, five controls without RT and five treatments with RT. The samples are made up as follows:

- Sample 1: control without RT
- Sample 2: control with RT
- Sample 3: VPA positive control without RT
- Sample 4: VPA positive control with RT
- Sample 5: 0.2mM DHA treatment sample without RT
- Sample 6: 0.2mM DHA treatment sample with RT
- Sample 7: 0.4mM DHA treatment sample without RT
- Sample 8: 0.4mM DHA treatment sample with RT
- Sample 9: 0.6mM DHA treatment sample without RT
- Sample 10: 0.6mM DHA treatment sample with RT

These ten samples were placed in a 96-well plate and run through a thermal cycler, which converted the mRNA of the solutions into cDNA by harnessing the RT enzyme. In heat, RT converts the mRNA into cDNA. A final, rapid heating cycle denatures the RT enzyme so that it is not included in the sample.

qPCR. The yeast cDNA was then put through a quantitative polymerase chain reaction, which amplified the few cDNA strands obtained in the previous step into many strands of cDNA. qPCR happens in two simplified steps. In this reaction, cDNA strands are denatured by heating,

meaning that the double-stranded DNA was split into two single strands. Then, an *INM1* primer that was introduced to the samples during cDNA conversion attaches only to the single DNA strands that encode specifically for the *INM1* gene. This results in the creation of two new DNA molecules that carry the *INM1* primer. Thus, with repeated cycles of this process, the cDNA strands were multiplied exponentially, amplifying the cDNA molecules. During this amplification, a SYBR green fluorescent marker specific to *INM1* tags all of the DNA strands carrying the *INM1* primer, causing those DNA strands to fluoresce, and at the end of the cycles, the amount of fluorescence is quantified to measure gene expression. RT-qPCR was also performed for the reference gene *TFC1* in order to compare gene expressions.

Gene expression calculation. After RT-qPCR, gene expression was quantified through relative quantification. This step compared of normalized the quantity of fluorescence of *INM1* to the quantify of fluorescence of *TFC1*. By normalizing *INM1* expression to the reference gene with known expression levels, *INM1* expression was calculated through a comparison. The relative quantification of *INM1* expression was then normalized to the control with the absence of treatment, where normalized expression ratio = $2^{-\Delta\Delta C_t}$.

Melt curve analysis. Following *INM1* expression quantification, the reliability of the expression data was observed through a melt curve analysis. As observed in figure 2, peaks around 75-80 degrees Celsius would have indicated contamination through primer dimers, which would be due to two *INM1* primers sticking together and causing SYBR green to tag it as a DNA strand encoding for *INM1*. Figures 2 and 3 both show peaks around 80 degrees Celsius which is the indicator *INM1* expression, and figure 3 shows the ideal Melt peak graph indicating little to no contamination.

