

THE PROTECTIVE EFFECT OF A DIET RICH IN FISH OIL IN AN
AMPHETAMINE TOXICITY MODEL OF PARKINSON'S DISEASE

by

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ABSTRACT OF THE DISSERTATION

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Parkinson's Disease

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Parkinson's disease is a neurodegenerative disorder that damages the dopaminergic neurons of the substantia nigra and their axonal projections to the striatum. This cell death results in significant motor deficits that include muscular rigidity, resting tremor, and akinesia. Although there is no known cure for Parkinson's disease, evidence from epidemiological studies suggests that consumption of fish oil, which is rich in omega-3 polyunsaturated fatty acids (PUFAs), may help to reduce the risk of this debilitating disorder. Furthermore, research using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) models of Parkinson's disease supports this conclusion. Consequently, this dissertation examined the potential protective effect of fish oil in an amphetamine-toxicity model of Parkinson's disease. In Experiment 1, mice were administered a diet rich in either corn oil or fish oil for one week and then were treated with either amphetamine or saline. After sacrifice 72 hours later, striatal tissue was assayed for neurochemical content using HPLC. It was determined that fish oil

protects against amphetamine-induced depletions of dopamine and its metabolites. Given the role of oxidative stress in amphetamine toxicity, this protection may be a result of the antioxidant properties of fish oil. Experiment 2, in addition to successfully replicating this effect, extended the protective effect of fish oil to behavioral and physiological measures. More specifically, a diet rich in fish oil significantly altered amphetamine's impact on behaviors including oral dyskinesia, self biting, stereotypy, and backwards walking; it also mitigated amphetamine-induced changes in dermal temperature. These results suggest that fish oil can moderate the elevated dopaminergic activity caused by amphetamine administration. Experiment 3 was designed to examine the time course of protection afforded by the fish oil-rich diet, and it was discovered that the protective effect of fish oil develops between 1 and 3 days of consumption. Experiment 4 was performed to determine whether fish oil alters amphetamine-induced increases in oxidative stress and dopamine release. Fish oil did not impact these measures, indicating that other mechanisms may be responsible for the observed protection. Collectively, these findings indicate that the consumption of fish oil offers protection against amphetamine toxicity in a model of Parkinson's disease.

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THE PROTECTIVE EFFECT OF A DIET RICH IN FISH OIL IN AN AMPHETAMINE TOXICITY MODEL OF PARKINSON'S DISEASE

Parkinson's Disease

Parkinson's disease is a neurodegenerative disorder characterized by muscular rigidity, resting tremor, and akinesia. These symptoms are a result of the degeneration of neuromelanin-containing dopaminergic neurons of the substantia nigra and their neuronal projections to the striatum (Figure 1). This cell death leads to a decrease in striatal dopamine, a loss of dopamine transport pumps, and a decrease in tyrosine hydroxylase activity. Once approximately 80% of the dopaminergic projections to the striatum have been lost, the movement symptoms associated with the disorder begin to manifest (Kita et al., 2003). In addition to its effects on the dopaminergic system, Parkinson's disease is also marked by a serotonergic lesion, which is exhibited by decreased striatal serotonin (Politis and Loane, 2011).

Parkinson's disease affects approximately 17 individuals per 100,000, as reported by Twelves et al. (2003), who examined incidence rates of Parkinson's disease reported in studies from a number of different countries. Twelves et al. (2003) report that there is an increased risk for developing the disease with age, with onset usually around the age of 60. In addition, several studies that they examined reported a 1.5- to 2-fold increased incidence of Parkinson's disease in males. Similarly, Mitchell et al. (1996) also reported that age and sex are risk factors for the disease. In a longitudinal study of nursing home residents over the age of 55, Mitchell et al. (1996) observed an increased prevalence of Parkinson's disease in both older individuals and male residents.

There are two forms of Parkinson's disease: familial and sporadic. A case of Parkinson's disease is considered familial when an individual has a first-degree relative who suffers from the disease. This accounts for approximately 15% of all of the cases of Parkinson's disease. The remaining instances are referred to as sporadic, indicating that the individual does not have any first-degree relatives with the disease. Familial Parkinson's disease often has a much earlier onset than the sporadic form of the disease. Baba et al. (2006) compared the familial and sporadic phenotypes of Parkinson's disease and found a number of commonalities. Of particular interest, they found a higher incidence in men in both forms of the disease. Because the sex distributions of Parkinsonism are equal for the familial and sporadic forms, they suggest that the difference in incidence between sexes may be the result of a neuroprotective effect in women rather than an increased risk in men. The effects of estrogen, along with other hormonal influences, may contribute to this sex disparity.

Although the cause of Parkinson's disease is not entirely apparent, it is clear that genetic factors play a role. There are several genetic polymorphisms that have been implicated in Parkinson's disease. Parkin gene mutations, for example, are the most common cause of autosomal recessive early-onset Parkinsonism (Kitada et al., 1998). The protein parkin is involved in the ubiquitin-proteasome pathway, which is crucial to protein processing and degradation within cells. Mutations in the parkin gene result in a loss of function of ubiquitin-protein ligase. Subsequently, this leads to the buildup of substrates for parkin within dopaminergic neurons, resulting in cell death (Hattori and Mizuno, 2004). Other genes that have been linked to recessive forms of familial Parkinson's disease include DJ-1 and PINK1 (Hakansson et al., 2008).

As for dominantly inherited forms of the disease, leucine-rich repeat kinase 2 (LRRK2) mutations are the most common genetic cause of familial Parkinson's disease (Hakansson et al., 2008). In addition, LRRK2 mutations contribute to the development of the sporadic form of Parkinsonism. LRRK2 is a unique and complex protein involved in a number of functions. Most LRRK2 mutations result in enhanced GTPase and kinase activity, which contributes to the neuronal toxicity associated with Parkinson's disease (Moore, 2008).

Mutations to the α -synuclein gene have also been linked to the disorder. Although the normal functions of α -synuclein have remained somewhat unclear, it is believed that α -synuclein acts as a molecular chaperone of synaptic proteins which are crucial in maintaining synaptic activity and integrity (Bonini and Giasson, 2005). In addition to maintenance of synapses, it may also act as an antioxidant, preventing the oxidation of unsaturated lipids (Zhu et al., 2006). When mutations to α -synuclein occur, however, it has a propensity to misfold into toxic conformations. Immunocytochemistry has demonstrated that α -synuclein is an abundant component of Lewy bodies, a pathological feature of Parkinson's disease (Olanow and Tatton, 1999). Furthermore, animal models of α -synuclein misregulation have demonstrated that it can play a role in neuronal cell death, dystrophic neurites, and alterations in dopamine metabolism and release (Maguire-Zess, 2008).

These are just a few of the genes that have been implicated in Parkinson's disease. In addition to these genetic factors, a number of studies have provided evidence associating toxicant exposure and an increased risk for developing Parkinson's disease. For example, case reports have linked the self-administration of 1-methyl-4-phenyl-

1,2,3,6-tetrahydropyridine (MPTP) to Parkinsonian symptoms. Langston et al. (1983) reported that drug users who took MPTP developed a syndrome that both clinically and pathologically resembled Parkinson's disease. MPTP induces toxicity through its conversion to the pyridinium ion (MPP^+) by monoamine oxidase type B. Then, MPP^+ is taken up by dopamine neurons and causes mitochondrial impairment (Olanow and Tatton, 1999). MPP^+ also induces hydroxyl free radical formation when injected into the striatum of rats and increases striatal lipid peroxidation when administered intracerebroventricularly in mice, indicating that oxidative stress is involved in its neurotoxic effects. Not only does MPTP lead to oxidative stress, it also disrupts the brain's ability to limit neuronal damage by reducing levels of glutathione, an endogenous tripeptide that has antioxidant properties (Olanow and Tatton, 1999). MPTP has been shown to deplete glutathione levels in the substantia nigra in mice, reducing their ability to prevent neural damage caused by oxidative stress (Desole et al., 1995).

Another toxicant that has been linked to Parkinson's disease is dieldrin, a chlorinated cyclodiene compound that was commonly used as a pesticide until the mid 1970s. Several studies have found traces of the pesticide in postmortem brain tissue samples of people who suffered from Parkinson's disease (Fleming et al., 1994; Corrigan et al., 2000). Kitazawa et al. (2001) revealed that dieldrin induced the release of dopamine, resulting in the depletion of intracellular dopamine. In addition, cytometric analysis revealed the generation of reactive oxygen species and increases in lipid peroxidation. Hatcher et al. (2007) observed that exposing mice to low levels of dieldrin for an extended period of time resulted in oxidative stress, as well as reduced levels of glutathione.

It is clear that there are a number of environmental factors that play a role in the development of Parkinson's disease. Because no particular environmental toxicant has been identified as a primary cause of Parkinson's disease, it is very likely that exposure to a combination of any number of toxicants could lead to oxidative stress, and ultimately the neuronal degeneration responsible for the disorder. Because there are also a number of genetic factors that appear to be involved in Parkinson's disease, it is most likely some combination of genetic factors that interact to predispose an individual to the disease. That is, genetic factors may make an individual more vulnerable to the neuronal damage caused by environmental toxicants.

Oxidative Stress in Parkinson's Disease

Both MPTP and dieldrin exert their neurotoxic effects via oxidative stress, which may be the primary mechanism by which neurodegeneration occurs in Parkinson's disease. Oxidative stress reflects an imbalance in the production of reactive oxygen species, which can result in tissue damage and cell death, and the ability to detoxify these free radicals. The oxidative metabolism of dopamine has the potential to yield hydrogen peroxide and other reactive oxygen species, which can result in oxidative stress and, in turn, cell death. Olanow and Tatton (1999) indicate that such neuronal death can occur under several circumstances; for one, when there is increased dopamine turnover, perhaps due to administration of a drug or exposure to a toxicant, the end result is excess peroxide formation. Oxidative stress can also occur when there is an increase in reactive iron, which promotes hydroxyl radical formation. Post mortem studies of Parkinson's disease brains have revealed increased iron levels. It is unclear whether this iron accumulation is primary or secondary, as MPTP treatment and 6-hydroxydopamine lesions have been

shown to increase iron in the substantia nigra, but in either instance, the increased iron can still contribute to the cell death associated with the disease. A third circumstance that can contribute to oxidative stress is a deficiency of glutathione. Jenner et al. (1992) provide evidence that suggests there is an impairment of the glutathione pathway in the substantia nigra of individuals with Parkinson's disease. Glutathione acts as an antioxidant, and a reduction in glutathione levels may impair the removal of hydrogen peroxide and other reactive oxygen species. Thus, such a deficit can contribute to the oxidative stress that has been observed in that brain region in Parkinson's disease.

In addition to glutathione and iron abnormalities, there are several other factors that may mediate oxidative stress associated with Parkinson's disease. A number of studies have revealed the role of mitochondrial dysfunction in Parkinson's disease and the potential impact this dysfunction has on oxidative damage. Bender et al. (2005) showed that there are high levels of deleted mitochondrial DNA in individuals with Parkinson's disease; these deletions can result in dysfunction. For example, defects in mitochondrial complex I in the substantia nigra of Parkinson's disease patients have been reported (Olanow and Tatton, 1999; Parker and Swerdlow, 1998). Shadrina and Slominsky (2008) examined the involvement of mitochondrial dysfunction and oxidative stress in the pathology of Parkinson's disease. They suggest that, in addition to dopamine oxidation, such a defect could be a source of the oxidative stress associated with the disorder. Parker and Swerdlow (1998) put forth a similar hypothesis, stating that mitochondria constitute a major site of reactive oxygen species production. The oxidative stress that results from this dysfunction, as well as the damage from dopamine oxidation, can then in

turn lead to further mitochondrial impairment and additional production of reactive oxygen species.

Excitotoxicity, neuronal cell death due to glutamate overactivity, also plays a role in free radical formation and oxidative damage, and has been implicated in Parkinsonian neurodegeneration. Substantia nigra dopaminergic neurons are rich in glutamate, making them very susceptible to excitotoxic damage (Olanow and Tatton, 1999). Excessive stimulation of N-methyl-D-aspartate (NMDA) receptors by glutamate results in a massive influx of extracellular Ca^{2+} , which activates a number of enzymes involved in catabolic processes. In addition, the calcium influx leads to the formation of nitric oxide (NO), which reacts with superoxide radicals to form peroxynitrite, a powerful oxidizing agent. NO may also contribute to neuronal degeneration by affecting mitochondrial function and displacing iron from ferritin binding sites, allowing the iron to react with hydrogen peroxide (Koutsilieri and Rieder, 2007; Olanow and Tatton, 1999).

Amphetamine Administration as a Model of Parkinson's Disease

The aforementioned factors are just some of the influences that may act to mediate the oxidative damage that occurs in the substantia nigra of individuals with Parkinson's disease. Our amphetamine administration animal model of Parkinson's disease demonstrates the role of these mediators in oxidative damage. Not only does the model provide an accurate representation of the disorder's pathology, but it captures many of the epidemiological features of Parkinson's disease as well.

Amphetamine and methamphetamine are central nervous system stimulants that have been used clinically to treat obesity, minimal brain dysfunction, narcolepsy, depression, and to counter fatigue. They also have become a common drug of abuse.

Amphetamines are indirect agonists of the catecholaminergic system, causing the release of norepinephrine and dopamine, blocking their reuptake from the synapse, and disrupting their breakdown by inhibiting monoamine oxidase (Kita et al., 2003). Although initially this will result in an increase in the synaptic levels of these neurotransmitters, the administration of amphetamines has been shown to cause long-lasting depletions of monoamines indicative of permanent neuronal damage. More specifically, there is a significant and long-lasting decrease in striatal dopamine and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), which has been observed in a number of different species, using various doses of amphetamine or methamphetamine, and at various time points following administration. For example, Seiden, Fischman, and Schuster (1976) demonstrated that methamphetamine-treated rhesus monkeys, six months following the last drug treatment, showed significantly reduced dopamine and norepinephrine levels compared to control monkeys; this lasting depletion is indicative of permanent neuronal damage. Striatal dopamine depletion associated with methamphetamine administration has been demonstrated in rats and guinea pigs as well. Doses up to 50 mg/kg of methamphetamine were administered to the animals, which were sacrificed two weeks later. The drug-treated animals showed a 50% depletion in striatal dopamine levels compared to controls (Wagner, Seiden, and Schuster, 1979). The dopaminergic depletion seen in these studies strongly resembles the neurochemical changes seen in Parkinson's disease, making amphetamine and methamphetamine administration a relevant model of the disorder.

Not only is Parkinson's disease marked by dopamine depletion, there is also a decrease in tyrosine hydroxylase activity, as well as a loss of dopamine transport pumps.

These neurochemical alterations are also represented in our amphetamines model (Kita et al., 2003). Studies indicate that the high doses of methamphetamine can result in a long-lasting decrease in tyrosine hydroxylase activity (Gibb and Kogan, 1979; Kogan, Nichols, and Gibb, 1976). Wagner et al. (1980) found that the repeated administration of methamphetamine to rats results in a significant decrease in the number of dopamine transport pumps.

Amphetamines alter the serotonergic system as well. Methamphetamine causes long-lasting serotonin depletions, affects tryptophan hydroxylase activity, and decreases the number of serotonin transport pumps (Kita et al., 2003). Trulson and Jacobs (1979, 1980) observed that repeated administration of amphetamine to cats results in serotonin depletion in a number of brain regions. Ricaurte, Schuster, and Seiden (1980) described a similar occurrence in rats, noting that the dopamine and serotonin depletion was greatest in the striatum. This striatal serotonergic lesion is particularly relevant to Parkinson's disease, as individuals with the disorder show dysfunction of the serotonergic system (Politis and Loane, 2011).

The aforementioned effects of amphetamines on the dopaminergic and serotonergic systems are indicative of permanent cell damage. Studies have shown that treatment with the amphetamines results in nerve terminal degeneration. For example, Ricaurte et al. (1984) have demonstrated that a single injection of amphetamine can result in long-lasting dopamine deficits in iprindole-treated rats by the destruction of dopaminergic nerve terminals. Sonsalla et al. (1996) found that there was approximately 45% loss of cell bodies in the substantia nigra, resulting from the administration of high doses of methamphetamine to mice. This damage corresponded to a 90% depletion of

striatal dopamine. These studies provide evidence that nerve cell degeneration does occur following treatment with amphetamines, making it a viable model of Parkinson's disease.

A primary mechanism by which this neurodegeneration occurs in Parkinson's disease is oxidative stress, and the damage caused by amphetamines is produced in the same manner (Figure 2). Acikgoz et al. (1998, 2000) demonstrated that methamphetamine treatment can increase lipid peroxidation levels in the striatum. The excess dopamine released due to amphetamine and methamphetamine administration is believed to encourage the formation of 6-hydroxydopamine (6-OHDA) by the auto-oxidation of dopamine (Kita et al., 2003). Seiden and Vosmer (1984) reported the formation of 6-OHDA in the caudate nucleus of the rat brain after an injection of methamphetamine. In addition, dopamine-related reactive oxygen species (ROS), such as superoxide and hydroxyl radicals, are also formed (Kita et al., 2003; Kita et al., 2009).

Like oxidative damage associated with Parkinson's disease, oxidative stress induced by amphetamines is mediated by excitotoxicity. For example, methamphetamine administration has been shown to increase the release of striatal glutamate (Mark, Soghomonian, and Yamamoto, 2004; Ohmori, Abekawa, and Koyama, 1996). As mentioned earlier, glutamate overactivity results in the overstimulation of NMDA receptors, which in turn leads to calcium influx, NO formation, and ultimately the formation of peroxynitrite. Pretreatment with NMDA receptor antagonists have been shown to attenuate methamphetamine-induced neurotoxicity (Mark, Soghomonian, and Yamamoto, 2004; Sonsalla, Nicklas, and Heikkila, 1989). In addition, neural nitric oxide synthase (nNOS) knock-out mice, animals that lack an enzyme involved in the synthesis

of NO, show protection against methamphetamine's neurotoxic effects (Kita et al., 2003). This collection of evidence indicates that glutamate activity plays an important role in mediating the neurotoxic effects of amphetamines.

Mitochondrial dysfunction plays a role in the neurotoxic effects of amphetamines as well. Mitochondria, the primary source of cellular adenosine triphosphate (ATP), form ROS as a secondary effect of ATP generation. Amphetamines have been shown to inhibit complexes within the mitochondrial electron transport chain (ETC), resulting in increased production of ROS (Brown and Yamamoto, 2003). More specifically, Brown, Quinton, and Yamamoto (2005) report that methamphetamine selectively inhibits complex II of the mitochondrial ETC in the striatum of rats. The ROS accumulation associated with this dysfunction can lead to neuronal damage. Such inhibition of the ETC has been reported in individuals with Parkinson's disease (Olanow and Tatton, 1999; Parker and Swerdlow, 1998; Brown and Yamamoto, 2005) and may contribute to neurodegeneration.

The amphetamines model of Parkinson's disease, in addition to effectively representing some of the pathological features of the disease, exhibits the disorder's epidemiological factors. There are sex-dependent effects associated with amphetamine and methamphetamine administration. As noted, there is a greater incidence of Parkinson's disease in men than women, and this trend is represented in the amphetamines model. Male mice have been observed to be more sensitive to the deleterious effects of amphetamines than female mice (Wagner, Tekirian, and Cheo, 1993; Yu and Liao, 2000). In addition to sex-dependent effects, it appears that younger animals are partially protected from amphetamine-induced damage. For example, rat

pups treated with methamphetamine show striatal depletions of DA that are reduced when compared to depletions in adults (Wagner, Schuster, and Seiden, 1981). Not only do young animals appear to be somewhat protected from the neurotoxic effects of amphetamines, but aged mice show an increased sensitivity to their effects. Wagner and Walsh (1991) treated both young adult and aged mice with four 12.5 mg/kg injections of methamphetamine, and sacrificed the mice two weeks later. They observed a greater depletion of dopamine in the aged mice than the younger adults. This protection in younger animals fits in quite well to our amphetamine toxicity model of Parkinson's disease, as aged people are at a greater risk for developing the disorder than younger individuals.

There are clearly a number of parallels that can be drawn between Parkinson's disease and our animal model. For example, the administration of amphetamines produces both dopaminergic and serotonergic depletions, similar to those seen in the disorder. This damage occurs in a manner comparable to the damage associated with Parkinson's disease, as oxidative stress is a key mechanism in both. Finally, the model accurately represents some epidemiological factors of Parkinson's disease, such as age- and sex-dependent influences. The model does have some flaws, however. For one, the amphetamines model of Parkinson's disease fails to address the formation of Lewy bodies (Kita et al., 2003). Unfortunately, other animal models of Parkinson's disease, such as the MPTP model, also fall short in this regard (Halliday et al., 2009; Shimoji et al., 2005). In addition, we use acute, high dose exposure to amphetamines; such exposure is not typical, as repeated, low dose exposure to toxicants is more common in humans. Even considering these flaws, the aforementioned neuropathological and epidemiological

factors that are represented in the model make amphetamines a useful tool to study Parkinson's disease.

Omega-3 Polyunsaturated Fatty Acids and Parkinson's Disease

Fatty acids consist of a carboxyl group (-COOH) and a tail of carbon atoms attached to hydrogen atoms. The carbon atom tail is typically between 16 and 26 carbon atoms in length, and these atoms are connected to each other by single or double bonds. If the tail only contains single bonds between carbons, the fatty acid is referred to as saturated. If a single double bond is present, the fatty acid is described as monounsaturated, and if there are multiple double bonds, it is described as polyunsaturated (Figure 3). Omega-3 and omega-6 polyunsaturated fatty acids (PUFAs), named for the location of the first double bond in the carbon atom tail, are essential components of our diet as they are not endogenously synthesized by the human body. The omega-3 variety, in particular, which is found abundantly in fish, has been associated with a number of health benefits including the protection against cardiovascular disease, inflammatory diseases such as rheumatoid arthritis, and mental health disorders such as depression, dementia, and Alzheimer's disease (Ruxton et al., 2004).

Although the findings of previous epidemiological studies investigating the role of dietary fat intake in Parkinson's disease have been inconsistent, several studies provide support for a protective role exerted by regular dietary intake of unsaturated fat. For example, in a longitudinal examination of dietary patterns in both men and women, an inverse association between a prudent diet (characterized by high intakes of fruit, vegetables, and fish) and Parkinson's disease risk was observed (Gao et al., 2007). The authors suggest that such a diet provides ample amounts of antioxidants and folate as well

as a limited amount of saturated fat, with both factors reducing the risk of the disease. In a related study assessing dietary habits of individuals ages 55 and older, it was found that intake of PUFAs was significantly associated with a decreased risk of Parkinson's disease (de Lau et al., 2005). More specifically, consumption of both omega-3 and omega-6 PUFAs were associated with this reduced risk.

Omega-3 PUFAs and Animal Models of Parkinson's Disease

The protective effects of omega-3 PUFAs have previously been observed in animal models of Parkinson's disease. Chronic supplementation of a diet with fish oil (rich in omega-3 PUFAs) in rats with unilaterally 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal pathway reduced apomorphine-induced rotational behavior (Delattre et al., 2009). Furthermore, mice fed a diet rich in omega-3 PUFAs prior to treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) were protected against its neurotoxic effects, completely preventing the MPTP-induced cell death in the substantia nigra and reducing the MPTP-induced decreases in nigral dopamine transporter mRNA expression, as well as reductions in mRNA levels of Nurr1, a nuclear receptor important for the regulation dopaminergic neuron survival (Bousquet et al., 2008). The omega-3 PUFA-rich diet also provided protection from decreases in striatal dopamine and dihydroxyphenylacetic acid (DOPAC) caused by the MPTP treatment. Finally, a dietary supplement of docosahexaenoic acid, an omega-3 PUFA, delayed the development and decreased the severity of levodopa-induced dyskinesias in MPTP-treated monkeys (Samadi et al., 2006).

In addition to 6-OHDA and MPTP, high dose administration of amphetamine serves as an effective model of Parkinson's disease, reducing striatal dopamine levels as

well as the number of striatal dopamine transporters, and reducing the number of substantia nigra neurons (Kita et al., 2000; Kita et al., 2003; Sonsalla et al., 1996). It has been shown that omega-3 PUFA deficiency augments sensitization to amphetamine treatment. More specifically, mice with a chronic dietary deficiency of omega-3 PUFAs exhibited increased sensitization to daily amphetamine treatment, as evidenced by increased locomotor activity. Furthermore, omega-3 PUFA deficiency led to altered striatal concentrations of dopamine and its metabolites following treatment with amphetamine (McNamara et al., 2008).

Rationale for the Dissertation

The aforementioned epidemiological evidence suggests that the consumption of omega-3 PUFAs has a wide range of health benefits, including a possible reduction in the risk of Parkinson's disease. In addition, omega-3 PUFAs have been protective in various animal models of the disorder. Furthermore, omega-3 PUFA deficiency has been shown to modulate amphetamine-induced neurochemical changes. Given this information, the following experiments were designed to assess the protective role of a diet rich in fish oil, a source of omega-3 PUFAs, in an amphetamine toxicity model of Parkinson's disease. More specifically, the initial experiments examine its potential protective effect against amphetamine-induced alterations in neurochemistry, behavior, and body temperature. Follow-up experiments investigated issues including how long the fish oil-rich diet must be administered before protection is conferred and the diet's impact on oxidative stress and dopamine turnover, used as an indicator of dopamine release.

