2022

Gut dysbiosis: the effects of a high-fat diet on brain-derived neurotrophic factor (BDNF) and pro-BDNF expression in the brain-gut axis

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

GUT DYSBIOSIS: THE EFFECTS OF A HIGH-FAT DIET ON BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF) AND PRO-BDNF EXPRESSION IN THE BRAIN-GUT AXIS

by

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B.A., Boston University, 2020

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2022

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DEDICATION

I would like to dedicate this work to my loving and supportive parents.

ACKNOWLEDGMENTS

I would like to thank my entire family for their unyielding love and support. I would like to acknowledge all the people that have helped me get to this point in my academic career and have pushed me to strive for excellence—all of my professors, advisors, mentors, and peers. I would like to especially acknowledge my thesis readers, Dr. Wisco and Dr. Joseph, for their patience and guidance throughout this entire process.

GUT DYSBIOSIS: THE EFFECTS OF A HIGH-FAT DIET ON BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF) AND PRO-BDNF EXPRESSION IN THE BRAIN-GUT AXIS

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ABSTRACT

The human microbiome and its role in the 'brain-gut' bi-directional axis are becoming increasingly relevant to our understanding of the effects of gastro-intestinal microbiota on brain development and on various neurological diseases. One protein that may mediate the effects of this brain-gut axis is brain-derived neurotrophic factor (BDNF). BDNF is found not only in the brain but also in peripheral tissues. BDNF is essential in learning and memory through stabilization of long-term potentiation (LTP). Research has shown that its expression levels have been found to be greatly affected by diet and exercise. In events such as aging, decreased levels of BDNF has been associated with cognitive decline and increased susceptibility to neurodegenerative diseases such as Alzheimer's disease (AD). This study aims to explore how the human microbiome maintained in a Western diet (high protein, high fat, high carbohydrate), affects BDNF expression and its levels in the 'brain-gut' bi-directional axis. Understanding BDNF and its relation to the brain-gut axis may prove invaluable towards further research to prevent the cognitive decline that comes with decreased BDNF expression.

In this project, we compared BDNF and its precursor, proBDNF, levels in the brain, jejunum, and distal colon of mice. The mice used were necropsied at either three or six months and were either on a normal or high fat diet. Protein levels were stained and

analyzed using MANOVAs, correlations, and regressions to observe any statistically significant relationship between the two proteins in their tissue location of origin. The results confirmed the hypothesized: proBDNF increased with age and was higher in a high fat diet versus a normal diet, while BDNF decreased with age. There was also a strong, positive correlation found between brain proBDNF and brain BDNF.

These results suggest an intricate, yet complex relationship between proBDNF and BDNF and that proBDNF may play a significant role in gut dysbiosis. On this basis, future studies need to be done to further understand this relationship in the brain-gut axis. An important focus looking at the vagus nerve and these proteins and their high-affinity receptors, TrkB and p75, will uncover key information that will aid in preventative medicine for cognitive decline and neurodegenerative diseases.

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LIST OF ABBREVIATIONS

BDNF	Brain-derived neurotrophic factor
CREB	Cyclic AMP response element-binding
GF	Germ-free
LTD	Long-term depression
LTP	Long-term potentiation
NMDAR	N-methyl-D-aspartate receptor
TrkB	Tropomyosin receptor kinase B

INTRODUCTION

BDNF: Overview

Brain-derived neurotrophic factor (BDNF) is one of the more widely studied neurotrophic growth factors that are found in the brain and the peripheral tissues. It has many functions that include differentiation, maturation, and neuroprotection of neurons (Bathina & Das, 2015). It is a key molecule that is involved in plastic changes that occur during learning and formation of memory (Miranda et al., 2019). BDNF is postulated to increase memory storage by stabilizing hippocampal long-term potentiation (LTP) resulting in stronger synapses and altering neuronal structure. BDNF is encoded by the BDNF gene and is synthesized as the precursor proBDNF, which can be stored in either dendrites or axons (Lessmann et al., 2003). Precursor proBDNF then undergoes cleavage intra- or extra-cellularly to produce the mature BDNF protein (Lee et al., 2001; Mowla et al., 2001). Although the location is still unclear, its cleavage is important because proBDNF and mature BDNF have different and opposing functions. Additionally, BDNF is released in an activity-dependent manner as a mixture of the two forms (Pang et al., 2004). ProBDNF can ameliorate long-term depression (LTD) induced by low frequency stimulation of chemical synapses and plays a role in inducing apoptosis. ProBDNF is found in both pathological and non-pathological conditions. In contrast, mature BDNF facilitates LTP induced by high frequency stimulation of chemical synapses and expression is found to decrease in pathological conditions (Nagappan et al., 2009). High BDNF levels help regulate both excitatory and inhibitory synaptic transmission (Tyler et

al., 2002; Wardle & Poo, 2003). The expression of BDNF is important for changes in synaptic plasticity and thus must be highly regulated.

BDNF expression is highly regulated during transcription and translation. During transcription, it is modulated by different promoters that determine activity-dependent and tissue-specific expression (W. G. Chen et al., 2003; Timmusk et al., 1993). In rats, at least four BDNF promoters have been identified, each one driving the transcription of mRNAs that may contain one of the 8 non-coding exons spliced to the common 30 coding exons (Timmusk et al., 1993). BDNF has a complex genome that includes a range of promoters and at least eight 5' exons and one 3' exon. The promoters are involved in the production and distribution of highly specific transcripts of BDNF across the brain, resulting in its diverse functions, including neuronal survival and differentiation (Adachi et al., 2014).

The BDNF pathway is signaled through two membrane-bound receptors: p75 and Tropomyosin receptor kinase B (TrkB) (Chao, 2003). ProBDNF binds with the p75 receptor to facilitate LTD and apoptosis. Mature BDNF binds to tyrosine kinase receptors to promote cell survival and facilitate LTP. It has been demonstrated that when p75 is co-expressed with TrkB, there is an increase in neurotrophin binding affinity, resulting in easier ligand discrimination (Bibel et al., 1999). ProBDNF is thought to help regulate mature BDNF activity in non-pathological conditions. The expression levels of specific BDNF exons and ultimately mature BDNF may be susceptible to environmental influence through different epigenetic mechanisms (Lubin et al., 2008). Different levels

of expressions for BDNF and its precursors are associated with varying degrees of cognitive dysfunction.

The BDNF gene has a single nucleotide polymorphism that involves a valine to methionine amino acid substitution at the codon 66 position (Val66Met). This impacts the packaging process of mature BDNF to secretory vesicles and decreases its secretion and may be associated with numerous psychiatric and neurological disorders such as schizophrenia (Maqsood & Stone, 2016). Val66Met is correlated to lower serum BDNF levels as well as volumetric decreases in specific brain regions such as the hippocampus, supporting the idea that BDNF plays a role in memory performance and cognition. Numerous studies could not find an association between the Val66Met genotype and memory performance specifically, but the polymorphism may confer disadvantages in cognitive performance and episodic memory (Tsai et al., 2008),(Houlihan et al., 2009; Karnik et al., 2010). This polymorphism may help explain the effects of BDNF on normal brain function and aging.

BDNF and the Brain

Age is the major risk factor for the development of many neurodegenerative diseases. Throughout the entire aging process, cognitive performance declines as synaptic plasticity is reduced in brain regions important for cognition. The most consistent deficit seen with aging is the decline in neurotrophic signaling, most notably BDNF-associated LTP signaling. Therefore, it is inferred that age-related cognitive deficits can be correlated to the decrease in BDNF expression in the affected brain regions seen in aging (Mattson et al., 2004). These decreased BDNF levels are also accompanied by reductions

in the expression and activation of TrkB receptors and the increase in levels of proBDNF and p75 associated with LTD and apoptosis (Miranda et al., 2019). TrkB receptors are activated by BDNF and are an important part of a pathway that mediates cell survival and proliferation. The TrkB signaling pathway can be further divided into three main pathways: the MAPK/ERK pathway, the PI3k/Akt pathway, and the PLC-γ pathway (Wurzelmann et al., 2017). These three pathways work together to regulate important cognitive processes, explaining why decreased expression of BDNF affects cognitive ability and memory formation.

The MAPK/ERK pathway is responsible mainly for promoting neuronal growth, synaptic plasticity, cell survival, and differentiation (T. Chen et al., 2017). The MAPK and ERK proteins regulate the cyclic AMP response element-binding protein (CREB), which is important for transcriptional regulation for a multitude of proteins associated with neuronal survival and LTP. In a Western Diet, BDNF is downregulated, likely as a result of gut dysbiosis.

The PI3k/Akt pathway is important for the inhibition of high glucose induced apoptosis. The pathway also reverses the effects of high glucose levels on the downregulation of synaptic plasticity-related proteins in hippocampal neurons (Zhong et al., 2018). Through the TrkB receptor, BDNF upregulates the PI3K and Akt pathway to inhibit the forkhead, Bad, and Pro-caspase 9 apoptosis proteins (Wurzelmann et al., 2017). PI3K/Akt also increases CREB activation to help with neuronal survival (Zhong et al., 2018).

The PLC-γ pathway is important for calcium signaling and synaptic plasticity (Numakawa et al., 2009). It is essential for calcium homeostasis. The levels of calcium ions maintained by the pathway activate kinases (calcium-calmodulin dependent kinases), that in turn activate transcription factors including CREB that help regulate neuroplasticity and neurogenesis (Gulyaeva, 2017).

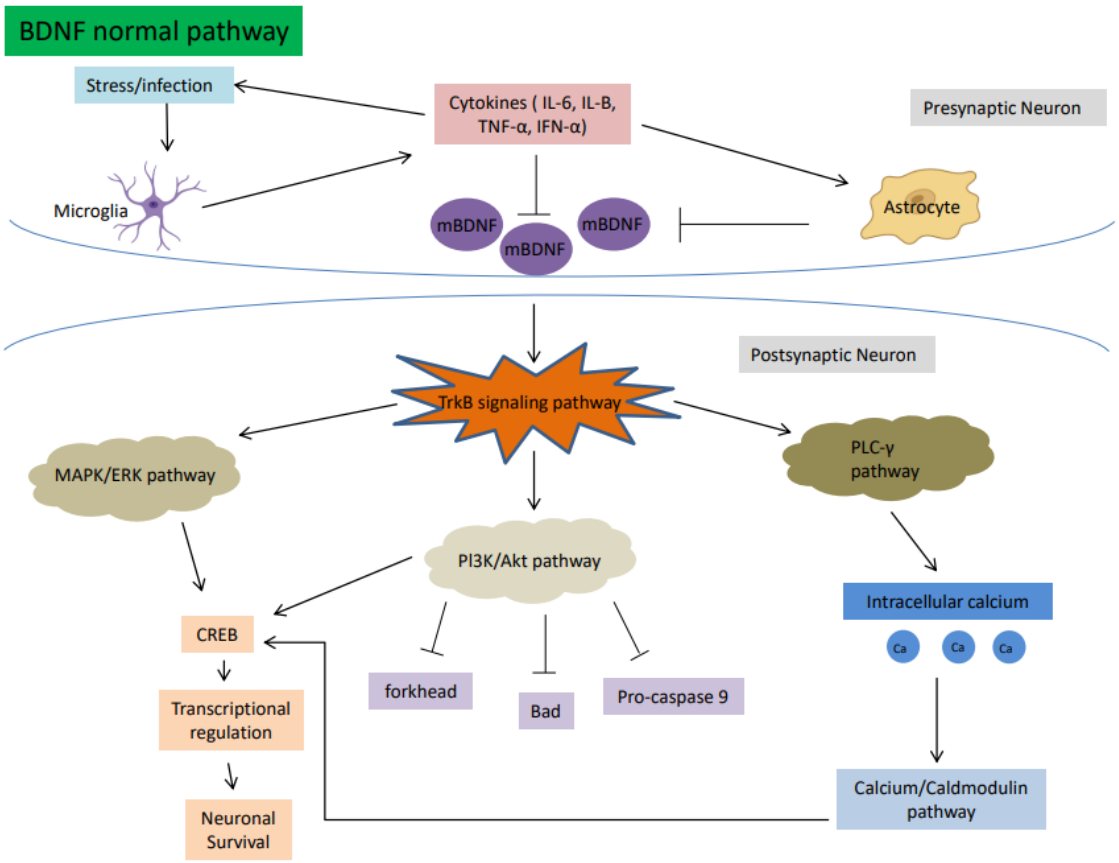


Figure 1. Normal BDNF signaling pathway. BDNF activates the TrkB signaling pathway which in turn activates the MAPK/ERK, PI3K/Akt, and PLC- γ pathways. These pathways work together to regulate important cognitive functions such as LTP formation and neurogenesis.

BDNF is important not only for synaptic plasticity, but also in neuronal survival. It exerts various trophic effects on hippocampal neurons to counteract the noxious effects of neuronal cell death (Almeida et al., 2005). In adult humans, high levels of BDNF in the hippocampus have been correlated with the survival and differentiation of dentate gyrus progenitor cells (Pencea et al., 2001; Shetty et al., 2004). In aged animals, low BDNF levels have been associated with deficient neurogenesis (Apple et al., 2017). More importantly, BDNF has been found to play a role in antioxidant defense during aging and its levels increase with oxidative stress (Mattson et al., 2004). One study on patients with schizophrenia implicated both oxidative stress and decreased BDNF levels in the pathology of the disease. The inverse association between BDNF and oxidative stress in schizophrenia may be the underlying mechanism that affects cognitive impairment (Zhang et al., 2015). The analysis of the brains of patients with psychiatric conditions and neurocognitive disorders showed decreased BDNF functions when compared with healthy individuals. It is however still unclear whether this decrease directly causes cognitive decline.

BDNF and the Brain-Gut Bi-directional Axis

The 'brain-gut' bi-directional axis is becoming increasingly relevant in our understanding of the effects of gastro-intestinal microbiota on brain development and neurological diseases such as Alzheimer's disease. In the brain, high levels of BDNF can be found in the hippocampus, amygdala, cerebellum and cerebral cortex with the highest levels found in hippocampal neurons (Hofer et al., 1990; Timmusk et al., 1993). BDNF is also expressed in other peripheral tissues, including that of the gastrointestinal tract, some

with higher levels than in the brain (Biddinger & Fox, 2014). In our knowledge of the brain-gut axis, it may be interesting to see how levels of BDNF in the gut may be associated with BDNF levels in the brain: Do the levels of BDNF in the gut correlate with those in the brain? Do they modulate the levels of BDNF in the brain or even the brain itself?

N-methyl-D-aspartate receptors (NMDAR) are important for regulating neuroplasticity and cognitive function. BDNF is believed to be associated with these NMDAR receptors (Maqsood & Stone, 2016). In the absence of gut microbiota seen in germ-free (GF) mice, BDNF levels in their central nervous system are reduced and thus inhibit NMDAR production. The reduced NMDAR input on GABA inhibitory interneurons causes the disinhibition of glutamatergic output, leading to many cognitive deficits (Maqsood & Stone, 2016). These observations suggest that the presence of gut microbiota affect the levels of BDNF in the brain and their corresponding effects on the brain.

Western diet, consisting of processed foods with abundant sugar, salt and chemicals, has been associated with cognitive deficits in psychiatric diseases and dementia in humans (Hansen et al., 2018). Such impairments may be attributed to a decrease in BDNF expression levels as well as increased oxidative stress. Some studies reported no effect or even positive effects on brain function with a Western diet regimen (Leffa et al., 2015; McNeilly et al., 2015). However, the Hansen et al. study on the effects of long term, high fat diet-induced dyslipidemia in a non-obsese guinea pig model on brain oxidative stress and potential regional alterations of BDNF showed decreased

hippocampal BDNF expression levels (Hansen et al., 2018). They concluded that these neurological changes occurred in the absence of obesity, suggesting a direct effect of an adverse diet on markers of brain function. The decreased levels of BDNF associated with a Western diet may have a chain reaction that ultimately affects the TrkB signaling pathway, resulting in cognitive dysfunction. In a Western Diet, decreased BDNF downregulates the TrkB receptor and ultimately the MAPK/ERK, PI3k/Akt, and PLC-γ pathways, leading to increased apoptosis and dysregulation of CREB, increased susceptibility for neuronal cell death, disruption of calcium ion homeostasis causing increased oxidative stress, apoptosis, and dysregulation of CREB and transcription factors that decrease LTP and neurogenesis (Shaito et al., 2020).

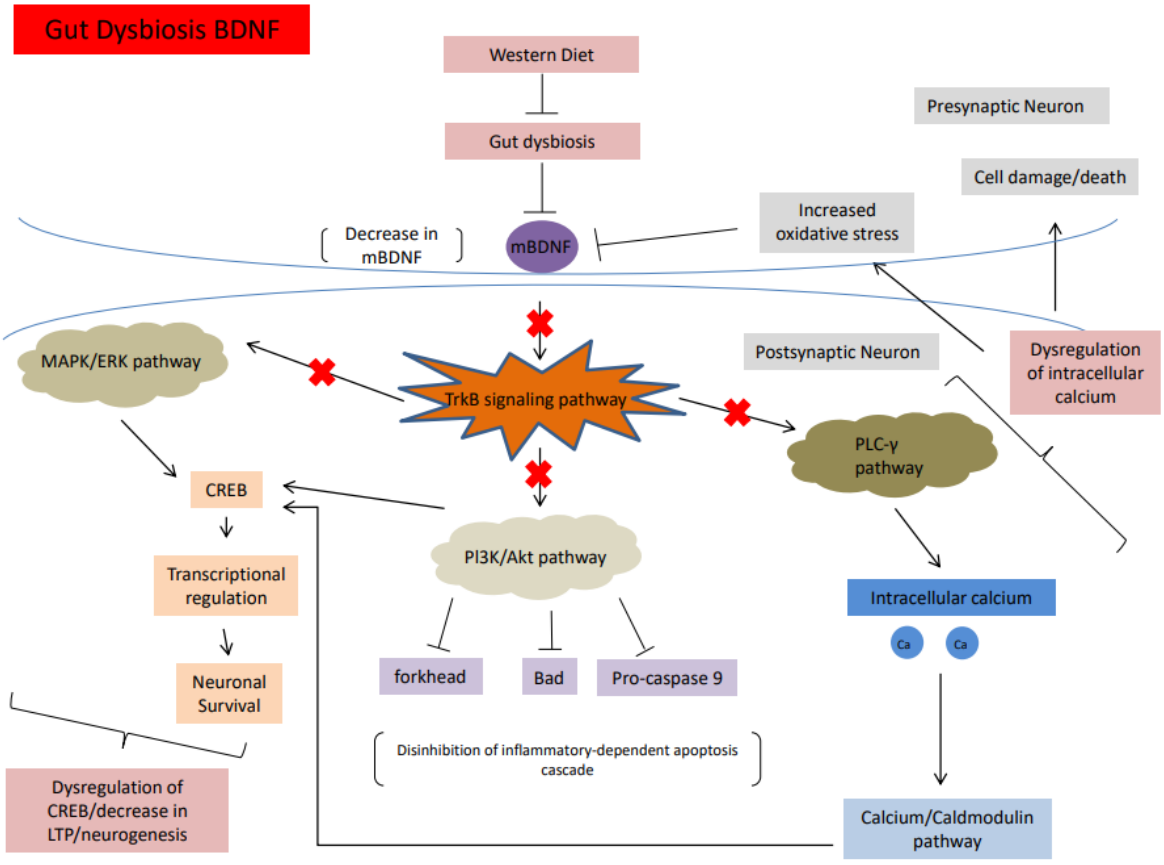


Figure 2. BDNF signaling pathway under Western Diet conditions. A Western Diet causes gut dysbiosis, which decreases levels of BDNF. These decreased BDNF levels downregulate the TrkB signaling pathway and ultimately the MAPK/ERK, PI3K/Akt, and PLC- γ pathways, causing a multitude of problems such as increased susceptibility to cell death, dysregulation of CREB and transcription factors, as well as a decrease in LTP and neurogenesis.