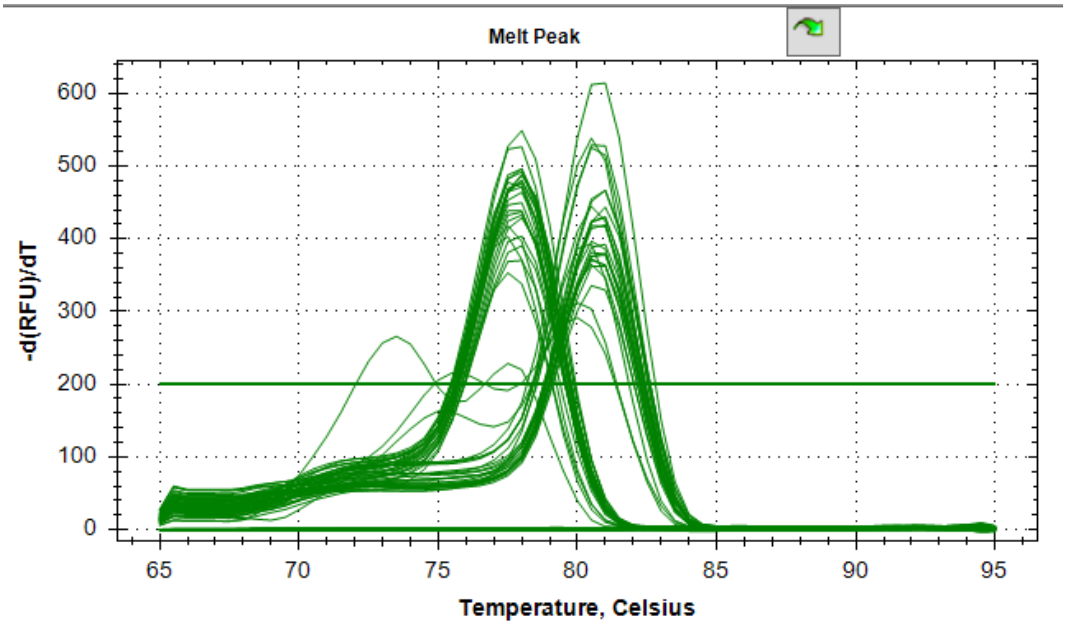

Fig. 2 Melt curve analysis showing instances of contamination in gene expression, as observed in the peaks at approximately 78 degrees Celsius. Data collected from previous trials not pertaining to the trials mentioned in this research.

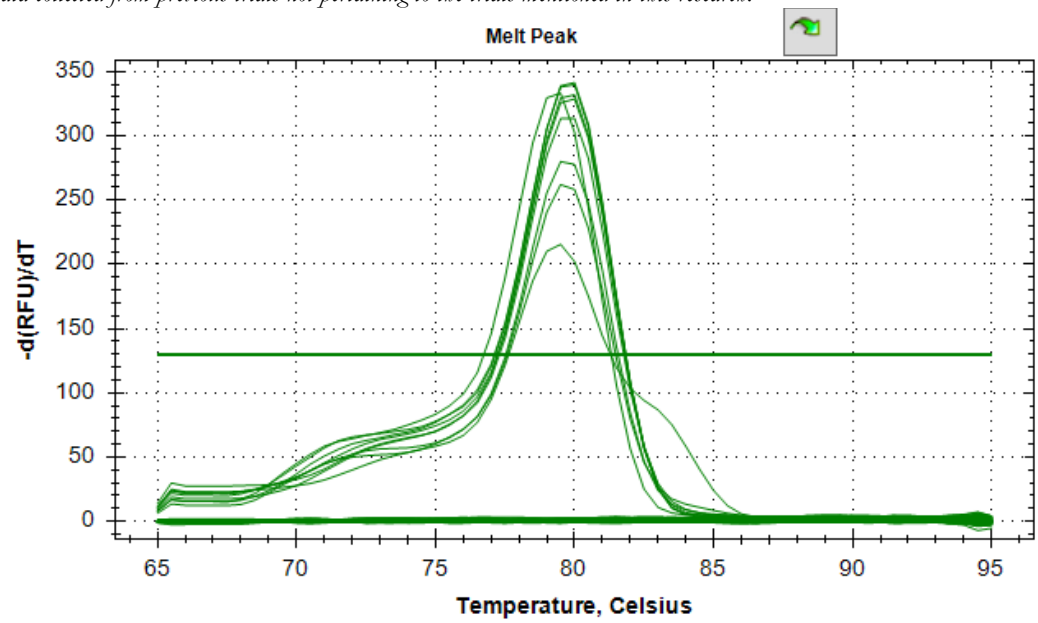

Fig. 3 Melt curve analysis showing little to no contamination. A peak around 80 degrees Celsius indicates successful INM1 expression.

Results

As seen in figure 4, the data, averaged out of five trials, show positive *INM1* expression for all variable samples. Each variable group was normalized to the control group to observe the increase or decrease in expression in the samples treated with either VPA or DHA dosages compared to the control that did not contain treatment and therefore acted to produce the "normal" amount of *INM1* expression. Figure 5 shows the raw data of *INM1* expression in each sample.

Cells treated with VPA showed highest *INM1* expression, with *INM1* expression increasing with increasing DHA concentrations.