Figure 1.

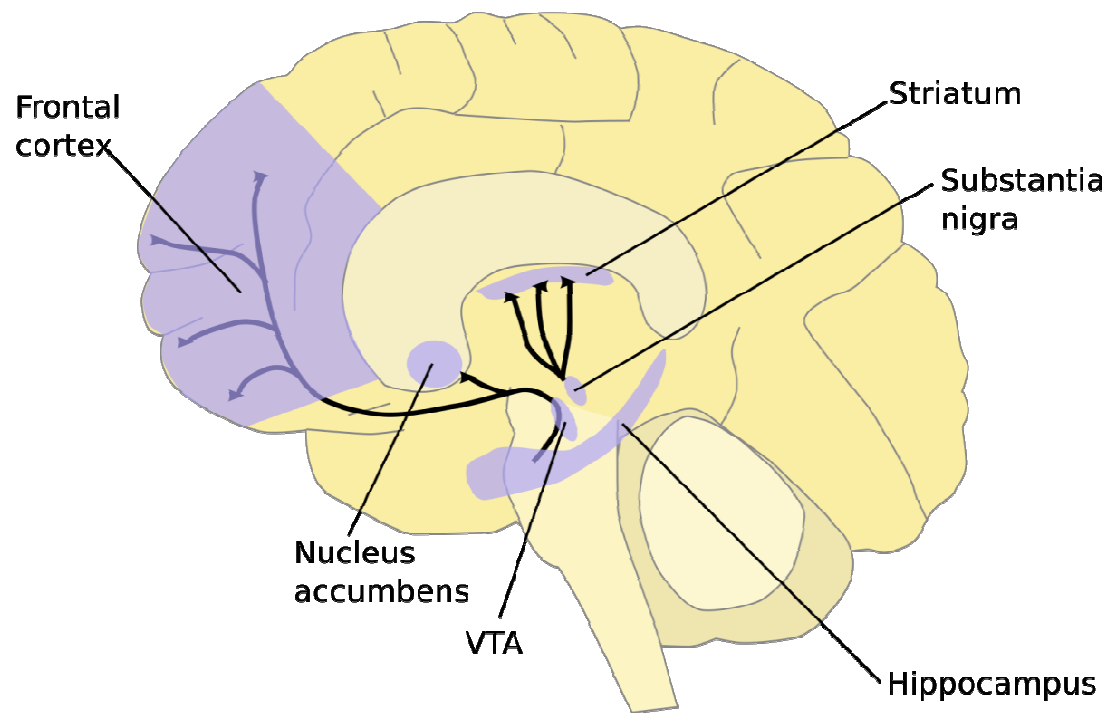


Figure 1. Dopamine pathways. The loss of cell bodies in the substantia nigra, along with their axonal projections to the striatum, is a key component in the pathology of Parkinson's disease.

Figure 2.

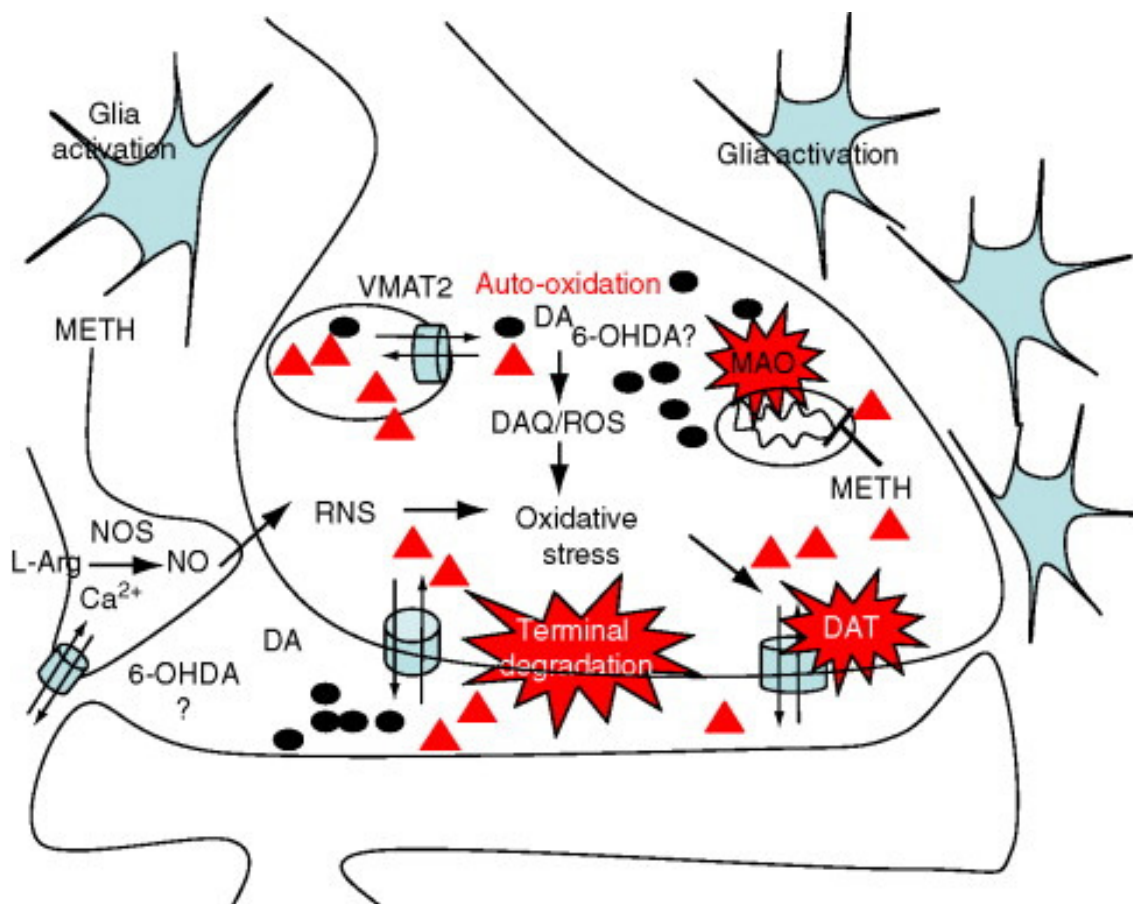


Figure 2. Schematic model of methamphetamine-induced neurotoxicity in the striatum.

Obtained from Kita et al. (2009) with permission. DA, dopamine; DAT, dopamine transporter; DAQ, dopamine quinone; NO, nitric oxide; MAO, monoamine oxidase; METH, methamphetamine; ROS, reactive oxygen species; RNS, reactive nitrogen species; VMAT, vesicular monoamine transporter.

Figure 3.

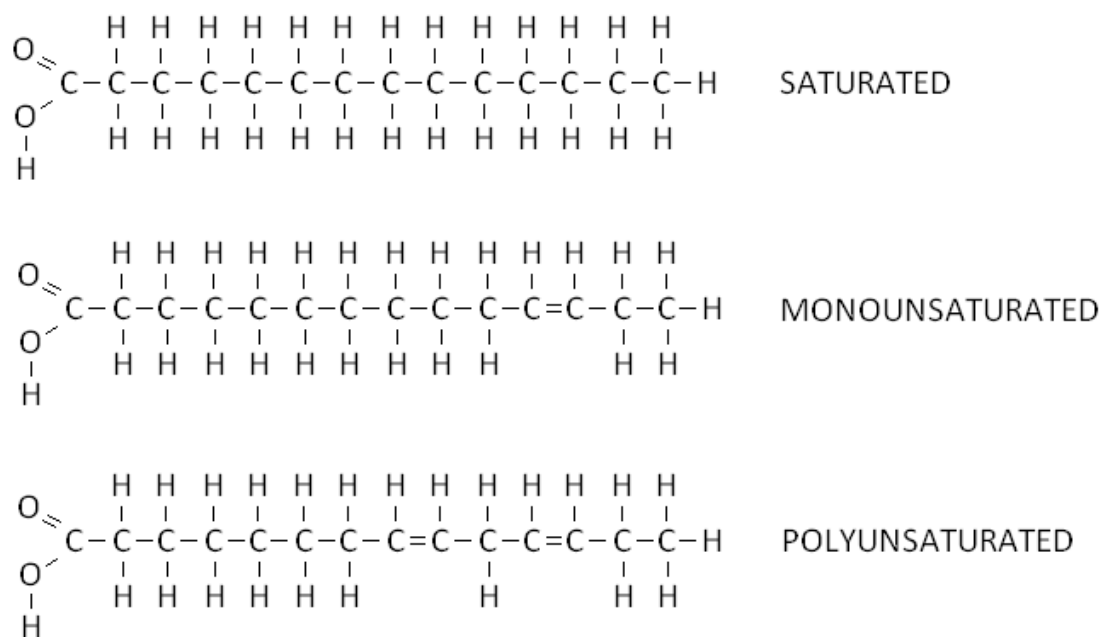


Figure 3. Fatty acid structures. Saturated fatty acids do not have any double bonds in the carbon tail, while monounsaturated and polyunsaturated fats have one or multiple double bonds, respectively. Please note that the structures displayed above are not intended to represent any particular fatty acids. Rather, the image was produced to highlight the difference between the saturated, monounsaturated, and polyunsaturated fat.

EXPERIMENT 1

Rationale

Although it is clear that a deficiency in dietary omega-3 PUFAs can affect amphetamine-induced behavior and neurochemistry, the effect of consuming a diet rich in omega-3 PUFAs in an amphetamine model of Parkinson's disease has not yet been assessed. Experiment 1 was performed to determine whether a diet containing fish oil will mitigate amphetamine-induced neurochemical lesion in the striatum as compared to a control diet containing corn oil. Mice were placed on a fish oil- or corn oil-rich diet for 7 days prior to treatment with amphetamine or saline, and 72 hours later, they were sacrificed. Striatal tissue was assayed for neurochemical content using HPLC. It was hypothesized that mice fed a diet rich in fish oil would express diminished neurochemical lesions, indicated most notably by reductions in amphetamine-induced striatal dopamine depletion.

Methods

Forty, 9-week old, male BALB/c mice (Jackson Laboratories, Bar Harbor, Maine), were individually housed in a temperature and humidity-regulated colony room with a 12:12 hour light:dark cycle. Mice were provided with unlimited access to both water and a liquid diet (described below). All procedures were approved by the Animal Care Committee and are in accordance with AAALAC guidelines.

The mice were provided with unlimited access to a liquid diet that contained either corn oil or fish oil (menhaden oil, with 200 parts-per-million tert-butylhydroquinone). The powder premix for these diets was obtained from Research Diets, New Brunswick, NJ, and this premix was mixed with water, maltodextrin, and oil.

Each kg of diet contained 42 g casein, 0.6 g DL-methionine, 15 g sucrose, 1 g cellulose, 3 g xanthan gum, 2.1 g vitamin mix, 7.3 g mineral mix, 0.4 g choline bitartrate, 0.254 g sodium saccharin, 803.346 g water, 35 g maltodextrin, and 90 g oil (either corn oil or fish oil). The diets were prepared daily, and bottles were weighed each day to measure diet consumption. Mice also had unlimited access to water throughout the study.

The animals were maintained on the liquid diet for one week prior to treatment with either amphetamine or saline. Mice received four subcutaneous injections, each separated by two hours, of either 12.5 mg/kg amphetamine (50 mg/kg total dose) or saline. The amphetamine (obtained from Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% saline to a concentration of 1.25 mg/ml. Access to the liquid diets and water was provided until sacrifice 72 hours following the first injection.

Following sacrifice, brains were removed and striatum was dissected, frozen in liquid nitrogen, and stored until homogenization in 0.3 ml of 0.4 N perchloric acid with 0.1 mM ethylenediaminetetraacetic acid (EDTA). Homogenized samples were centrifuged at 20,000 x g for 20 min at 4°C and the supernatant was frozen in liquid nitrogen until analyzed. Supernatant was assayed for dopamine, serotonin, and their metabolites using HPLC-electrochemical detection (Bioanalytical Systems, West Lafayette, Indiana). Samples were delivered through a high-pressure (Rheodyne) valve fitted with a 20 µl sample loop onto a Biophase ODS C-18 reverse-phase column (5 mm, 250 x 4.6 mm i.d.), and oxidized with a +.72 V potential between the glassy carbon electrode and the Ag/AgCl reference electrode. The mobile phase consisted of 0.1375 M sodium phosphate (dibasic), 0.0625 M citric acid, 5.0mg EDTA, and 14% methanol with a flow rate of 0.7ml/min. Quantification was measured against external standards

injected between every six samples. Neurotransmitter and metabolite levels are reported in micrograms per gram of tissue.

All data were analyzed using Statview statistical software. ANOVAs for amphetamine treatment and diet were performed. Fisher's PLSD *post hoc* tests were performed on statistically significant main effects. *P* values < .05 were considered statistically significant.

Results

Intake of the corn oil and fish oil diets were averaged across three time points: days 1-4 (i.e. days associated with diet acquisition), days 5-7 (i.e. diet intake before treatment), and days 8-10 (i.e. diet intake following treatment with either saline or amphetamine) (Figure 4). There was a significant main effect of diet, as well as a significant interaction between time point and diet [$F(1,68) = 31.04, p < .01$ and $F(2,68) = 5.06, p < .01$, respectively]. *Post hoc* analysis revealed that mice in the fish oil group consumed significantly less diet than mice in the corn oil group during the first four days. By days 5-7, however, there was no longer a significant difference between diet groups, as the intake of liquid diet in the fish oil group increased significantly from days 1-4. That is, there were no significant differences in the intake between the corn oil and fish oil groups at the time of treatment. Following either amphetamine or saline (days 8-10), there were significant main effects of both treatment and diet on intake [$F(1,32) = 14.67, p < .01$ and $F(1,32) = 19.48, p < .01$, respectively] (Figure 5). *Post hoc* tests showed that mice in the fish oil group treated with saline consumed significantly less diet than those in the corn oil group. This difference did not occur in amphetamine-treated mice. In

addition, in corn oil-fed mice, those treated with amphetamine consumed significantly less diet than their saline-treated counterparts.

Analysis of body weights revealed significant main effects of diet and time point, as well as a significant interaction of diet and time point [$F(1,68) = 12.18, p < .01$, $F(2,68) = 13.14, p < .01$, and $F(2,68) = 38.30, p < .01$, respectively] (Figure 6). *Post hoc* analysis revealed that mice in the corn oil and fish oil groups weighed the same at the start of the study, but fish oil-fed mice weighed significantly less than corn-oil fed mice at the time of treatment and at the end of the study. Following either amphetamine or saline, there were significant main effects of treatment and diet on body weight [$F(1,32) = 6.72, p = .01$ and $F(1,32) = 35.28, p < .01$, respectively] (Figure 7). *Post hoc* tests showed that in both saline- and amphetamine-treated mice, those fed the fish oil diet weighed significantly less than those in the corn oil group. Additionally, within the fish oil groups, mice treated with amphetamine weighed significantly less at sacrifice than those treated with saline.

Neurochemical analysis revealed a significant main effect of amphetamine treatment as well as a treatment by diet interaction for dopamine levels [$F(1, 32) = 56.46, p < .01$ and $F(1,32) = 9.18, p < .01$, respectively] (Figure 8). *Post hoc* analysis revealed that in both the corn oil and fish oil groups, mice treated with amphetamine had significantly less dopamine than those in the saline condition. Additionally, of the mice treated with amphetamine, mice that were fed the fish oil diet had significantly more dopamine than those in the corn oil condition.

There was a significant main effect of amphetamine treatment and a significant amphetamine by diet interaction on 3,4-dihydroxyphenylacetic acid (DOPAC) levels

[$F(1, 32) = 58.61, p < .01$ and $F(1,32) = 12.39, p < .01$, respectively] (Figure 8). *Post hoc* analysis showed that for both the corn oil and fish oil groups, mice treated with amphetamine had significantly less DOPAC than their saline-treated counterparts. In the saline condition, mice in the fish oil group had significantly less DOPAC than those in the corn oil group. However, this effect was reversed in the amphetamine condition, as mice in the fish oil group had significantly more DOPAC than mice in the corn oil group.

There were also significant main effects of amphetamine treatment and diet as well as a significant amphetamine by diet interaction on levels of homovanillic acid (HVA) [$F(1, 32) = 21.12, p < .01$, $F(1, 32) = 6.02, p = .02$, and $F(1,32) = 9.57, p < .01$, respectively] (Figure 8). *Post hoc* analysis revealed there was significantly less HVA in amphetamine-treated mice in the corn oil group, but there was no significant difference in the fish oil group. Furthermore, in amphetamine-treated groups, mice in the fish oil group had significantly more HVA than those in the corn oil group.

Analysis of striatal DOPAC/DA turnover revealed a significant main effect of amphetamine and a significant amphetamine by diet interaction [$F(1, 32) = 42.50, p < .01$ and $F(1,32) = 4.92, p = .03$, respectively] (Figure 9). *Post hoc* analysis showed that in both the corn oil and fish oil groups, amphetamine-treated mice had significantly higher DOPAC/DA turnover than their saline-treated counterparts.

Finally, there was also a significant main effect of amphetamine treatment for HVA/DA turnover [$F(1, 32) = 51.44, p < .01$] (Figure 9). *Post hoc* analysis revealed that amphetamine-treated mice had significantly higher HVA/DA turnover than saline-treated animals in both the corn oil and fish oil groups.

Neurochemical analysis did not reveal any significant effects for serotonin levels but did show significant main effects of amphetamine treatment and diet for 5-hydroxyindolacetic acid (5-HIAA) levels [$F(1, 32) = 10.69, p < .01$ and $F(1,32) = 4.27, p = .05$, respectively] (Figure 8). Animals treated with amphetamine had significantly higher levels of 5-HIAA than those treated with saline. In addition, mice fed the fish oil-rich diet had significantly more striatal 5-HIAA than their corn oil-fed counterparts.

Neurochemical analysis revealed a significant main effect of amphetamine treatment [$F(1, 32) = 58.61, p < .01$] on 5-HIAA/5-HT turnover (Figure 9). *Post hoc* analysis showed that mice treated with amphetamine had significantly higher serotonin turnover than those treated with saline.

Conclusions

Results indicate that a number of the neurochemical alterations caused by amphetamine were mitigated by the fish oil diet. The most notable neurochemical protection observed in fish oil-fed mice is evidenced by alterations in striatal dopamine and its metabolites. Although amphetamine treatment led to decreased dopamine levels in both the corn oil and fish oil groups, the depletion was significantly reduced in mice fed the fish oil diet. More specifically, in amphetamine-treated mice, there was approximately an 85% reduction in dopamine in the corn oil group and only about a 48% reduction in the fish oil group. Like dopamine, amphetamine treatment caused a decrease in DOPAC levels in both corn oil- and fish oil-fed animals. However, the DOPAC depletion was diminished in the fish oil group (36%) relative to the corn oil group (75%). Additionally, the amphetamine treatment led to a 61% decrease in HVA in the corn oil group but did not significantly alter HVA in the fish oil group.

There are a number of mechanisms by which the omega-3-rich diet may confer neuroprotection. Omega-3 PUFAs have been shown to protect against oxidative damage (Wu et al., 2004; Yavin et al., 2002). It has been suggested that amphetamine-induced release of dopamine leads to the formation of reactive oxygen species (Kita et al., 2003; Yamamoto & Raudensky, 2008). Therefore, it is possible that omega-3 PUFAs reduced the amphetamine-induced toxicity through an antioxidant mechanism. This conclusion was reached in studies using other dopaminergic toxins, such as 6-OHDA and MPTP (Bousquet et al., 2008; Delattre et al., 2009). Other potential mechanisms of neuroprotection of omega-3 PUFAs include reduction of neuroinflammation (Lu et al., 2010), inhibition of apoptosis (Kim et al., 2001), the modulation of brain derived neurotrophic factor, the activation of nuclear receptors that operate as transcription factors, and its incorporation into neuronal membranes (Bousquet et al., 2009; Bousquet et al., 2008). It is likely that a number of these mechanisms are interacting to result in the neuroprotective effect observed in the fish oil-fed mice. Experiment 4 of the dissertation was performed to shed light on the possible mechanism of protection. More specifically, it investigated the potential antioxidant effect of the fish oil-rich diet.

There are several potential confounding factors to consider. Although both corn oil and fish oil are composed primarily of PUFAs with the fish oil being rich in the omega-3 variety, there are other differences in the composition of these oils. For example, the oils contain different percentages of monounsaturated, polyunsaturated, and saturated fats. Therefore, the beneficial effects of the fish oil cannot entirely be ascribed to the presence of the omega-3 PUFAs. Rather, we can only say that a fish oil-rich diet is protective in this model.

A second confounding factor to consider is the dose of fish oil consumed in this experiment. With an average consumption of 10 g of diet per day and an average body weight of 24 g, the approximate daily dose of fish oil was 37.5 g/kg body weight. Approximately 30% of the fatty acids in the fish oil are of the omega-3 variety, therefore the dose of omega-3 PUFAs in these mice is 11.25 g/kg. Most pill supplements consist of 1 g of oil (300 mg of omega-3 PUFAs). Consequently, the volume of omega-3 PUFAs consumed daily in both the mice and humans taking the supplement is comparable, but certainly the g/kg dose is considerably smaller in humans. When you consider intake from other sources as well, such as fish, plants, nuts, and seeds, the average daily intake of omega-3 PUFAs in Americans is 1.6 g (Kris-Etherton et al., 2003). Therefore, Americans, who on average weigh close to 80 kg, consume a dose of 0.02 g/kg. Even in humans who ingest relatively high amounts of omega-3 PUFAs, their dose is substantially smaller than the dose consumed by the mice in this experiment.

Another factor to consider is that the animals in the corn oil group weighed more on average than those in the fish oil group at the time of injections. This weight difference is likely due to higher consumption of the corn oil diet than the fish oil diet during the first few days of the study, perhaps because of an initial taste neophobia. Although all the mice received the same mg/kg dose of amphetamine, the heavier animals received a larger absolute dose. Consequently, it is possible that more of the drug reached the brains of mice in the corn oil group than the fish oil group. This confound will need to be ruled out before we can confidently attribute the observed protection to the effect of the fish oil-rich diet.

Lastly, tert-butylhydroquinone (tBHQ), an antioxidant used as a preservative, was added by the manufacturer to the fish oil. Without this antioxidant, menhaden oil has been shown to oxidize appreciably within 24 hours at room temperature. The addition of tBHQ prevents this deterioration for at least 72 hours (Fritsche & Johnston, 1988). This antioxidant has also been shown to activate nuclear factor erythroid 2-related factor and protect against both 6-OHDA- and MPTP-induced neurotoxicity (Abdel-Wahab, 2005; de Vries et al., 2008; Jakel et al., 2005; Kraft et al., 2004; Siebert et al., 2009). Therefore, it is possible that the tBHQ could have contributed to the neuroprotection observed in the fish oil group. However, it is unlikely that this agent is accounting for any appreciable amount of protection as there was only 18 mg of tBHQ per kg of diet. In a mouse weighing 24 g and consuming 10 g of fish oil diet per day, the daily dose of tBHQ would be approximately 7 mg/kg. This dose is considerably less than the 100 mg/kg oral dose that was shown to protect against MPTP-induced neurotoxicity (Abdel-Wahab, 2005).

Figure 4.