Gut microbiota can modulate BDNF expression and function in the brain. The exact mechanism, however, remains unclear. In one study, BDNF levels are found to be lower in the cortex and hippocampus of germ-free (GF) mice compared to the controls, suggesting that gastrointestinal microbiota may play some role in elevating the brain BDNF levels (Sudo et al., 2004). It is known that brain BDNF is associated with learning and memory and is important for preserving cognitive abilities. However, it is not known

with certainty how the gut microbiota affect the BDNF levels in the brain and other peripheral tissues. This study presents the effects of a Western on the BDNF expression levels in the brain-gut axis, specifically comparing the BDNF and its precursor, proBDNF, levels in the brain, jejunum, and distal colon of mice.

MATERIALS AND METHODS

Animals

Mice (n=26) with the APP NLGF strain (both male and female) were fed either a Western diet (13% protein = 0.54 kcal/g, 14.5% fat = 1.30 kcal/g, 52.9% carbohydrate = 2.12 kcal/g; n = 9) or normal diet (n = 17) (Saito et al., 2014). Groups were divided into 3 months (n = 18) and 6 months (n = 8) old. The mice were necropsied and their brain and organs removed and washed with PBS. Biopsies were later taken to create tissue samples for the brain, jejunum, and distal colon and prepared for immunohistochemistry.

Immunohistochemistry

Brain, jejunum, and distal colon histological tissue samples were fixed in formalin solution and sent to iHisto (Salem, MA) for tissue processing. Tissue samples were paraffin-embedded, cut at 6 microns, and serial sections were stained with H&E, anti-BDNF (1:200) and anti-proBDNF (1:200) through iHisto. These antibodies were purchased from Novus Biologicals (Catalog #NB100-98682 and #NB100-98756, respectively). A secondary antibody was tagged with a 3,3’ - Diaminobenzidine (DAB) chromagen kit (VectorLab’s ImmPRESS Kit) then reacted with the primary antibody. Stained sections were observed at 30x magnification using QuPath and screen captured.

A montage of images for each stained section was produced in Adobe Photoshop Creative Suite (San Jose, CA), correcting for image distortion and lighting inhomogeneity. Each montage served as the input for positive pixel count quantitative analysis.

Positive Pixel Count

A positive pixel count of strong positive and positive was performed on the IHC-stained images to quantify the amount of protein in the brain, jejunum, and distal colon samples.

To segment the DAB chromogen staining "brown," a parameter was set that collected all the desired pixel coordinates based on the red pixel value and the absolute value of the difference between the red and the blue pixel value. For these purposes, the red pixel value must be larger than the blue, or the difference between the red and blue pixel value must be greater than 20 for the pixel to be considered brown. Once all the pixel coordinates and their values are known, a 95% confidence interval was set on the red, green, and blue pixel values. The max and min of the resulting pixel values are used as the basis for what is considered weak positive, positive, and strong positive. The range is divided into thirds for each category we set into place. Everything that is part of the first one-third- this has smaller pixel values, is considered a strong positive. Positive is considered everything in the second-third and weak positive is considered in the last-third for this range. These labels allowed us to highlight the regions of confidence on the original image. The addition of a boundary image is also utilized to establish different

regions of interest. As a final result, an image was generated showing color coding of areas of strong positive and positive for different regions of the image.

Statistical Analysis

After completion of the positive pixel count on the images, we used SPSS to calculate three principal statistical models: 1) MANOVA to determine statistical significance of the effect of age, sex, and diet on the amount of proBDNF or BDNF stained; 2) bivariate correlations to determine the existence of relationships between proBDNF and BDNF; 3) linear regression to determine if age (days) could predict the value of the amount of proBDNF or BDNF stained. All positive pixel count values were normalized to the parenchymal region of interest area: hippocampus (mid-sagittal slice, including CA1, CA2, CA3, and CA4 subfields); jejunum (biopsied near proximally, near duodenum; including mucosa, submucosa, and muscularis externa); distal colon (biopsied in either rectum or sigmoid colon; including mucosa, submucosa, and muscularis externa).

RESULTS

ProBDNF

MANOVA: Normalized Strong Positive

Table 1 (below) reports normalized strong positive staining of proBDNF. We found statistically significant main effects for age [$F(3, 14) = 5.587$, $p = 0.010$; Wilks' $\Lambda = 0.455$, partial $\eta 2 = 0.545$, observed power = 0.859] and diet [$F(3, 14) = 8.906$, $p = 0.001$; Wilks' $\Lambda = 0.344$, partial $\eta 2 = 0.656$, observed power = 0.975], but not for sex or

any interactions. No statistically significant main effects were found for normalized positive staining of proBDNF or normalized total staining of proBDNF.

Table 1. MANOVA of Normalized Strong Positive Staining of proBDNF with Age, Sex, Diet, and Interactions

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[c]
Intercept	Pillai's Trace	.990	477.583[b]	3.000	14.000	<.001	.990	1432.749	1.000
	Wilks' Lambda	.010	477.583[b]	3.000	14.000	<.001	.990	1432.749	1.000
	Hotelling's Trace	102.339	477.583[b]	3.000	14.000	<.001	.990	1432.749	1.000
	Roy's Largest Root	102.339	477.583[b]	3.000	14.000	<.001	.990	1432.749	1.000
Agemo	Pillai's Trace	.545	5.587[b]	3.000	14.000	.010	.545	16.760	.859
	Wilks' Lambda	.455	5.587[b]	3.000	14.000	.010	.545	16.760	.859
	Hotelling's Trace	1.197	5.587[b]	3.000	14.000	.010	.545	16.760	.859
	Roy's Largest Root	1.197	5.587[b]	3.000	14.000	.010	.545	16.760	.859
Diet	Pillai's Trace	.656	8.906[b]	3.000	14.000	.001	.656	26.719	.975
	Wilks' Lambda	.344	8.906[b]	3.000	14.000	.001	.656	26.719	.975
	Hotelling's Trace	1.909	8.906[b]	3.000	14.000	.001	.656	26.719	.975
	Roy's Largest Root	1.909	8.906[b]	3.000	14.000	.001	.656	26.719	.975
Sex	Pillai's Trace	.088	.451[b]	3.000	14.000	.721	.088	1.352	.118
	Wilks' Lambda	.912	.451[b]	3.000	14.000	.721	.088	1.352	.118
	Hotelling's Trace	.097	.451[b]	3.000	14.000	.721	.088	1.352	.118
	Roy's Largest Root	.097	.451[b]	3.000	14.000	.721	.088	1.352	.118
Agemo * Diet	Pillai's Trace	.101	.527[b]	3.000	14.000	.671	.101	1.581	.131
	Wilks' Lambda	.899	.527[b]	3.000	14.000	.671	.101	1.581	.131
	Hotelling's Trace	.113	.527[b]	3.000	14.000	.671	.101	1.581	.131
	Roy's Largest Root	.113	.527[b]	3.000	14.000	.671	.101	1.581	.131
Agemo * Sex	Pillai's Trace	.143	.780[b]	3.000	14.000	.524	.143	2.341	.176
	Wilks' Lambda	.857	.780[b]	3.000	14.000	.524	.143	2.341	.176
	Hotelling's Trace	.167	.780[b]	3.000	14.000	.524	.143	2.341	.176
	Roy's Largest Root	.167	.780[b]	3.000	14.000	.524	.143	2.341	.176
Diet * Sex	Pillai's Trace	.061	.306[b]	3.000	14.000	.821	.061	.917	.095
	Wilks' Lambda	.939	.306[b]	3.000	14.000	.821	.061	.917	.095
	Hotelling's Trace	.065	.306[b]	3.000	14.000	.821	.061	.917	.095
	Roy's Largest Root	.065	.306[b]	3.000	14.000	.821	.061	.917	.095
Agemo * Diet * Sex	Pillai's Trace	.115	.603[b]	3.000	14.000	.623	.115	1.810	.144
	Wilks' Lambda	.885	.603[b]	3.000	14.000	.623	.115	1.810	.144
	Hotelling's Trace	.129	.603[b]	3.000	14.000	.623	.115	1.810	.144
	Roy's Largest Root	.129	.603[b]	3.000	14.000	.623	.115	1.810	.144

a. Design: Intercept + Agemo + Diet + Sex + Agemo * Diet + Agemo * Sex + Diet * Sex + Agemo * Diet * Sex

b. Exact statistic

c. Computed using alpha = .05

Table 2. Tests of Between-Subjects Effects for Normalized Strong Positive Staining of proBDNF with Age, Sex, Diet, and Interactions

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[d]
Corrected Model	Brain proBDNF Normalized Strong	.009[a]	7	.001	5.364	.003	.701	37.551	.972
	DC proBDNF Normalized Strong	.009[b]	7	.001	.654	.707	.222	4.576	.200
	JJ proBDNF Normalized Strong	.004[c]	7	.001	2.191	.092	.489	15.337	.629
Intercept	Brain proBDNF Normalized Strong	.223	1	.223	922.579	<.001	.983	922.579	1.000
	DC proBDNF Normalized Strong	.314	1	.314	154.932	<.001	.906	154.932	1.000
	JJ proBDNF Normalized Strong	.191	1	.191	662.868	<.001	.976	662.868	1.000
Agemo	Brain proBDNF Normalized Strong	.004	1	.004	15.536	.001	.493	15.536	.959
	DC proBDNF Normalized Strong	.001	1	.001	.709	.412	.042	.709	.124
	JJ proBDNF Normalized Strong	.001	1	.001	2.050	.171	.114	2.050	.270
Diet	Brain proBDNF Normalized Strong	.002	1	.002	7.353	.015	.315	7.353	.721
	DC proBDNF Normalized Strong	.005	1	.005	2.332	.146	.127	2.332	.300
	JJ proBDNF Normalized Strong	.003	1	.003	8.923	.009	.358	8.923	.801
Sex	Brain proBDNF Normalized Strong	.000	1	.000	.919	.352	.054	.919	.147
	DC proBDNF Normalized Strong	5.751E-5	1	5.751E-5	.028	.868	.002	.028	.053
	JJ proBDNF Normalized Strong	.000	1	.000	.446	.514	.027	.446	.096
Agemo * Diet	Brain proBDNF Normalized Strong	.000	1	.000	1.165	.296	.068	1.165	.174
	DC proBDNF Normalized Strong	8.712E-6	1	8.712E-6	.004	.949	.000	.004	.050
	JJ proBDNF Normalized Strong	.000	1	.000	.456	.509	.028	.456	.097
Agemo * Sex	Brain proBDNF Normalized Strong	2.136E-6	1	2.136E-6	.009	.926	.001	.009	.051
	DC proBDNF Normalized Strong	.000	1	.000	.164	.691	.010	.164	.067
	JJ proBDNF Normalized Strong	.001	1	.001	1.930	.184	.108	1.930	.257
Diet * Sex	Brain proBDNF Normalized Strong	1.281E-5	1	1.281E-5	.053	.821	.003	.053	.055
	DC proBDNF Normalized Strong	1.021E-5	1	1.021E-5	.005	.944	.000	.005	.051
	JJ proBDNF Normalized Strong	.000	1	.000	.923	.351	.055	.923	.148
Agemo * Diet * Sex	Brain proBDNF Normalized Strong	6.826E-5	1	6.826E-5	.282	.603	.017	.282	.079
	DC proBDNF Normalized Strong	.002	1	.002	.982	.337	.058	.982	.154
	JJ proBDNF Normalized Strong	.000	1	.000	.623	.441	.038	.623	.115
Error	Brain proBDNF Normalized Strong	.004	16	.000					
	DC proBDNF Normalized Strong	.032	16	.002					
	JJ proBDNF Normalized Strong	.005	16	.000					
Total	Brain proBDNF Normalized Strong	.406	24						
	DC proBDNF Normalized Strong	.520	24						
	JJ proBDNF Normalized Strong	.273	24						
Corrected Total	Brain proBDNF Normalized Strong	.013	23						
	DC proBDNF Normalized Strong	.042	23						
	JJ proBDNF Normalized Strong	.009	23						

a. R Squared = .701 (Adjusted R Squared = .571)

b. R Squared = .222 (Adjusted R Squared = -.118)

c. R Squared = .489 (Adjusted R Squared = .266)

d. Computed using alpha = .05

Age: Normalized Strong Positive

For normalized strong positive staining of brain proBDNF, we found statistically significant between-subject effects for age [p=0.001, partial η2 = 0.493, observed power = 0.959], but not for strong positive staining of distal colon or jejunum proBDNF (Table 2).

In pairwise comparisons (Table 3), brain proBDNF mean difference for age (3 mo. - 6 mo.) was 0.31 [std. error = 0.008, 95% CI lower = 0.14, CI upper= 0.47]. Figure 3 is a quantified representation of these results.

Table 3. Pairwise Comparisons for Normalized Strong proBDNF and Age (mo)

Pairwise Comparisons

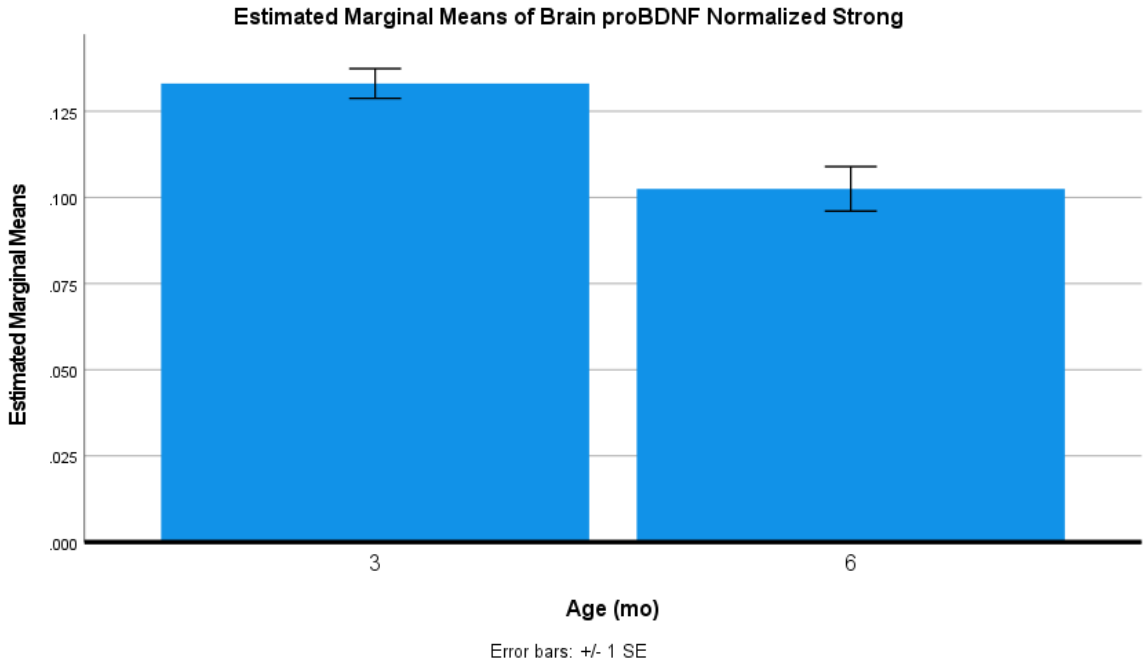
Dependent Variable	(I) Age (mo)	(J) Age (mo)	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
Brain proBDNF Normalized Strong	3	6	.031*	.008	.001	.014	.047
	6	3	-.031*	.008	.001	-.047	-.014
DC proBDNF Normalized Strong	3	6	.019	.022	.412	-.029	.067
	6	3	-.019	.022	.412	-.067	.029
JJ proBDNF Normalized Strong	3	6	-.012	.008	.171	-.030	.006
	6	3	.012	.008	.171	-.006	.030

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Figure 3. Estimated Marginal Means of Brain proBDNF Normalized Strong and Age (mo)



Diet: Normalized Strong Positive

For between-subject effects for diet (Table 2), statistical significance was found for normalized strong positive staining of brain proBDNF [p=0.015, partial η2 = 0.315, observed power = 0.721] and jejunum proBDNF [p=0.009, partial η2 = 0..358, observed power = 0.801], but not for normalized strong positive staining of distal colon proBDNF.

In pairwise comparisons (Table 4), brain proBDNF mean difference for diet (normal - high fat) was 0.21 [std. error = 0.008, CI lower = 0.005, CI upper = 0.37]. For jejunum proBDNF, mean difference for diet (high fat - normal) was 0.25 [std. error = 0.008, CI lower = 0.007, CI upper = 0.043]. Figures 4 and 5 quantify these results respectively.

