AMPHETAMINE-INDUCED DOPAMINERGIC TOXICITY:

A SINGLE DOSE ANIMAL MODEL OF PARKINSON'S DISEASE

By

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A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

and

The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Joint Graduate Program in Toxicology

written under the direction of

George C. Wagner, Ph.D.

and approved by

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New Brunswick, New Jersey

October 2008

ABSTRACT OF THE DISSERTATION

Amphetamine-induced dopaminergic toxicity: a single dose animal model of Parkinson's disease

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Parkinson's disease affects millions of people worldwide and is characterized by loss of dopaminergic neurons of the nigro-striatal pathway. Although many mechanisms have been postulated to account for the destruction of these cells, no clear cause has been elucidated. The hypothesis that oxidative stress plays an important role in dopamine depletion in Parkinson's disease was examined through use of amphetamine, a dopaminergic toxicant known to act through oxidative stress. First, a thorough characterization of a single high dose of amphetamine was completed as a new model of Parkinson's disease. Then, antioxidant and anti-inflammatory treatments were used to protect against amphetamine's neurotoxic effects. The antioxidant ascorbic acid was successful in attenuating amphetamine-induced dopamine depletion, while others tested, including Trolox and EGCG, did not attenuate dopaminergic toxicity. In addition, an end product of lipid peroxidation, malondialdehyde, was measured in response to amphetamine treatment and evaluated with the time course of amphetamine-induced

dopamine depletion. Studies have shown increased levels of malondialdehyde in the blood and brains of Parkinson's patients.

Finally, the behavior and sensitivity of mice with selective deletions of genes coding for GSTM1, PAK5, PAK6, or both PAK5 and PAK6 to amphetamine was examined. Multiple genes have been implicated in the etiology of Parkinson's disease, some of which may be associated with oxidative stress response, mitochondrial function, protein kinase function and/or neuronal survival mechanisms. A null mutation in GSTM1 has been associated with Parkinson's disease and plays a role as an antioxidant in the brain. Mice lacking the GSTM1 gene did not show an abnormal behavioral phenotype compared to controls and were not sensitive to amphetamine toxicity. The p21-activated kinases (PAKs) are highly expressed in the brain as well and have been implicated in several neurological disorders, including Parkinson's disease. Mice lacking one or more of the PAK genes showed motoric similarities to Parkinson's disease, although they were relatively resistant to amphetamine toxicity. Collectively, these experiments explored the role of oxidative stress, antioxidant function and related genetic components in a single dose, amphetamine animal model of Parkinson's disease.

Acknowledgments

To my parents, Melvin and Gwendolyn Jobes, and my siblings Aaron and Melissa: Thank you for a lifetime of absolute love and unwavering support. You have always believed in me and were never reserved in your pride and love for me. Your support of my goals and dreams is one of the greatest gifts you could ever give and for that I thank you from the bottom of my heart. I love you all so much and I hope that I continue to make you proud.

To my fiancé Thomas B. Howell IV: I almost do not know where to begin. Thank you for always supporting and believing in me. You have been there for me every day since the start of my graduate career and I am forever grateful for your steadfast encouragement, understanding and love throughout this journey. Even when faced with the idea of moving out of state away from friends and family, you did not hesitate for a second in your support of me and my future and have put me first for many years. For that and so much more, I am forever grateful. You are my best friend and one of the smartest and most loving people I know. I love you so much and greatly look forward to calling you my husband soon and spending the rest of our lives together filled with love and happiness.

To the JGPT faculty and fellow RATS: Thank you all so very much for your support and friendship during my years with you. This journey was challenging but incredibly

rewarding and I owe much of my success to all of you and the solid foundation that was built over the years by many outstanding faculty and student scientists.

To Bernadine Chmielowicz: Without you much of the JGPT would be lost, myself included. From day one, you were one of my biggest advocates and never failed to go above and beyond when I needed your help. You have made an incredible impact upon me personally and as a student in JGPT. I don't know what I would have done without you. Thank you for all of your love and support and know that it is returned at least 10 fold.