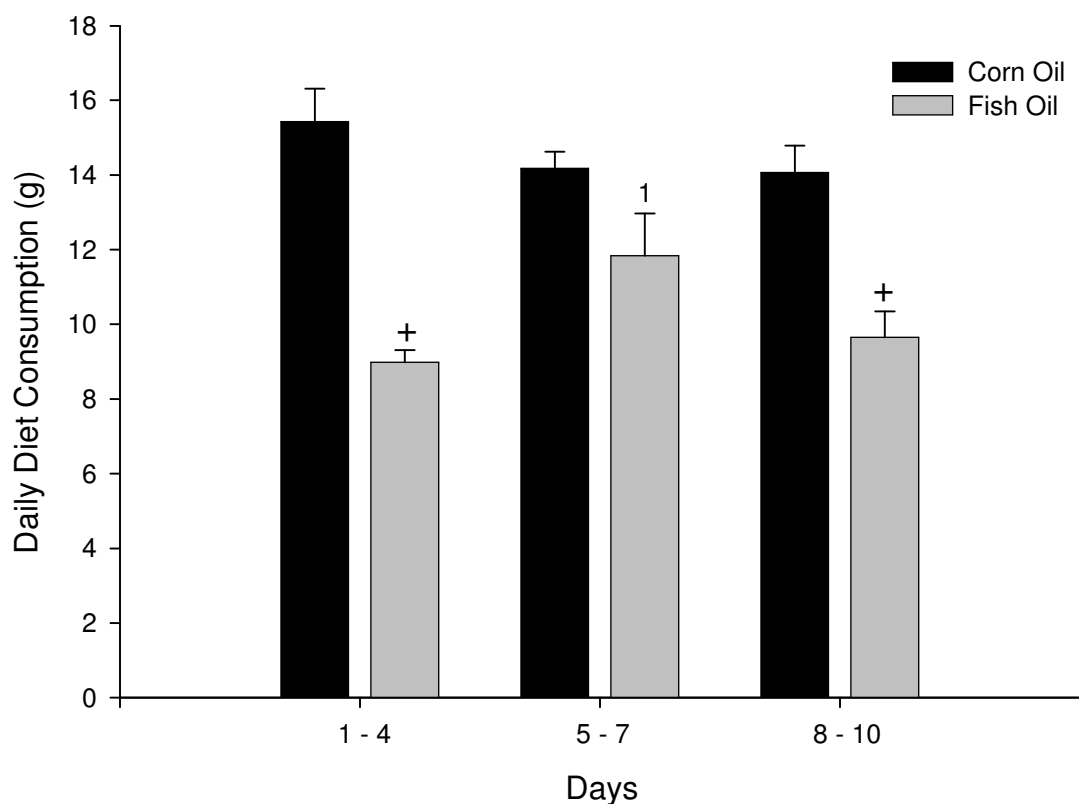


Figure 4. Daily consumption of a liquid diet rich in either corn oil or fish oil averaged across three time points: days 1-4 (i.e. days associated with diet acquisition), days 5-7 (i.e. diet intake before treatment), and days 8-10 (i.e. diet intake following treatment with either saline or amphetamine). Data shown includes both amphetamine- and saline-treated mice collapsed across treatment condition. ⁺ indicates significantly different from corn oil at that time point, $p < .05$; ¹ indicates significantly different from days 1-4, within diet, $p < .05$; error bars indicate SEM.

Figure 5.

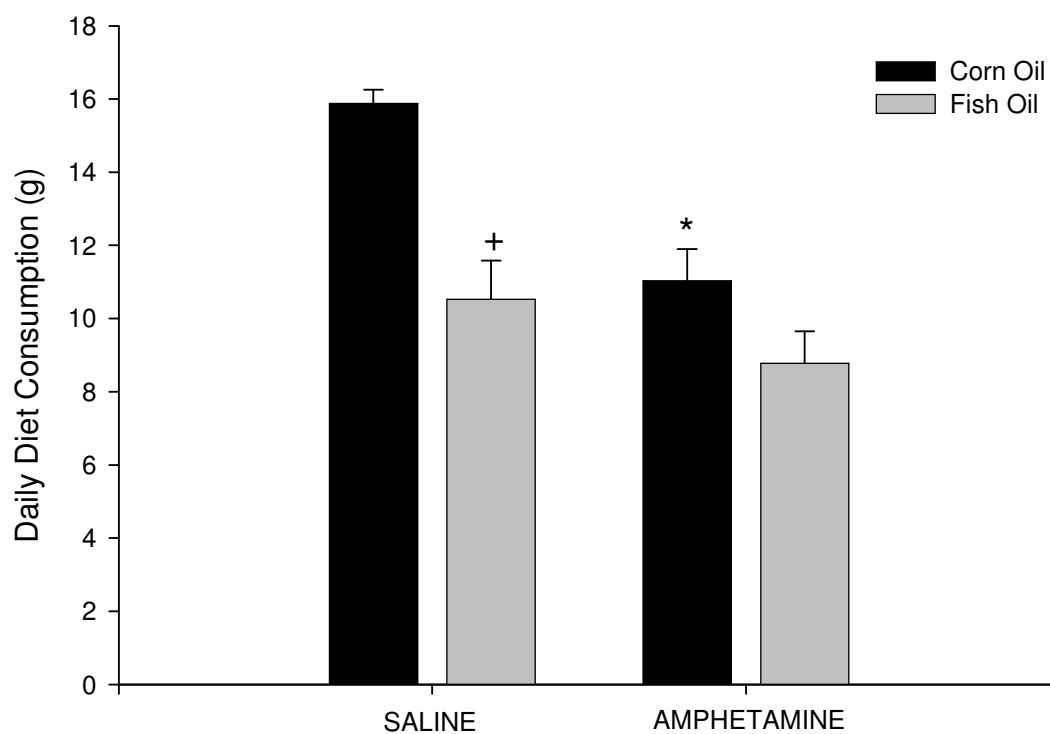


Figure 5. Daily consumption of a liquid diet rich in either corn oil or fish oil averaged across days 8-10 of the experiment (i.e. diet intake following treatment with either saline or amphetamine). * indicates significantly different from saline, within diet, $p < .05$; + indicates significantly different from corn oil, within treatment, $p < .05$; error bars indicate SEM.

Figure 6.

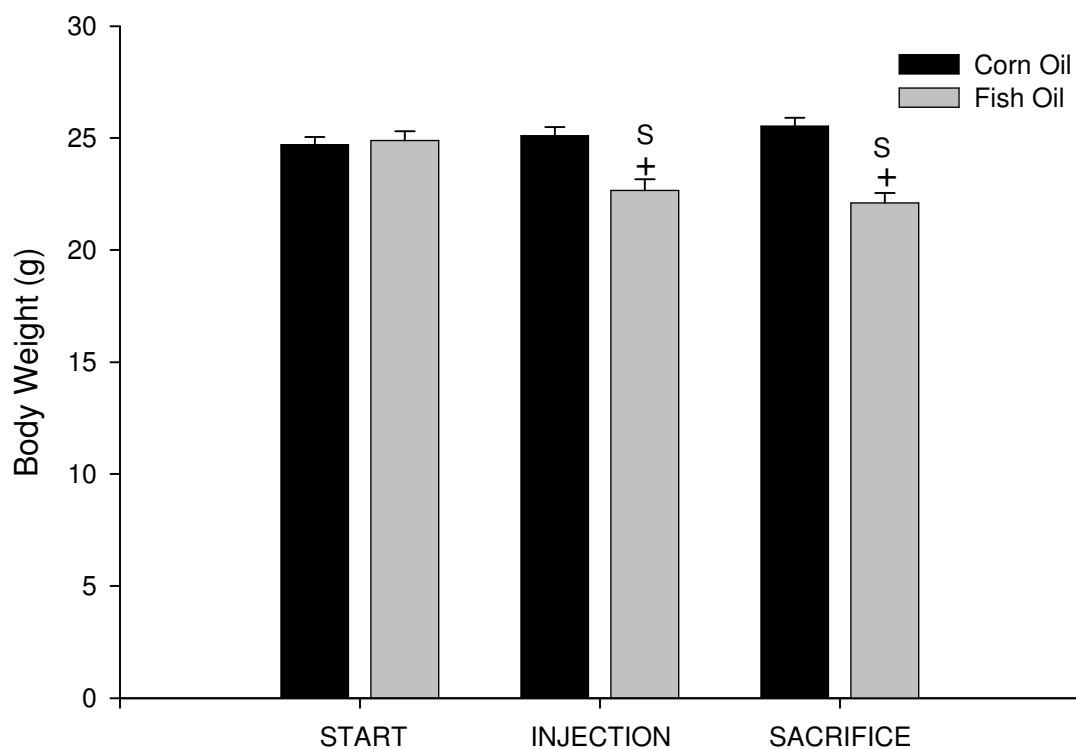


Figure 6. Body weight at the start of the experiment, on the day of injections, and at sacrifice in mice fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. Data shown includes both amphetamine- and saline-treated mice collapsed across treatment condition. + indicates significantly different from corn oil at that time point, $p < .05$; S indicates significantly different from the start of the experiment, within diet, $p < .05$; error bars indicate SEM.

Figure 7.

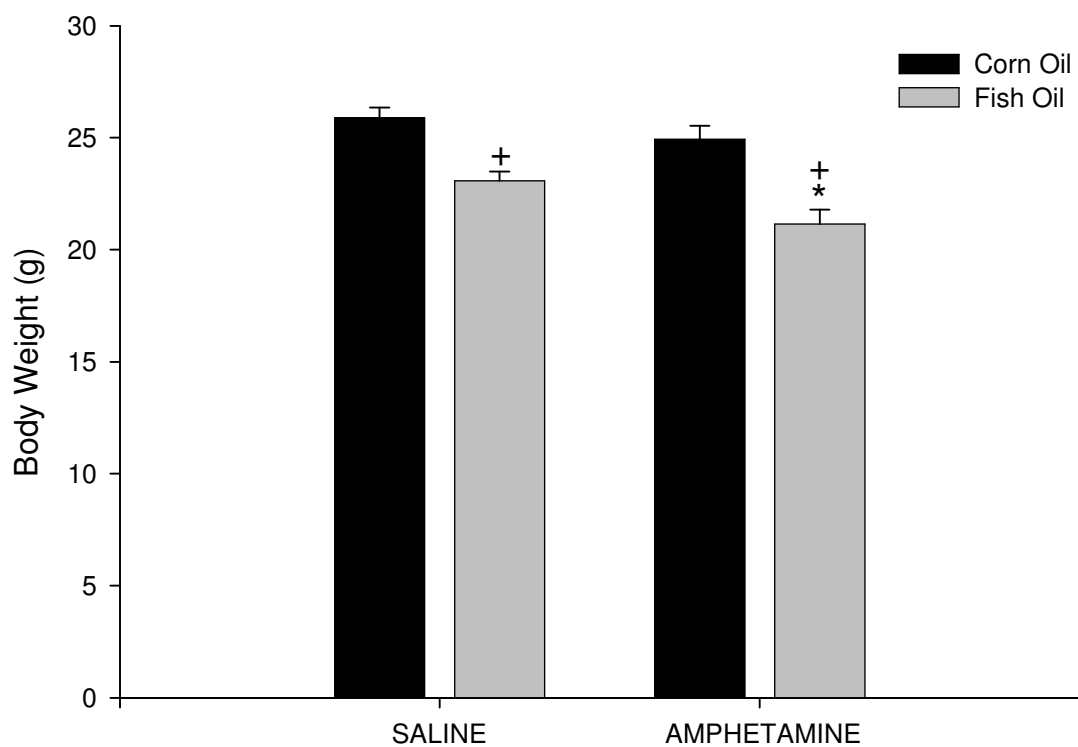


Figure 7. Body weight at sacrifice in mice fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. * indicates significantly different from saline, within diet, $p < .05$; + indicates significantly different from corn oil, within treatment, $p < .05$; error bars indicate SEM.

Figure 8.

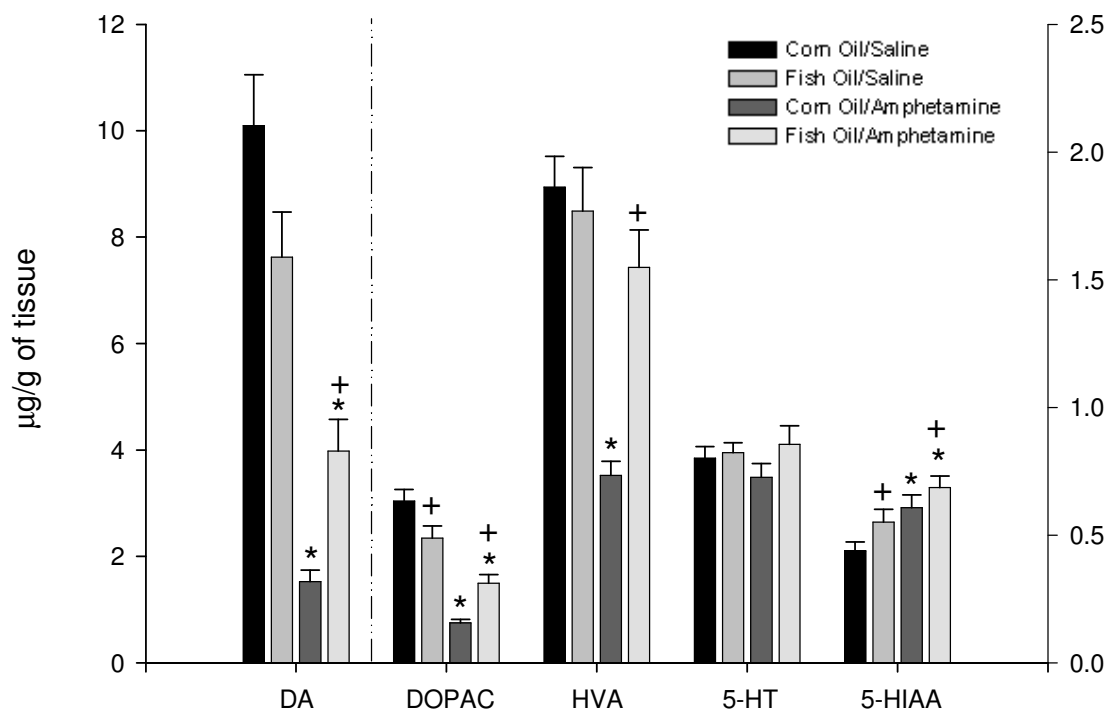


Figure 8. Striatal concentrations of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindolacetic acid (5-HIAA) in mice fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. Dopamine concentration is given by the axis to the left of the dashed line, while the other concentrations are given by the axis on the right. * indicates significantly different from saline, within-diet, $p < .05$; + indicates significantly different from corn oil, within-treatment, $p < .05$; error bars indicate SEM.

Figure 9.

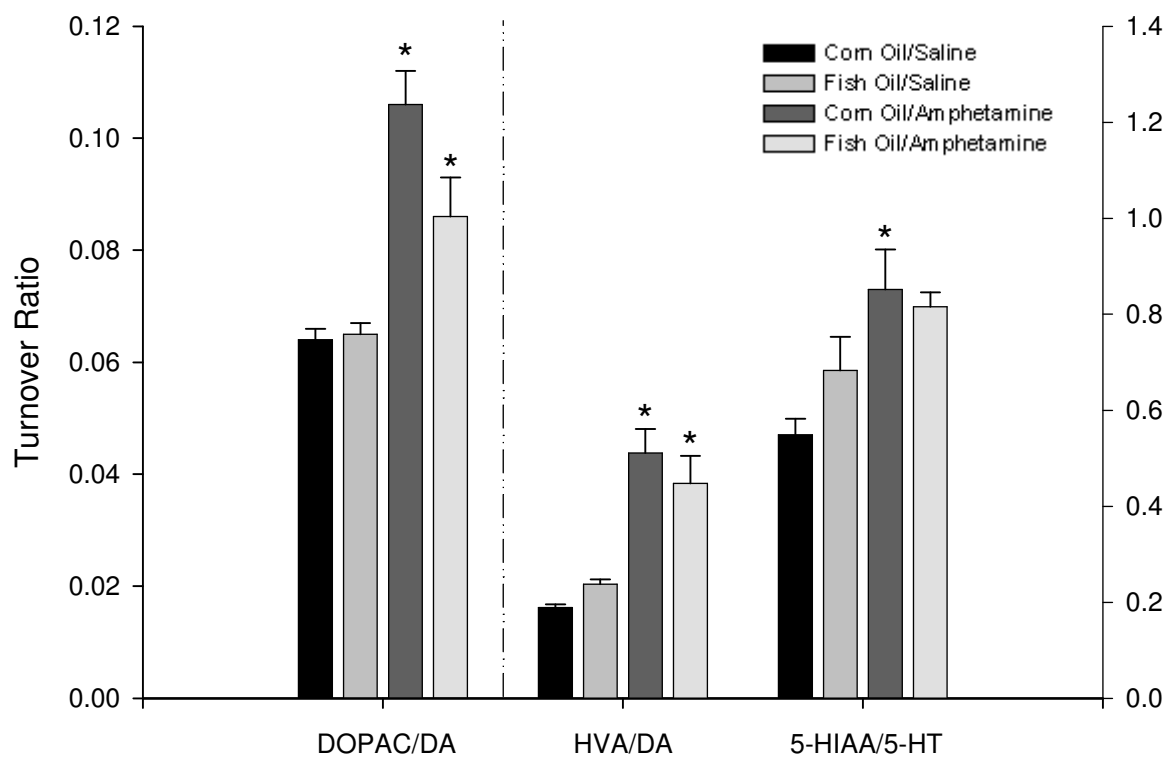


Figure 9. Striatal turnover ratios in mice fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. DOPAC/DA turnover ratio is given by the axis to the left of the dashed line, while the other turnover ratios are given by the axis on the right. * indicates significantly different from saline, within-diet, $p < .05$; error bars indicate SEM.

EXPERIMENT 2

Rationale

As stated earlier, in Experiment 1, animals in the fish oil condition weighed significantly less than their counterparts in the corn oil condition at the time of amphetamine treatment. This is due to reduced consumption of diet during the first few days of the study, which is likely a result of a taste neophobia to the fish oil diet. Although each mouse that received amphetamine received a 50 mg/kg total dose, those mice in the corn oil group received a larger absolute dose. This is potentially troublesome because depending upon how amphetamine is distributed throughout the body, it's possible that the larger absolute dose may result in more amphetamine reaching the brain. If this is the case, the difference in the lesion could simply be due to this difference in absolute dose and not because fish oil is neuroprotective. This confound must be ruled out before it can be conclusively determined that the fish oil-rich diet confers protection. Consequently, the animals in Experiment 2 were divided into groups based upon weight; the heavier animals were placed into the fish oil condition, and the lighter animals were placed into the corn oil condition. This change in methods was implemented to eliminate the weight difference previously observed at the time of amphetamine treatment. It was hypothesized that the fish oil-rich diet would still confer protection, indicated by a diminished neurochemical lesion, after this weight difference is removed.

It is clear that selecting groups based upon weight was not an ideal solution to remedy the weight difference issue as biased groups were being created. Despite this fact, this method was preferable to other possibilities. One such option would have been

to administer a daily dose of oil using oral gavage. However, this method is stressful for the mice, and there is evidence suggesting that chronic stress can alter an animal's response to amphetamine (Deroche et al., 1992; Herman et al., 1984). A second possible option would have been to restrict the consumption of diet in the corn oil condition to match the consumption of diet in the fish oil condition. However, food restriction can also be stressful, and in this case, we would have been restricting intake in only one group, creating an additional difference between the two cohorts of mice.

In addition to the neurochemical lesion induced by amphetamine administration, the drug also results in a number of acute behavioral and physiological changes that occur while the drug is active. For example, methamphetamine has been shown to induce hyperactivity and self-injurious behavior (Kita et al., 2000; Kita et al., 2003). In addition to these behavioral alterations, hyperthermia also occurs as a result of methamphetamine treatment (Kita et al., 2003). In fact, it has been suggested that this hyperthermia may be related to the neurotoxicity, as the increased body temperature correlates with the magnitude of dopamine depletion (Bowyer et al., 1992). Following the hyperthermic effect, body temperature drops below baseline and hypothermia is observed. Given that the preliminary study suggests that a fish oil-rich diet attenuates the neurochemical lesion induced by amphetamine, it is likely that the diet will alter the acute impact of the drug as well. This issue is examined in the following experiment. More specifically, dermal temperature, locomotor activity, several amphetamine-induced behaviors, such as oral dyskinesia, self-biting, head bobbing, backwards walking, and stereotypy, was assessed. It was hypothesized that administration of a diet that is rich in fish oil would result in reduced hyperactivity, self-injurious behaviors, and stereotypic behaviors as compared to

their corn oil counterparts. Furthermore, the magnitude of the initial hyperthermia and subsequent hypothermia was expected to be diminished.

It has been demonstrated that amphetamine administration can result in striatal nerve terminal degeneration and subsequent cell death of substantia nigra cell bodies (Sonsalla et al., 1996), and this neurodegeneration results in permanent behavioral deficits (Kita et al., 2003). For example, animals that experience neurochemical damage induced by amphetamine exhibit motor deficits evidenced by poor performance on active avoidance and balance beam tasks (Walsh and Wagner, 1992). If fish oil confers protection against the lesion, it's likely that these behavioral alterations are mitigated. Consequently, Experiment 2 assessed the impact of amphetamine treatment and fish oil-rich diet on the motor capabilities, as indicated by locomotor activity levels and rotarod performance, 72 hours after treatment. It was expected that the amphetamine-induced striatal lesion would result in motor deficits evidenced by both decreased locomotor activity and shorter latency to fall off a rotating rod relative to saline-treated animals. In addition, it was hypothesized that of the mice that were treated with amphetamine, mice that were fed the fish oil diet would perform better than those fed the control diet; that is, they would be more active and exhibit longer latencies to fall.

Methods

Thirty-two male, 9-week old BALB/c mice (Jackson Laboratories, Bar Harbor, Maine) were used for this experiment. The methods associated with animal housing, diet, amphetamine treatment, and neurochemical analysis were similar to those of Experiment 1. As stated earlier, however, the mice were not randomly placed into the diet and treatment groups. Rather, in order to control for the weight difference observed in the

previous study, the heavier animals were chosen for the fish oil condition while the lighter animals were placed into the corn oil group.

Locomotor activity was assessed using an “Opto-Varimex Minor” activity chamber (Columbus Instruments) during the amphetamine treatment. This apparatus measures the animals’ horizontal movements by infrared beams. Fifteen minutes after each injection, the mice were placed in the activity chamber for a period of 30 minutes. Halfway through each 30 minute session, evaluation of amphetamine-induced behaviors, including measures of hyperactivity and self-injurious behavior (SIB), was conducted by an experimenter blind to the treatment conditions. The behaviors that were assessed include oral dyskinesia (jaw tremor, licking into the air, or vacuous chewing), self-biting (biting at paws, limbs or chest area), head bobbing, backward walking, and stereotypy (any repetitive activity like circling, grooming, biting, digging, and jumping). The behaviors were determined to be either present or absent. This binary designation was used as these behaviors are difficult to quantify in terms of intensity.

A Fluke 62 infrared temperature gun (obtained from Cole-Parmer) was used to measure the dermal temperature of the mice during the amphetamine treatment. In mice, dermal temperature taken from various locations on the chest and abdomen measured using an infrared thermometer has been shown to correlate with rectal temperature (Ochiai et al., 2007). Prior to each injection and at the end of each 30 minute locomotor activity session, dermal temperature of the upper abdomen was measured and recorded (as described by Ochiai et al., 2007).

On the day of sacrifice (72 hours after drug treatment), locomotor activity was measured using the same activity chamber for a period of 30 minutes. In addition, motor

capabilities were assessed by a rotorod challenge. Mice were placed on a rotating rod (rotating at 12 rotations per minute) and the latency to fall was recorded. Each mouse was placed on the rod three times, with a maximum latency of 60 seconds per trial.

Given the findings from Experiment 1 and preliminary analyses from this experiment, dietary condition does not have a major impact on neurochemistry, body temperature, or behavior in saline treated animals. Consequently, all saline-treated subjects from the fish oil and corn oil dietary conditions were collapsed into a single control group, which was then compared against the amphetamine-treated animals from each dietary condition. Locomotor activity data from both injection and sacrifice day, rotorod activity data, dermal temperature data, and brain chemistry data were analyzed using Statview Statistical software. ANOVAs comparing treatment groups were performed. Repeated measures ANOVAs were used to analyze the results of post-injection locomotor activity and dermal temperature. Fisher's PLSD post hoc tests were performed on statistically significant main effects (P values $\leq .05$ were considered statistically significant). Amphetamine-induced behavioral data was analyzed using non-parametric tests. If the expected frequencies were greater than or equal to 5, chi-square tests for independence were used, but if the expected frequencies were less than 5, Fisher Exact tests were used.

Results

Analysis of consumption data showed significant main effects of both treatment group and time point [$F(2,46) = 11.14, p < .01$ and $F(2,46) = 7.77, p < .01$, respectively] (Figure 10). *Post hoc* tests revealed that mice in the corn oil/amphetamine group ate significantly more than their fish oil-fed counterparts across all time points. In addition,

consumption of diet was significantly reduced following treatment (days 8 through 10) relative to the first two time points in all groups.

An ANOVA for body weight revealed a significant main effect of day as well as a significant interaction between day and treatment group [$F(2,50) = 18.93, p < .01$ and $F(4,50) = 6.21, p < .01$, respectively] (Figure 11). *Post hoc* analysis showed that mice in the corn oil/amphetamine group weighed significantly more than their fish oil-fed counterparts at the beginning of the experiment. At the time of injections however, mice from all groups weighed the same; mice in the fish oil/amphetamine group lost a significant amount of weight while the other groups did not change significantly. There were no changes in body weight between the time of injections and the time of sacrifice in any of the groups.

Analysis of locomotor activity revealed significant main effects of group and trial, as well as a significant interaction [$F(2,63) = 6.83, p < .01$, $F(3,63) = 25.23, p < .01$, and $F(6,63) = 4.43, p < .01$, respectively] (Figure 12). *Post hoc* analysis revealed that activity levels remained constant in saline treated animals throughout the four trials, while changes in activity were observed in both amphetamine-treated groups.