Table 4. Pairwise Comparisons for Normalized Strong proBDNF and Diet

Pairwise Comparisons

Dependent Variable	(I) Diet	(J) Diet	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
Brain proBDNF Normalized Strong	High fat	Normal	-.021*	.008	.015	-.037	-.005
	Normal	High fat	.021*	.008	.015	.005	.037
DC proBDNF Normalized Strong	High fat	Normal	.034	.022	.146	-.013	.082
	Normal	High fat	-.034	.022	.146	-.082	.013
JJ proBDNF Normalized Strong	High fat	Normal	.025*	.008	.009	.007	.043
	Normal	High fat	-.025*	.008	.009	-.043	-.007

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Figure 4. Estimated Marginal Means of Brain proBDNF Normalized Strong and Diet

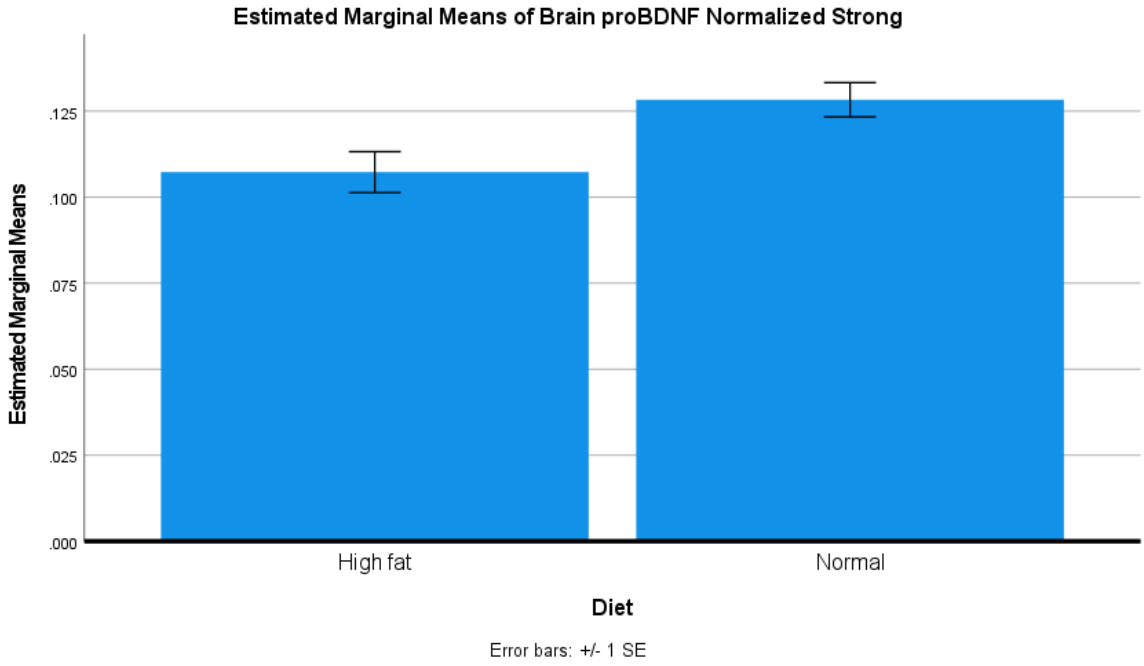
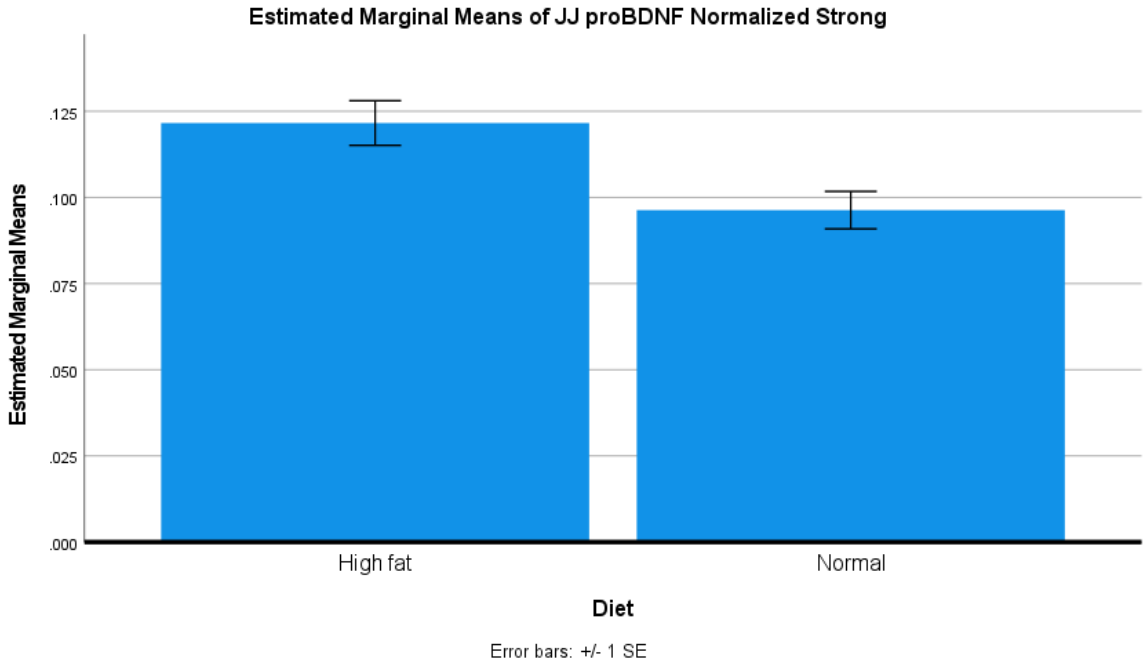


Figure 5. Estimated Marginal Means of Jejunum proBDNF Normalized Strong and Diet



Linear Regression

A linear regression established that age in days significantly predicted the amount of brain proBDNF normalized strong staining, $F(1,23) = 30.056$, $p < 0.001$, and age in days accounted for 54.8% of the explained variability in the amount of brain proBDNF normalized strong staining (Table 5). The regression equation was: predicted amount of brain proBDNF normalized strong staining = 0.18 - 3.64E-4*x (age in days) (Figure 6).

Table 5. Linear Regression Model Summary for Age (days) and Normalized Strong Brain proBDNF

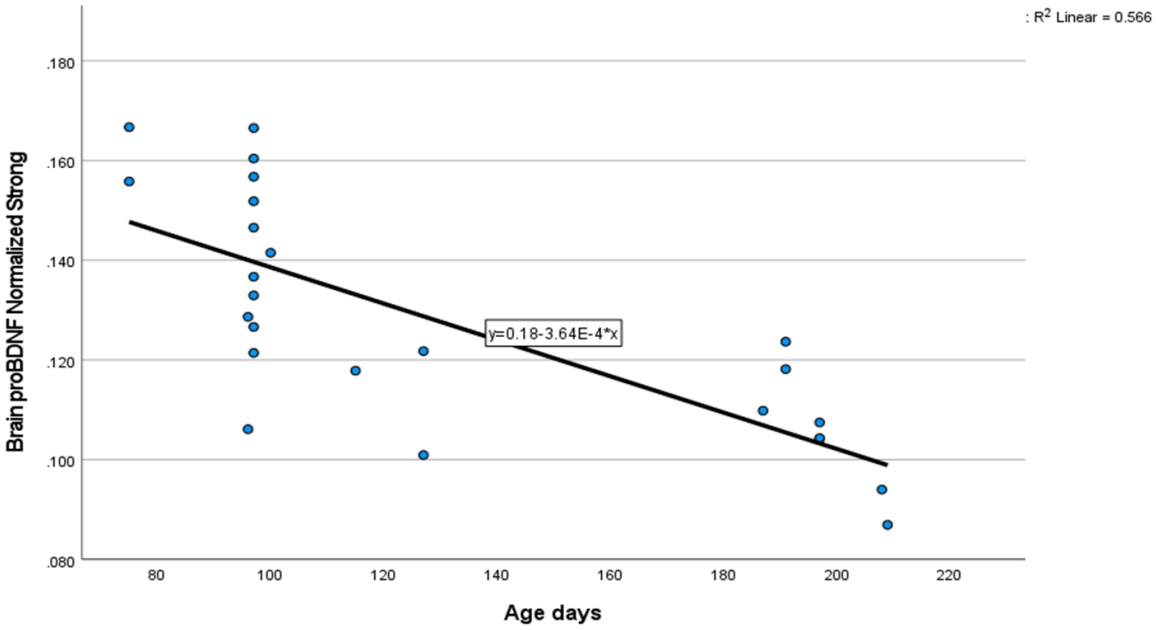
Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.753[a]	.566	.548	.015690	.566	30.056	1	23	<.001

a. Predictors: (Constant), Age days

b. Dependent Variable: Brain proBDNF Normalized Strong

Figure 6. Linear Regression Plot for Age (days) and Normalized Strong Brain proBDNF



A linear regression also established that age in days significantly predicted the amount of brain proBDNF normalized total staining, $F(1,23) = 5.874$, $p = 0.024$, and age in days accounted for 16.9% of the explained variability in the amount of brain proBDNF normalized total staining (Table 6). The regression equation was: predicted amount of brain proBDNF normalized total staining = 0.49 - 1.04E-3*x (age in days) (Figure 7).

Table 6. Linear Regression Model Summary for Age (days) and Normalized Total Brain proBDNF

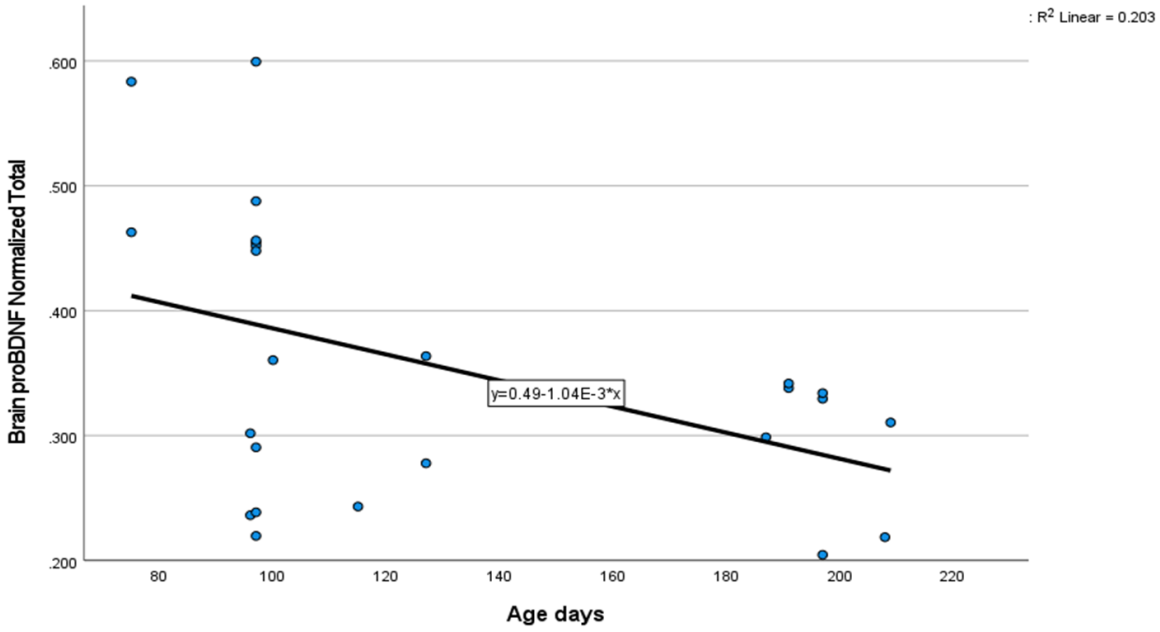
Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics			
					R Square Change	F Change	df1	df2	Sig. F Change
1	.451[a]	.203	.169	.101732	.203	5.874	1	23	.024

a. Predictors: (Constant), Age days

b. Dependent Variable: Brain proBDNF Normalized Total

Figure 7. Linear Regression Plot for Age (days) and Normalized Total Brain proBDNF



A linear regression also established that age in days significantly predicted the amount of distal colon proBDNF normalized positive staining, $F(1,22) = 7.011$, $p = 0.015$, and age in days accounted for 20.7% of the explained variability in the amount of distal colon proBDNF normalized positive staining (Table 7). The regression equation was: predicted amount of distal colon proBDNF normalized positive staining = 0.082 + 3.02E-4*x (age in days) (Figure 8).

Table 7. Linear Regression Model Summary for Age (days) and Normalized Positive Distal Colon proBDNF

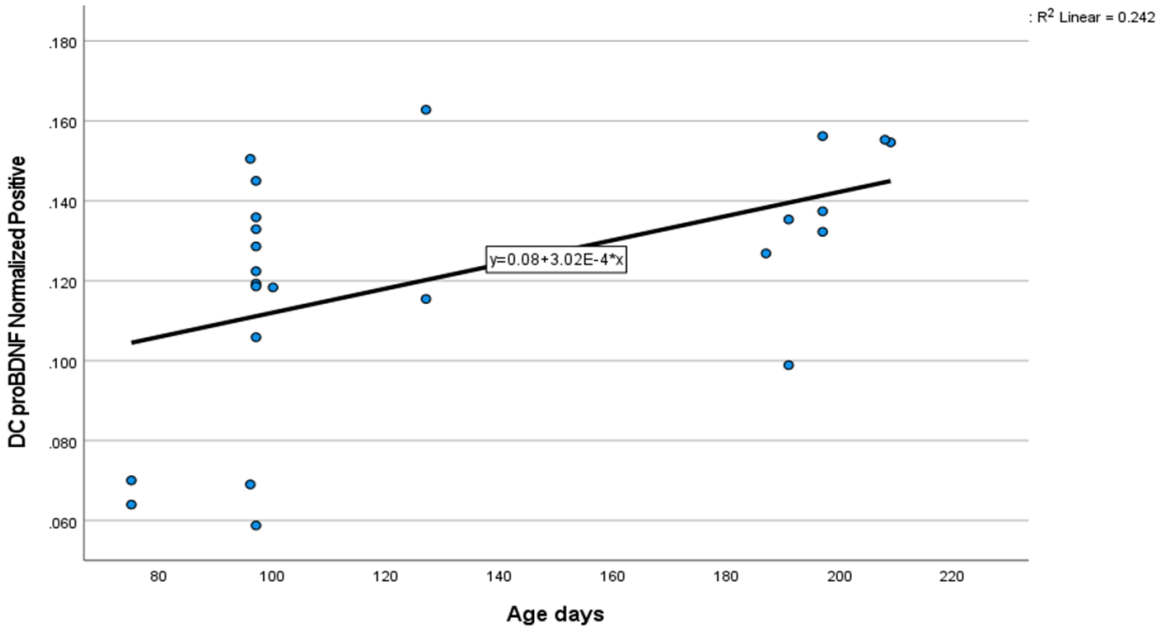
Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.492[a]	.242	.207	.026892	.242	7.011	1	22	.015

a. Predictors: (Constant), Age days

b. Dependent Variable: DC proBDNF Normalized Positive

Figure 8. Linear Regression Plot for Age (days) and Normalized Positive Distal Colon proBDNF



A linear regression also established that age in days significantly predicted the amount of jejunum proBDNF normalized strong staining, $F(1,22) = 5.090$, $p = 0.034$, and age in days accounted for 15.1% of the explained variability in the amount of jejunum proBDNF normalized strong staining (Table 8). The regression equation was: predicted

amount of jejunum proBDNF normalized strong staining = 0.08 + 1.75E-4*x (age in days) (Figure 9).

Table 8. Linear Regression Model Summary for Age (days) and Normalized Strong Jejunum proBDNF

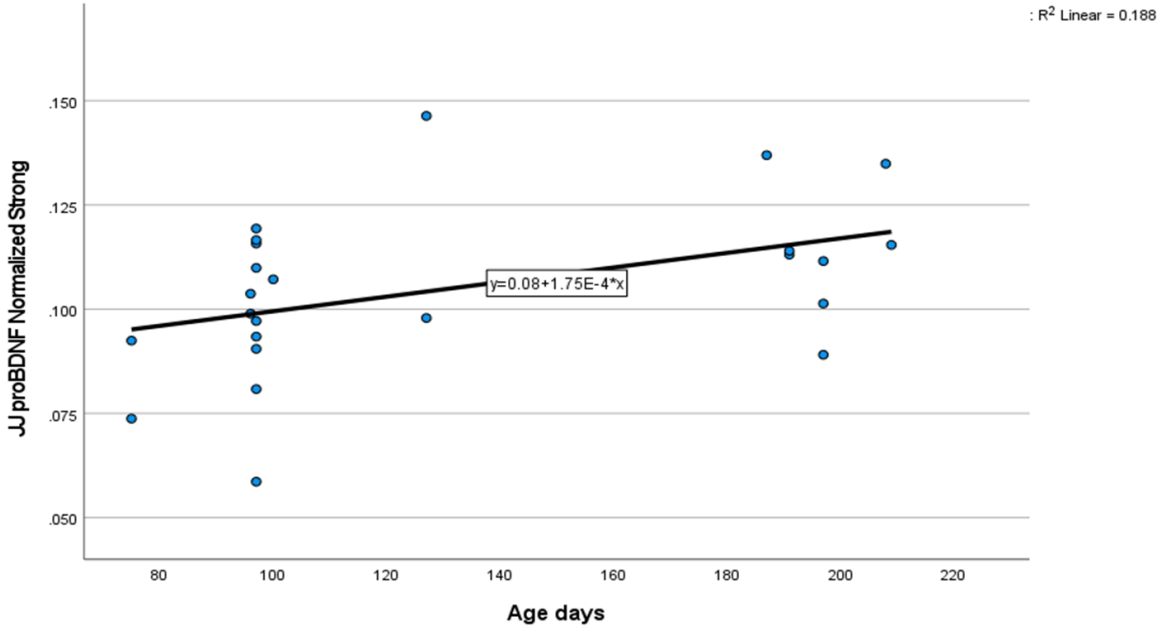
Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.433[a]	.188	.151	.018264	.188	5.090	1	22	.034

a. Predictors: (Constant), Age days

b. Dependent Variable: JJ proBDNF Normalized Strong

Figure 9. Linear Regression Plot for Age (days) and Normalized Strong Jejunum proBDNF



A linear regression also established that age in days significantly predicted the amount of jejunum proBDNF normalized total staining, $F(1,22) = 5.149$, $p = 0.033$, and age in days accounted for 15.3% of the explained variability in the amount of jejunum proBDNF normalized total staining (Table 9). The regression equation was: predicted

amount of jejunum proBDNF normalized total staining = 0.19 + 4.55E-4*x (age in days) (Figure 10).

Table 9. Linear Regression Model Summary for Age (days) and Normalized Total Jejunum proBDNF

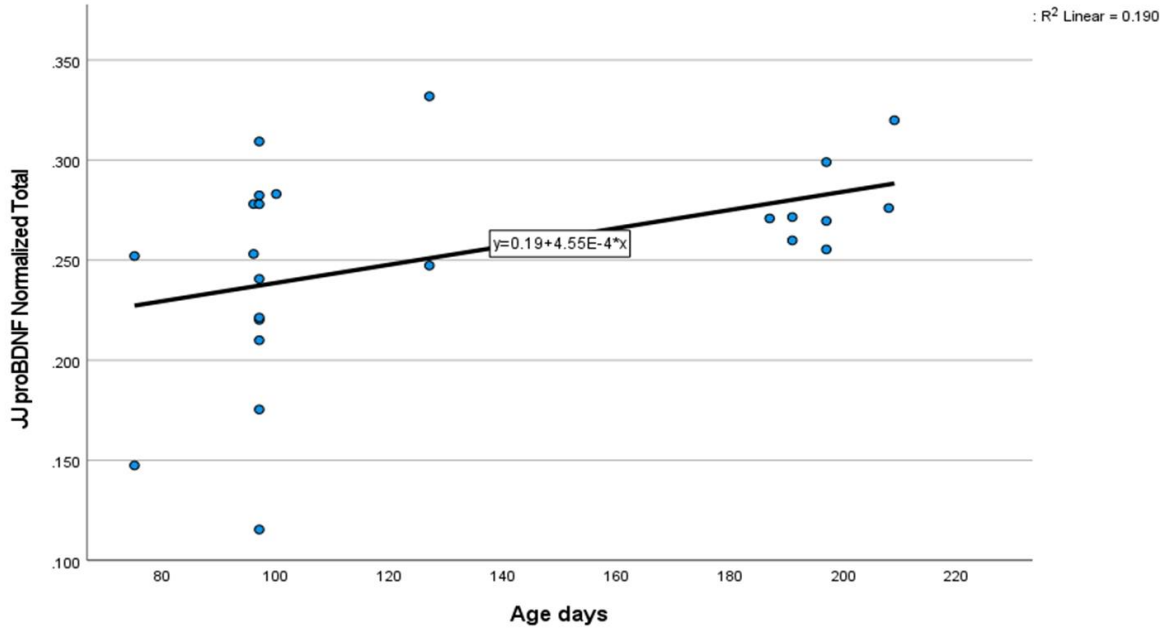
Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.435[a]	.190	.153	.047304	.190	5.149	1	22	.033

a. Predictors: (Constant), Age days

b. Dependent Variable: JJ proBDNF Normalized Total

Figure 10. Linear Regression Plot for Age (days) and Normalized Total Jejunum proBDNF



All other brain, distal colon, and jejunum proBDNF normalized data were not statistically significant to establish a linear relationship with age in days.