To Harry Ting and Melody Furnari: Thank you so much for all of your friendship, hard work and assistance with my research. It was truly my pleasure to work with two incredibly intelligent, talented and motivated students such as yourselves. I wish the best of luck to both of you in graduate school and in your future careers. I am so proud of your accomplishments and look forward to many years as friends and colleagues.

To Anne Sokolowski and the Psychology Department: Thank you for treating me like one your own students and always being there to answer all my questions. You have been a great help to me over years and I am grateful.

To Bonnie Nolan: Thank you for all of our long talks about life, science, our work and the future. You inspire me in many ways and I cherish our friendship. I will miss you tremendously but look forward to a lifetime of long talks and continued friendship.

To Daniella Urbach-Ross: I cannot say enough how fortunate I am to have met you while in college and then happen to see your familiar face on one of my first days in lab. You have been one of my best friends ever since and have supported me through some tough times and been there for some of the happiest moments in my life. I am so glad I have been able to share these experiences with you and your love and friendship means more to me than I can express. I love you very much and look forward to sharing many more wonderful times together in life and science.

To the Wagner lab: Thank you to all members past and present. I have enjoyed your company and collaborations greatly. The work that the lab has done over the years is so valuable and I am honored to have been a part of it.

To my committee Dr. Kenneth Reuhl, Dr. Alexander Kusnecov, Dr. Mona Thiruchelvam and Dr. Alycia Halladay: Thank you for all of your support and guidance throughout this process. I appreciate all of your feedback on my work and have benefited so much from each of you. Thank you for everything.

To Dr. Kenneth Reuhl: Thank you for all of your support over the years and for always pushing me to work harder and be a better scientist. You are an amazing teacher and I learned so much from you over the years. Your guidance and effort put into each of the students in JGPT is incredible and I am grateful to have been one of them. Thank you

also for your kind words and encouragement as I finished up. I hope I continue to make you proud.

Most importantly, to my advisor Dr. George C. Wagner: Thank you for believing in me and supporting me throughout my graduate career. You have been an extraordinary role model and your knowledge, guidance, integrity and patience have helped mature me into a true scientist. You have been the best teacher, both in the classroom and behind the bench, that I have ever had and I am forever in debt to you for all the things you have taught me over the years. I look forward to continued shared laughter, friendship and collaborations. I hope I have made you proud and continue to do so in the future.

Table of Contents

List of figures

AMPHETAMINE-INDUCED DOPAMINERGIC TOXICITY: A SINGLE DOSE ANIMAL MODEL OF PARKINSON'S DISEASE

Parkinson's disease affects millions of people worldwide, out of whom most are over the age of 55. It is a disease characterized by loss of the dopaminergic neurons of the nigro-striatal pathway. While many mechanisms that have been postulated to account for the destruction of these cells, no clear cause has been elucidated. Furthermore, Parkinson's disease reveals itself clinically only after the majority of damage has occurred. Today, there is much research being done to determine therapeutic interventions as well as determine at-risk individuals and detect the onset of Parkinson's disease before irreversible damage has been done. Oxidative stress is widely believed to be a key pathogenic mechanism in Parkinson's disease and increased markers of it found peripherally may be able to predict or indicate future or coexisting neurotoxicity. First, the hypothesis that oxidative stress plays an important role in dopamine depletion in human Parkinson's disease as well as in animal models of Parkinson's disease will be examined through a novel dose of amphetamine, a dopaminergic toxicant known to act through oxidative stress. While the high dose amphetamine model has been used previously to induce dopamine lesions, there have been no previous studies to characterize the acute dose-response and time course of the neurochemical changes following the single high dose of amphetamine. Furthermore, an attempt will be made to counteract this depletion with known antioxidant or anti-inflammatory treatments. In addition, as part of this first hypothesis, an end product of lipid peroxidation, malondialdehyde, that is formed throughout the body, will be measured in the liver to

determine if peripheral levels of it might serve as a marker for predicting later neurotoxicity in mice comparable to that seen in Parkinson's disease. Lipid peroxidation, consequent to oxidative stress, is a constant occurrence in the body. Detrimental effects can arise when lipid peroxidation increases beyond normal, when the body's defense mechanisms are reduced or compromised, or when a combination of the above occur. It is predicted that events such as toxicant exposure that cause loss of central dopaminergic neurons through oxidative stress will also increase levels of peripheral malondialdehyde.