There was no significant difference in activity levels between the groups during trial 1, although the corn oil-fed, amphetamine-treated animals showed activity that trended towards elevated levels relative to saline-treated mice ($p = .08$). In trial 2, activity levels significantly decreased in both amphetamine-treated groups compared to the first trial. Additionally, mice in the fish oil/amphetamine group were significantly less active than both the saline group and their corn oil-fed, amphetamine-treated counterparts. Activity levels in trial 3 are similar to those in trial 2, and mice in the fish oil/amphetamine group

remained significantly less active than the other groups. In trial 4, the locomotor activity of the corn oil/amphetamine group dropped to a level similar to that of fish oil-fed, amphetamine-treated mice. Consequently, these two groups were significantly less active than the saline group in the final trial.

An ANOVA for dermal temperature revealed a significant main effect of time point [$F(4,108) = 4.31, p < .01$] and a significant interaction between time point and group [$F(8,108) = 3.148, p < .01$] (Figure 13). *Post hoc* analysis indicated that dermal temperature in mice from the corn oil/amphetamine group increased significantly from trial 1 to trial 2. In trial 3, the corn oil/amphetamine group had a significantly higher dermal temperature than the saline condition. Furthermore, the temperature in the fish oil/amphetamine group during trial 3 was significantly higher than trial 1, within group. By trial 4, temperature of the corn oil/amphetamine mice decreased to a level significantly lower than at the start of the experiment, trial 2, and trial 3, within group. In addition, the dermal temperature of the corn oil/amphetamine group was significantly lower than the fish oil/amphetamine group during trial 4.

Analysis of oral dyskinesia behavior showed that during the first trial, a significantly greater proportion of mice in both amphetamine-treated groups displayed the behavior as compared to their saline-treated counterparts (Figure 14). In addition, the proportion of mice in the corn oil/amphetamine group was significantly higher than in the fish oil/amphetamine group. By trial 2, the proportion of mice displaying this behavior in the corn oil/amphetamine group decreased significantly to a level comparable to the fish oil/amphetamine condition. Both of these groups remained significantly higher than saline in trial 2, however. By the third trial, there were no significant differences between

the three treatment groups. The proportion of mice displaying oral dyskinesia in the corn oil/amphetamine group remained significantly lower than in trial 1, and the proportion of mice in the fish oil/amphetamine group was significantly lower than in trial 2. In the fourth trial, the proportion of mice in the fish oil/amphetamine group increased significantly from the previous trial, and that proportion was significantly higher than both the corn oil/amphetamine and saline groups. There was no change in the proportion of mice displaying this behavior in the corn oil/amphetamine group, and it remained significantly lower than in trial 1.

Nonparametric tests performed on self-biting indicated that there was no significant self-biting behavior displayed during trial 1 (Figure 15). By the second trial, the proportion of mice showing self-biting increased significantly in the fish oil/amphetamine condition to a level significantly higher than the saline group. The proportion of mice in the corn oil/amphetamine group was not significantly higher than the saline group in this trial, although this difference approached significance ($p = .06$). In both the third and fourth trials, the proportion of mice displaying self-biting in the fish oil/amphetamine group remained significantly higher than in the first trial; it also remained higher than the saline group, within trials. Furthermore, in trial 4, the proportion of mice in the fish oil/amphetamine group was significantly higher than the corn oil group.

Analysis of stereotypic behavior revealed that a significantly higher proportion of mice in the corn oil/amphetamine group displayed this behavior during trial 1 than in the other treatment groups (Figure 16). By trial 2, the proportion of mice displaying stereotypy in the fish oil/amphetamine group increased to a level significantly higher than

the saline group and similar to their corn oil-fed counterparts. In the third trial, both amphetamine-treated groups remained significantly higher than the saline group, and the proportion of mice in the fish oil/amphetamine group increased slightly to a level that is significantly higher than in trial 1. In the final trial, the proportion of mice displaying this behavior in the corn oil/amphetamine group decreased significantly such that it was significantly lower than in the first two trials. In addition, the proportion of mice displaying stereotypy in the corn oil/amphetamine group was significantly lower than the fish oil amphetamine group in trial 4.

Nonparametric tests indicated that the proportion of mice displaying backwards walking in the corn oil/amphetamine condition was significantly higher than the other two treatments groups (Figure 17). The proportion of animals in the fish oil/amphetamine group increased significantly from trial 1 to trial 2 to a level comparable to the corn oil/amphetamine group. In the remaining two trials, the proportion of mice displaying backwards walking in the corn oil/amphetamine group was similar to the saline group and significantly lower than the fish oil/amphetamine condition. Furthermore, the fish oil/amphetamine group remained significantly higher than in trial 1, within group.

An ANOVA performed for locomotor activity on sacrifice day revealed a significant effect of treatment group [$F(2,25) = 4.58, p = .02$] (Figure 18). *Post hoc* analysis revealed that mice that were fed a diet rich in fish oil and treated with amphetamine were significantly less active than saline-treated animals. There were no significant differences between the corn oil/amphetamine group and the other treatment groups. Another measure performed on sacrifice day, rotorod performance, was not

significantly impacted by treatment group (Figure 19). It should be noted that four mice in the corn oil/amphetamine group died prior to sacrifice day and consequently, could not be included in these analyses. Nonparametric tests performed on mortality rate revealed that a significantly higher proportion of mice from the corn oil/amphetamine group died as compared to either the saline group or the fish oil/amphetamine group. There was no difference in mortality between the fish oil/amphetamine condition and saline-treated mice (Figure 20).

Neurochemical analysis of dopamine content in the striatum revealed a significant effect of treatment group [$F(2,24) = 47.36, p < .01$] (Figure 21). *Post hoc* tests showed that striatal dopamine levels were significantly higher in the saline group than both of the other treatment conditions. In addition, there was significantly more striatal dopamine in the fish oil/amphetamine group than the corn oil/amphetamine group.

As for DOPAC levels, analysis revealed a significant effect of treatment group [$F(2,24) = 52.76, p < .01$] (Figure 21). *Post hoc* analysis showed a similar pattern to the dopamine results. That is, DOPAC levels in the corn oil/amphetamine group were significantly lower than in the fish oil/amphetamine group, and both of these amphetamine-treated groups were lower than the saline-treated condition.

Neurochemical analysis of HVA levels revealed a significant effect of group [$F(2,24) = 20.86, p < .01$] (Figure 21). Like dopamine and DOPAC, *post hoc* tests indicated that HVA content in the corn oil/amphetamine group were significantly lower than in the fish oil/amphetamine group, and both of these amphetamine-treated groups were lower than the saline-treated condition.

Analysis of serotonin levels revealed a significant effect of treatment group [$F(2,24) = 6.34, p = .01$] (Figure 21). *Post hoc* analysis showed that serotonin levels in both amphetamine-treated groups were significantly lower than the saline group. Analysis of 5-HIAA levels revealed no significant differences between treatment groups.

An ANOVA performed on DOPAC/DA turnover indicated that there was a significant effect of treatment group [$F(2, 24) = 23.64, p < .01$] (Figure 22). *Post hoc* analysis showed that mice in the corn oil/amphetamine group had significantly higher turnover than their fish oil-fed counterparts. Furthermore, the fish oil/amphetamine group had a significantly higher turnover than the saline group.

As for HVA/DA turnover, analysis revealed a significant effect of group [$F(2, 24) = 35.28, p < .01$] (Figure 22). Like DOPAC/DA turnover, *post hoc* analysis revealed that the corn oil/amphetamine group had significantly higher HVA/DA turnover than the fish oil/amphetamine group, which had higher turnover than the saline group.

There was also a significant effect of group on 5-HIAA/5-HT turnover [$F(2, 24) = 15.33, p < .01$] (Figure 22). *Post hoc* analysis revealed that both amphetamine-treated groups had significantly higher turnover than the saline-treated group.

Conclusions

The neurochemistry results from Experiment 2 mirror those from the first experiment. Amphetamine treatment resulted in significant depletions of dopamine and its metabolites, DOPAC and HVA, and those depletions were mitigated by fish oil. More specifically, a 93% depletion of dopamine in the corn oil/amphetamine condition was reduced to 61% in the fish oil group. An 81% decrease in DOPAC was reduced to 61%, and a 68% decrease in HVA was reduced to 36%. Furthermore, the fish oil diet

significantly reduced the impact of amphetamine treatment on turnover rates relative to the corn oil diet. More specifically, relative to the saline group, DOPAC/DA turnover was increased by 170% in the corn oil/amphetamine group but only increased by 58% in the fish oil/amphetamine group. Similarly, HVA/DA turnover was increased by 364% in the corn oil/amphetamine group and increased by 123% in the fish oil/amphetamine condition. Collectively, these results indicate that the fish oil-rich diet conferred significant neuroprotection from amphetamine-induced depletions of dopamine and its metabolites, as well as increases in dopamine turnover.

Consumption of diet in this experiment was comparable to consumption in the first experiment. Consequently, significant weight loss in the fish oil-fed mice occurred, likely due to an initial taste neophobia. However, because the heaviest mice were placed into the fish oil/amphetamine group at the start of the experiment, there were no significant differences in body weight on the day of injections. As stated earlier, a concern from the first experiment was that it was a difference in body weight mediating the differential response to amphetamine and not the diet consumption. The results of this experiment, however, indicate that the diet condition, not the difference in body weight, was responsible for the observed protection.

The second experiment was also designed to examine the impact of a fish oil-rich diet on amphetamine-induced changes in behavior and dermal temperature. The results suggest that diet does influence the effect of amphetamines on a number of measures. For example, mice in the corn oil/amphetamine and fish oil/amphetamine treatment groups showed a different pattern of locomotor activity on injection day. It was expected that amphetamine treatment would result in a period of hyperactivity initially followed by

hypoactivity; it was also hypothesized that fish oil would mitigate these effects. Mice in the corn oil/amphetamine group appeared to be more active than saline-treated animals after the first injection, although this effect only approached significance. Surprisingly, mice from the fish oil/amphetamine condition were less active starting after the second injection and persisting through the last trial, whereas hypoactivity was only observed in corn-oil fed mice after the fourth injection. The locomotor activity results were somewhat unexpected as hyperactivity was predicted to be more pronounced, and although diet had an impact on amphetamine-induced activity, a protective effect of fish oil was not evident.

Protection by the fish oil-rich diet was apparent, however, in the dermal temperature results. Although there was an increase in dermal temperature in both the corn oil/amphetamine and fish oil/amphetamine groups, hyperthermia occurred earlier in the corn-oil fed mice. More specifically, an increase in temperature was observed during the second trial in the corn oil/amphetamine group, but the effect wasn't present in the fish oil/amphetamine group until the third trial. This means that the dose of amphetamine necessary to produce hyperthermia was lower in the corn oil-fed mice (two injections for a total dose of 25 mg/kg) than the fish oil-fed animals (three injections for a total dose of 37.5 mg/kg). Not only did the fish oil delay amphetamine-induced hyperthermia, but it also prevented the hypothermia that was present in the corn oil/amphetamine group following the fourth injection. This protective effect of fish oil against amphetamine-induced temperature alterations is important, as the hyperthermic response can help to predict the severity of the neurochemical lesion (Kita et al., 2003).

It is clear that a fish oil-rich diet can have a significant impact on amphetamine-induced behaviors, as corn oil and fish oil had different effects on oral dyskinesia, self biting, stereotypy, and backwards walking. Further examination revealed a similar pattern amongst these behaviors. More specifically, with the exception of self biting, a greater proportion of mice from the corn oil/amphetamine group displayed the behaviors early during the injection regimen than those from the fish oil/amphetamine group. In addition, this effect was reversed later on during the day; more mice from the fish oil/amphetamine group showed these behaviors during the later trials than did mice in the corn oil/amphetamine condition. Thus, it appears that fish oil delays the onset of these behaviors within this injection schedule. Because an injection occurred prior to each trial, this delay indicates that a larger dose of amphetamine was required to produce these behaviors in the fish oil-fed mice than in their corn oil-fed counterparts. Given that these behaviors are related to the striatal dopaminergic activity, it is likely that fish oil alters some aspect of dopamine activity, such as release, reuptake, or breakdown. This issue is examined further in Experiment 4.

Perhaps one of the most obvious indicators of the protection afforded by the fish oil-rich diet was the mortality rate of mice following the injection regimen. Four mice died following treatment, and all of them were in the corn-oil/amphetamine group. Again, this suggests that a diet rich in fish oil can reduce the severity of an animal's reaction to amphetamine. Although it's unclear what physiological response to the amphetamine treatment is directly responsible for their death, it is apparent that fish oil can combat this mechanism.

Inconsistent with expectations, diet did not appear to alter the impact of amphetamine on rotorod and locomotor activity on the day of sacrifice. It was hypothesized that the substantial striatal dopaminergic lesion caused by the amphetamine treatment would result in motor deficits that would be apparent in these behaviors. Furthermore, it was expected that the fish oil-rich diet would mitigate these deficits. The reasons for such an outcome are unclear. A dopamine depletion of 93% in the corn oil/amphetamine condition should have been sufficient to produce motor deficits, which suggests perhaps that the choice of behavioral measures was not appropriate to reveal a significant effect. Maybe using other tasks that rely on motor capabilities and are impacted by amphetamine, such as active avoidance and balance beam (Walsh & Wagner, 1992; Halladay et al., 2000), would have produced the expected results. Finally a difference in the mortality rate between corn oil/amphetamine and fish oil/amphetamine groups could have played a factor in these tests. Four of the mice in the corn oil/amphetamine condition died prior to sacrifice day and consequently, could not be included in these measures. If it were assumed that the reason these four animals from the corn oil/amphetamine condition died was because they had the most severe response to the amphetamine injections, one might also assume that they would display the greatest behavioral deficits. If this were true, and these mice could have been included in the sacrifice day measures, the averages for motor activity and latency to fall in the corn oil/amphetamine group would be lower, making a significant effect more likely to be observed.

Figure 10.

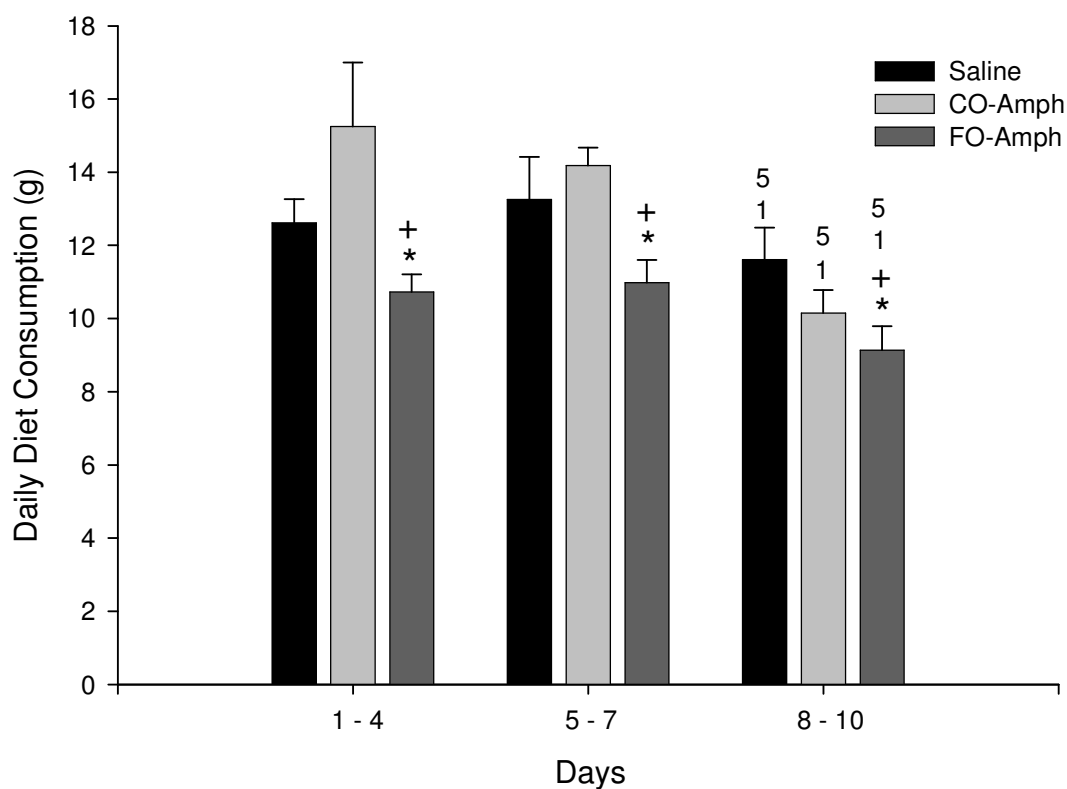


Figure 10. Daily consumption of a liquid diet rich in either corn oil or fish oil averaged across three time points: days 1-4 (i.e. days associated with diet acquisition), days 5-7 (i.e. diet intake before treatment), and days 8-10 (i.e. diet intake following treatment with either saline or amphetamine). Saline group consists of both corn oil- and fish oil-fed mice. * indicates significantly different from saline group, within time point, $p < .05$; + indicates significantly different from corn oil/amphetamine group, within time point, $p < .05$; I indicates significantly different from days 1-4, within group, $p < .05$; 5 indicates significantly different from Days 5-7, within group, $p < .05$; error bars indicate SEM.

Figure 11.

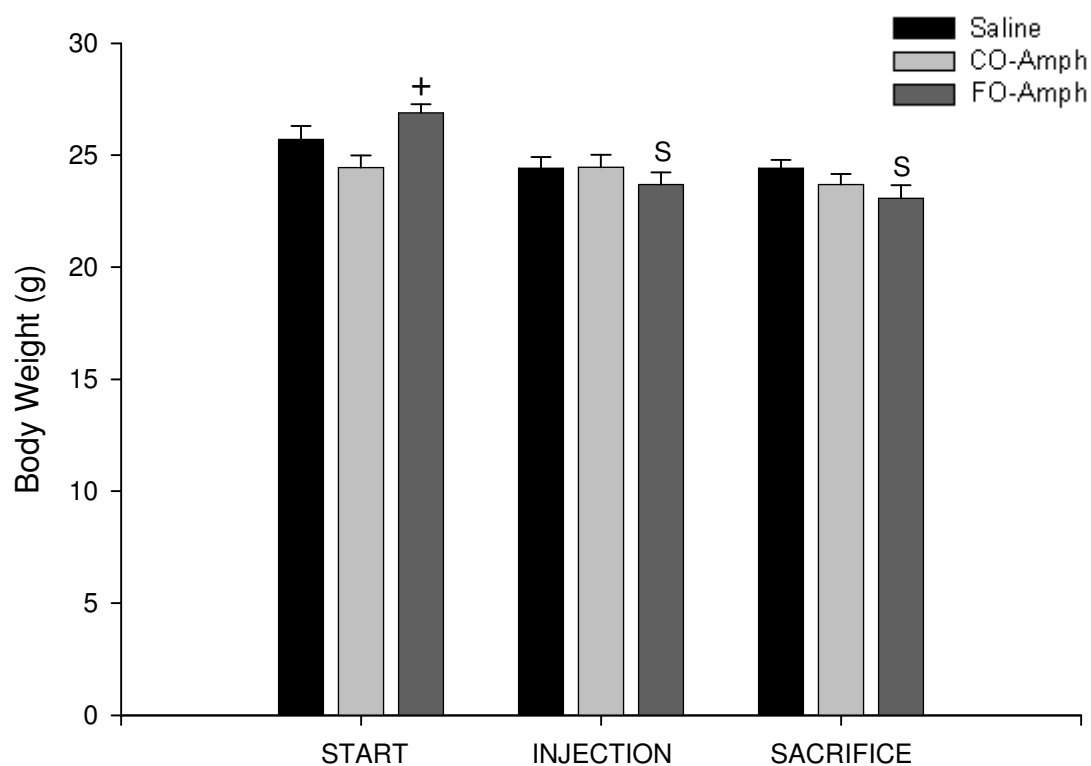


Figure 11. Body weight at the start of the experiment, on the day of injections, and at sacrifice. Mice were fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. Saline group consists of both corn oil- and fish oil-fed mice. ⁺ indicates significantly different from corn oil/amphetamine group, within time point, $p < .05$; S indicates significantly different from the start of the experiment, within group, $p < .05$; error bars indicate SEM.

Figure 12.

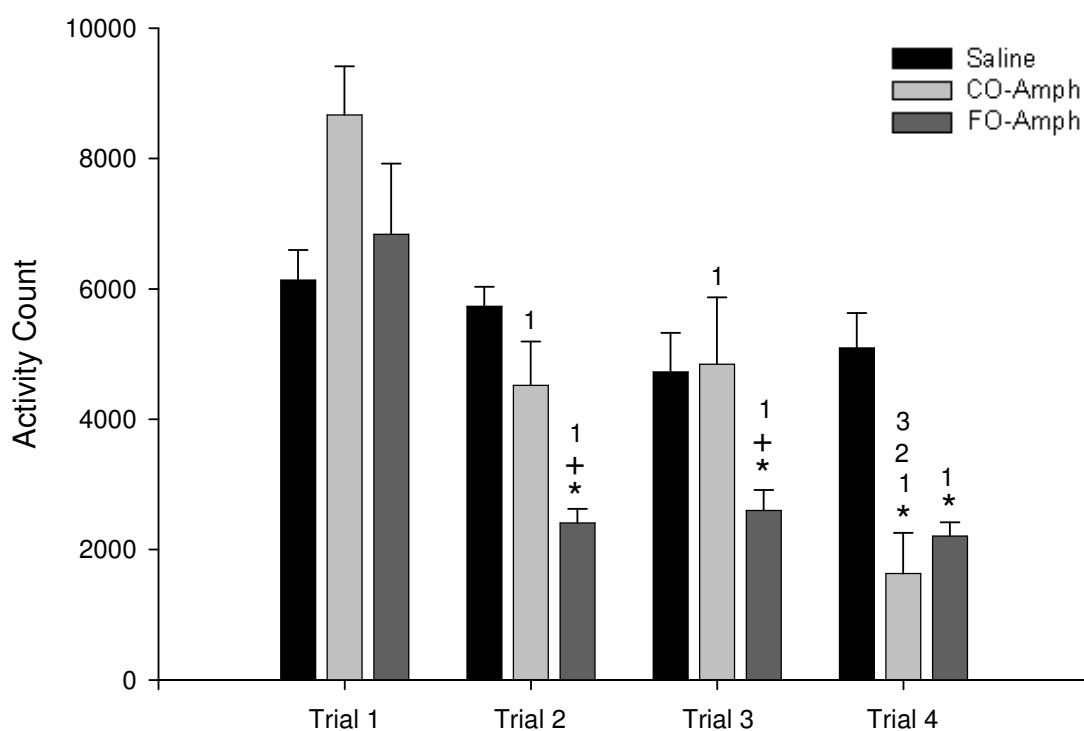


Figure 12. Locomotor activity, as indicated by infrared beam breaks, on injection day in mice fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. Saline group consists of both corn oil- and fish oil-fed mice. * indicates significantly different from saline group, within trial, $p < .05$; + indicates significantly different from corn oil/amphetamine group, within trial, $p < .05$; the numbers above the bars indicate significantly different from that trial, within group, $p < .05$; error bars indicate SEM.

Figure 13.

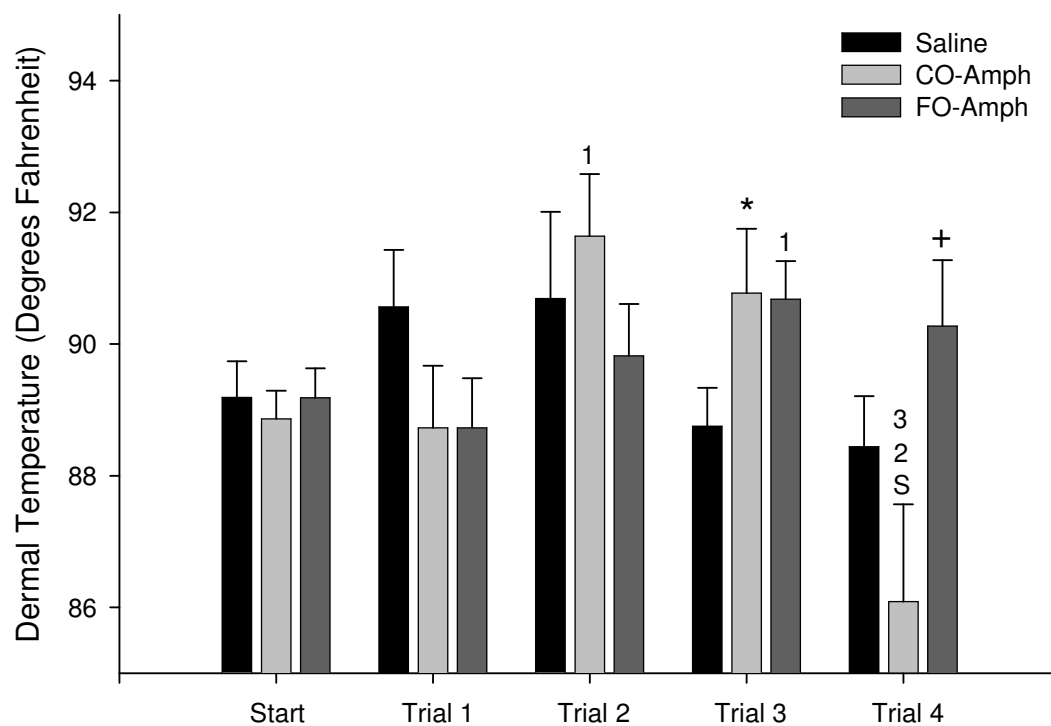


Figure 13. Dermal temperature on injection day in mice fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. Saline group consists of both corn oil- and fish oil-fed mice. * indicates significantly different from saline group, within trial, $p < .05$; + indicates significantly different from corn oil/amphetamine group, within trial, $p < .05$; the numbers above the bars indicate significantly different from that trial, within group, $p < .05$; S indicates significantly different from start, within group, $p < .05$; error bars indicate SEM.