BDNF

MANOVA: Normalized Strong Positive

For normalized strong positive staining of BDNF, we found statistically significant main effects for age [$F(3, 13) = 8.978$, $p = 0.002$; Wilks' $\Lambda = 0.326$, partial $\eta 2 = 0.674$, observed power = 0.973] and diet [$F(3, 13) = 9.287$, $p = 0.002$; Wilks' $\Lambda = 0.318$, partial $\eta 2 = 0.682$, observed power = 0.977], but not for sex or any interactions (Table 10).

Table 10. MANOVA of Normalized Strong Positive Staining of BDNF with Age, Sex, Diet, and Interactions

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[c]
Intercept	Pillai's Trace	.988	365.392[b]	3.000	13.000	<.001	.988	1096.176	1.000
	Wilks' Lambda	.012	365.392[b]	3.000	13.000	<.001	.988	1096.176	1.000
	Hotelling's Trace	84.321	365.392[b]	3.000	13.000	<.001	.988	1096.176	1.000
	Roy's Largest Root	84.321	365.392[b]	3.000	13.000	<.001	.988	1096.176	1.000
Agemo	Pillai's Trace	.674	8.978[b]	3.000	13.000	.002	.674	26.935	.973
	Wilks' Lambda	.326	8.978[b]	3.000	13.000	.002	.674	26.935	.973
	Hotelling's Trace	2.072	8.978[b]	3.000	13.000	.002	.674	26.935	.973
	Roy's Largest Root	2.072	8.978[b]	3.000	13.000	.002	.674	26.935	.973
Diet	Pillai's Trace	.682	9.287[b]	3.000	13.000	.002	.682	27.862	.977
	Wilks' Lambda	.318	9.287[b]	3.000	13.000	.002	.682	27.862	.977
	Hotelling's Trace	2.143	9.287[b]	3.000	13.000	.002	.682	27.862	.977
	Roy's Largest Root	2.143	9.287[b]	3.000	13.000	.002	.682	27.862	.977
Sex	Pillai's Trace	.332	2.150[b]	3.000	13.000	.143	.332	6.449	.425
	Wilks' Lambda	.668	2.150[b]	3.000	13.000	.143	.332	6.449	.425
	Hotelling's Trace	.496	2.150[b]	3.000	13.000	.143	.332	6.449	.425
	Roy's Largest Root	.496	2.150[b]	3.000	13.000	.143	.332	6.449	.425
Agemo * Diet	Pillai's Trace	.394	2.819[b]	3.000	13.000	.080	.394	8.458	.539
	Wilks' Lambda	.606	2.819[b]	3.000	13.000	.080	.394	8.458	.539
	Hotelling's Trace	.651	2.819[b]	3.000	13.000	.080	.394	8.458	.539
	Roy's Largest Root	.651	2.819[b]	3.000	13.000	.080	.394	8.458	.539
Agemo * Sex	Pillai's Trace	.154	.789[b]	3.000	13.000	.521	.154	2.367	.175
	Wilks' Lambda	.846	.789[b]	3.000	13.000	.521	.154	2.367	.175
	Hotelling's Trace	.182	.789[b]	3.000	13.000	.521	.154	2.367	.175
	Roy's Largest Root	.182	.789[b]	3.000	13.000	.521	.154	2.367	.175
Diet * Sex	Pillai's Trace	.022	.099[b]	3.000	13.000	.959	.022	.297	.064
	Wilks' Lambda	.978	.099[b]	3.000	13.000	.959	.022	.297	.064
	Hotelling's Trace	.023	.099[b]	3.000	13.000	.959	.022	.297	.064
	Roy's Largest Root	.023	.099[b]	3.000	13.000	.959	.022	.297	.064
Agemo * Diet * Sex	Pillai's Trace	.058	.265[b]	3.000	13.000	.849	.058	.796	.088
	Wilks' Lambda	.942	.265[b]	3.000	13.000	.849	.058	.796	.088
	Hotelling's Trace	.061	.265[b]	3.000	13.000	.849	.058	.796	.088
	Roy's Largest Root	.061	.265[b]	3.000	13.000	.849	.058	.796	.088

a. Design: Intercept + Agemo + Diet + Sex + Agemo * Diet + Agemo * Sex + Diet * Sex + Agemo * Diet * Sex

b. Exact statistic

c. Computed using alpha = .05

Table 11. Tests of Between-Subjects Effects for Normalized Strong Positive Staining of BDNF with Age, Sex, Diet, and Interactions

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[d]
Corrected Model	Brain BDNF Normalized Strong	.008[a]	7	.001	2.607	.057	.549	18.250	.706
	DC BDNF Normalized Strong	.103[b]	7	.015	15.246	<.001	.877	106.723	1.000
	JJ BDNF Normalized Strong	.020[c]	7	.003	3.342	.024	.609	23.397	.825
Intercept	Brain BDNF Normalized Strong	.213	1	.213	469.180	<.001	.969	469.180	1.000
	DC BDNF Normalized Strong	.362	1	.362	376.635	<.001	.962	376.635	1.000
	JJ BDNF Normalized Strong	.167	1	.167	199.351	<.001	.930	199.351	1.000
Agemo	Brain BDNF Normalized Strong	.001	1	.001	2.882	.110	.161	2.882	.356
	DC BDNF Normalized Strong	.018	1	.018	19.212	<.001	.562	19.212	.983
	JJ BDNF Normalized Strong	.007	1	.007	8.030	.013	.349	8.030	.755
Diet	Brain BDNF Normalized Strong	.002	1	.002	3.469	.082	.188	3.469	.414
	DC BDNF Normalized Strong	.023	1	.023	24.334	<.001	.619	24.334	.996
	JJ BDNF Normalized Strong	.002	1	.002	2.252	.154	.131	2.252	.290
Sex	Brain BDNF Normalized Strong	8.640E-5	1	8.640E-5	.190	.669	.013	.190	.069
	DC BDNF Normalized Strong	.004	1	.004	4.197	.058	.219	4.197	.483
	JJ BDNF Normalized Strong	.001	1	.001	1.491	.241	.090	1.491	.208
Agemo * Diet	Brain BDNF Normalized Strong	.001	1	.001	1.957	.182	.115	1.957	.258
	DC BDNF Normalized Strong	.004	1	.004	3.651	.075	.196	3.651	.432
	JJ BDNF Normalized Strong	.003	1	.003	2.989	.104	.166	2.989	.367
Agemo * Sex	Brain BDNF Normalized Strong	.001	1	.001	1.697	.212	.102	1.697	.231
	DC BDNF Normalized Strong	6.812E-5	1	6.812E-5	.071	.794	.005	.071	.057
	JJ BDNF Normalized Strong	.000	1	.000	.319	.581	.021	.319	.083
Diet * Sex	Brain BDNF Normalized Strong	6.065E-7	1	6.065E-7	.001	.971	.000	.001	.050
	DC BDNF Normalized Strong	.000	1	.000	.171	.685	.011	.171	.067
	JJ BDNF Normalized Strong	.000	1	.000	.233	.636	.015	.233	.074
Agemo * Diet * Sex	Brain BDNF Normalized Strong	9.661E-5	1	9.661E-5	.213	.651	.014	.213	.072
	DC BDNF Normalized Strong	.000	1	.000	.137	.717	.009	.137	.064
	JJ BDNF Normalized Strong	.000	1	.000	.397	.538	.026	.397	.091
Error	Brain BDNF Normalized Strong	.007	15	.000					
	DC BDNF Normalized Strong	.014	15	.001					
	JJ BDNF Normalized Strong	.013	15	.001					
Total	Brain BDNF Normalized Strong	.355	23						
	DC BDNF Normalized Strong	.836	23						
	JJ BDNF Normalized Strong	.338	23						
Corrected Total	Brain BDNF Normalized Strong	.015	22						
	DC BDNF Normalized Strong	.117	22						
	JJ BDNF Normalized Strong	.032	22						

a. R Squared = .549 (Adjusted R Squared = .338)

b. R Squared = .877 (Adjusted R Squared = .819)

c. R Squared = .609 (Adjusted R Squared = .427)

d. Computed using alpha = .05

Age: Normalized Strong Positive

We found statistically significant between-subject effects for normalized strong positive staining of distal colon BDNF and age [$p < 0.001$, partial $\eta 2 = 0.562$, observed power = 0.938] and normalized strong positive staining of jejunum BDNF and age [$p = 0.013$, partial $\eta 2 = 0.349$, observed power = 0.755], but not for strong positive staining of brain BDNF (Table 11).

In pairwise comparisons (Table 12), distal colon BDNF mean difference for age (3 mo. – 6 mo.) was 0.068 [std. error = 0.016, 95% CI lower = 0.035, CI upper= 0.102]. The jejunum BDNF mean difference for age (3 mo. – 6 mo.) was 0.041 [std. error = 0.015, 95% CI lower = 0.010, CI upper= 0.072]. These results are quantified in Figures 11 and 12.

Table 12. Pairwise Comparisons for Normalized Strong BDNF and Age (mo)

Pairwise Comparisons

Dependent Variable	(I) Age (mo)	(J) Age (mo)	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
Brain BDNF Normalized Strong	3	6	.018	.011	.110	-.005	.041
	6	3	-.018	.011	.110	-.041	.005
DC BDNF Normalized Strong	3	6	.068*	.016	<.001	.035	.102
	6	3	-.068*	.016	<.001	-.102	-.035
JJ BDNF Normalized Strong	3	6	.041*	.015	.013	.010	.072
	6	3	-.041*	.015	.013	-.072	-.010

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Figure 11. Estimated Marginal Means of Normalized Strong Distal Colon BDNF and Age (mo)

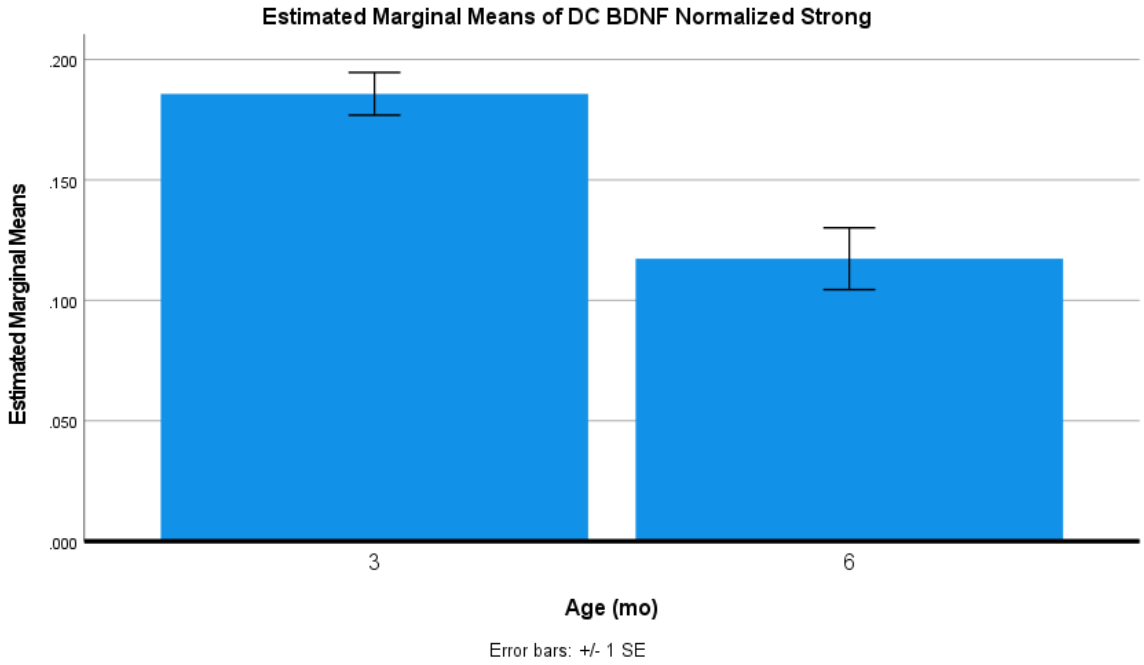
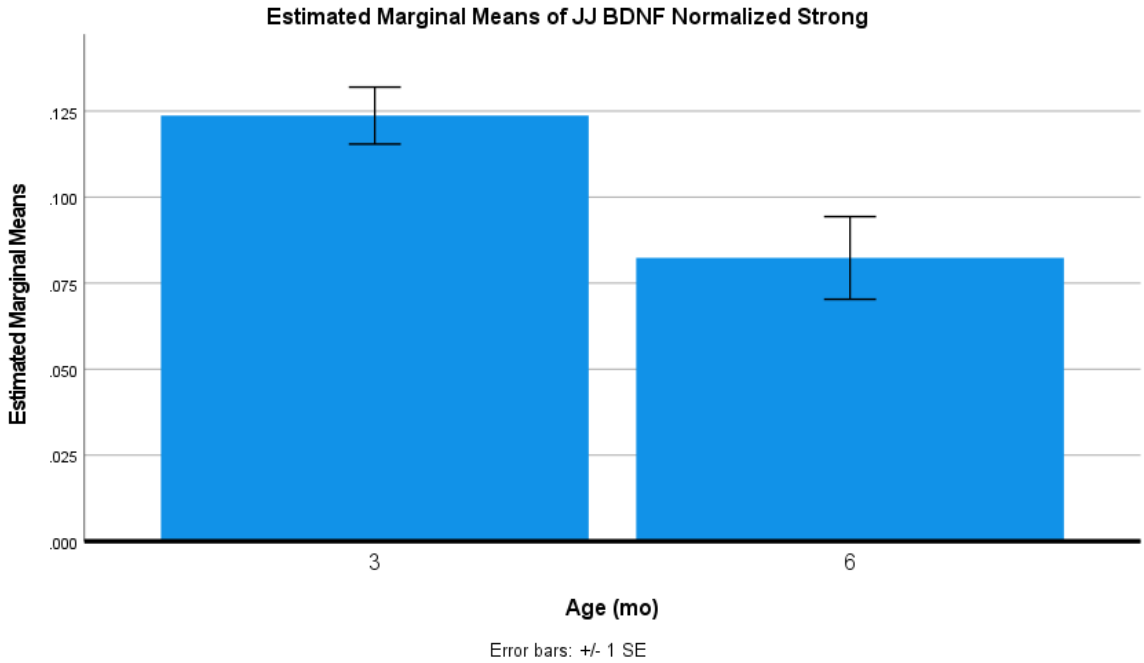


Figure 12. Estimated Marginal Means of Normalized Strong Jejunum BDNF and Age (mo)



Diet: Normalized Strong Positive

For normalized strong positive staining of distal colon BDNF, we found statistically significant between-subject effects for diet [$p < 0.001$, partial $\eta 2 = 0.619$, observed power = 0.996], but not for normalized strong positive staining of brain or jejunum proBDNF (Table 11).

In pairwise comparisons (Table 13), distal colon BDNF mean difference for diet (normal – high fat) was 0.77 [std. error = 0.016, CI lower = 0.044, CI upper = 0.110].

Table 13. Pairwise Comparisons for Normalized Strong BDNF and Diet

Pairwise Comparisons

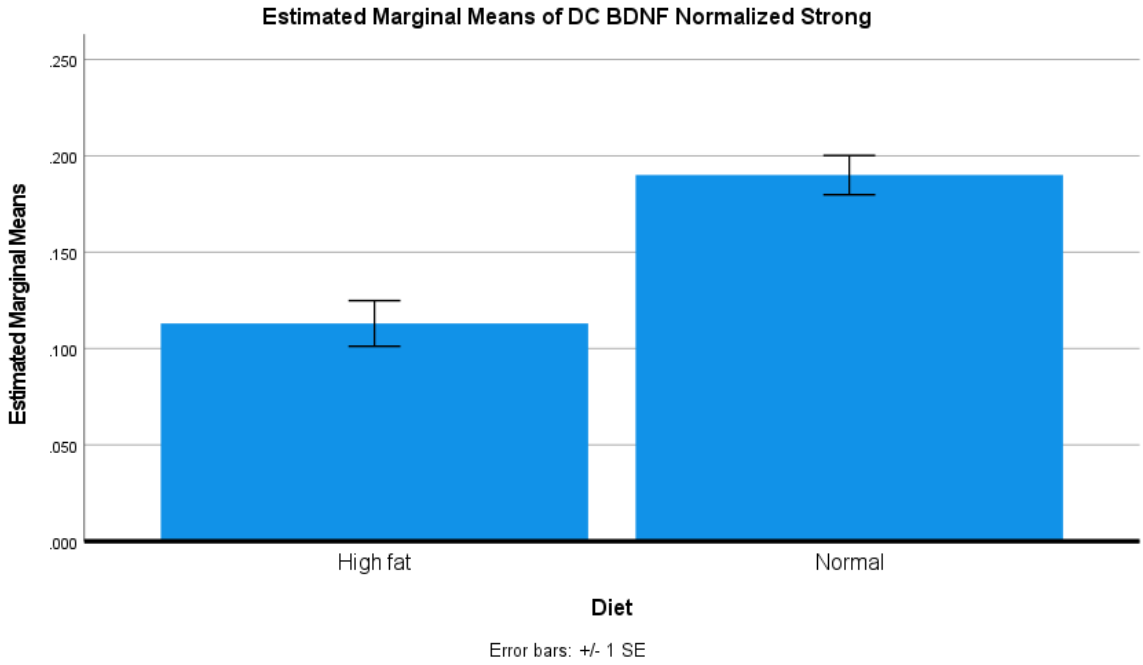
Dependent Variable	(I) Diet	(J) Diet	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
Brain BDNF Normalized Strong	High fat	Normal	-.020	.011	.082	-.043	.003
	Normal	High fat	.020	.011	.082	-.003	.043
DC BDNF Normalized Strong	High fat	Normal	-.077*	.016	<.001	-.110	-.044
	Normal	High fat	.077*	.016	<.001	.044	.110
JJ BDNF Normalized Strong	High fat	Normal	-.022	.015	.154	-.053	.009
	Normal	High fat	.022	.015	.154	-.009	.053

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Figure 13. Estimated Marginal Means of Normalized Strong Distal Colon BDNF and Diet



MANOVA: Normalized Positive

For normalized positive staining of BDNF, we found statistically significant main effects for age [$F(3, 13) = 42.771$, $p < 0.001$; Wilks' $\Lambda = 0.092$, partial $\eta 2 = 0.908$, observed power = 1.000], diet [$F(3, 13) = 20.486$, $p < 0.001$; Wilks' $\Lambda = 0.175$, partial $\eta 2 = 0.825$, observed power = 1.000], and age*sex [$F(3, 13) = 4.358$, $p = 0.025$; Wilks' $\Lambda = 0.499$, partial $\eta 2 = 0.501$, observed power = 0.745], but not for sex or any other interactions (Table 14).

For age*sex, we found statistically significant between-subject effects for normalized positive staining of brain BDNF [$p = 0.044$, partial $\eta 2 = 0.244$, observed power = 0.540], but not for normalized positive staining of distal colon or jejunum BDNF (Table 15).