Secondly, the hypothesis that mice with selective deletions of genes coding for GSTM1, PAK5, PAK6, or both PAK5 and PAK6 will show enhanced sensitivity to amphetamine and behavioral deficits similar to the Parkinson's disease motoric phenotype is examined. Multiple genes have been implicated in the etiology of Parkinson's disease and include *Parkin*, *SNCA*, *PINK1*, *DJ-1* and *LRRK2*. Recently, some of these genes linked to familial Parkinson's disease have also been linked to idiopathic Parkinson's disease. Currently, the specific functions of these genes are unknown but have been suggested to be associated with oxidative stress response, mitochondrial function, protein kinase function and/or neuronal survival mechanisms. A null mutation in glutathione-s-transferase mu 1 (GSTM1) has been associated with the increased incidence of Parkinson's disease. The p21-activated kinases (PAKs) are highly expressed in the brain as well and have been implicated in several neurological disorders. Recently, a mutation in one PAK enzyme has been associated with oligomers of α-synuclein, a soluble protein also associated with Parkinson's disease. Collectively, these experiments will explore the role of oxidative stress, antioxidant function and related genetic components in an animal model of Parkinson's disease.

PART I. BIOMARKERS AND MODELS OF PARKINSON'S DISEASE

I. PARKINSON'S DISEASE

Parkinson's disease is characterized by degeneration of the dopaminergic neurons of the substantia nigra. The substantia nigra, a key component of the extrapyramidal motor system, resides in the midbrain and is involved in certain aspects of movement and attention. It is made up of two regions – the pars compacta and the pars reticulata. The pars compacta contains densely packed neurons that, in the human brain, are pigmented by neuromelanin. It is these neurons that are preferentially damaged in Parkinson's disease. The majority of the neurons in the pars compacta send their axons along the nigrostriatal pathway to the striatum where they release dopamine. The striatum is a subcortical region of the telencephalon that consists of the caudate nucleus (which helps to control voluntary movement) and the putamen (which is involved in sensory-motor coordination). The substantia nigra projects to the putamen in the striatum, which, in turn, projects to the premotor and supplementary motor areas of the cortex. Overall, the striatum's main role is in the planning and modulation of movement pathways and is also involved in a variety of other cognitive processes involving executive function.

Symptoms of Parkinson's disease result from the severe loss of dopaminergic cells located in the substantia nigra along with dopamine depletion in the striatum, reduced tyrosine hydroxylase activity, a depletion of dopamine transporter pumps, as well as increases in dopamine turnover and postsynaptic receptor proliferation. The behavioral and motor signs/symptoms do not present themselves until approximately 80% of the dopaminergic neurons have been lost. These signs and symptoms include tremor, rigidity, bradykinesia, akinesia and postural instability. As the dopaminergic

cells die, the neuromelanin is also lost, resulting in a bleaching of the substantia nigra in the human brain. In idiopathic Parkinson's disease, this usually occurs after age 55 (Jankovic, 2008).

Oxidative Stress

It is widely believed that persistent oxidative stress is a key pathogenic mechanism in Parkinson's disease. Oxidative stress occurs when the amount of free radicals, highly reactive molecules with unpaired electrons, present exceeds the normal antioxidant capacity of a cell, leading to cell damage and possibly death. Free radicals, more appropriately termed reactive species, are continuously produced *in vivo* in all body tissues. Their levels are kept in a tightly regulated and subtle balance by the body's antioxidant defenses. As such, reactive species are valuable in some normal cellular and biochemical functions but quickly can become deleterious (Kohen and Nyska, 2002). This may happen when events generating high levels of reactive species exceed the body's antioxidant defenses or the levels of endogenous antioxidants are diminished and normal amounts of reactive species are overwhelming.