Figure 14.

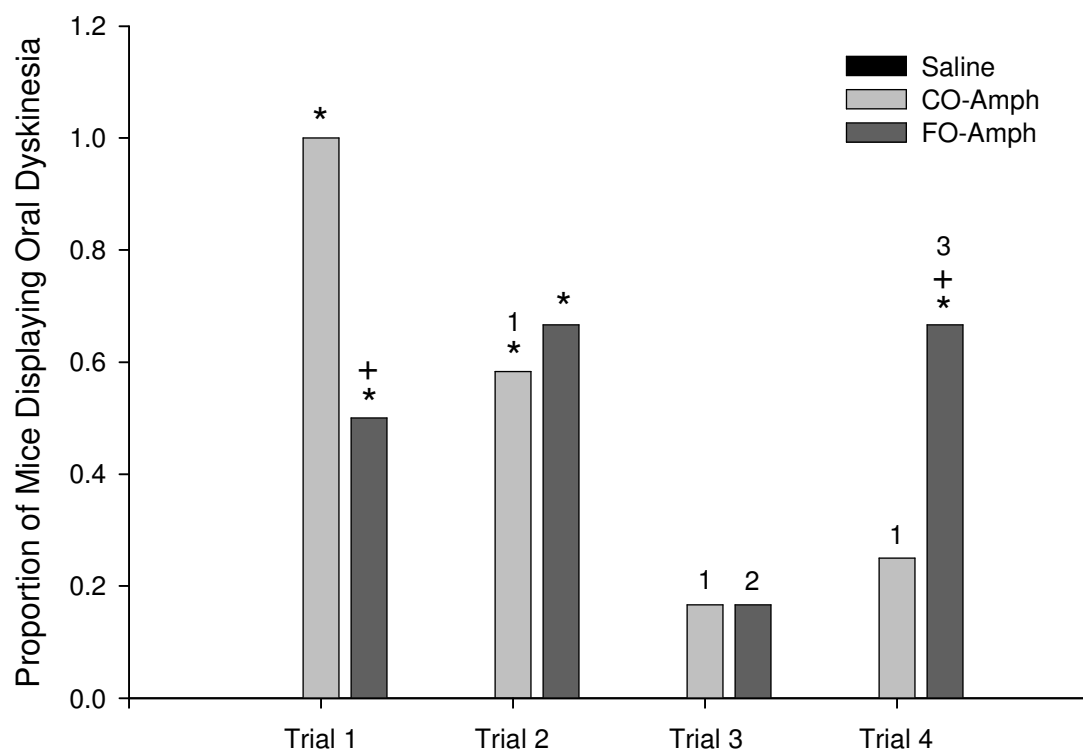


Figure 14. Proportion of mice displaying oral dyskinesia on injection day. Mice were fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. Saline group consists of both corn oil- and fish oil-fed mice. * indicates significantly different from saline group, within trial, $p < .05$; + indicates significantly different from corn oil/amphetamine group, within trial, $p < .05$; the numbers above the bars indicate significantly different from that trial, within group, $p < .05$; error bars indicate SEM.

Figure 15.

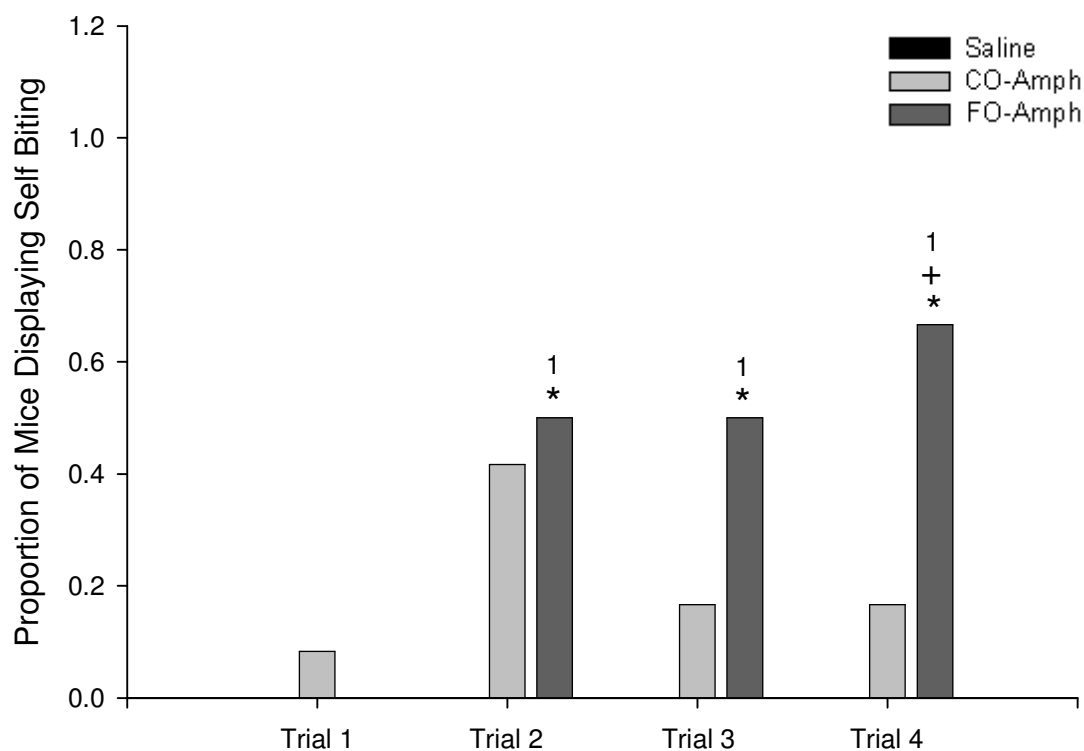


Figure 15. Proportion of mice displaying self biting on injection day. Mice were fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine.

Saline group consists of both corn oil- and fish oil-fed mice. * indicates significantly different from saline group, within trial, $p < .05$; + indicates significantly different from corn oil/amphetamine group, within trial, $p < .05$; the numbers above the bars indicate significantly different from that trial, within group, $p < .05$; error bars indicate SEM.

Figure 16.

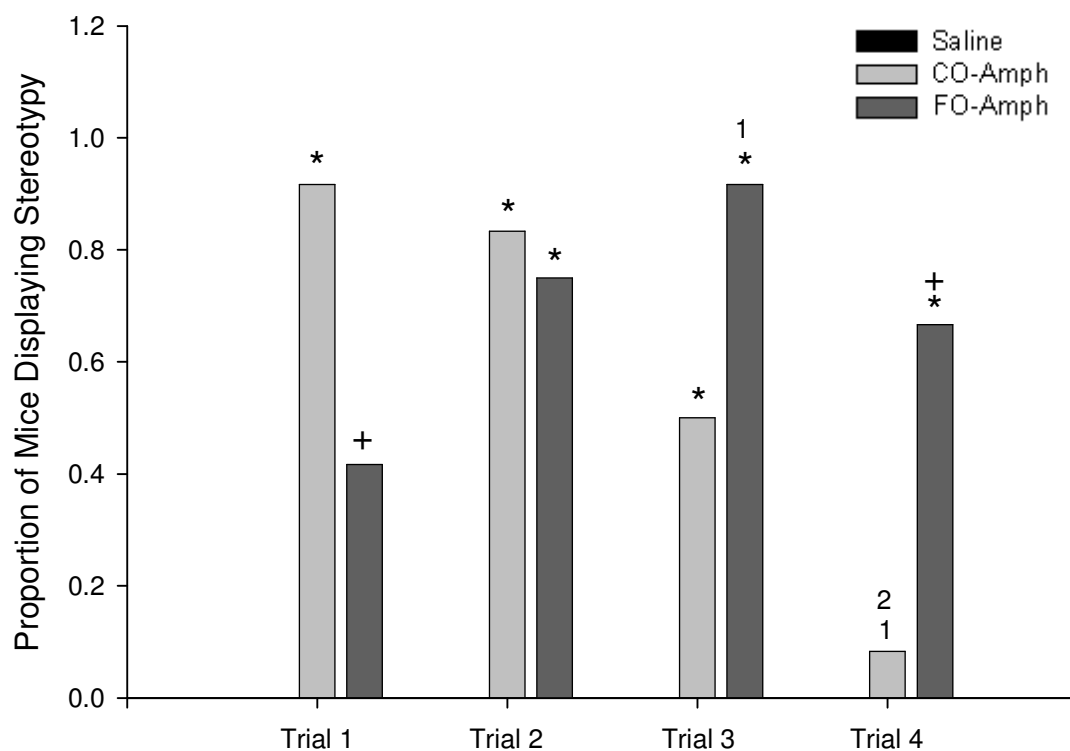


Figure 16. Proportion of mice displaying stereotypy on injection day. Mice were fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine.

Saline group consists of both corn oil- and fish oil-fed mice. * indicates significantly different from saline group, within trial, $p < .05$; + indicates significantly different from corn oil/amphetamine group, within trial, $p < .05$; the numbers above the bars indicate significantly different from that trial, within group, $p < .05$; error bars indicate SEM.

Figure 17.

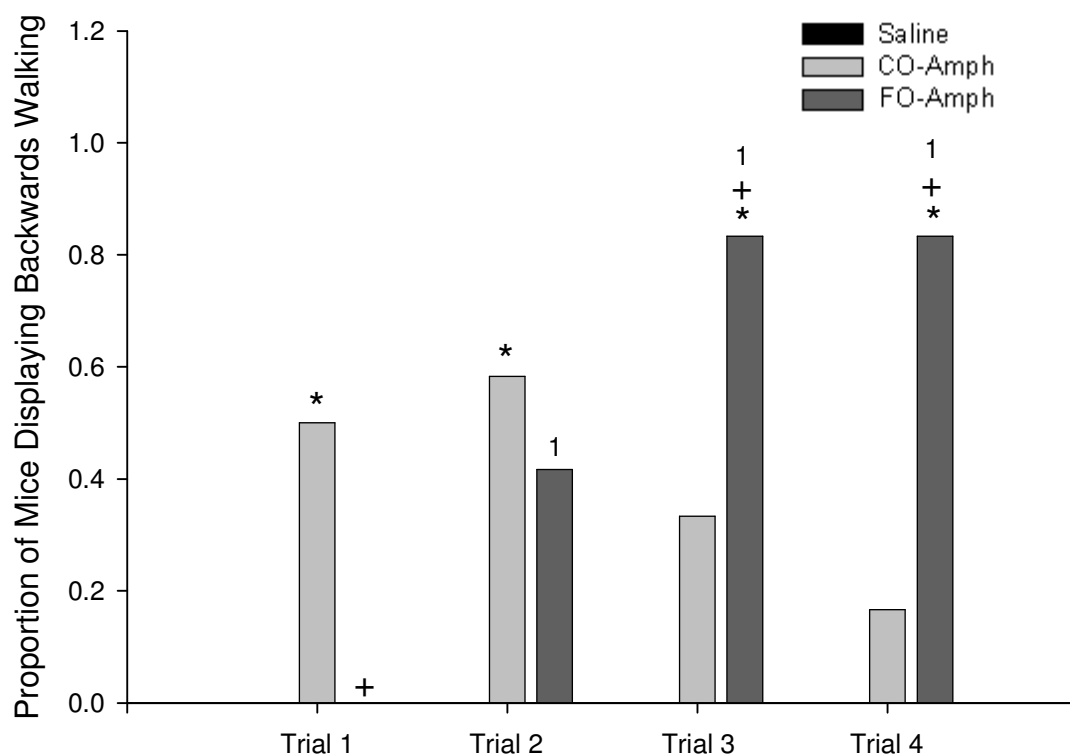


Figure 17. Proportion of mice displaying backwards walking on injection day. Mice were fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. Saline group consists of both corn oil- and fish oil-fed mice. * indicates significantly different from saline group, within trial, $p < .05$; + indicates significantly different from corn oil/amphetamine group, within trial, $p < .05$; the numbers above the bars indicate significantly different from that trial, within group, $p < .05$; error bars indicate SEM.

Figure 18.

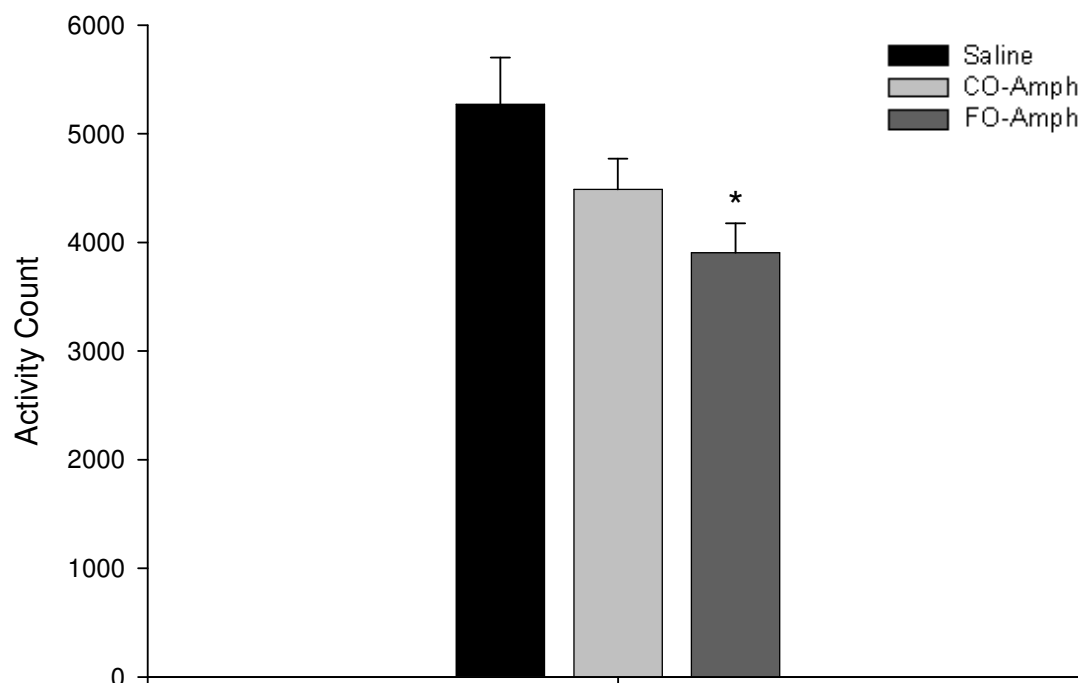


Figure 18. Locomotor activity, as indicated by infrared beam breaks, on day of sacrifice. Mice were fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. Saline group consists of both corn oil- and fish oil-fed mice. * indicates significantly different from saline group, $p < .05$; error bars indicate SEM.

Figure 19.

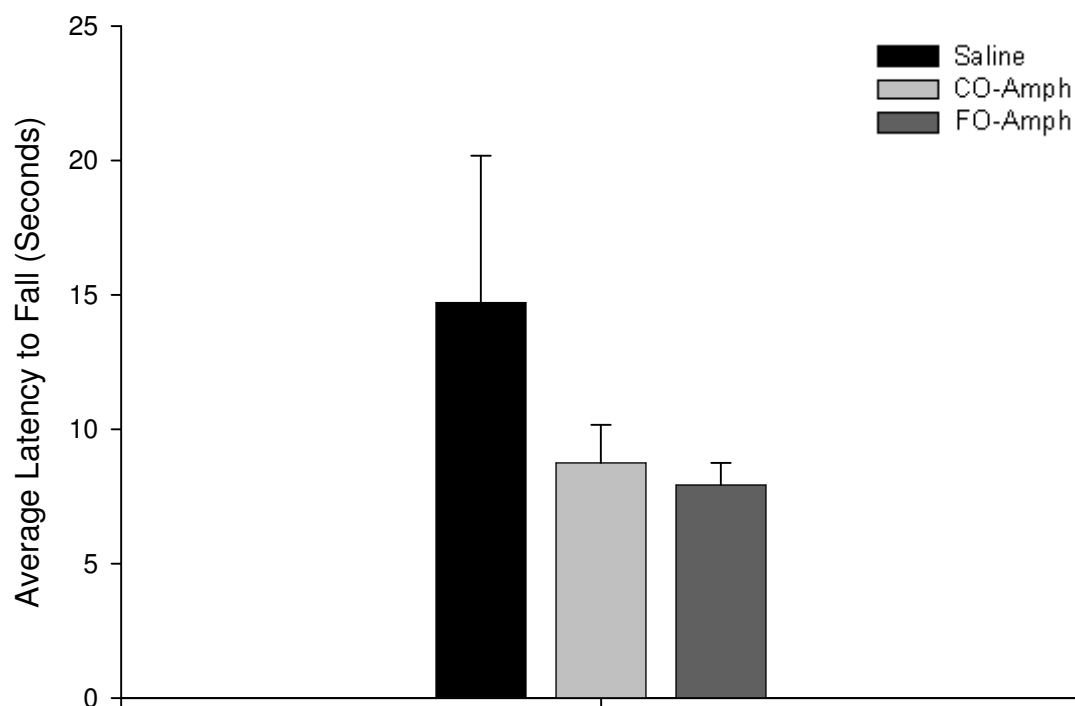


Figure 19. Latency to fall off of a rod rotating at a speed of 12 rotations per minute. Mice were fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. Saline group consists of both corn oil- and fish oil-fed mice. Error bars indicate SEM.

Figure 20.

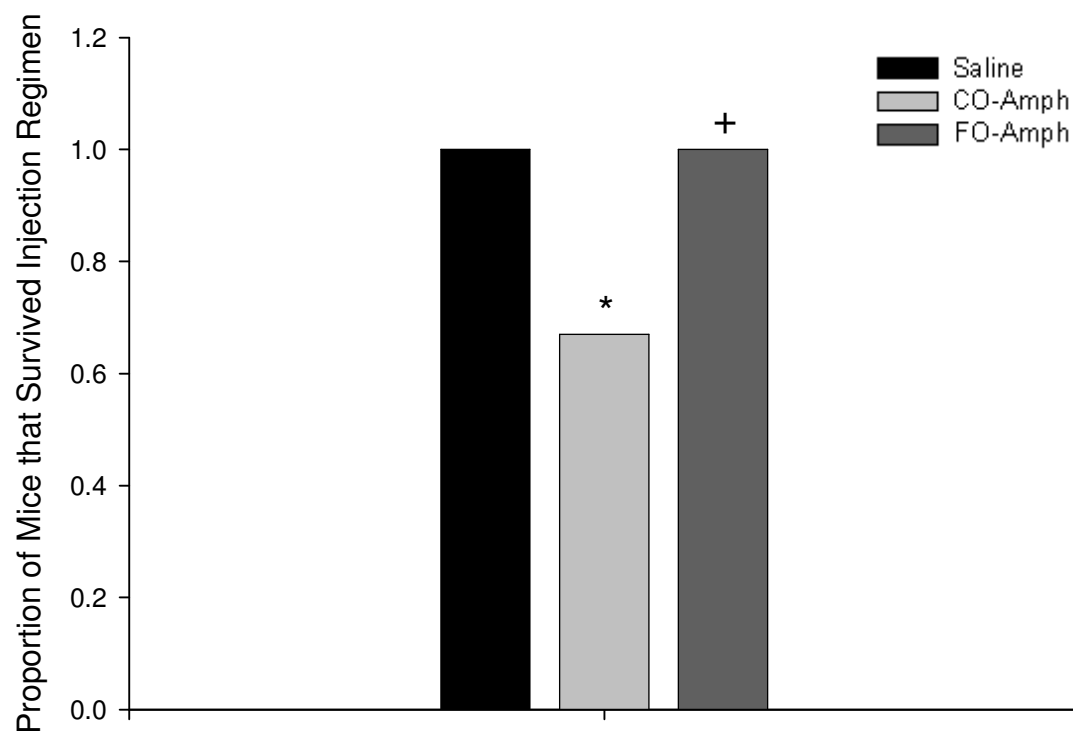


Figure 20. Proportion of mice that survived the injection regimen assessed on the day of sacrifice. Mice were fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. Saline group consists of both corn oil- and fish oil-fed mice. * indicates significantly different from saline group, $p < .05$; + indicates significantly different from corn oil/amphetamine group, $p < .05$; error bars indicate SEM.

Figure 21.

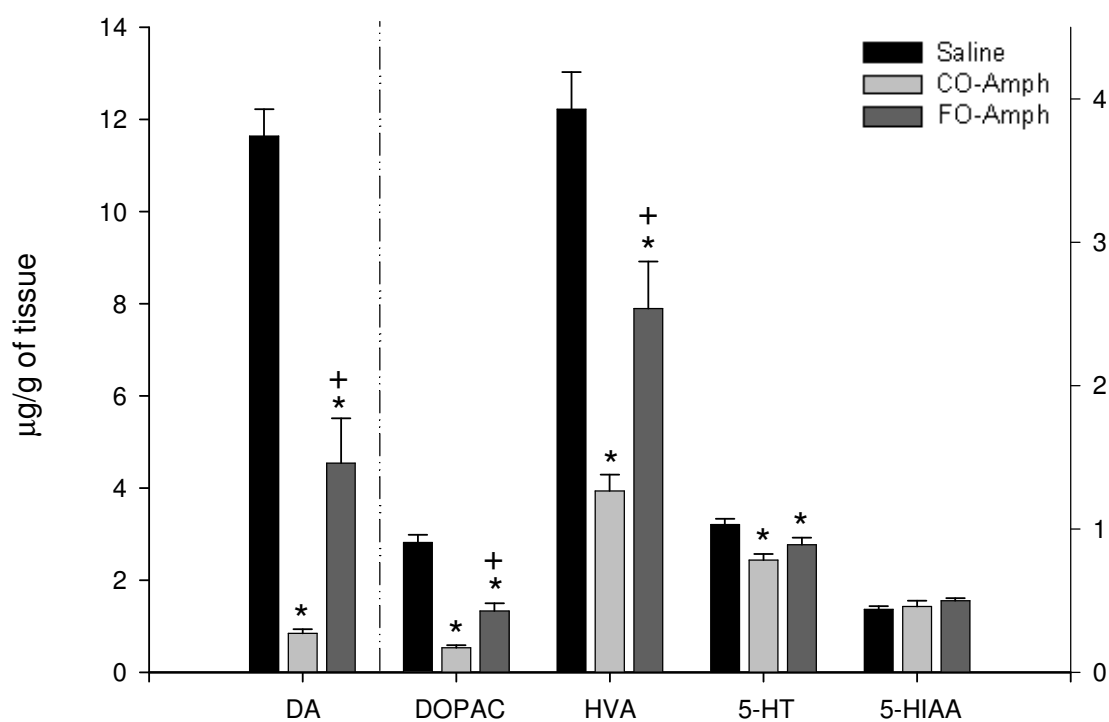


Figure 21. Striatal concentrations of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindolacetic acid (5-HIAA) in mice fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. Saline group consists of both corn oil- and fish oil-fed mice. Dopamine concentration is given by the axis to the left of the dashed line, while the other concentrations are given by the axis on the right. * indicates significantly different from saline group, $p < .05$; + indicates significantly different from corn oil/amphetamine group, $p < .05$; error bars indicate SEM.

Figure 22.