Table 14. MANOVA of Normalized Positive Staining of BDNF with Age, Sex, Diet, and Interactions

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[c]
Intercept	Pillai's Trace	.996	1053.154[b]	3.000	13.000	<.001	.996	3159.461	1.000
	Wilks' Lambda	.004	1053.154[b]	3.000	13.000	<.001	.996	3159.461	1.000
	Hotelling's Trace	243.035	1053.154[b]	3.000	13.000	<.001	.996	3159.461	1.000
	Roy's Largest Root	243.035	1053.154[b]	3.000	13.000	<.001	.996	3159.461	1.000
Agemo	Pillai's Trace	.908	42.771[b]	3.000	13.000	<.001	.908	128.313	1.000
	Wilks' Lambda	.092	42.771[b]	3.000	13.000	<.001	.908	128.313	1.000
	Hotelling's Trace	9.870	42.771[b]	3.000	13.000	<.001	.908	128.313	1.000
	Roy's Largest Root	9.870	42.771[b]	3.000	13.000	<.001	.908	128.313	1.000
Diet	Pillai's Trace	.825	20.486[b]	3.000	13.000	<.001	.825	61.457	1.000
	Wilks' Lambda	.175	20.486[b]	3.000	13.000	<.001	.825	61.457	1.000
	Hotelling's Trace	4.727	20.486[b]	3.000	13.000	<.001	.825	61.457	1.000
	Roy's Largest Root	4.727	20.486[b]	3.000	13.000	<.001	.825	61.457	1.000
Sex	Pillai's Trace	.322	2.059[b]	3.000	13.000	.155	.322	6.177	.409
	Wilks' Lambda	.678	2.059[b]	3.000	13.000	.155	.322	6.177	.409
	Hotelling's Trace	.475	2.059[b]	3.000	13.000	.155	.322	6.177	.409
	Roy's Largest Root	.475	2.059[b]	3.000	13.000	.155	.322	6.177	.409
Agemo * Diet	Pillai's Trace	.432	3.296[b]	3.000	13.000	.055	.432	9.887	.612
	Wilks' Lambda	.568	3.296[b]	3.000	13.000	.055	.432	9.887	.612
	Hotelling's Trace	.761	3.296[b]	3.000	13.000	.055	.432	9.887	.612
	Roy's Largest Root	.761	3.296[b]	3.000	13.000	.055	.432	9.887	.612
Agemo * Sex	Pillai's Trace	.501	4.358[b]	3.000	13.000	.025	.501	13.073	.745
	Wilks' Lambda	.499	4.358[b]	3.000	13.000	.025	.501	13.073	.745
	Hotelling's Trace	1.006	4.358[b]	3.000	13.000	.025	.501	13.073	.745
	Roy's Largest Root	1.006	4.358[b]	3.000	13.000	.025	.501	13.073	.745
Diet * Sex	Pillai's Trace	.295	1.817[b]	3.000	13.000	.194	.295	5.452	.365
	Wilks' Lambda	.705	1.817[b]	3.000	13.000	.194	.295	5.452	.365
	Hotelling's Trace	.419	1.817[b]	3.000	13.000	.194	.295	5.452	.365
	Roy's Largest Root	.419	1.817[b]	3.000	13.000	.194	.295	5.452	.365
Agemo * Diet * Sex	Pillai's Trace	.129	.643[b]	3.000	13.000	.601	.129	1.929	.149
	Wilks' Lambda	.871	.643[b]	3.000	13.000	.601	.129	1.929	.149
	Hotelling's Trace	.148	.643[b]	3.000	13.000	.601	.129	1.929	.149
	Roy's Largest Root	.148	.643[b]	3.000	13.000	.601	.129	1.929	.149

a. Design: Intercept + Agemo + Diet + Sex + Agemo * Diet + Agemo * Sex + Diet * Sex + Agemo * Diet * Sex

b. Exact statistic

c. Computed using alpha = .05

Table 15. Tests of Between-Subjects Effects for Normalized Positive Staining of BDNF with Age, Sex, Diet, and Interactions

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[d]
Corrected Model	Brain BDNF Normalized Positive	.047[a]	7	.007	1.474	.249	.408	10.321	.429
	DC BDNF Normalized Positive	.030[b]	7	.004	10.708	<.001	.833	74.957	1.000
	JJ BDNF Normalized Positive	.110[c]	7	.016	8.564	<.001	.800	59.945	.999
Intercept	Brain BDNF Normalized Positive	1.795	1	1.795	393.183	<.001	.963	393.183	1.000
	DC BDNF Normalized Positive	.576	1	.576	1438.080	<.001	.990	1438.080	1.000
	JJ BDNF Normalized Positive	1.062	1	1.062	577.794	<.001	.975	577.794	1.000
Agemo	Brain BDNF Normalized Positive	.025	1	.025	5.473	.034	.267	5.473	.590
	DC BDNF Normalized Positive	.014	1	.014	35.694	<.001	.704	35.694	1.000
	JJ BDNF Normalized Positive	.056	1	.056	30.473	<.001	.670	30.473	.999
Diet	Brain BDNF Normalized Positive	.005	1	.005	1.188	.293	.073	1.188	.175
	DC BDNF Normalized Positive	.005	1	.005	13.533	.002	.474	13.533	.930
	JJ BDNF Normalized Positive	.036	1	.036	19.584	<.001	.566	19.584	.985
Sex	Brain BDNF Normalized Positive	.009	1	.009	1.873	.191	.111	1.873	.249
	DC BDNF Normalized Positive	.000	1	.000	.422	.526	.027	.422	.093
	JJ BDNF Normalized Positive	.005	1	.005	2.588	.128	.147	2.588	.325
Agemo * Diet	Brain BDNF Normalized Positive	.001	1	.001	.140	.713	.009	.140	.064
	DC BDNF Normalized Positive	6.079E-6	1	6.079E-6	.015	.904	.001	.015	.052
	JJ BDNF Normalized Positive	.013	1	.013	6.891	.019	.315	6.891	.690
Agemo * Sex	Brain BDNF Normalized Positive	.022	1	.022	4.854	.044	.244	4.854	.540
	DC BDNF Normalized Positive	.000	1	.000	.850	.371	.054	.850	.139
	JJ BDNF Normalized Positive	.004	1	.004	2.316	.149	.134	2.316	.297
Diet * Sex	Brain BDNF Normalized Positive	.001	1	.001	.125	.729	.008	.125	.063
	DC BDNF Normalized Positive	.001	1	.001	1.950	.183	.115	1.950	.258
	JJ BDNF Normalized Positive	.008	1	.008	4.577	.049	.234	4.577	.517
Agemo * Diet * Sex	Brain BDNF Normalized Positive	.003	1	.003	.576	.460	.037	.576	.110
	DC BDNF Normalized Positive	.001	1	.001	1.361	.262	.083	1.361	.194
	JJ BDNF Normalized Positive	.000	1	.000	.152	.702	.010	.152	.065
Error	Brain BDNF Normalized Positive	.068	15	.005					
	DC BDNF Normalized Positive	.006	15	.000					
	JJ BDNF Normalized Positive	.028	15	.002					
Total	Brain BDNF Normalized Positive	3.157	23						
	DC BDNF Normalized Positive	.735	23						
	JJ BDNF Normalized Positive	1.283	23						
Corrected Total	Brain BDNF Normalized Positive	.116	22						
	DC BDNF Normalized Positive	.036	22						
	JJ BDNF Normalized Positive	.138	22						

a. R Squared = .408 (Adjusted R Squared = .131)

b. R Squared = .833 (Adjusted R Squared = .755)

c. R Squared = .800 (Adjusted R Squared = .706)

d. Computed using alpha = .05

Age: Normalized Positive

We found statistically significant between-subject effects (Table 15) for normalized positive staining of brain BDNF and age [$p = 0.034$, partial $\eta 2 = 0.267$, observed power = 0.590], normalized positive staining of distal colon BDNF and age [$p < 0.001$, partial $\eta 2 = 0.704$, observed power = 1.000], and normalized positive staining of jejunum BDNF and age [$p < 0.001$, partial $\eta 2 = 0.670$, observed power = 0.999].

In pairwise comparisons (Table 16), normalized positive brain BDNF mean difference for age (3 mo. - 6 mo.) was 0.080 [std. error = 0.034, 95% CI lower = 0.007, CI upper= 0.152]. The normalized positive distal colon BDNF mean difference for age (3 mo. - 6 mo.) was 0.060 [std. error = 0.010, 95% CI lower = 0.039, CI upper= 0.082]. The normalized positive jejunum BDNF mean difference for age (3 mo. - 6 mo.) was 0.119 [std. error = 0.022, 95% CI lower = 0.073, CI upper= 0.165]. These results are quantified in Figures 14, 15, and 16.

Table 16. Pairwise Comparisons for Normalized Positive BDNF and Age (mo)

Pairwise Comparisons

Dependent Variable	(I) Age (mo)	(J) Age (mo)	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
Brain BDNF Normalized Positive	3	6	.080*	.034	.034	.007	.152
	6	3	-.080*	.034	.034	-.152	-.007
DC BDNF Normalized Positive	3	6	-.060*	.010	<.001	-.082	-.039
	6	3	.060*	.010	<.001	.039	.082
JJ BDNF Normalized Positive	3	6	-.119*	.022	<.001	-.165	-.073
	6	3	.119*	.022	<.001	.073	.165

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Figure 14. Estimated Marginal Means of Normalized Positive Brain BDNF and Age (mo)

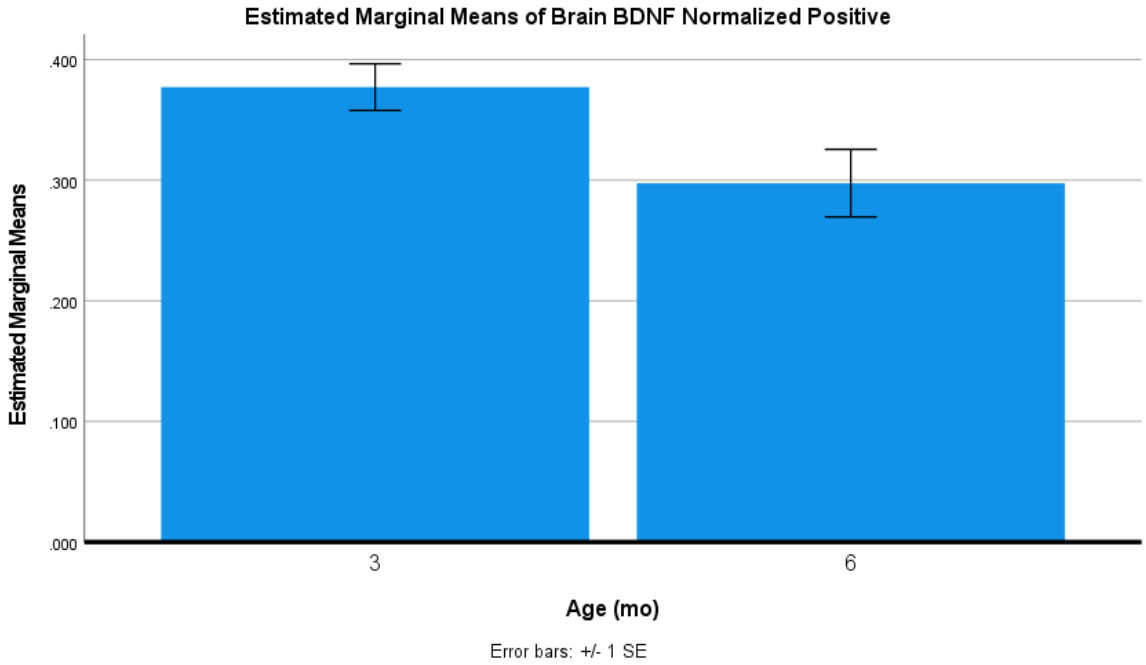


Figure 15. Estimated Marginal Means of Normalized Positive Distal Colon BDNF and Age (mo)

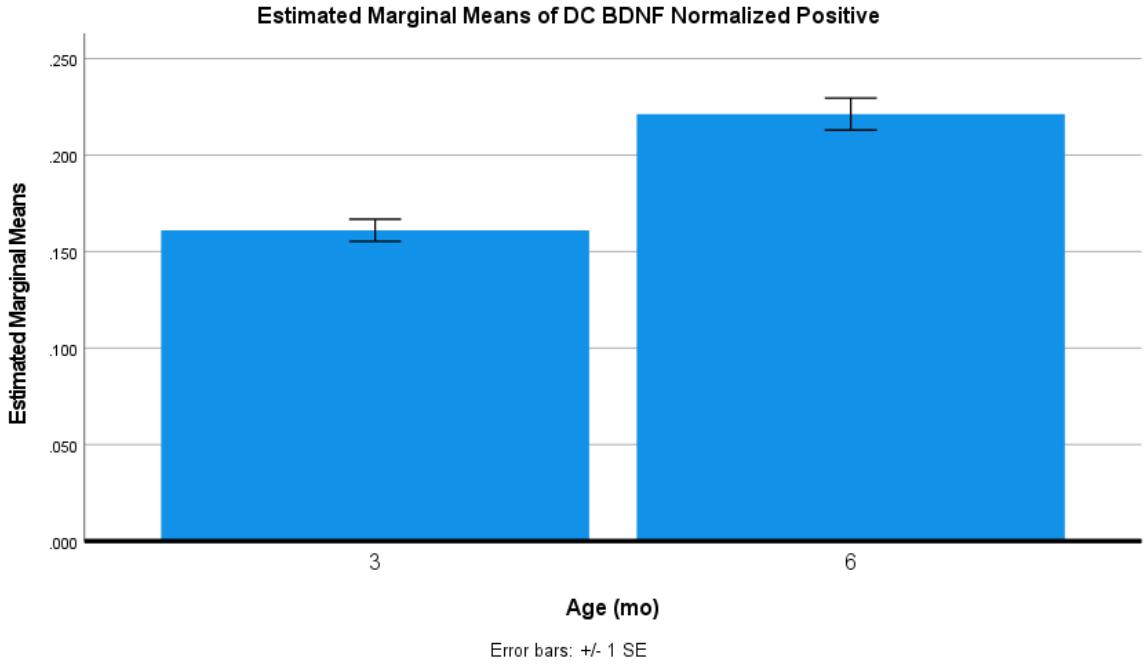
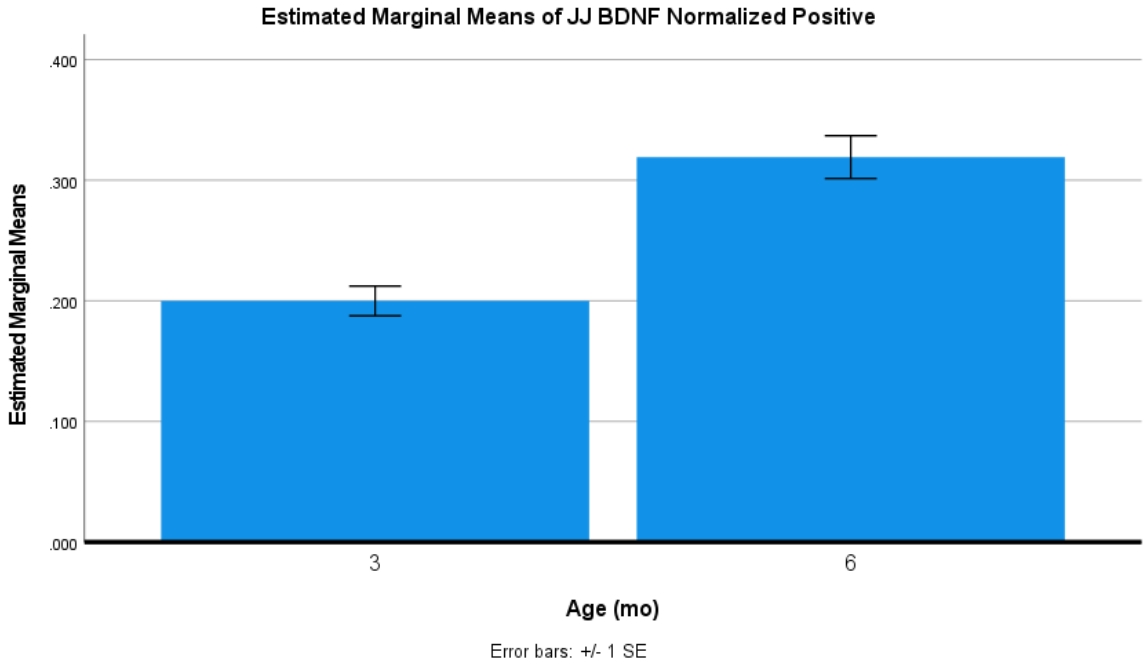


Figure 16. Estimated Marginal Means of Normalized Positive Jejunum BDNF and Age (mo)



Diet: Normalized Positive

For diet, we found statistically significant between-subject effects for normalized positive staining of distal colon BDNF [$p = 0.002$, partial $\eta 2 = 0.474$, observed power = 0.930] and normalized positive staining of jejunum BDNF [$p < 0.001$, partial $\eta 2 = 0.566$, observed power = 0.985], but not for normalized positive staining of brain BDNF.

In pairwise comparisons (Table 17), normalized positive distal colon BDNF mean difference for diet (normal - high fat) was 0.37 [std. error = 0.010, CI lower = 0.016, CI upper = 0.059]. The normalized positive jejunum BDNF mean difference for diet (normal - high fat) was 0.096 [std. error = 0.022, CI lower = 0.050, CI upper = 0.142]. These results are quantified in Figures 17 and 18.