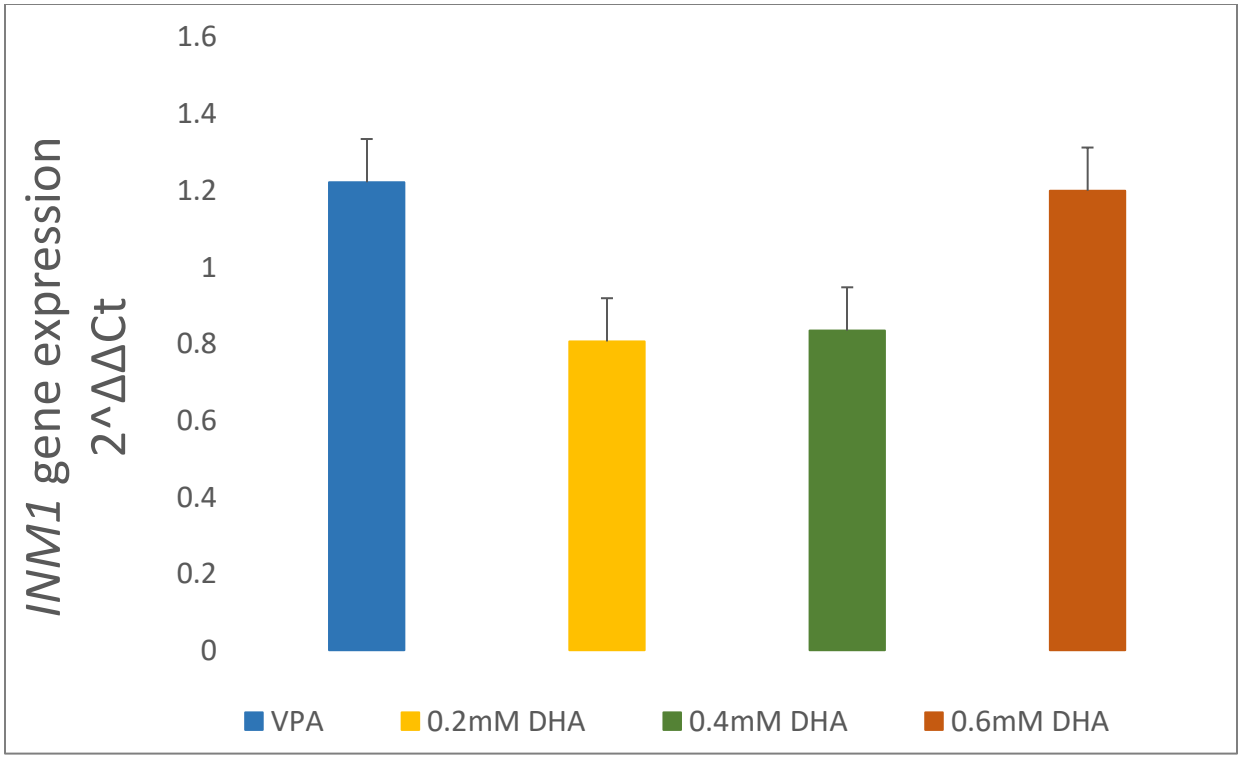

Fig. 4 Average INM1 expression. Cells were grown for 24 hours in the variable concentrations indicated. Gene expression was measured by RT-qPCR and normalized to non-treatment control. n = 5, VPA = positive control.

	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
VPA	1.126	1.734	0.996	1.215	1.032
0.2 mM DHA	0.845	0.973	0.563	0.872	0.774
0.4 mM DHA	0.907	0.942	0.610	0.898	0.812
0.6 mM DHA	1.026	1.754	0.982	1.226	1.004

Fig. 5 Raw data of INM1 expression in each trial conducted for each variable sample. Values indicate gene expression normalized to non-treatment control.

Conclusion

The results show that VPA and the three DHA concentrations expressed more *INM1* compared to the control group, as indicated by the positive values of expression seen in figures 4 and 5. A negative value would have indicated less *INM1* expression in the variable group compared to the control. Positive expression values in the VPA treated sample supports Vaden *et al.* and their previous research where VPA was found to increase *INO1* expression as well (2001).

Vaden and colleagues have found in previous experiments that *INO1* expression is decreased in the presence of DHA (2001); however, the data in this study show an increase in *INM1* expression for the various DHA samples. This result shows indications for increased intracellular inositol production in yeast cells treated with DHA, hinting that *INM1* expression and *INO1* expression might be influenced in different ways by omega-3 fatty acids. Furthermore, this finding indicates that DHA does not work as a feedback loop as observed in the effects of VPA on inositol production, but rather as a sort of transcription activator where *INM1* activation results in increased inositol production. However, further studies would need to be conducted in order to support this hypothesis.

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