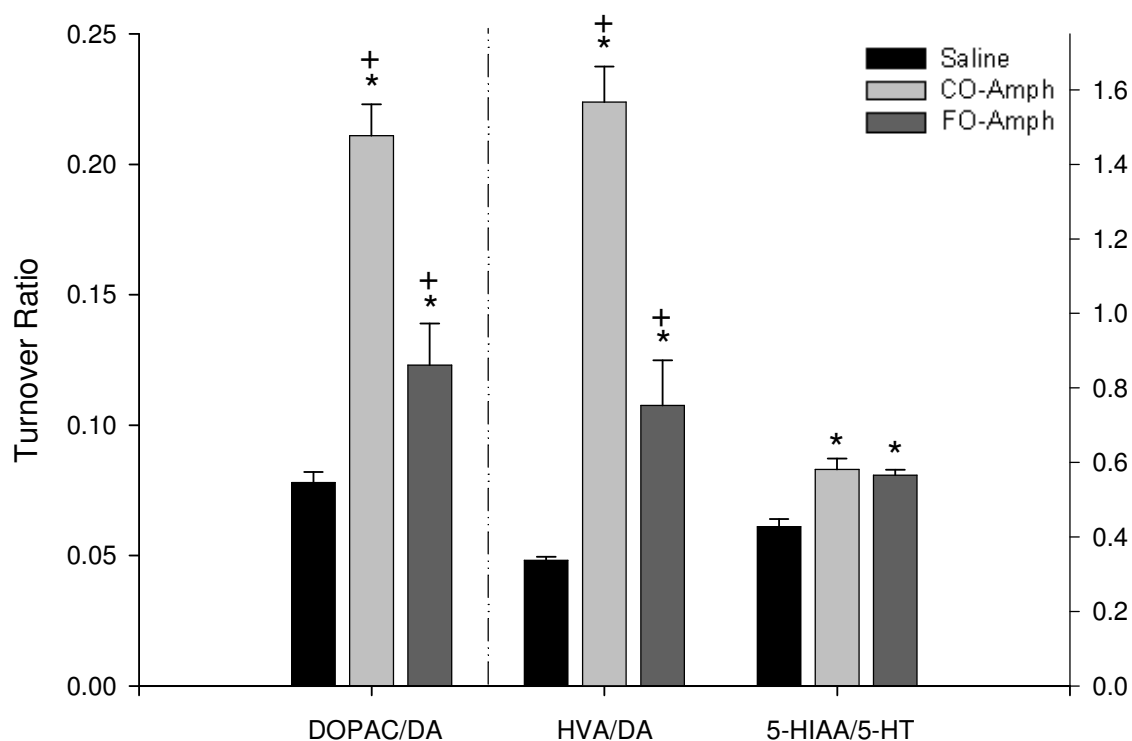


Figure 22. Striatal turnover ratios in mice fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. Saline group consists of both corn oil- and fish oil-fed mice. DOPAC/DA turnover ratio is given by the axis to the left of the dashed line, while the other turnover ratios are given by the axis on the right. * indicates significantly different from saline group, $p < .05$; + indicates significantly different from corn oil/amphetamine group, $p < .05$; error bars indicate SEM.

EXPERIMENT 3

Rationale

Experiments 1 and 2 indicate that consumption of a fish oil-rich diet for 7 days prior to drug treatment is sufficient time for the oil to confer a measurable level of protection against amphetamine-induced damage. It's possible that the protective impact of the fish oil builds gradually with repeated daily consumption, or perhaps this level of protection can be achieved by fish oil being consumed just prior to amphetamine exposure. Consequently, the purpose of this experiment is to assess the timeline for which this protection develops. Mice in this experiment were administered diet for 1, 3, or 7 days prior to amphetamine treatment. It was hypothesized that the protective effect of fish oil develops gradually, and the longer a mouse is fed a fish oil-rich diet, the greater potential for protection against amphetamine exposure. In other words, it was expected that the greatest amount of protection would occur in the animals fed the diet for 7 days before treatment. Additionally, it was expected that little protection would be evident in the 1 day group, and a moderate amount would be observed in the 3 day group.

Methods

Sixty, 8-week old Swiss Webster (ND4) mice (Harlan Laboratories, Indianapolis, Indiana) were used for this experiment. Swiss Webster mice, like Balb/c mice, are sensitive to amphetamine toxicity (Myers et al., 1999). In this experiment, mice were administered a diet rich in either fish oil or corn oil for a period of 1, 3, or 7 days prior to treatment with either saline or amphetamine. These mice were sacrificed 72 hours after treatment, and striatal neurochemical content was assessed using HPLC. Like Experiment 2, the saline group consisted of both corn oil- and fish oil-fed mice, and the

heaviest mice were placed into the fish oil groups to account for the projected weight loss. In addition, both the saline group and the corn oil/amphetamine group contained mice from each of the day conditions; that is, the mice in these two treatment groups were on the diet for either 1, 3, or 7 days prior to treatment. The groups were collapsed in this manner in an effort to reduce the number of subjects required. The remaining methods associated with animal housing, diet, amphetamine treatment, sacrifice HPLC, and statistical analysis are identical to those in the second experiment.

Results

Analysis of consumption data from the 1 day condition revealed that there was a significant main effect of treatment group as well as a significant interaction between group and time point [$F(2,16) = 34.10, p < .01$ and $F(2,16) = 3.95, p = .04$, respectively] (Figure 23). *Post hoc* tests indicated that on day 1, the day prior to treatment, consumption in both the corn oil/amphetamine group and the saline group was significantly higher than in the fish oil/amphetamine group. Furthermore, the corn oil/amphetamine group's consumption was higher on the first day than the saline group. After treatment (days 2 through 4), consumption in the corn oil/amphetamine group remained significantly higher than the fish oil/amphetamine group. The fish oil/amphetamine condition also remained lower than the saline group in the days after treatment.

An ANOVA performed on consumption for the 3 day condition revealed a significant main effect of treatment group [$F(2,17) = 5.67, p = .01$] and a significant interaction between group and time point [$F(2,17) = 11.98, p < .01$] (Figure 24). *Post hoc* analysis showed that prior to treatment (days 1 through 3), consumption of diet in the

corn oil/amphetamine group was significantly higher than the two other treatment groups. Moreover, consumption in the saline group was significantly higher than the fish oil/amphetamine group. Following treatment (days 4 to 6), consumption in the saline group was significantly higher than the fish oil/amphetamine group.

Analysis of consumption in the 7 day condition revealed significant main effects of treatment group and time point, as well as significant interaction between group and time point [$F(2,34) = 8.11, p < .01$, $F(2,34) = 6.373, p < .01$, and $F(4,34) = 2.90, p = .04$, respectively] (Figure 25). *Post hoc* tests indicated that during the first four days, fish oil/amphetamine mice ate significantly less than both the corn oil/amphetamine group and the saline group. By days 5 to 7, just prior to treatment, consumption in the fish oil/amphetamine group had increased significantly to levels comparable to the other groups. Following treatment (days 8 to 10), the consumption in the fish oil/amphetamine group decreased significantly such that it was significantly lower than the consumption of the saline group.

Analysis of body weight data showed that there were significant main effects of treatment group and time point, as well as a significant interaction between group and time point [$F(4,106) = 7.19, p < .01$, $F(2,106) = 170.30, p < .01$, and $F(8,106) = 25.99, p < .01$, respectively] (Figure 26). *Post hoc* tests revealed that at the start of the experiment, the three fish oil/amphetamine groups weighed significantly more than both the corn oil/amphetamine and saline groups. Additionally, the 1 day fish oil/amphetamine group weighed significantly more than the 3 day fish oil/amphetamine group, which in turn weighed significantly more than 7 day fish oil/amphetamine group. By the day of injections, all three fish oil/amphetamine groups had lost a significant

amount of weight. At this time point, the 3 day and 7 day fish oil/amphetamine groups weighed significantly less than corn oil. The 1 day fish oil/amphetamine group remained significantly heavier than the 3 day fish oil/amphetamine group, which still weighed more than those in the 7 day condition. In addition, the mice of the 1 day fish oil/amphetamine group weighed significantly more than the saline group, and the saline group weighed significantly more than the 7 day fish oil/amphetamine group. By the time of sacrifice, the three fish oil/amphetamine conditions had again lost a significant amount of weight. At this time point, both the corn oil/amphetamine group and saline group weighed significantly more than all three fish oil/amphetamine conditions. Furthermore, the 1 day fish oil/amphetamine group weighed significantly more than the other fish oil/amphetamine conditions.

An ANOVA performed on striatal dopamine levels revealed significant effect of treatment group [$F(4,53) = 27.57, p < .01$] (Figure 27). *Post hoc* analysis indicated that both the corn oil/amphetamine group and the 1 day fish oil/amphetamine group had significantly lower dopamine levels than the three other treatment conditions. Furthermore, the striatal dopamine content of mice in the 3 day and 7 day fish oil/amphetamine groups was significantly lower than that of the saline group.

Neurochemical analysis of DOPAC levels in the striatum revealed a significant effect of treatment group [$F(4,53) = 29.49, p < .01$] (Figure 27). *Post hoc* tests showed that like the dopamine results, DOPAC levels in the corn oil/amphetamine group and 1 day fish oil/amphetamine group were significantly lower than the other treatment groups. Also, DOPAC levels in the saline group were significantly higher than the 3 day and 7 day fish oil/amphetamine conditions.

Analysis of striatal HVA also revealed a significant impact of treatment group [$F(4,52) = 15.47, p < .01$] (Figure 27). *Post hoc* analysis showed that striatal HVA, like dopamine and its metabolite, was significantly reduced in the corn oil/amphetamine group and 1 day fish oil/amphetamine group relative to the other groups. Moreover, HVA levels were significantly higher in the saline group than the 3 day and 7 day fish oil/amphetamine conditions.

Neurochemical analysis of serotonin revealed that there was no significant effect of treatment group. In addition, there was no effect of group on levels of serotonin's metabolite, 5-HIAA (Figure 27).

An ANOVA performed on DOPAC/DA turnover revealed a significant effect of treatment group [$F(4,53) = 10.23, p < .01$] (Figure 28). *Post hoc* analysis indicated that there was elevated turnover in both the corn oil/amphetamine and 1 day fish oil/amphetamine groups relative to the three other conditions.

Analysis of HVA/DA turnover revealed a significant effect of treatment group as well [$F(4,53) = 11.87, p < .01$] (Figure 28). Like the effect observed for DOPAC/DA turnover, HVA/DA turnover was increased significantly in both the corn oil/amphetamine group and the 1 day fish oil/amphetamine group as compared to the other treatment groups.

Unlike the dopamine turnover rates, analysis of 5-HIAA/5-HT turnover did not reveal a significant effect of treatment condition (Figure 28).

Conclusions

The neurochemistry results from this experiment indicate that amphetamine caused a significant striatal dopaminergic lesion in mice fed a corn oil-rich diet, and this

effect was reduced in both the 3 day and 7 day fish oil/amphetamine groups. More specifically, an 85% lesion in the corn oil/amphetamine group was reduced to an average of 38% in the 3 day and 7 day fish oil/amphetamine groups. A similar effect was observed in the metabolites of dopamine, DOPAC and HVA. DOPAC levels were reduced by 74% in the corn oil/amphetamine group and only 34% in the 3 day and 7 day fish oil/amphetamine groups. HVA was decreased by 64% in the corn oil/amphetamine group; this decrease was reduced to 26% on average in the 3 day and 7 day fish oil/amphetamine conditions. Protection was not apparent in the 1 day fish oil/amphetamine group for dopamine, DOPAC, and HVA.

The amphetamine treatment also resulted in a compensatory increase in dopamine turnover that was prevented by the fish oil. More specifically, DOPAC/DA turnover was increased by 96% in corn oil-fed mice, while there was no increase in mice administered a fish oil-rich diet for either 3 days or 7 days prior to injections. Similarly, HVA/DA turnover was increased by 174% in the corn oil/amphetamine group, and there was no increase observed in the 3 day and 7 day fish oil/amphetamine conditions. Mice in the 1 day fish oil/amphetamine group had turnover rates comparable to the corn oil/amphetamine group.

Collectively, these results suggest that the neurochemical protection afforded by fish oil is similar in the 3 day and 7 day groups. In other words, consumption of the fish oil-rich diet produces protection against amphetamine's neurotoxicity when administered between 3 and 7 days prior to treatment. On the other hand, administration of the diet for a single day before treatment is not sufficient to mitigate the lesion. Therefore, it appears that having fish oil in your system at the time of neural injury is not sufficient to yield a

protective effect. Rather, you should consume fish oil for an extended period of time prior to insult in order to achieve any level of protection. Although increased protection was not afforded to mice consuming the diet for 7 days relative to 3 days, it is unclear whether a longer time on the diet, such as two weeks or a month, would enhance the beneficial impact of fish oil.

Like the second experiment, body weights were biased so that each of the fish oil-fed, amphetamine-treated groups was heavier than the corn oil/amphetamine and saline groups at the start of the experiment. Unlike the second experiment, however, this did not result in statistically similar body weights on the injection day. More specifically, mice from the 3 day fish oil/amphetamine group weighed significantly less than animals from the 1 day fish oil/amphetamine and corn oil/amphetamine conditions. In addition, mice from the 7 day fish oil/amphetamine group weighed significantly less than all the other groups. The reason for this effect is unclear, as the weight differences were sufficiently eliminated by the time of injections in the previous experiment. In addition, the pattern of consumption is comparable to that of the first two experiments. It could be due to the differences between the experimental designs. For example, in Experiment 3, the corn oil/amphetamine group consisted of animals fed the diet for 1, 3, and 7 days before injections while in Experiment 2, all the mice were administered the diet for 7 days prior to treatment. This is unlikely, however, when the body weight results from Experiment 4 are considered. Briefly, the results of Experiment 4 revealed a significant difference in body weight at the time of injections between the corn oil/amphetamine and fish oil/amphetamine groups despite the fact that the pre-injection methods were identical to Experiment 2; that is, body weights were biased by placing the heavier animals into the

fish oil/amphetamine condition, and the diet was administered for 7 days prior to treatment. Instead, perhaps strain differences between Balb/c mice (Experiments 1 and 2) and Swiss Webster mice (Experiments 3 and 4) could explain the inability to replicate the body weight results from the second experiment. Regardless of the reason for this effect, the concern regarding body weight altering the impact of amphetamine-induced neurotoxicity was alleviated in the second experiment. Furthermore, in this experiment, the 3 day fish oil/amphetamine group weighed more than the 7 day fish oil/amphetamine group at the time of injections, yet there was no difference between the magnitudes of their lesions.

Figure 23.

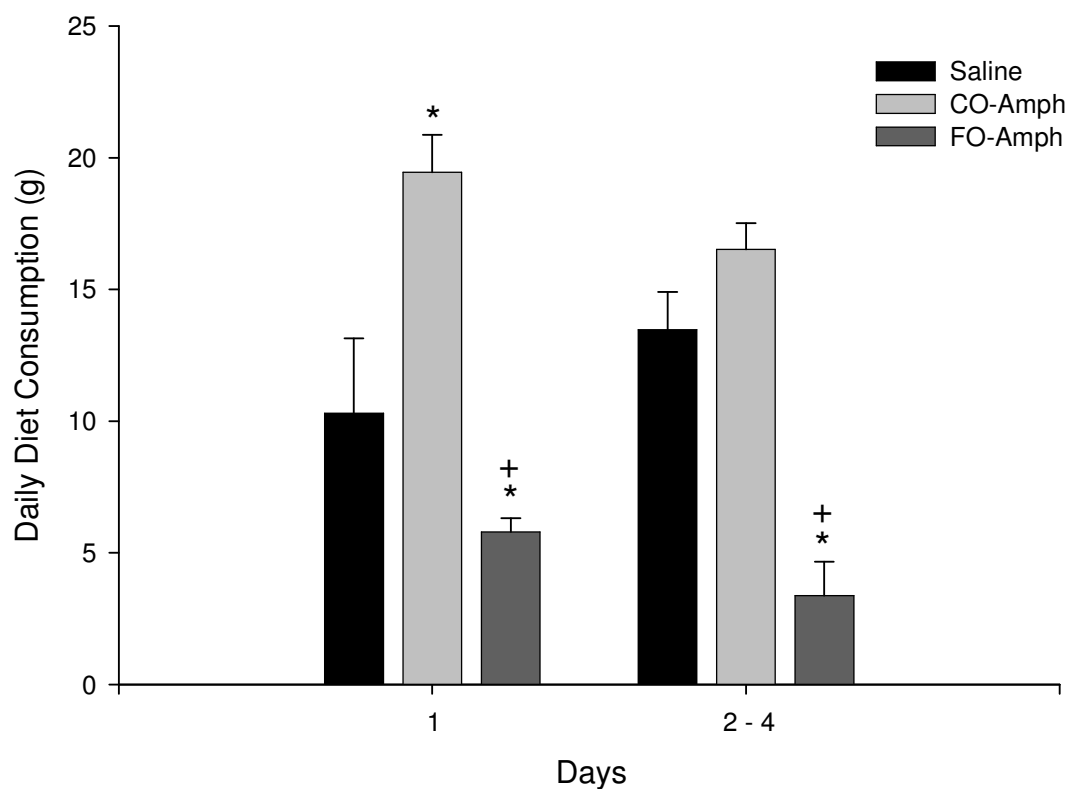


Figure 23. Daily consumption of a liquid diet rich in either corn oil or fish oil in the 1 day condition averaged across two time points: day 1 (i.e. diet intake prior to treatment) and days 2-4 (i.e. diet intake following treatment with either saline or amphetamine). Saline group consists of both corn oil- and fish oil-fed mice. * indicates significantly different from saline group, within time point, $p < .05$; + indicates significantly different from corn oil/amphetamine, within time point, $p < .05$; error bars indicate SEM.

Figure 24.

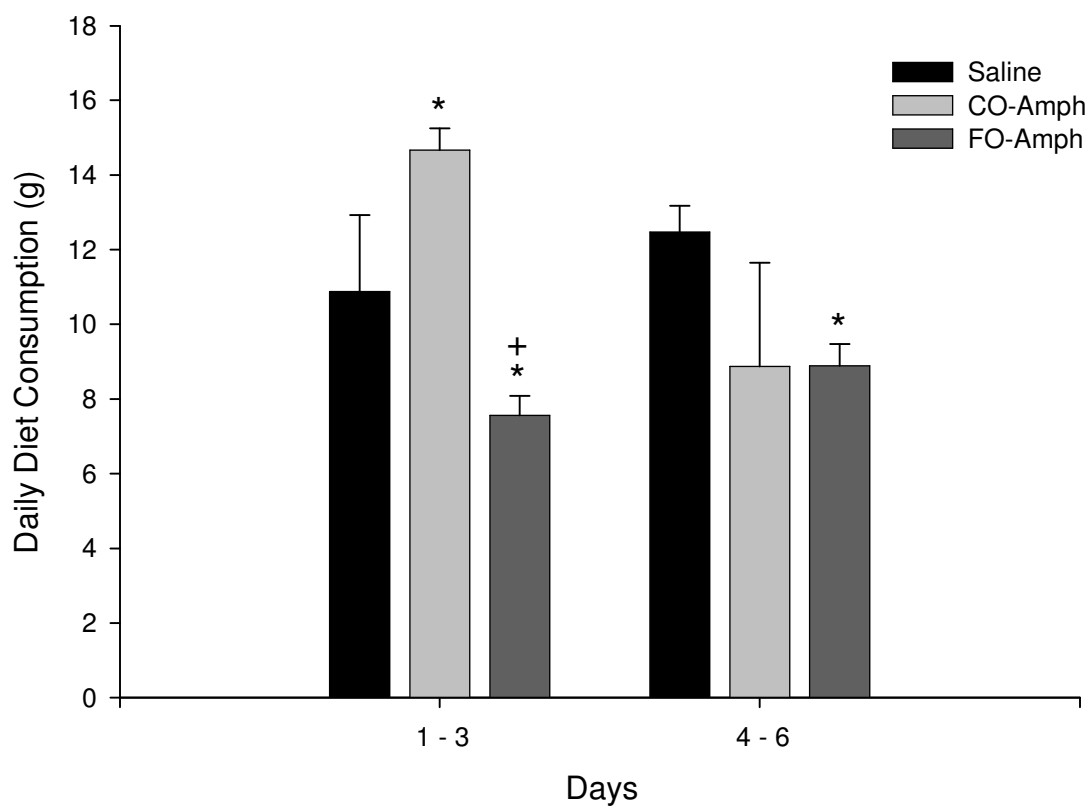


Figure 24. Daily consumption of a liquid diet rich in either corn oil or fish oil in the 3 day condition averaged across two time points: days 1-3 (i.e. diet intake prior to treatment) and days 4-6 (i.e. diet intake following treatment with either saline or amphetamine). Saline group consists of both corn oil- and fish oil-fed mice. * indicates significantly different from saline group, within time point, $p < .05$; + indicates significantly different from corn oil/amphetamine, within time point, $p < .05$; error bars indicate SEM.

Figure 25.

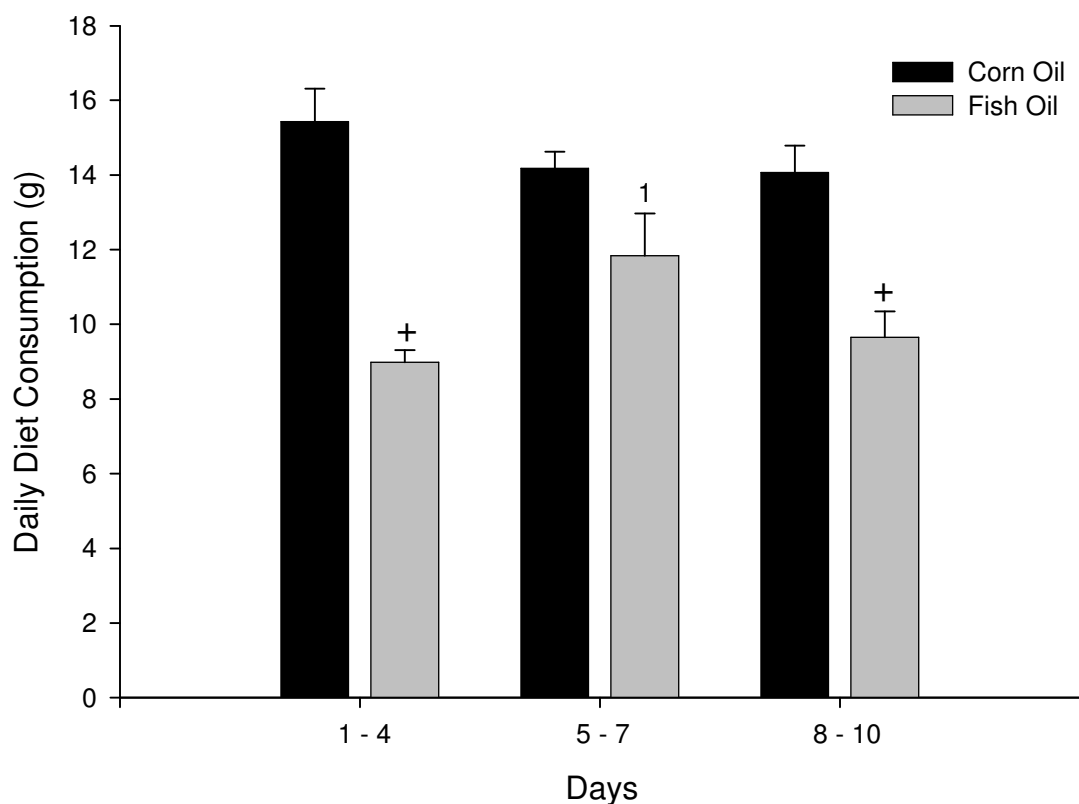


Figure 25. Daily consumption of a liquid diet rich in either corn oil or fish oil in the 7 day condition averaged across three time points: days 1-4 (i.e. days associated with diet acquisition), days 5-7 (i.e. diet intake before treatment), and days 8-10 (i.e. diet intake following treatment with either saline or amphetamine). Saline group consists of both corn oil- and fish oil-fed mice. * indicates significantly different from saline group, within time point, $p < .05$; + indicates significantly different from corn oil/amphetamine, within time point, $p < .05$; 1 indicates significantly different from days 1-4, within group, $p < .05$; 5 indicates significantly different from days 5-7, within group, $p < .05$; error bars indicate SEM.

Figure 26.

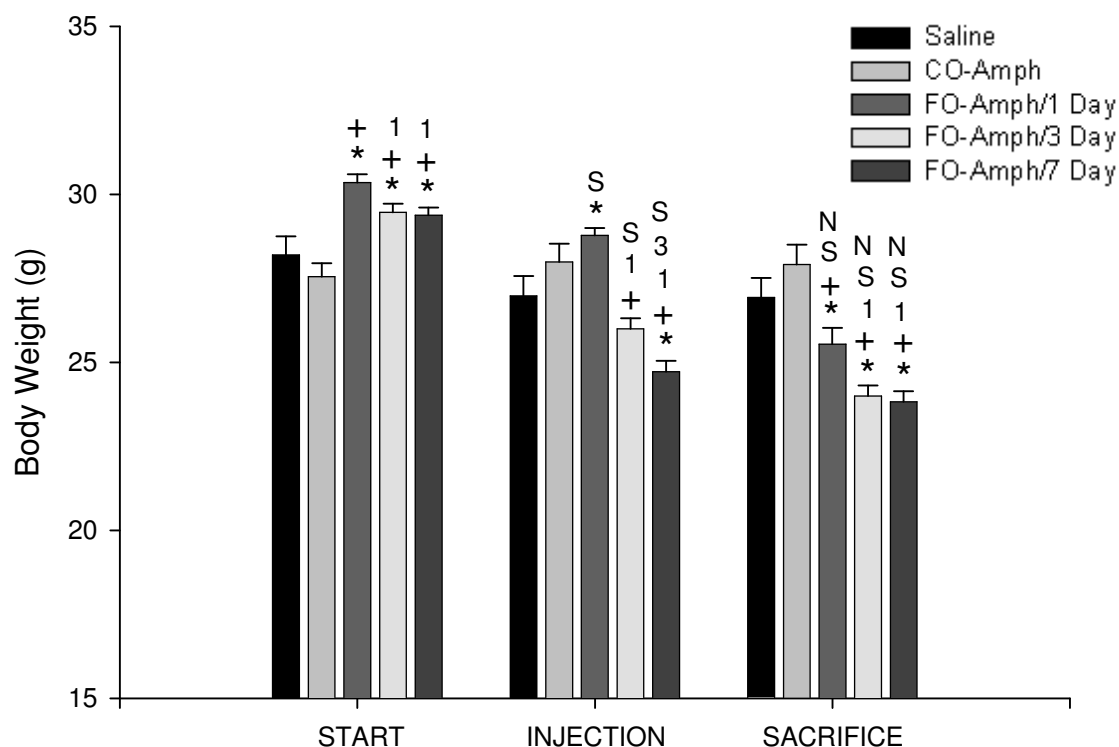


Figure 26. Body weight at the start of the experiment, on the day of injections, and at sacrifice. Mice were fed a diet rich in either corn oil or fish oil for 1, 3 or 7 days prior to treatment. Saline group consists of both corn oil- and fish oil-fed mice from each of the day conditions. Corn oil/amphetamine group consists of mice from each of the day conditions. * indicates significantly different from saline group, $p < .05$, within time point; + indicates significantly different from corn oil/amphetamine group, within time point, $p < .05$; numbers above the bars indicate significantly different from that fish oil-fed time point group, $p < .05$; S indicates significantly different from the start of the experiment, within group, $p < .05$; N indicates significantly different from the injection time point, within group, $p < .05$; error bars indicate SEM.