Table 17. Pairwise Comparisons for Normalized Positive BDNF and Diet

Pairwise Comparisons

Dependent Variable	(I) Diet	(J) Diet	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
Brain BDNF Normalized Positive	High fat	Normal	-.037	.034	.293	-.110	.035
	Normal	High fat	.037	.034	.293	-.035	.110
DC BDNF Normalized Positive	High fat	Normal	.037*	.010	.002	.016	.059
	Normal	High fat	-.037*	.010	.002	-.059	-.016
JJ BDNF Normalized Positive	High fat	Normal	.096*	.022	<.001	.050	.142
	Normal	High fat	-.096*	.022	<.001	-.142	-.050

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Figure 17. Estimated Marginal Means of Normalized Positive Distal Colon BDNF and Diet

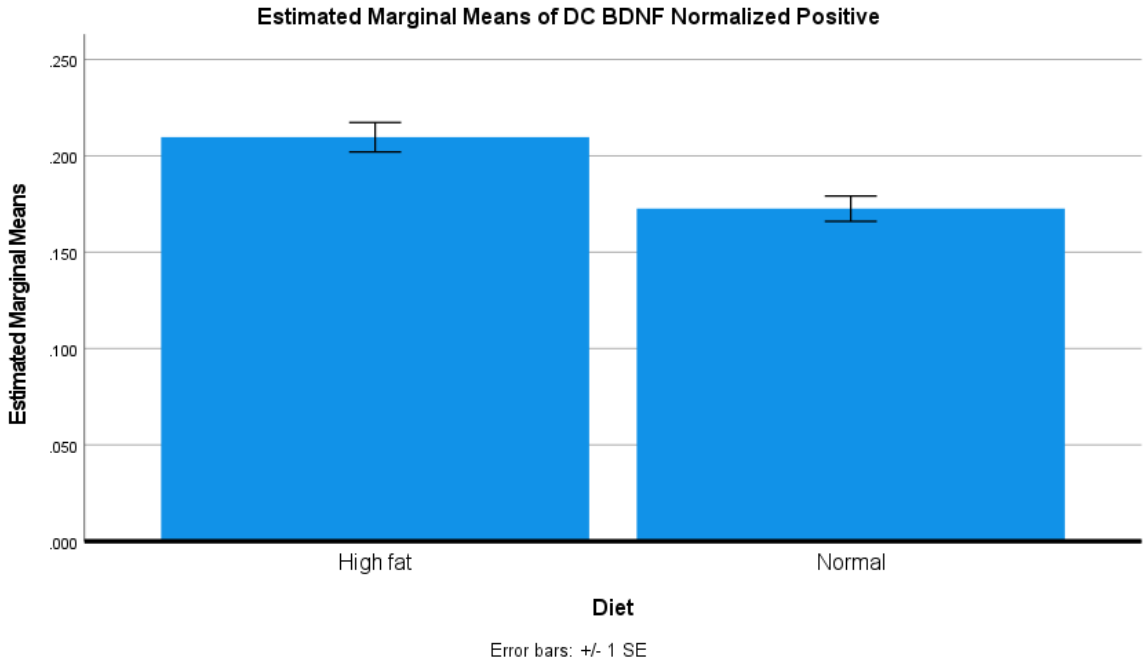
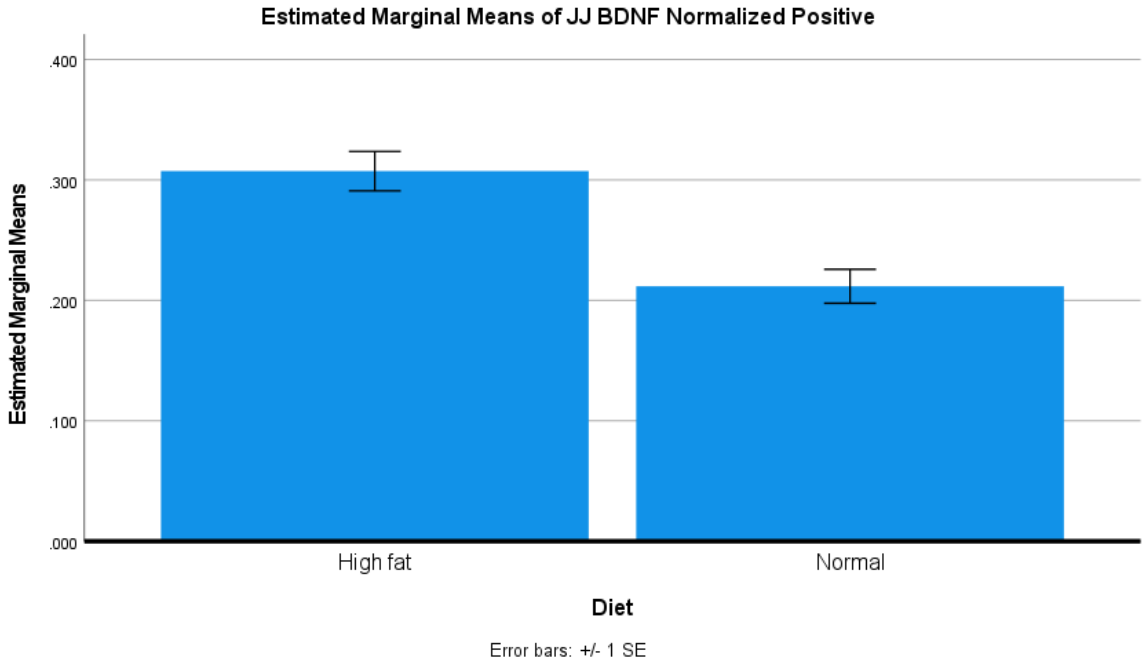


Figure 18. Estimated Marginal Means of Normalized Positive Jejunum BDNF and Diet



MANOVA: Normalized Total

For normalized total staining of BDNF, we found statistically significant main effects for age [$F(3, 13) = 7.346$, $p = 0.004$; Wilks' $\Lambda = 0.371$, partial $\eta 2 = 0.629$, observed power = 0.936], diet [$F(3, 13) = 5.131$, $p = 0.015$; Wilks' $\Lambda = 0.458$, partial $\eta 2 = 0.542$, observed power = 0.817], and age*diet [$F(3, 13) = 4.738$, $p = 0.019$; Wilks' $\Lambda = 0.478$, partial $\eta 2 = 0.522$, observed power = 0.783], but not for sex or any other interactions (Table 18).

For age*diet (Table 19), we found statistically significant between subjects effects for normalized total staining of jejunum BDNF [$p = 0.05$, partial $\eta 2 = 0.426$, observed power = 0.876], but not for normalized total staining of distal colon or brain BDNF.

Table 18. MANOVA of Normalized Total Staining of BDNF with Age, Sex, Diet, and Interactions

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[c]
Intercept	Pillai's Trace	.995	890.335[b]	3.000	13.000	<.001	.995	2671.006	1.000
	Wilks' Lambda	.005	890.335[b]	3.000	13.000	<.001	.995	2671.006	1.000
	Hotelling's Trace	205.462	890.335[b]	3.000	13.000	<.001	.995	2671.006	1.000
	Roy's Largest Root	205.462	890.335[b]	3.000	13.000	<.001	.995	2671.006	1.000
Agemo	Pillai's Trace	.629	7.346[b]	3.000	13.000	.004	.629	22.037	.936
	Wilks' Lambda	.371	7.346[b]	3.000	13.000	.004	.629	22.037	.936
	Hotelling's Trace	1.695	7.346[b]	3.000	13.000	.004	.629	22.037	.936
	Roy's Largest Root	1.695	7.346[b]	3.000	13.000	.004	.629	22.037	.936
Diet	Pillai's Trace	.542	5.131[b]	3.000	13.000	.015	.542	15.392	.817
	Wilks' Lambda	.458	5.131[b]	3.000	13.000	.015	.542	15.392	.817
	Hotelling's Trace	1.184	5.131[b]	3.000	13.000	.015	.542	15.392	.817
	Roy's Largest Root	1.184	5.131[b]	3.000	13.000	.015	.542	15.392	.817
Sex	Pillai's Trace	.266	1.570[b]	3.000	13.000	.244	.266	4.710	.319
	Wilks' Lambda	.734	1.570[b]	3.000	13.000	.244	.266	4.710	.319
	Hotelling's Trace	.362	1.570[b]	3.000	13.000	.244	.266	4.710	.319
	Roy's Largest Root	.362	1.570[b]	3.000	13.000	.244	.266	4.710	.319
Agemo * Diet	Pillai's Trace	.522	4.738[b]	3.000	13.000	.019	.522	14.215	.783
	Wilks' Lambda	.478	4.738[b]	3.000	13.000	.019	.522	14.215	.783
	Hotelling's Trace	1.093	4.738[b]	3.000	13.000	.019	.522	14.215	.783
	Roy's Largest Root	1.093	4.738[b]	3.000	13.000	.019	.522	14.215	.783
Agemo * Sex	Pillai's Trace	.373	2.583[b]	3.000	13.000	.098	.373	7.750	.500
	Wilks' Lambda	.627	2.583[b]	3.000	13.000	.098	.373	7.750	.500
	Hotelling's Trace	.596	2.583[b]	3.000	13.000	.098	.373	7.750	.500
	Roy's Largest Root	.596	2.583[b]	3.000	13.000	.098	.373	7.750	.500
Diet * Sex	Pillai's Trace	.200	1.083[b]	3.000	13.000	.391	.200	3.250	.228
	Wilks' Lambda	.800	1.083[b]	3.000	13.000	.391	.200	3.250	.228
	Hotelling's Trace	.250	1.083[b]	3.000	13.000	.391	.200	3.250	.228
	Roy's Largest Root	.250	1.083[b]	3.000	13.000	.391	.200	3.250	.228
Agemo * Diet * Sex	Pillai's Trace	.077	.361[b]	3.000	13.000	.782	.077	1.084	.103
	Wilks' Lambda	.923	.361[b]	3.000	13.000	.782	.077	1.084	.103
	Hotelling's Trace	.083	.361[b]	3.000	13.000	.782	.077	1.084	.103
	Roy's Largest Root	.083	.361[b]	3.000	13.000	.782	.077	1.084	.103

a. Design: Intercept + Agemo + Diet + Sex + Agemo * Diet + Agemo * Sex + Diet * Sex + Agemo * Diet * Sex

b. Exact statistic

c. Computed using alpha = .05

Table 19. Tests of Between-Subjects Effects for Normalized Total Staining of BDNF with Age, Sex, Diet, and Interactions

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[d]
Corrected Model	Brain BDNF Normalized Total	.069[a]	7	.010	2.743	.048	.561	19.203	.732
	DC BDNF Normalized Total	.032[b]	7	.005	3.398	.022	.613	23.789	.833
	JJ BDNF Normalized Total	.064[c]	7	.009	3.872	.013	.644	27.107	.884
Intercept	Brain BDNF Normalized Total	3.245	1	3.245	896.924	<.001	.984	896.924	1.000
	DC BDNF Normalized Total	1.852	1	1.852	1373.752	<.001	.989	1373.752	1.000
	JJ BDNF Normalized Total	2.073	1	2.073	871.762	<.001	.983	871.762	1.000
Agemo	Brain BDNF Normalized Total	.038	1	.038	10.430	.006	.410	10.430	.855
	DC BDNF Normalized Total	.000	1	.000	.198	.663	.013	.198	.070
	JJ BDNF Normalized Total	.024	1	.024	10.051	.006	.401	10.051	.842
Diet	Brain BDNF Normalized Total	.013	1	.013	3.551	.079	.191	3.551	.422
	DC BDNF Normalized Total	.006	1	.006	4.667	.047	.237	4.667	.524
	JJ BDNF Normalized Total	.021	1	.021	8.998	.009	.375	8.998	.800
Sex	Brain BDNF Normalized Total	.007	1	.007	1.913	.187	.113	1.913	.254
	DC BDNF Normalized Total	.003	1	.003	1.893	.189	.112	1.893	.252
	JJ BDNF Normalized Total	.001	1	.001	.475	.501	.031	.475	.099
Agemo * Diet	Brain BDNF Normalized Total	2.012E-5	1	2.012E-5	.006	.942	.000	.006	.051
	DC BDNF Normalized Total	.004	1	.004	2.825	.113	.158	2.825	.350
	JJ BDNF Normalized Total	.026	1	.026	11.126	.005	.426	11.126	.876
Agemo * Sex	Brain BDNF Normalized Total	.015	1	.015	4.054	.062	.213	4.054	.470
	DC BDNF Normalized Total	.000	1	.000	.077	.785	.005	.077	.058
	JJ BDNF Normalized Total	.007	1	.007	2.800	.115	.157	2.800	.347
Diet * Sex	Brain BDNF Normalized Total	.001	1	.001	.148	.706	.010	.148	.065
	DC BDNF Normalized Total	.002	1	.002	1.234	.284	.076	1.234	.180
	JJ BDNF Normalized Total	.006	1	.006	2.541	.132	.145	2.541	.320
Agemo * Diet * Sex	Brain BDNF Normalized Total	.002	1	.002	.475	.501	.031	.475	.099
	DC BDNF Normalized Total	.000	1	.000	.105	.751	.007	.105	.061
	JJ BDNF Normalized Total	.001	1	.001	.515	.484	.033	.515	.103
Error	Brain BDNF Normalized Total	.054	15	.004					
	DC BDNF Normalized Total	.020	15	.001					
	JJ BDNF Normalized Total	.036	15	.002					
Total	Brain BDNF Normalized Total	5.538	23						
	DC BDNF Normalized Total	2.888	23						
	JJ BDNF Normalized Total	2.734	23						
Corrected Total	Brain BDNF Normalized Total	.124	22						
	DC BDNF Normalized Total	.052	22						
	JJ BDNF Normalized Total	.100	22						

a. R Squared = .561 (Adjusted R Squared = .357)

b. R Squared = .613 (Adjusted R Squared = .433)

c. R Squared = .644 (Adjusted R Squared = .478)

d. Computed using alpha = .05

Age: Normalized Total

We found statistically significant between-subject effects for normalized total staining of brain BDNF and age [p = 0.006, partial η2 = 0.410, observed power = 0.855], and normalized total staining of jejunum BDNF and age [p = 0.006, partial η2 = 0.401, observed power = 0.842], but not for normalized total staining of distal colon BDNF (Table 19).

In pairwise comparisons (Table 20), normalized total brain BDNF mean difference for age (3 mo. - 6 mo.) was 0.098 [std. error = 0.030, 95% CI lower = 0.033, CI upper= 0.162]. The normalized total jejunum BDNF mean difference for age (3 mo. - 6 mo.) was 0.078 [std. error = 0.025, 95% CI lower = 0.026, CI upper= 0.130]. These results are quantified in Figures 19 and 20.

Table 20. Pairwise Comparisons for Normalized Total BDNF and Age (mo)

Pairwise Comparisons

Dependent Variable	(I) Age (mo)	(J) Age (mo)	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
Brain BDNF Normalized Total	3	6	.098*	.030	.006	.033	.162
	6	3	-.098*	.030	.006	-.162	-.033
DC BDNF Normalized Total	3	6	.008	.018	.663	-.031	.048
	6	3	-.008	.018	.663	-.048	.031
JJ BDNF Normalized Total	3	6	-.078*	.025	.006	-.130	-.026
	6	3	.078*	.025	.006	.026	.130

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Figure 19. Estimated Marginal Means of Normalized Total Brain BDNF and Age (mo)

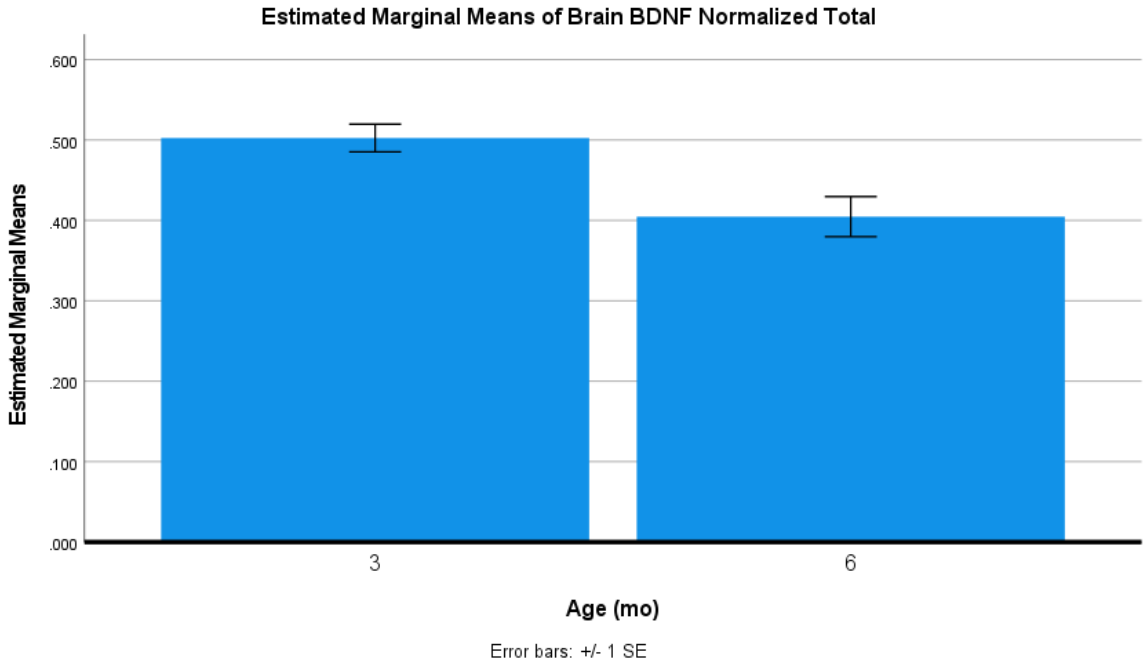
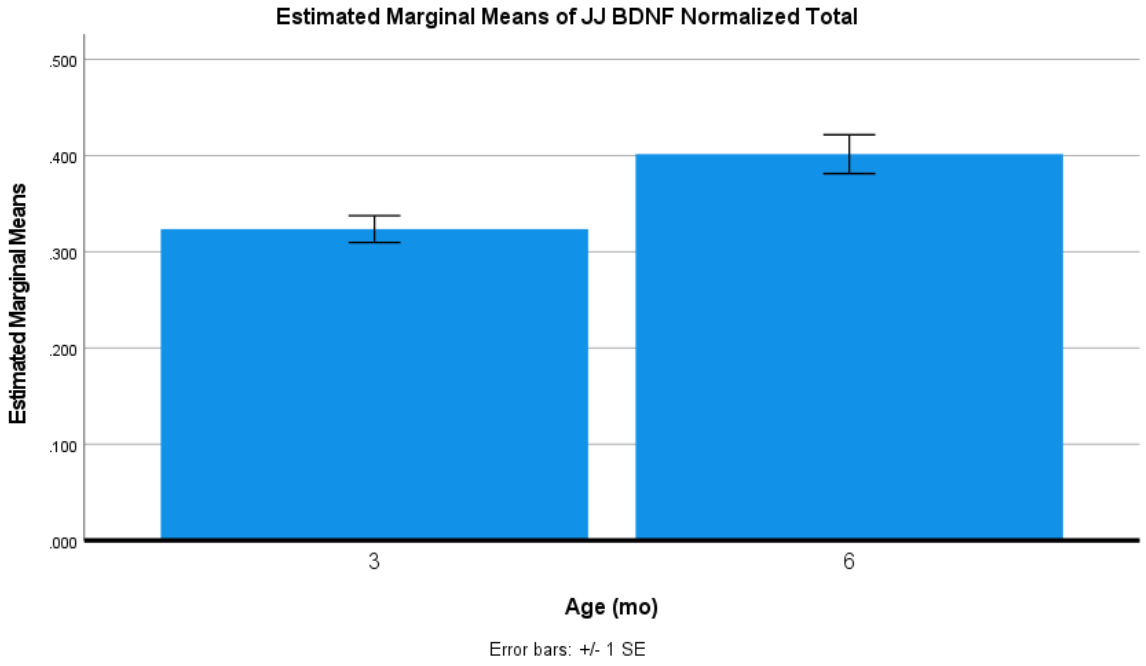


Figure 20. Estimated Marginal Means of Normalized Total Jejunum BDNF and Age (mo)



Diet: Normalized Total

For diet, we found statistically significant between-subject effects for normalized total staining of distal colon BDNF [p = 0.047, partial η2 = 0.237, observed power = 0.524] and normalized total staining of jejunum BDNF [p = 0.009, partial η2 = 0.375, observed power = 0.800], but not for normalized total staining of brain BDNF.

In pairwise comparisons (Table 21), normalized total distal colon BDNF mean difference for diet (normal - high fat) was 0.40 [std. error = 0.018, CI lower = 0.001, CI upper = 0.079]. The normalized total jejunum BDNF mean difference for diet (normal - high fat) was 0.074 [std. error = 0.025, CI lower = 0.021, CI upper = 0.126]. These results are quantified in Figures 21 and 22.

Table 21. Pairwise Comparisons for Normalized Total BDNF and Diet

Pairwise Comparisons

Dependent Variable	(I) Diet	(J) Diet	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
Brain BDNF Normalized Total	High fat	Normal	-.057	.030	.079	-.122	.007
	Normal	High fat	.057	.030	.079	-.007	.122
DC BDNF Normalized Total	High fat	Normal	-.040*	.018	.047	-.079	-.001
	Normal	High fat	.040*	.018	.047	.001	.079
JJ BDNF Normalized Total	High fat	Normal	.074*	.025	.009	.021	.126
	Normal	High fat	-.074*	.025	.009	-.126	-.021

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Figure 21. Estimated Marginal Means of Normalized Total Distal Colon BDNF and Diet

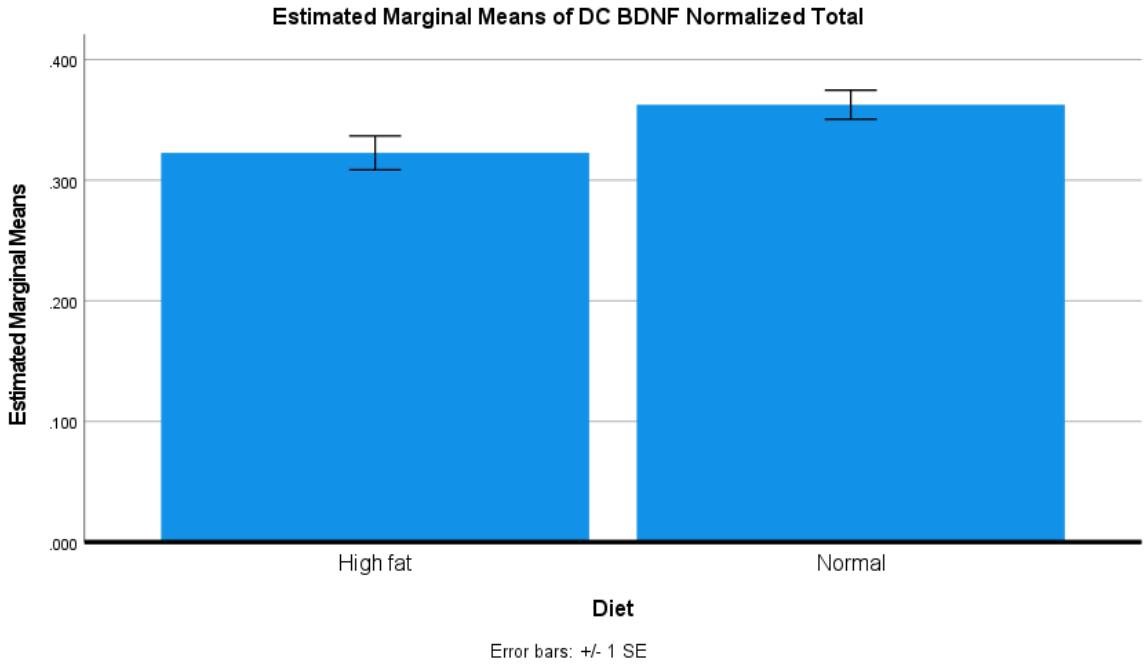
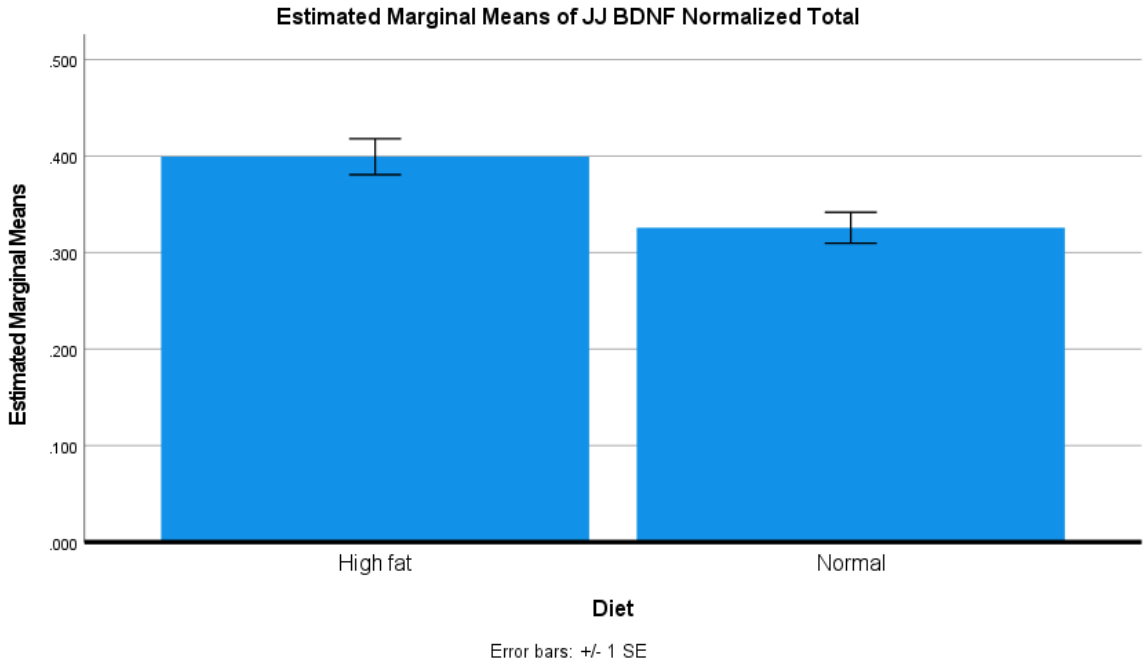


Figure 22. Estimated Marginal Means of Normalized Total Jejunum BDNF and Diet



Linear Regression

A linear regression established that age in days could significantly predicted the amount of brain BDNF normalized strong staining, $F(1,23) = 6.435$, $p = 0.018$ and age in days accounted for 18.5% of the explained variability in the amount of brain BDNF normalized strong staining (Table 23). The regression equation was: predicted amount of brain BDNF normalized strong staining = 0.15 - 2.62E-4*x (age in days) (Table 22).

Table 22. Linear Regression Model Summary for Age (days) and Normalized Strong Brain BDNF

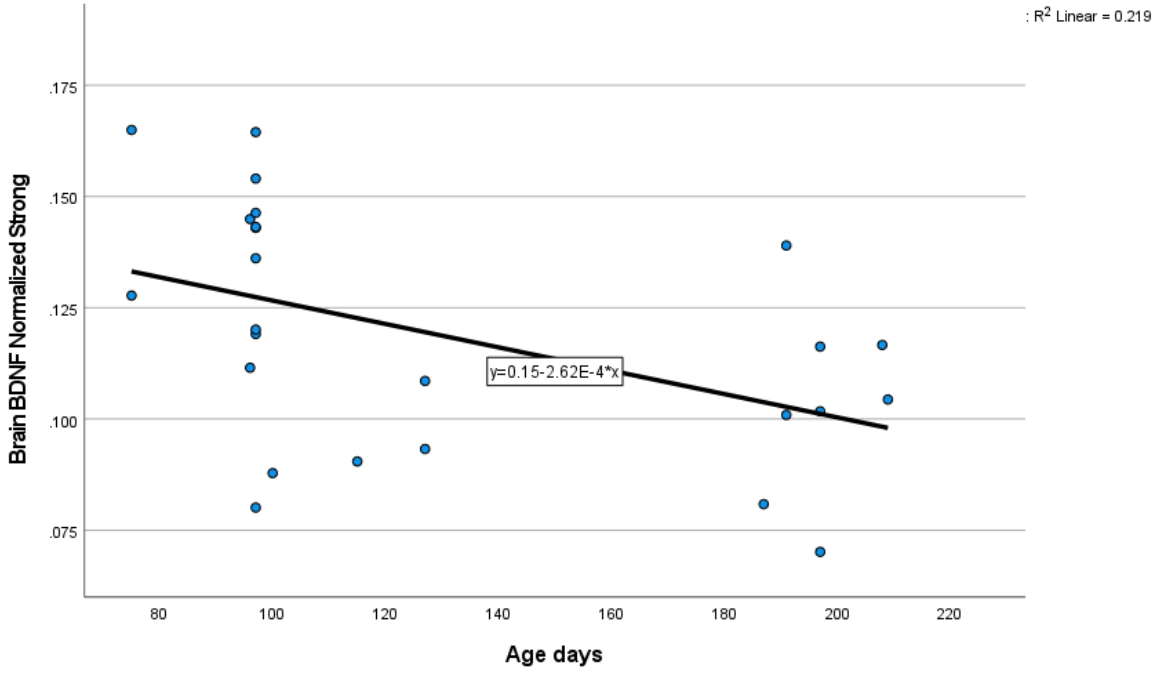
Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.468[a]	.219	.185	.024429	.219	6.435	1	23	.018

a. Predictors: (Constant), Age days

b. Dependent Variable: Brain BDNF Normalized Strong

Figure 23. Linear Regression Plot for Age (days) and Normalized Strong Brain BDNF



A linear regression established that age in days significantly predicted the amount of brain BDNF normalized total staining, F(1,23) = 7.469, p = 0.012 and age in days accounted for 21.2% of the explained variability in the amount of brain BDNF normalized total staining (Table 23). The regression equation was: predicted amount of brain BDNF normalized total staining = 0.58 - 7.59E-4*x (age in days) (Figure 24).

Table 23. Linear Regression Model Summary for Age (days) and Normalized Total Brain BDNF

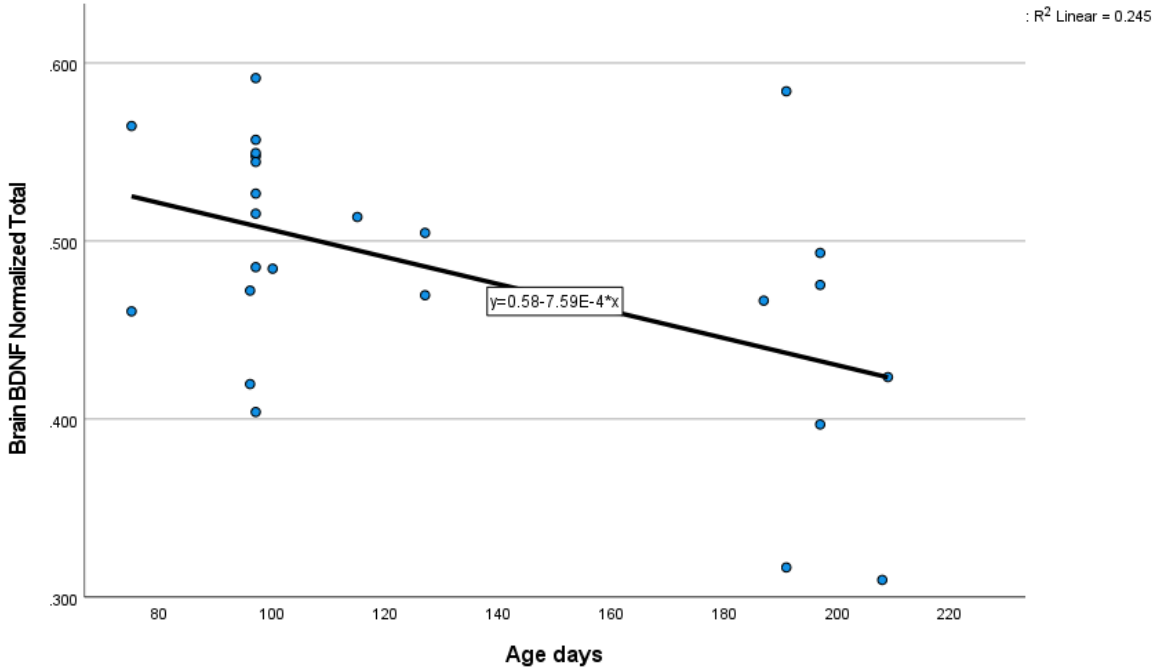
Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.495[a]	.245	.212	.065578	.245	7.469	1	23	.012

a. Predictors: (Constant), Age days

b. Dependent Variable: Brain BDNF Normalized Total

Figure 24. Linear Regression Plot for Age (days) and Normalized Total Brain BDNF



A linear regression established that age in days significantly predicted the amount of distal colon BDNF normalized strong staining, $F(1,21) = 14.987$, $p < 0.001$ and age in days accounted for 38.9% of the explained variability in the amount of distal colon BDNF normalized strong staining (Table 24). The regression equation was: predicted amount of distal colon BDNF normalized strong staining = 0.30 - 9.47E-4*x (age in days) (Figure 25).

Table 24. Linear Regression Model Summary for Age (days) and Normalized Strong Distal Colon BDNF

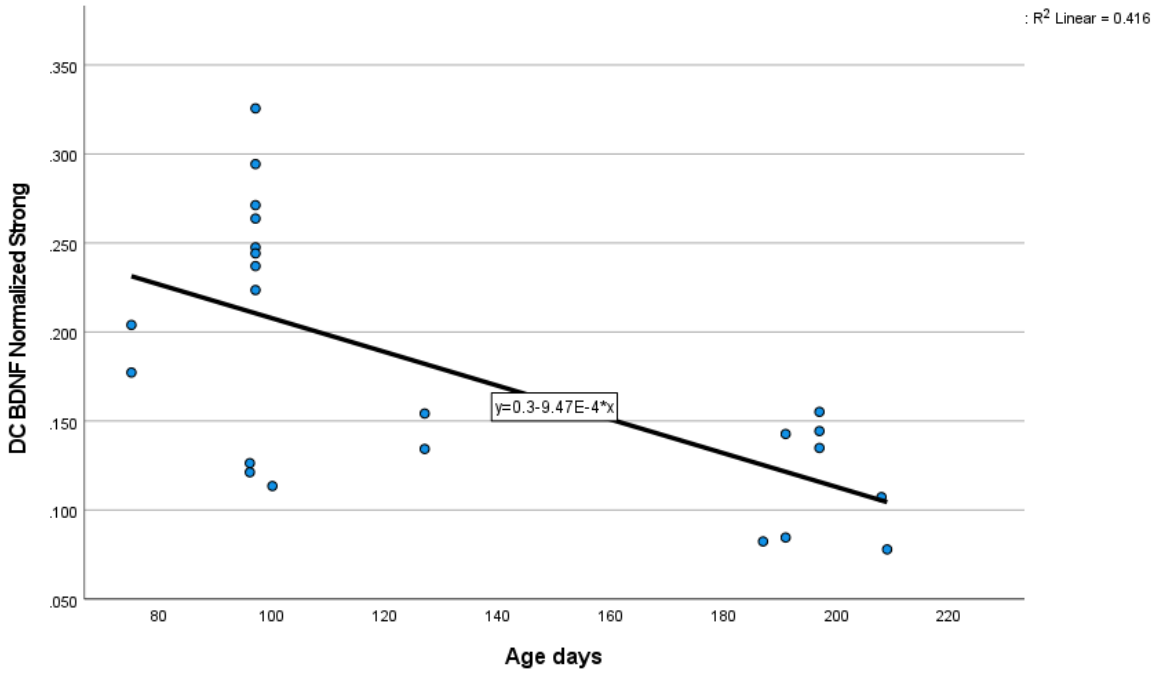
Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.645[a]	.416	.389	.057022	.416	14.987	1	21	<.001

a. Predictors: (Constant), Age days

b. Dependent Variable: DC BDNF Normalized Strong

Figure 25. Linear Regression Plot for Age (days) and Normalized Strong Distal Colon BDNF



A linear regression established that age in days significantly predicted the amount of distal colon BDNF normalized positive staining, $F(1,21) = 23.804$, $p < 0.001$ and age in days accounted for 50.9% of the explained variability in the amount of distal colon BDNF normalized positive staining (Table 25). The regression equation was: predicted amount of distal colon BDNF normalized positive staining = 0.1 + 5.94E-4*x (age in days) (Figure 26).

Table 25. Linear Regression Model Summary for Age (days) and Normalized Positive Distal Colon BDNF

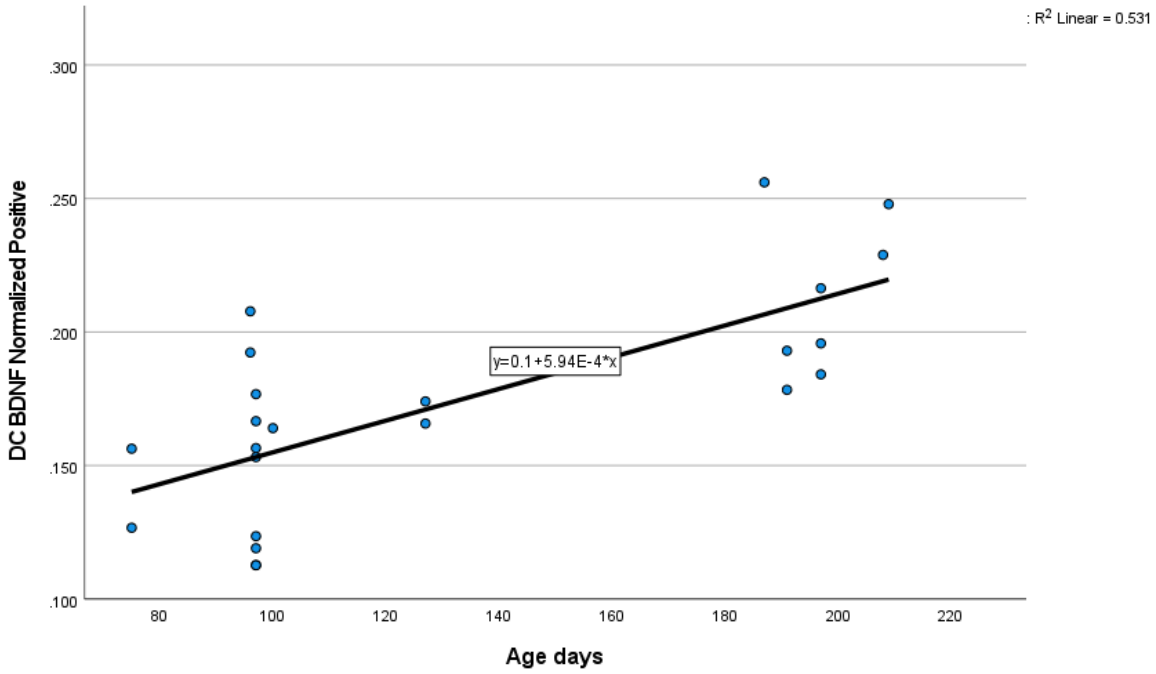
Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.729[a]	.531	.509	.028361	.531	23.804	1	21	<.001

a. Predictors: (Constant), Age days

b. Dependent Variable: DC BDNF Normalized Positive

Figure 26. Linear Regression Plot for Age (days) and Normalized Positive Distal Colon BDNF



A linear regression established that age in days significantly predicted the amount of jejunum BDNF normalized strong staining, $F(1,22) = 16.646$, $p < 0.001$ and age in days accounted for 40.5% of the explained variability in the amount of jejunum BDNF normalized strong staining (Table 26). The regression equation was: predicted amount of jejunum BDNF normalized strong staining = 0.18 - 5.02E-4*x (age in days) (Figure 27).