Figure 27.

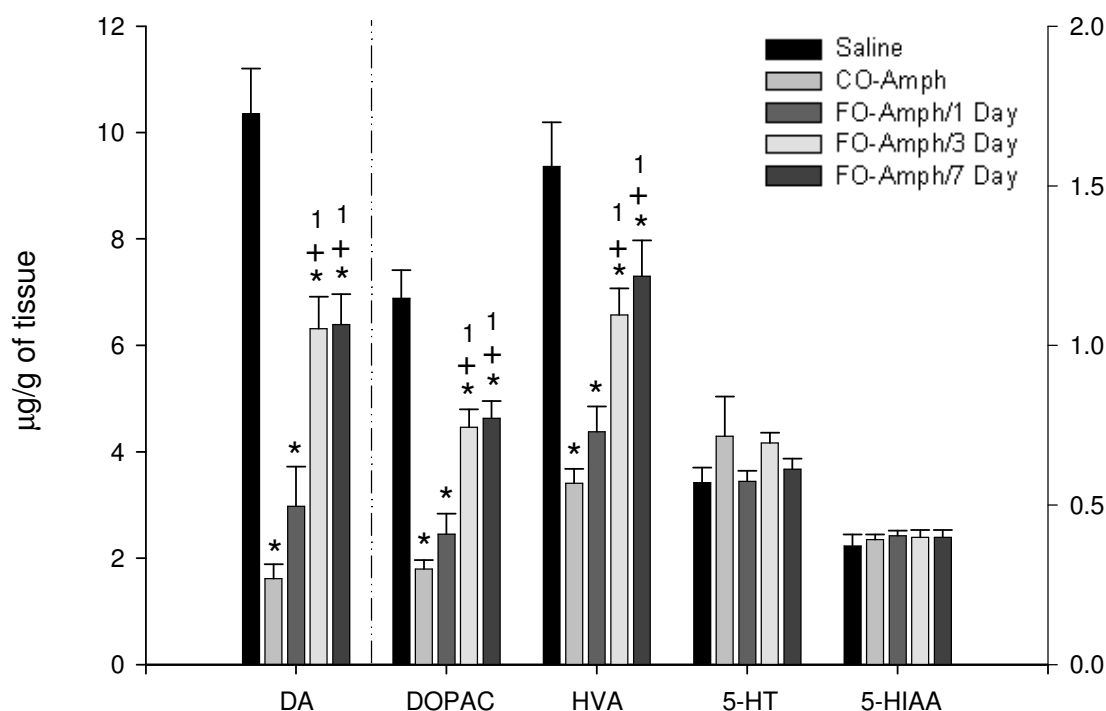


Figure 27. Striatal concentrations of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindolacetic acid (5-HIAA) in mice fed a diet rich in either corn oil or fish oil for 1, 3 or 7 days prior to treatment with either saline or amphetamine. Saline group consists of both corn oil- and fish oil-fed mice from each of the day conditions. Corn oil/amphetamine group consists of mice from each of the day conditions. Dopamine concentration is given by the axis to the left of the dashed line, while the other concentrations are given by the axis on the right. * indicates significantly different from saline group, $p < .05$; + indicates significantly different from corn oil/amphetamine group, $p < .05$; the numbers above the bars indicate significantly different from that fish oil-fed time point group, $p < .05$; error bars indicate SEM.

Figure 28.

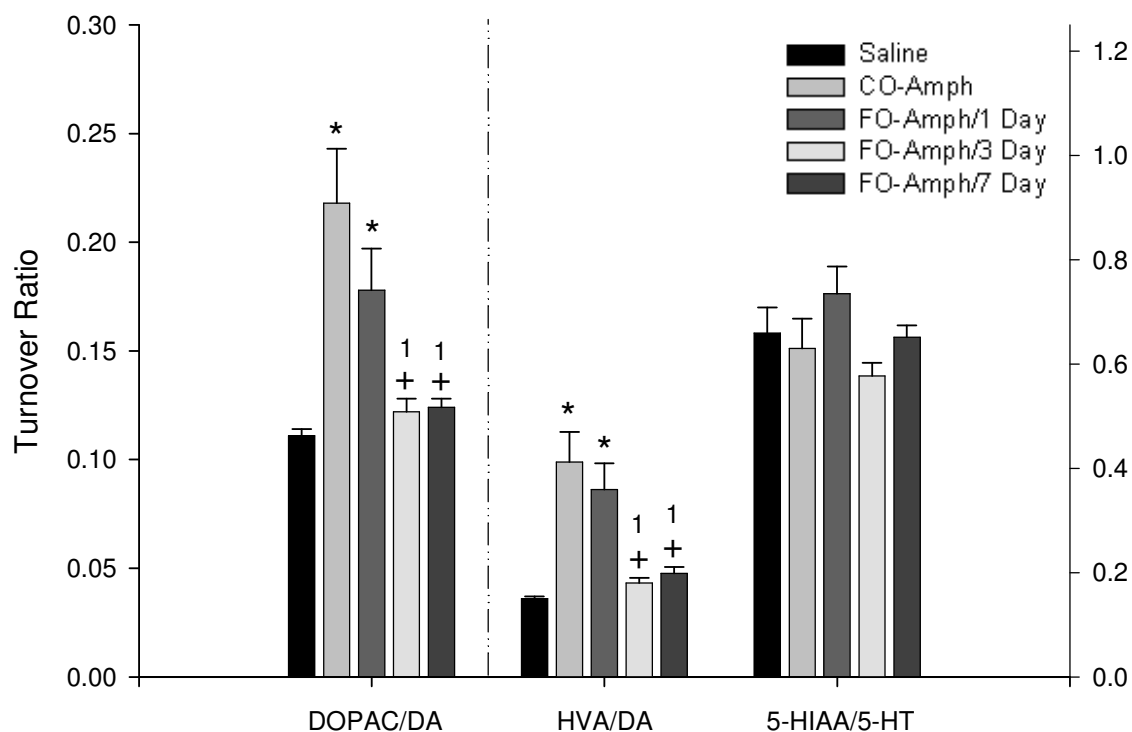


Figure 28. Striatal turnover ratios in mice fed a diet rich in either corn oil or fish oil for 1, 3 or 7 days prior to treatment with either saline or amphetamine. Saline group consists of both corn oil- and fish oil-fed mice from each time point. Corn oil/amphetamine group consists of mice from each time point. DOPAC/DA turnover ratio is given by the axis to the left of the dashed line, while the other turnover ratios are given by the axis on the right. * indicates significantly different from saline group, $p < .05$; + indicates significantly different from corn oil/amphetamine group, $p < .05$; the numbers above the bars indicate significantly different from that fish oil-fed time point group, $p < .05$; error bars indicate SEM.

EXPERIMENT 4

Rationale

Given the significant role that oxidative damage plays in amphetamine toxicity, it's possible that the fish oil-rich diet provides its protection through antioxidant activity. In addition, it has been suggested that the antioxidant properties of omega-3 PUFAs, a significant component of fish oil, are responsible for the observed protection in both MPTP (Bousquet et al., 2008) and 6-OHDA (Delattre et al., 2009) models of Parkinson's disease. Consequently, this experiment used a TBARS (thiobarbituric acid reactive substances) assay to measure malondialdehyde (MDA) levels in striatal tissue. MDA is a naturally occurring product of lipid peroxidation, an indicator of oxidative stress in cells and tissues. Under conditions of high temperature and high acidity, MDA reacts with thiobarbituric acid (TBA) to form an MDA-TBA adduct, and this adduct can be measured using spectrophotometry. This assay is a common measure of oxidative stress, and has been used to assess amphetamine-induced toxicity (Acikgoz et al., 1998; Acikgoz et al., 2000). It was hypothesized that the amphetamine treatment would result in a significant increase in oxidative stress as evidenced by increased levels of MDA found in the striatum. In addition, it was expected that a diet that is rich in fish-oil would reduce the level of MDA. Such a result would suggest that the reduction of oxidative stress is at least one of the mechanisms of protection afforded by the fish oil.

As mentioned previously, the amount of dopamine released in the striatum directly influences the magnitude of amphetamine-induced neurochemical lesions (Kita et al., 2003). It is possible, therefore, that any reduction of dopaminergic activity by fish oil could account for the diminished lesion. In fact, there is evidence that the

consumption of dietary PUFAs can alter the fatty acid composition of neuronal membranes and consequently, influence neurochemical activity. More specifically, it has been shown that deficiency of omega-3 and omega-6 PUFAs can alter the activity of several neurotransmitter systems, including dopamine and serotonin, by influencing the vesicular pools of these neurotransmitters, by modifying receptors, and interfering with monoamine oxidase activity (Chalon, 2006). Furthermore, the behavioral data from Experiment 2 suggests that a diet rich in fish oil does alter dopaminergic activity. This experiment utilized HPLC to determine if a diet rich in fish oil altered amphetamine-induced dopamine release as signified by dopamine turnover ratios, which have been used as an indicator of release (Kuczenski, 1980; Waldmeier et al., 1981). It was expected that the fish oil-rich diet would reduce the dopamine release following amphetamine treatment relative to the corn oil-fed mice. This change in dopamine release, if observed, would explain the altered amphetamine-induced behavior during the second experiment. Moreover, it would provide a possible mechanism of protection against the striatal lesions observed in the first three experiments.

Methods

Twenty four, 8-week old Swiss Webster (ND4) mice (Harlan Laboratories, Indianapolis, Indiana) were used for this experiment. In this experiment, mice were administered a diet rich in either fish oil or corn oil for 7 days prior to treatment with either saline or 50 mg/kg of amphetamine (four injections of 12.5 mg/kg, each separated by two hours). Mice were sacrificed one hour after the final injection (seven hours after the start of treatment). Striatal neurochemical content from one brain hemisphere was assessed using HPLC; the remaining striatum was assayed for a measure of oxidative

stress (see below). Like Experiment 2, the saline group consisted of both corn oil- and fish oil-fed mice, and the heaviest mice were placed into the fish oil groups to account for the projected weight loss. The remaining methods associated with animal housing, diet, amphetamine treatment, sacrifice, HPLC, and statistical analysis were identical to those in the second experiment.

A TBARS assay (Cayman Chemical Company, Ann Arbor, Michigan) was performed to assess MDA levels in the remaining striatal tissue. Tissue was homogenized in 100 μ l of common lysis buffer (50 mM HEPES, 150 mM, 150 mM NaCl, 5 mM EDTA, 1% Triton) with 1 % protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Missouri). Striatal tissue was sonicated for 15 seconds at 40V over ice. Next, the samples were centrifuged at 1,600 x g for 10 minutes at 4 degrees Celsius. Supernatant was removed and stored on ice. After eight MDA standards ranging in concentration from 0 to 5 μ M MDA were prepared, 10 μ l of each sample and standard was added to microcentrifuge tubes with 10 μ l of sodium dodecyl sulfate (SDS) solution along with 400 μ l of color reagent. The color reagent was prepared by dissolving 106 mg of TBA in 10 ml of an acetic acid solution (40 ml acetic acid diluted in 160 ml HPLC-grade water) and 10 ml of a sodium hydroxide solution (20 ml 10x sodium hydroxide diluted in 180 ml HPLC-grade water). The microcentrifuge tubes were put in foam holders and placed in boiling water for one hour. After an hour had passed, the tubes were put on ice for 10 minutes to stop the reaction. The samples were then centrifuged again at 1,600 x g for 10 minutes at 4 degrees Celsius. After spinning, 150 μ l of the supernatant from each sample was loaded in duplicate to a black, 96-well plate. The fluorescence was read at an

excitation wavelength of 530 nm and an emission wavelength of 550 nm. MDA concentrations were adjusted for protein concentration.

A Pierce BCA protein assay (Thermo Scientific, Rockford, IL) was performed to assess the concentration of protein in the samples. First, eight bovine serum albumin standards ranging from 0 to 2000 µg/ml were generated. Next, the working reagent was prepared by mixing 50 parts BCA reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide) with 1 part BCA reagent B (4% cupric sulfate). Twenty-five µl of each standard and sample (homogenate from the TBARS assay) was loaded in duplicate to a clear, 96-well plate. Then, 200 µl of the working reagent was loaded into each well and mixed using a plate shaker for 30 seconds. The plate was covered and incubated at 37 degrees Celsius for 30 minutes, after which the absorbance was read at a wavelength of 562 nm.

Results

Analysis of consumption data revealed a significant main effect of group [$F(2,21) = 9.64, p < .01$] (Figure 29). *Post hoc* tests indicated that the corn oil/amphetamine group ate significantly more than the fish oil/amphetamine group. In addition, the corn oil/amphetamine group consumed significantly more diet than the saline group.

An ANOVA performed for body weight revealed that there was a significant main effect of time point [$F(1,21) = 8.23, p = .01$], as well as a significant interaction between treatment group and time point [$F(2,21) = 17.17, p < .01$] (Figure 30). *Post hoc* analysis showed that at the start of the experiment, mice in the fish oil/amphetamine condition weighed significantly more than mice in the other two treatment groups. Additionally, mice in the corn oil/amphetamine group weighed significantly less than those in the

saline group. By the time of injections, mice in the corn oil/amphetamine group had gained a significant amount of weight, and mice in the fish oil/amphetamine group had lost a significant amount of weight. Consequently, on the day of injections, mice in the corn oil/amphetamine group weighed significantly more than their fish oil-fed counterparts.

An ANOVA performed on striatal MDA levels did not show a significant impact of treatment group (Figure 31).

Neurochemical analysis of dopamine levels in the striatum revealed a significant effect of treatment group [$F(2,21) = 14.43, p < .01$] (Figure 32). *Post hoc* tests indicated that there was significantly more dopamine in the saline group than mice in either of the other conditions. Furthermore, mice in the corn oil/amphetamine group had significantly less striatal dopamine than mice in the fish oil/amphetamine group.

An ANOVA performed for DOPAC levels also showed a significant effect of group [$F(2,21) = 12.90, p < .01$] (Figure 32). *Post hoc* tests revealed that DOPAC levels were significantly higher in the saline group than in either of the other two treatment groups.

Neurochemical analysis of striatal HVA levels revealed a significant effect of treatment group [$F(2,21) = 7.07, p < .01$] (Figure 32). *Post hoc* tests indicated that mice in both the corn oil/amphetamine and fish oil/amphetamine groups had significantly elevated levels of HVA as compared to the saline group.

Analysis of serotonin levels in the striatum also revealed a significant effect of group [$F(2,21) = 5.29, p = .01$] (Figure 32). *Post hoc* analysis indicated that the saline group had significantly less serotonin than the corn oil/amphetamine group.

An ANOVA performed for striatal 5-HIAA content indicated that there was a significant impact of treatment group [$F(2,21) = 12.35, p < .01$] (Figure 32). *Post hoc* tests revealed that mice in both the corn oil/amphetamine and fish oil/amphetamine groups had significantly higher levels of 5-HIAA than mice in the saline group.

Analysis of DOPAC/DA turnover did not reveal a significant effect of group, but there was an effect of treatment group on HVA/DA turnover [$F(2,21) = 5.43, p = .01$] (Figure 33). *Post hoc* tests indicated that the corn oil/amphetamine group had a significantly higher HVA/DA turnover rate than the saline group.

Neurochemical analysis indicated that there was a significant effect of treatment group on 5-HIAA/5-HT turnover [$F(2,21) = 8.33, p < .01$] (Figure 33). *Post hoc* tests showed that both the fish oil/amphetamine and corn oil/amphetamine groups had significantly elevated 5-HIAA/5-HT turnover relative to the saline group.

Conclusions

The previous three experiments examined neurochemistry 72 hours following amphetamine treatment. The fourth experiment, however, examined neurochemistry as well as oxidative stress, as indicated by MDA levels, on the day of injections. The TBARS assay showed that there was not an effect of treatment group on striatal MDA levels. In other words, there was no evidence that the amphetamine treatment increased oxidative stress, and consequently, no protective effect of fish oil could be observed. This outcome was not expected as elevated levels of lipid peroxidation in striatal tissue were detected using an identical injection schedule, sacrifice time point, and MDA assay, albeit rats and methamphetamine were used instead of mice and amphetamine (Acikgoz et al., 1998). The reason for the failure to observe a similar effect is unclear. It is

feasible that the differences between methamphetamine and amphetamine, and the doses administered, could explain the results. Acikgoz et al. (1998) administered four doses of 15 mg/kg methamphetamine for a total dose of 60 mg/kg. In this experiment, four doses of 12.5 mg/kg amphetamine were administered for a total dose of 50 mg/kg. It may appear that the higher dose of methamphetamine, which has been suggested to be more potent than amphetamine (Hall et al., 2008), could account for this discrepancy. However, this possibility seems highly unlikely considering the severity of the lesion obtained using 50 mg/kg amphetamine. This dose was able to produce a substantial dopaminergic lesion and even proved lethal in some of the corn oil-fed animals in Experiment 2. If oxidative stress is a mechanism involved in the amphetamine-induced neurotoxicity, it would certainly have occurred at this dose. A second possibility is that the time course for amphetamine-induced oxidative stress is different in the mice utilized in this study than the rats used by Acikgoz et al. (1998). Perhaps the time of sacrifice, seven hours after the first injection, was not the ideal time to observe elevated levels of MDA in this experiment. Using mice from a series of time points rather than just one could help to discover the appropriate time course. Finally, maybe the use of a TBARS assay is not appropriate for use in this study. Although TBARS is a very common measure of oxidative stress, and it has been used to assess amphetamine-induced oxidative stress (Acikgoz et al., 1998, Acikgoz et al., 2000), perhaps the fatty acid-rich diets employed in this experiment could interfere with the assay prior to amphetamine treatment. Again, the TBARS assay measures MDA levels, a marker of lipid peroxidation. Fish oil is known to oxidize readily while corn oil is less susceptible to peroxidation (Fritsche & Johnston, 1988). It's feasible that the presence of these oils

could lead to differential levels of lipid peroxidation, making it difficult to observe an effect of amphetamine and consequently, a protective effect. Future assessments of oxidative stress in an experiment employing lipid-rich diets should utilize measures other than those that detect lipid peroxidation. Such measures include those that assess DNA and RNA damage, protein oxidation, reactive oxygen species, and endogenous antioxidant activity.

The neurochemistry results suggest that the amphetamine treatment causes significant alterations in levels of dopamine, serotonin, and their metabolites. Most notably, amphetamine caused a 68% decrease in dopamine in the corn oil-fed mice and only a 28% increase in their fish oil counterparts. DOPAC levels were decreased by 50% by the amphetamine injections in both diet groups, while HVA levels were increased by 78%. Decreased dopamine and DOPAC along with increased HVA is consistent with amphetamine-induced dopamine release, as HVA is the end product of dopamine and DOPAC breakdown. Furthermore, dopamine turnover rates were also impacted by amphetamine. Although there were no differences in DOPAC/DA turnover between any of the groups, HVA/DA turnover was increased by an average of 393% in the corn oil/amphetamine and fish oil/amphetamine groups relative to saline-treated mice. Although the amphetamine-induced increase in turnover was expected, there was no difference between corn oil- and fish oil-fed mice, suggesting similar rates of dopamine release. If the activity of dopamine is similar between these two groups, an explanation for the differences in the acute behavioral measures in Experiment 2 is not apparent. However, it is possible that a more direct measure of dopamine release, such as microdialysis, would reveal decreased dopamine release in the mice fed a fish oil-rich

diet. Even if dopamine release is similar between the two groups, other aspects of dopamine activity could be altered by the diet. For example, dopamine reuptake or breakdown could be influenced by fish oil. Amphetamine is known to interfere with dopamine breakdown by inhibiting monoamine oxidase (Kita et al., 2009), and fish oil could potentially mitigate amphetamine's impact on monoamine oxidase activity. Such an outcome could account for the observed behavioral and neurochemical results.

Figure 29.

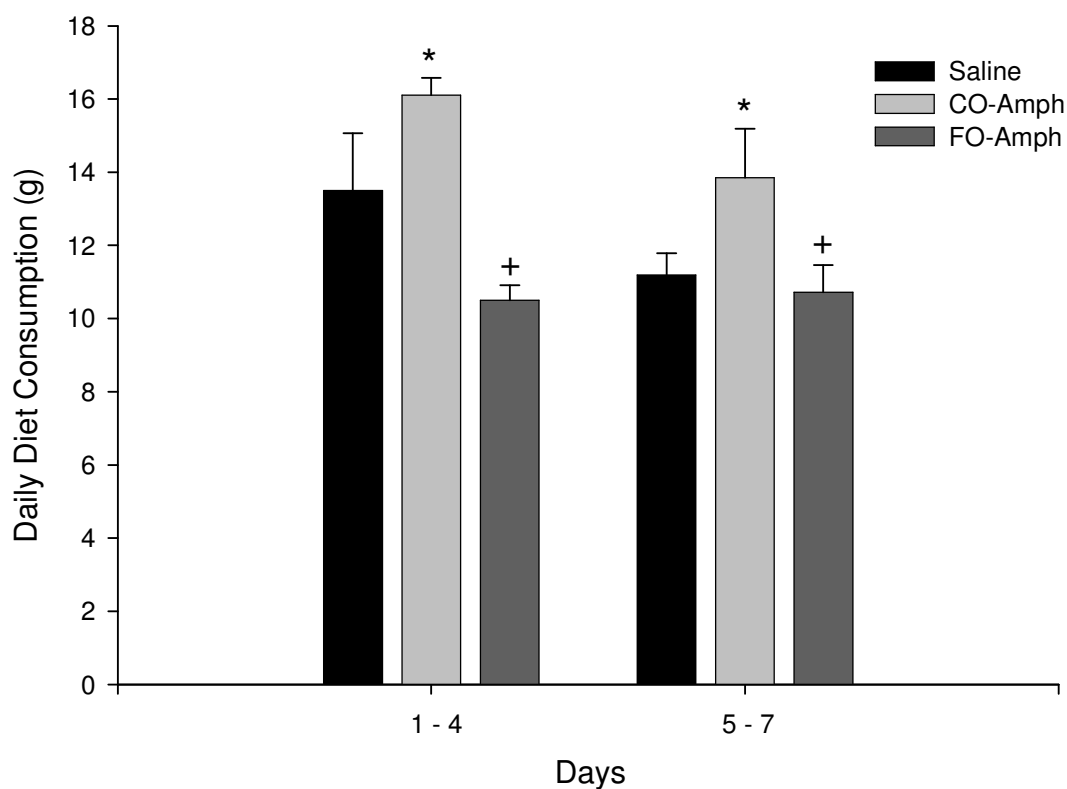


Figure 29. Daily consumption of a liquid diet rich in either corn oil or fish oil averaged across two time points: days 1-4 (i.e. days associated with diet acquisition) and days 5-7 (i.e. diet intake before treatment). Saline group consists of both corn oil- and fish oil-fed mice. * indicates significantly different from saline group, within time point, $p < .05$; + indicates significantly different from corn oil/amphetamine group, within time point, $p < .05$; error bars indicate SEM.

Figure 30.