Table 26. Linear Regression Model Summary for Age (days) and Normalized Strong Jejunum BDNF

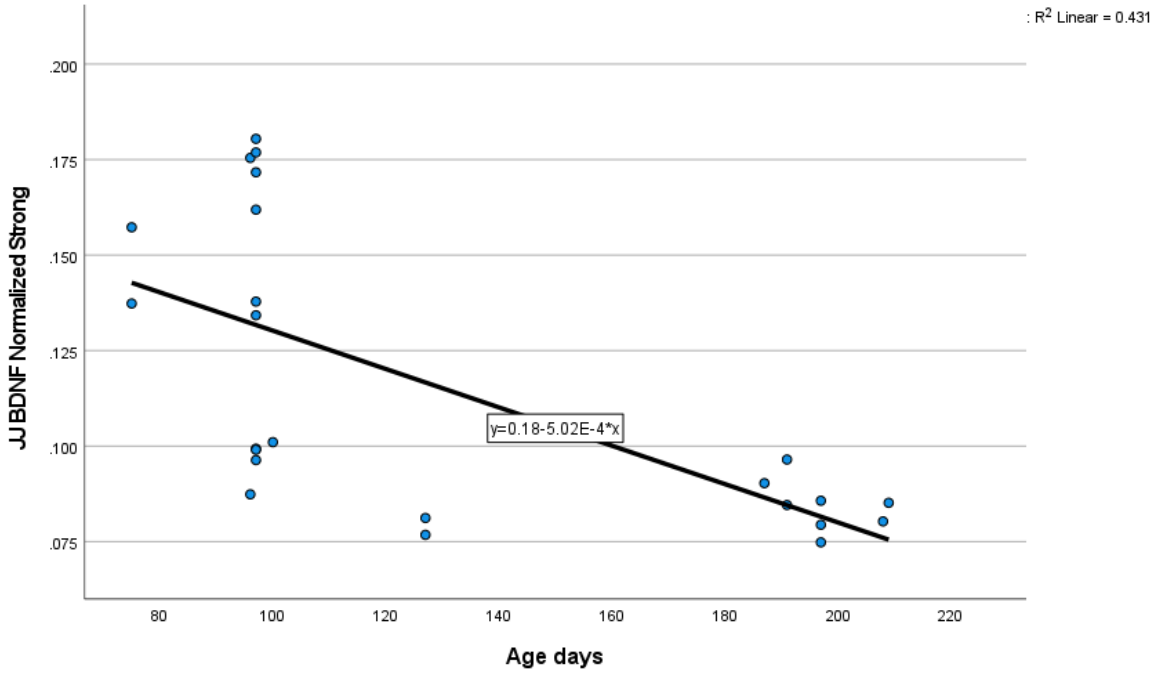
Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.656[a]	.431	.405	.028990	.431	16.646	1	22	<.001

a. Predictors: (Constant), Age days

b. Dependent Variable: JJ BDNF Normalized Strong

Figure 27. Linear Regression Plot for Age (days) and Normalized Strong Jejunum BDNF



A linear regression established that age in days significantly predicted the amount of jejunum BDNF normalized positive staining, $F(1,22) = 18.503$, $p < 0.001$ and age in days accounted for 43.2% of the explained variability in the amount of jejunum BDNF normalized positive staining (Table 27). The regression equation was: predicted amount of jejunum BDNF normalized positive staining = 0.08 + 1.08E-3*x (age in days) (Figure 28).

Table 27. Linear Regression Model Summary for Age (days) and Normalized Positive Jejunum BDNF

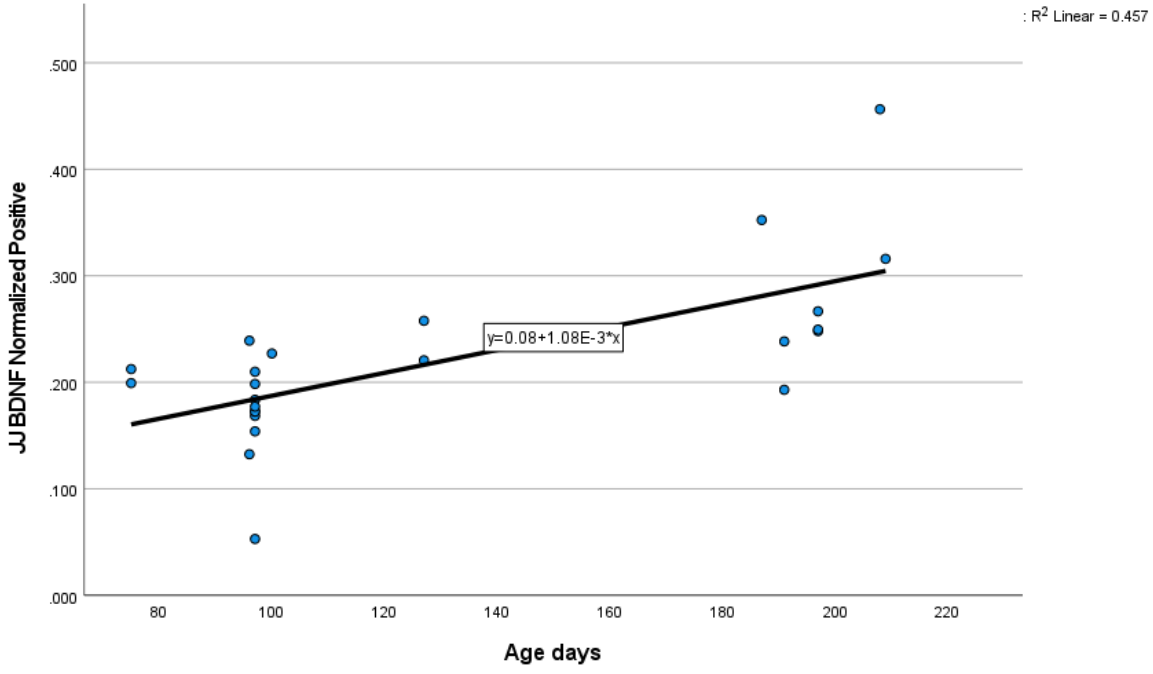
Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.676[a]	.457	.432	.058927	.457	18.503	1	22	<.001

a. Predictors: (Constant), Age days

b. Dependent Variable: JJ BDNF Normalized Positive

Figure 28. Linear Regression Plot for Age (days) and Normalized Positive Jejunum BDNF



A linear regression established that age in days significantly predicted the amount of jejunum BDNF normalized total staining, F(1,22) = 4.643, p = 0.042 and age in days accounted for 13.7% of the explained variability in the amount of jejunum BDNF normalized total staining (Table 28). The regression equation was: predicted amount of jejunum BDNF normalized total staining = 0.26 + 5.74E-4*x (age in days) (Figure 29).

Table 28. Linear Regression Model Summary for Age (days) and Normalized Total Jejunum BDNF

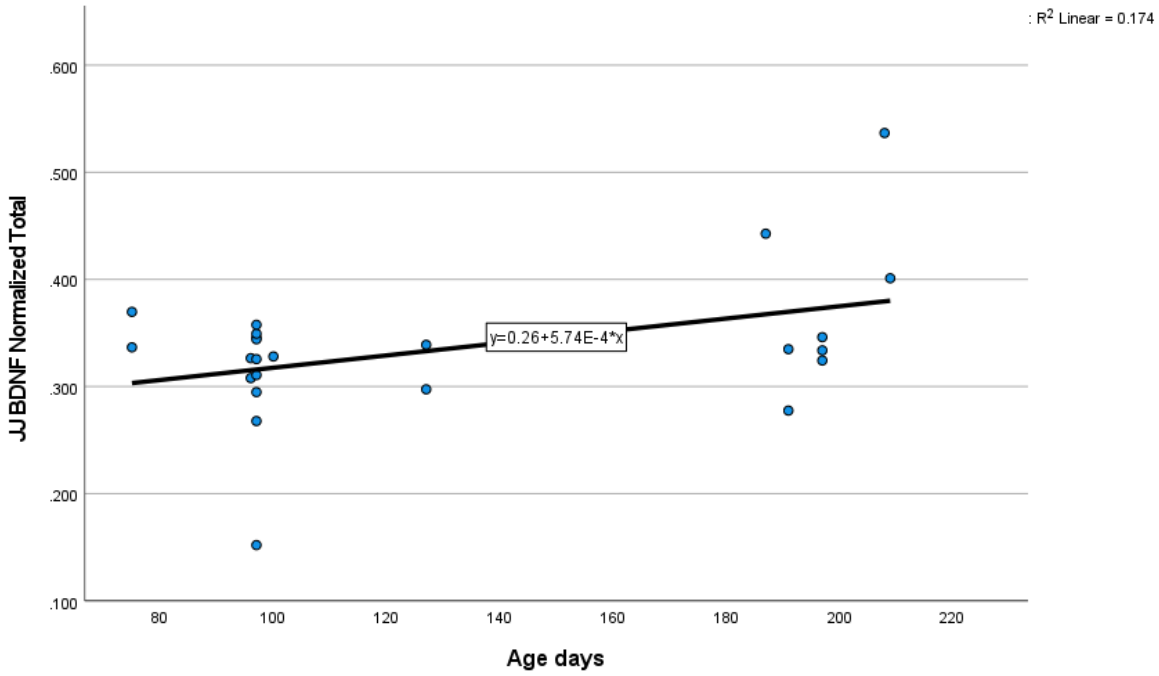
Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.417[a]	.174	.137	.062746	.174	4.643	1	22	.042

a. Predictors: (Constant), Age days

b. Dependent Variable: JJ BDNF Normalized Total

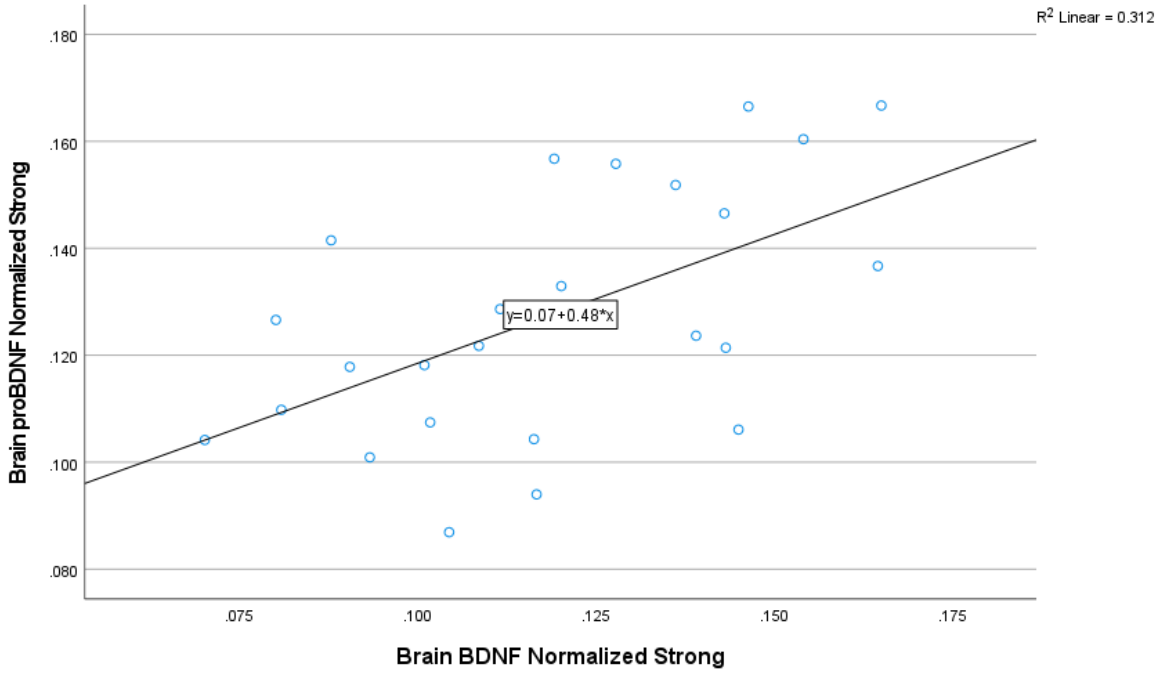
Figure 29. Linear Regression Plot for Age (days) and Normalized Total Jejunum BDNF



Bivariate Correlation

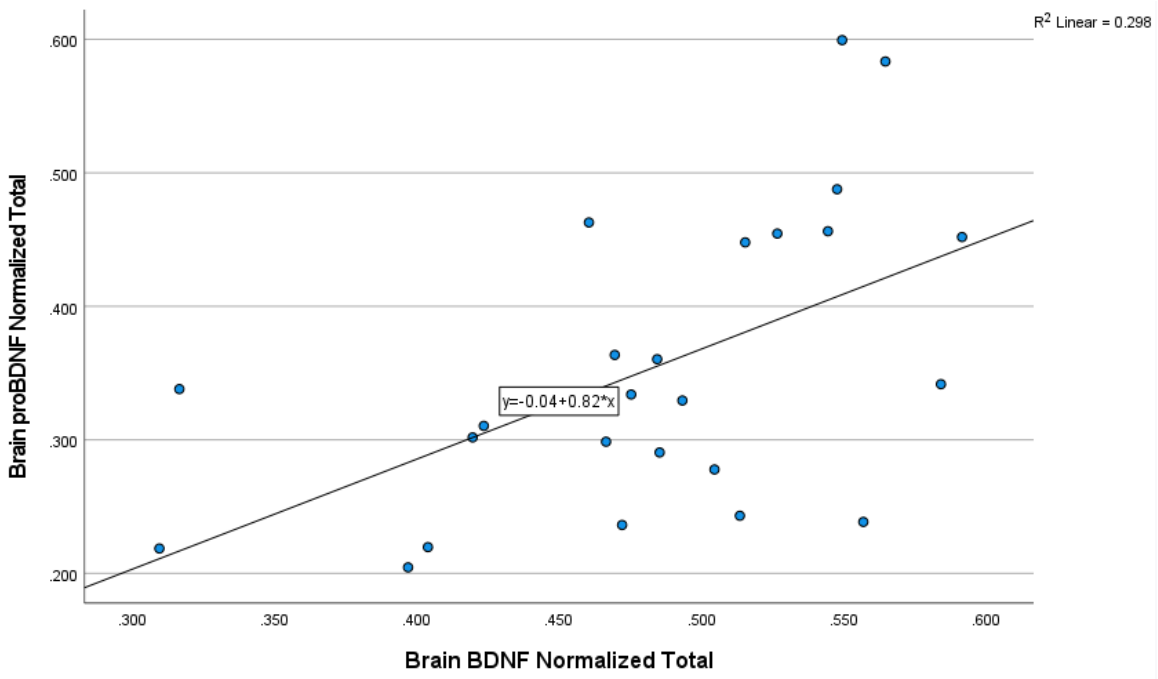
A Pearson product-moment correlation was run to determine the relationship between BDNF and proBDNF (normalized strong, normalized positive, and normalized total) in the brain, distal colon, and jejunum. There was a strong, positive correlation between normalized strong brain proBDNF and normalized strong brain BDNF, which was statistically significant (r = 0.559, n = 25, p = .004) (Figure 30).

Figure 30. Correlation Plot between Normalized Strong Brain proBDNF and BDNF



There was a strong, positive correlation between normalized total brain proBDNF and normalized total brain BDNF, which was statistically significant (r = 0.546, n = 25, p = .005) (Figure 31).

Figure 31. Correlation Plot between Normalized Total Brain proBDNF and BDNF



DISCUSSION

The central nervous system communicates with microbiota in the brain-gut axis via the vagus nerve through cholinergic signaling. The vagus nerve afferent fibers can be directly or indirectly stimulated by microbiota through gut endocrine cells. These endocrine cells are activated (by BDNF) or inhibited (by proBDNF), which in turn activate or inhibit vagal nerve communication to the brain. As a result, the presence of proBDNF and BDNF in the gut is important in regulating the brain-gut axis communication (Bonaz et al., 2018).

Our study demonstrated significant findings of positive pixel count of proBDNF and BDNF in the brain, distal colon, and jejunum in different cohorts of mice based on age (3 or 6 months), sex, and diet (high fat or normal). However, one limitation of the

study is that they are all APP mice. We expect these mice to have a higher systemic inflammatory response than normal mice, which would result in less staining of proBDNF and BDNF in wild type mice overall. Future studies would need to be done with normal wild type mice in order to observe these differences.

In our study, we found a decrease in normalized strong brain proBDNF as age increased, as well as in a high fat diet. This comes as expected as proBDNF is a precursor to BDNF. In human brains, BDNF has been seen to decrease in age as well as in a high fat diet. Interestingly, there was found to be an increase in normalized strong jejunum proBDNF in a high fat diet versus a normal diet. This may be due to proBDNF and its role in the gut to suppress communication of the BDNF pathway in the brain-gut axis via the vagus nerve, possibly playing into what may be seen in gut dysbiosis.

A linear regression was found to further support our finding of a decrease in brain proBDNF as age increased for both normalized strong positive and normalized total positive staining. Linear regressions for age in days to statistically significantly predict the amount of normalized strong as well as normalized total jejunum proBDNF also further supports its possible relation to gut dysbiosis and suppression of the BDNF pathway. As days increased, the amount of jejunum proBDNF increased.

Although the amount of normalized strong jejunum proBDNF increases as age increases, we saw a decrease in normalized strong jejunum BDNF as age increases. This suggests that less proBDNF became fully matured BDNF, which further implies that proBDNF could have a significant role in gut dysbiosis.

Unexpectedly, we saw an increase in normalized total jejunum BDNF in a high fat versus normal diet as well as with age in 6 months versus 3 months that conflicts with the decrease in normalized strong jejunum BDNF. This signifies that the normalized positive pixel count heavily outweighs the normalized strong positive pixel count for jejunum BDNF. It is possible that a single 2-Dimensional image of a 3-Dimensional organ slice may explain these findings: The amounts of normalized strong positive pixels are not ubiquitously distributed, and could be indicating areas of focal response.

Looking at correlations between proBDNF and BDNF, we found a strong, positive correlation between normalized strong and normalized total brain proBDNF and brain BDNF. These correlations confirm generally that high amounts of proBDNF can translate to high amounts of BDNF in the brain and vice versa. This is expected because proBDNF is a precursor to BDNF.

Interestingly, our results for the distal colon did not show any significant differences in age or diet. When thinking about the brain-gut axis, the vagus nerve does not innervate the entirety of the digestive tract. The distal end of the digestive tract, the colon and rectum, are innervated by the lumbar and sacral splanchnic nerves (Brierley et al., 2018). Therefore, we are not surprised to see no significant differences in age or diet for the distal colon staining.

Further studies are needed to explore gut BDNF and its relationship to the gut microbes specifically and the brain BDNF levels. An area of particular interest may be vagus nerve interactions with microbiota and gut BDNF in relation to the brain BDNF

and the brain itself. Elucidating this complex mechanism will provide another option to prevent or treat cognitive decline seen in many neurological diseases.

CONCLUSION

Even with our results confirming some relationships between proBDNF and BDNF with age and diet in mice, more data is needed to truly understand the mechanism that regulates those proteins and the role they play in the brain-gut axis. Looking forward, it may be helpful to do a similar study that includes the TrkB and p75 proteins, which are high-affinity receptors for BDNF and proBDNF respectively. Whether or not those receptors are upregulated is an essential part in knowing how BDNF and proBDNF function in the brain as well as in the gut. Wild type normal mice need to be included as a control in future studies so that these protein levels can be further compared. The vagus nerve is known to play an important role in connecting the brain and the gut through cholinergic signaling. It may be interesting to see how proBDNF, BDNF, and their receptors directly affect this signaling pathway of the vagus nerve to ultimately help with prevention or treatment of cognitive decline seen in many neurological diseases.

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