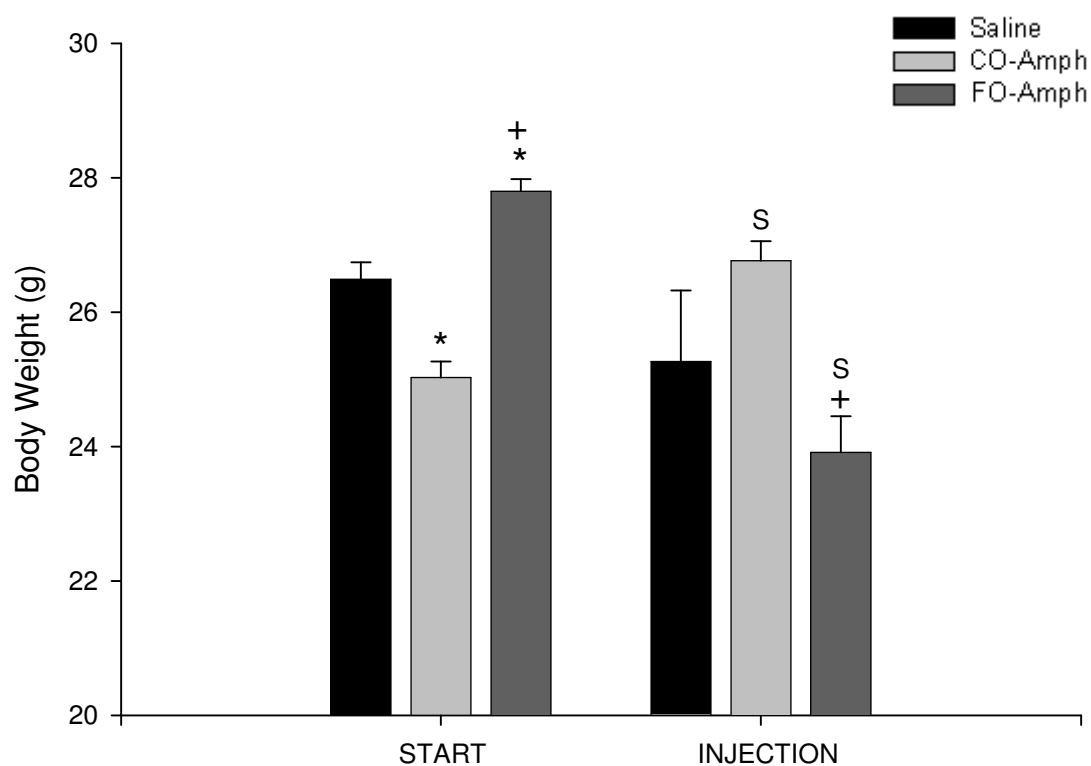


Figure 30. Body weight at the start of the experiment and on the day of injections/sacrifice. Mice were fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. Saline group consists of both corn oil- and fish oil-fed mice. * indicates significantly different from saline group, within time point, $p < .05$; + indicates significantly different from corn oil/amphetamine group, within time point, $p < .05$; S indicates significantly different from the start of the experiment, within group, $p < .05$; error bars indicate SEM.

Figure 31.

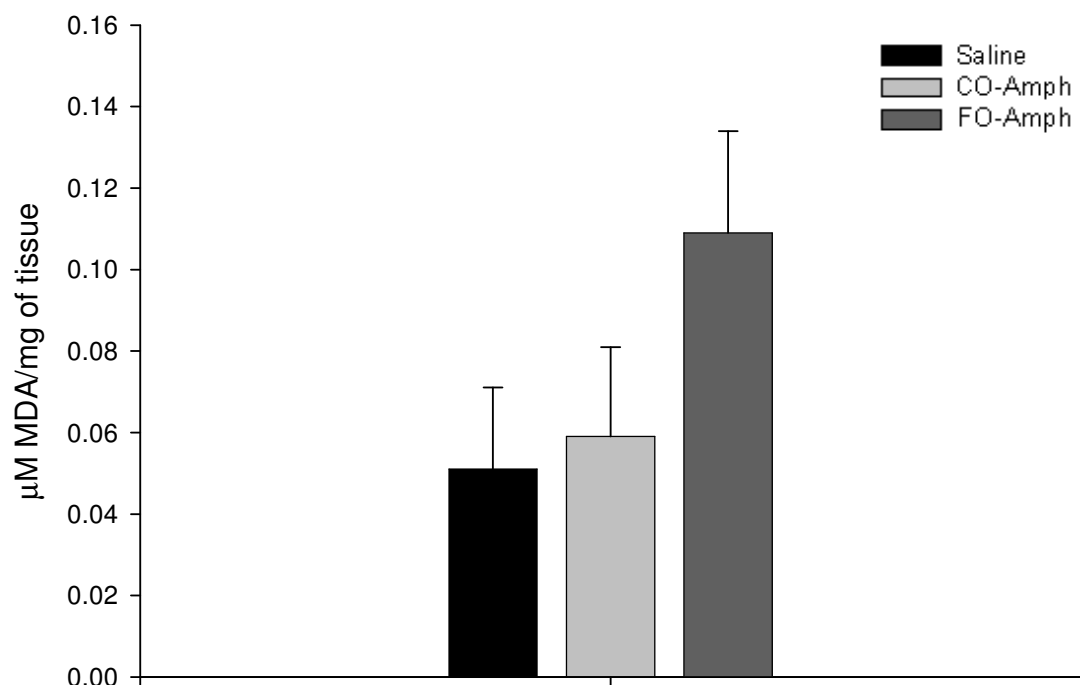


Figure 31. Malondialdehyde (MDA) levels in the striatum of mice fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. Saline group consists of both corn oil- and fish oil-fed mice. Error bars indicate SEM.

Figure 32.

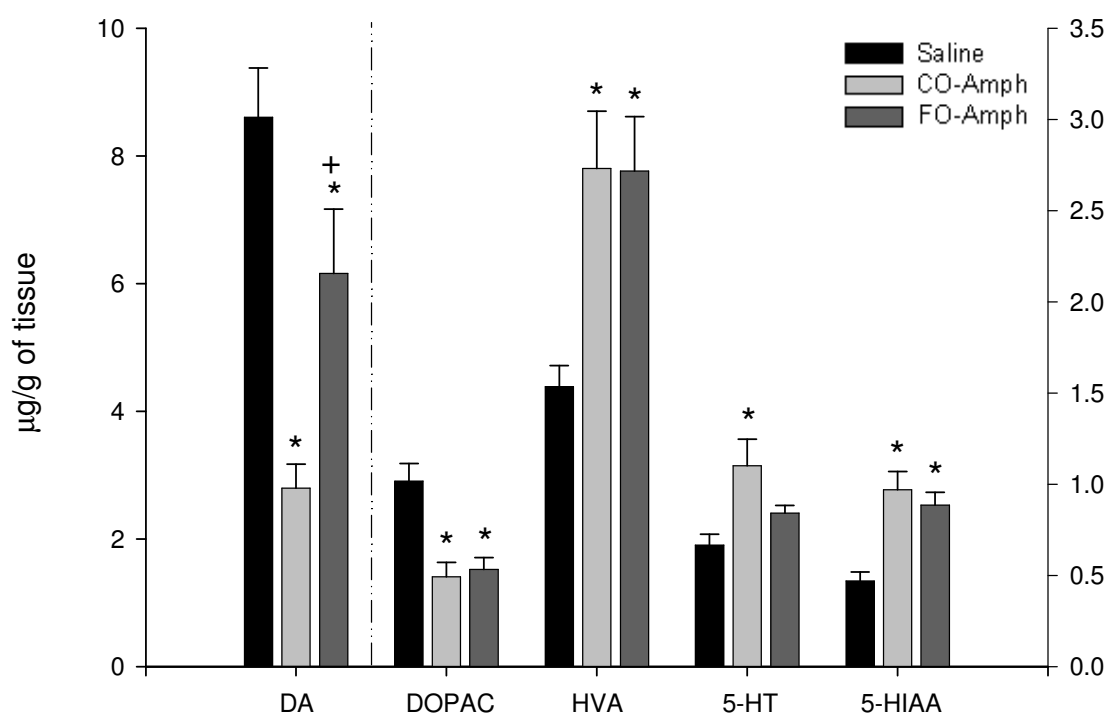


Figure 32. Striatal concentrations of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindolacetic acid (5-HIAA) in mice fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. Saline group consists of both corn oil- and fish oil-fed mice. Dopamine concentration is given by the axis to the left of the dashed line, while the other concentrations are given by the axis on the right. * indicates significantly different from saline group, $p < .05$; + indicates significantly different from corn oil/amphetamine group, $p < .05$; error bars indicate SEM.

Figure 33.

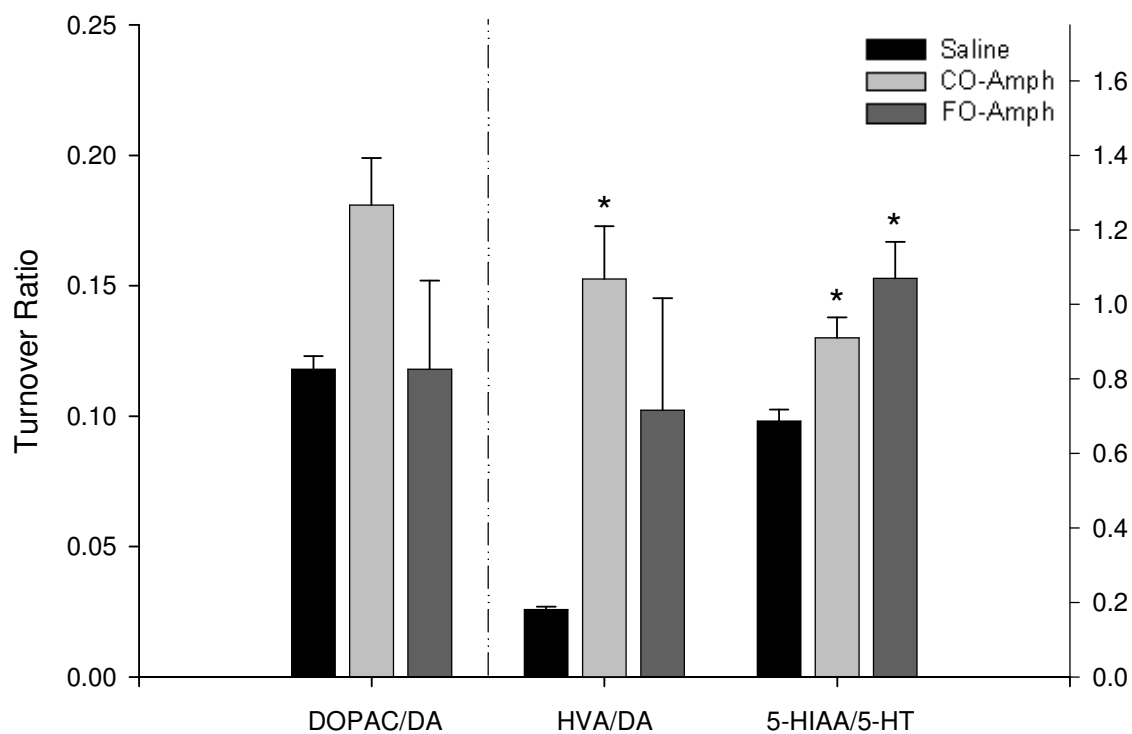


Figure 33. Striatal turnover ratios in mice fed a diet rich in either corn oil or fish oil and treated with either saline or amphetamine. Saline group consists of both corn oil- and fish oil-fed mice. DOPAC/DA turnover ratio is given by the axis to the left of the dashed line, while the other turnover ratios are given by the axis on the right. * indicates significantly different from saline group, $p < .05$; error bars indicate SEM.

GENERAL DISCUSSION

This series of experiments was undertaken to examine the possible protective effect of a diet rich in fish oil in an amphetamine toxicity model of Parkinson's disease. Collectively, these experiments have been successful in demonstrating the protective effect of fish oil in this model. More specifically, Experiments 1 and 2 showed definitively that administration of a liquid diet for one week prior to amphetamine treatment can significantly reduce a striatal dopaminergic lesion 72 hours following injections. These findings, in conjunction with research from other animal models (Delattre et al., 2009; Bousquet et al., 2008; Samadi et al., 2006), suggest that the consumption of a diet rich in fish oil may help to protect against the neural damage that is involved in Parkinson's disease. If true, increasing fish intake or taking a fish oil supplement could be an easy way to reduce one's risk of this debilitating disorder.

In addition to replicating the neuroprotection from the first experiment, Experiment 2 broadened the scope of protection to amphetamine-induced behaviors and temperature alterations. Fish oil impacted behaviors assessed during the injection regimen including oral dyskinesia, self-biting, stereotypy, and backwards walking. Moreover, fish oil delayed the onset of these behaviors, indicating that a larger dose of amphetamine is necessary to produce these behaviors than in the corn oil-fed mice. A similar effect was observed in dermal temperature, as the amphetamine-induced hyperthermia was delayed in the fish oil/amphetamine group relative to the corn oil/amphetamine condition, and the hypothermic effect observed in corn oil-fed mice at the end of the injection regimen was prevented altogether. This outcome is of particular interest because it has been suggested that the hyperthermic effect following

amphetamine administration is a predictor of the magnitude of the striatal neurochemical lesion (Kita et al., 2003). These effects were observed on the injection day, and an impact on behavior occurred just thirty minutes after the first injection. Consequently, unlike the neurochemistry and sacrifice day behavioral results, these outcomes should not be attributed to the magnitude of the amphetamine-induced lesion. Rather, it seems likely that the observed behavioral alterations are due to fish oil's ability to modulate dopaminergic activity, an issue that was assessed in the fourth experiment.

As for the sacrifice day behavior from Experiment 2, motor deficits were expected to be present in amphetamine-treated mice, and those deficits were predicted to be mitigated in fish oil-fed mice. However, amphetamine treatment did not have an impact on locomotor activity or rotorod performance. The substantial dopaminergic lesions achieved in these experiments most certainly would have produced motor deficits, and it's very likely that other behavioral measures would have detected these decrements. Consequently, future studies should use alternative assessments like active avoidance and balance beam, both of which are impacted by amphetamine-induced lesions (Walsh & Wagner, 1992; Halladay et al., 2000). It is expected that these motor deficits would be mitigated by the fish oil-rich diet. This protection would be directly related to fish oil's ability to reduce the striatal neurochemical lesion produced by amphetamine.

After demonstrating the protective effects of fish oil in the first two experiments, the third experiment was designed to ascertain how long the diet must be administered before these benefits develop. It was determined that eating the diet for 3 days or 7 days prior to treatment produced similar levels of protection, and no impact was present after a single day. This suggests that the protective effect of fish oil begins to develop between

1 and 3 days after the start of consumption. Thus, a fish oil rich diet should be maintained for an extended period of time prior to insult. In other words, protection may not be afforded by the omega-3 PUFA-rich fish oil if the diet is initiated after or just prior to developing the disorder. Consequently, long-term fish oil consumption should begin before one is at risk for developing Parkinson's disease and be maintained during senescence.

This protection time course could be examined further. For example, long-term consumption of fish oil could enhance its beneficial effects, and consequently, the impact of diet administration for several weeks or months should be assessed. Although it would be very difficult to determine how the time course of protection in mice would translate to humans, an estimate could certainly be developed. Another question that should be investigated is how long the protective effect lasts after termination of the fish oil consumption. It is quite likely that this protection will persist for some period of time after one stops ingesting fish oil. Therefore, perhaps the consumption of fish oil can occur intermittently, such as every other day or twice a week, and the protection will still be conferred; this time course should be ascertained.

Experiment 4 was performed to assess two possible mechanisms of protection, reduction in oxidative stress and modulation of dopamine release. Although it was hypothesized that fish oil reduces amphetamine-induced increases in oxidative stress, amphetamine did not affect striatal MDA levels. Of course, failure to observe an effect of treatment on oxidative stress levels prevented the assessment of fish oil's capacity to act as an antioxidant. It is believed that using a different measure of oxidative stress, as well as assaying at multiple time points, would reveal amphetamine-induced increases in

oxidative stress and a protective effect in the fish oil-fed animals. The TBARS assay performed in this experiment assessed lipid peroxidation, and because fish oil consists of readily oxidizable lipids (Clegand et al., 2005; Fritsche & Johnston, 1988), another measure of oxidative stress may have been more appropriate. The dopamine release results were similarly inconclusive, as dopamine turnover was altered in amphetamine-treated mice, but it was unclear if fish oil modulated this effect. Using a more direct measure of dopamine release, such as microdialysis, would reveal reduced dopamine release in the fish oil/amphetamine group as compared to the corn oil/amphetamine condition; this result would provide an explanation for the behavioral results from Experiment 2. Other potential mechanisms of protection that have been associated with omega-3 PUFAs, such as the reduction of neuroinflammation (Lu et al., 2010) and the inhibition of apoptosis (Kim et al., 2001), could also be investigated. It is quite plausible, if not likely, that a number of mechanisms are contributing to the protection observed in these experiments. Finally, only central mechanisms of protection were investigated in Experiment 4, but it is also possible that fish oil's benefits are conferred in the periphery. That is, it's feasible that a fish oil-rich diet could reduce the amount of amphetamine reaching the brain, ultimately resulting in a mitigated toxic effect; this possibility can also be assessed in future experiments.

There are a number of other issues that should be examined in future work. For example, the protective effect of a dose of fish oil that is comparable to the amount consumed by humans should be assessed with the amphetamine-toxicity model of Parkinson's disease. As stated earlier, the dose administered to the mice in the experiment is substantially larger than the typical dose ingested by humans. In order to

improve the validity of the model and determine whether the human dose of fish oil has the potential to be protective against parkinsonian-like damage, such a dose should be assessed. However, given the other observed benefits of fish oil in humans, which are discussed below, it is clear that typical levels of human consumption can confer protection against other diseases. Furthermore, a failure to find observe protection from amphetamine toxicity at a lower dose of fish oil should not rule out its possible benefit for the prevention of Parkinson's disease. In fact, it is quite likely that the human dose would not be protective in this model. One of the flaws of our amphetamine toxicity model of Parkinson's disease is that it utilizes an acute, high dose of the drug while chronic, low dose exposure to toxicants is typical in humans. Thus, a larger dose of fish oil is almost certainly necessary to protect in the amphetamine toxicity model than is necessary to protect humans from toxicant exposure.

A number of changes to the diet should be made in future work. For example, although it was suggested that the amount of tBHQ added to the fish oil was not enough to confer any appreciable amount of protection, an ideal control diet would contain tBHQ. Therefore, in future experiments, tBHQ should be added to the oil used in the control diet. A second concern is that corn oil may not be the best oil to use as a control. Because fish oil and corn oil contain different percentages of monounsaturated, polyunsaturated, and saturated fats, the beneficial effects of the fish oil cannot entirely be credited to the presence of the omega-3 PUFAs. The two oils utilized would ideally be identical to each other besides the presence of omega-3 PUFAs in the experimental oil. Finally, perhaps a pelleted diet can be used rather than a liquid diet. Of course, solid food is certainly more natural for the animals and may help to prevent the weight loss

observed in mice consuming the fish oil-rich liquid diet. For example, in an assessment of the impact omega-3 PUFA consumption on UV-induced skin cancer, Lou et al. (2011) administered pelleted diets rich in either omega-3 or omega-6 PUFAs and observed no differences in body weight between the two groups of mice. The use of such a diet would be ideal in our amphetamine toxicity model.

The neurochemical analysis in these experiments was limited to the striatum because of its relevance in our model of Parkinson's disease. However, the neurochemical impact of amphetamine extends beyond the striatum, as it has been reported that the prefrontal cortex, amygdala, and hippocampus appear to be involved in the toxicity and behavioral changes induced by the drug (Feier et al., 2012). Consequently, future work could investigate the impact of amphetamine, as well as the potential protective effect of fish oil, in these regions. It's quite likely that the benefits of fish oil are not limited to the striatum, and fish oil would modulate the effects of amphetamine throughout the brain.

If, in fact, fish oil consumption reduces the risk for developing Parkinson's disease, it would be another positive effect added to a long list of well-documented benefits. For example, fish oil has a number of neurological benefits including the prevention of recurrent depressive episodes. In elderly individuals, fish oil also may help to prevent dementia, cognitive decline, and Alzheimer's disease (Morris et al., 2003, 2005). Fish oil's effects are certainly not limited to the central nervous system, as one of its most notable benefits is the reduction in mortality associated with cardiovascular disease (Harrison et al., 2004). It also has been shown to reduce the progression of coronary atherosclerosis (Erkkila et al., 2004; Seierstad et al., 2005) as well as help

reduce high blood pressure (Ueshima et al., 2007). Additionally, fish oil has a beneficial effect on cholesterol, increasing levels of HDL, “good” cholesterol, and decreasing levels of LDL, “bad” cholesterol (Mostad et al., 2008). Furthermore, fish oil has been reported to reduce the risk for ischemic stroke (He et al., 2004). Fish oil also may benefit the cardiovascular system during exercise, as it has been reported that omega-3 PUFAs are readily incorporated into heart and skeletal muscle, lowering heart rate and decreasing the demand for oxygen without a decrement in performance (Peoples et al., 2008). Dietary fish oil also appears to benefit a number of inflammatory disorders; it has been reported to reduce the expression of genes involved in inflammation and help to restore the balance between pro- and anti-inflammatory cytokines (Bouwens et al., 2009).

Consequently, beneficial effects of consumption have been observed in individuals suffering from sepsis (Michaeli et al., 2007), rheumatoid arthritis (Lau et al., 1993), lupus (Duffy et al., 2004), Crohn’s disease (Belluzzi et al., 1996), and asthma (Dry & Vincent, 1991). Finally, fish oil may be able to prevent certain kinds of cancer. For example, fish oil has been shown to substantially reduce sunburn, a UV radiation-induced inflammatory response, which can in turn help to prevent skin cancer (Rhodes et al., 1995; Lou et al., 2005). Also, a diet rich in fish oil can reduce the risk for prostate cancer (Leitzmann et al., 2004).

It’s clear that fish oil has a wide range of benefits, and it confers these protective effects with very few side effects. However, there are a few safety concerns that should be mentioned. Beyond the minor fish oil intolerance issues, such as recurring taste, heartburn, diarrhea, and headache, it has been suggested that fish oil can lead to increased oxidative stress because it is readily oxidizable (Clegand et al., 2005). Although this is

theoretically possible, the authors suggest that this is quite unlikely as there is evidence that fish oil has the opposite effect, acting as an antioxidant. Perhaps a more legitimate safety concern is the contamination of fish oil by environmental contaminants. For example, methylmercury (MeHg), which has the ability to produce oxidative stress, is found in fish oil (Tur et al., 2012). MeHg has been associated with a number of issues including neurodevelopmental problems, ataxia, and parasthesia (Gochfeld, 2003). Although MeHg certainly has the potential to be toxic, when present in fish it is bound to cysteine, reducing its toxicity potential by approximately 90% (Jeejeebhoy, 2008; Harris et al., 2003). Additionally, MeHg is found primarily in large, predatory fish such as shark, tuna, and swordfish, so limiting the intake of these particular fish should keep MeHg consumption to low, nontoxic levels. Other possible contaminants include dioxins and polychlorinated biphenyls, which have been associated with an increased risk for cancer (Mozaffarian & Rimm, 2006). These contaminants, however, can be removed through molecular distillation and are present in very low levels in quality fish oil supplements (Melanson et al., 2005). Despite the aforementioned safety concerns, the numerous benefits conferred by fish oil certainly outweigh the possible negative effects.

Overall, the results of this series of experiments indicate that a diet-rich in fish oil is protective in an amphetamine-toxicity model of Parkinson's disease. More specifically, fish oil has the ability to mitigate amphetamine-induced striatal lesions, alterations in behavior, and changes in body temperature. This protective effect develops between 1 and 3 days after the start of consumption. Although the protection is readily apparent, the mechanism of protection remains unclear. These findings, along with other animal model work (Delattre et al., 2009; Bousquet et al., 2008; Samadi et al., 2006) and

epidemiological observations (Gao et al., 2007; de Lau et al., 2005) suggest a promising role for fish oil as a preventative agent against Parkinson's disease.

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Graduate Student, Dr. George Wagner's Lab, Rutgers University, Department of Psychology (Fall 2008 – Spring 2013)
 Senior Honors Thesis, Alcohol Lab, The College of New Jersey, Department of Psychology (Fall 2007 – Spring 2008)
 Research Assistant, Alcohol Lab, The College of New Jersey, Department of Psychology (Fall 2006 – Spring 2007)

Publications

Medvecky, C. M., Yochum, C. L., Nolan, B., Fisher, H., Wagner, G. C. (in preparation) Protection against amphetamine-induced striatal dopamine depletion in mice fed a liquid diet rich in fish oil.

Lou, Y. R., Peng, Q. Y., Li, T., Medvecky, C. M., Lin, Y., Shih, W. J., Conney, A. H., Shapses, S., Wagner, G. C., Lu, Y. P. (2011). Effects of high-fat diets rich in either omega-3 or omega-6 fatty acids on UVB-induced skin carcinogenesis in SKH-1 mice. *Carcinogenesis*, 32(7), 1078-1084.

Yochum, C. L., Medvecky, C. M., Cheh, M. A., Bhattacharya, P., Wagner, G. C. (2010). Differential development of central dopaminergic and serotonergic systems in BALB/c and C57BL/6J mice. *Brain Research*, 1349, 97-104.