Common reactive species include the superoxide radical, peroxide radical, iron, hydroxyl radicals, hydrogen peroxide, and various aldehydes (Halliwell, 2001; Kohen and Nyska, 2002). Given that most reactive species are relatively short-lived and thus react quickly with other molecules, their toxicity does not necessarily correlate with their tissue concentration. It is sometimes assumed that the longer the half-life of a molecule, the more time it has to cause damage, but that is not always the case. For example, superoxide anion has a comparatively long half-life and is able to diffuse to other

locations in order to interact with other molecules. On the other hand, the hydroxyl radical has a very short life span and is produced in the location where it can cause damage to its immediate surroundings and is many times more damaging than the superoxide anion (Kohen and Nyska, 2002). To prevent negative interactions between such reactive species and their surrounding biological targets, an antioxidant molecule in the same vicinity as the reactive species may detoxify the radical before it can interact with the target (Kohen and Nyska, 2002).

Superoxide anion can react with any compound or substrate that is capable of donating a hydrogen atom (Kohen and Nyska, 2002) and can also inactivate some enzymes (those containing Fe-S clusters) by interacting with their active sites while causing the release of iron. However, it cannot directly attack such molecules as lipids, DNA or protein (Halliwell, 2001). The dismutation of superoxide is its most significant reaction whereby two superoxide ions combine to yield hydrogen peroxide and oxygen as follows:

$$2\ O_2^{\bullet -} + 2\ H^+ \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide also does not seem to be able to directly oxidize lipids, DNA or most proteins (Halliwell and Gutteridge, 1999) but does easily penetrate biological membranes (Halliwell, 2001). It can cause the degradation of heme proteins at low levels and thus cause the release of iron ions (Halliwell, 2001; Kohen and Nyska, 2002). In addition, hydrogen peroxide serves as a good provider for more detrimental species like the hydroxyl radical and hypochlorous acid (Kohen and Nyska, 2002). Hydrogen peroxide is in fact a key substrate in the Fenton reaction. Here, iron in its ferrous form interacts with hydrogen peroxide to form a hydroxyl radical:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$

The hydroxyl radical formed by Fenton chemistry is a very powerful oxidizing agent that interacts with all types of molecules *in vivo.* Lipids, proteins, DNA and carbohydrates all can be attacked and damaged (Halliwell, 2001; Kohen and Nyska, 2002). Individual amino acid residues and DNA bases are attacked by the hydroxyl radical. Furthermore, these radicals also oxidize lipids and begin the lipid peroxidation chain reactions.

Lipid Peroxidation

Lipid peroxidation is a normally occurring, complex consequence of oxidative stress and is defined as the oxidative degeneration of lipids by free radical chain reaction. Membrane phospholipids are plentiful throughout biological systems and are thus easily accessible to all reactive species. In particular, reactive oxygen species are most damaging in tissues like the brain which has a high density of phospholipid membranes (de Zwart et al., 1999). Polyunsaturated fatty acids and other lipids are oxidized to form an array of bioactive molecules including conjugated dienes, lipid hydroperoxides and malondialdehyde (MDA) (Esterbauer et al., 1991).

Characteristically, lipid peroxidation occurs in three stages: initiation, propagation and termination (Kohen and Nyska, 2002). The initiation step of lipid peroxidation begins with the abstraction of a hydrogen atom from a $-CH_2-$ group of a polyunsaturated fatty acid. This weakens the bond between the H and C atoms so that it can be easily broken. The resulting carbon radical is then stabilized by a molecular rearrangement to a conjugated diene. A conjugated diene is a molecule with a single C-C bond flanked by

two double bonds. This conjugated diene can react with an oxygen molecule to form a peroxyl radical that can in turn react with hydroxyl radicals from other lipids or simply remove a hydrogen atom from a neighboring lipid as in the original initiation. This step is termed propagation as the chain reaction of lipid peroxidation is perpetuated. One initiation step can lead to the peroxidation of all the lipids in one membrane through propagation. Termination, the final step in lipid peroxidation, occurs when one of the peroxyl radicals interacts with another radical or an antioxidant molecule to stop the chain reaction (Kohen and Nyska, 2002; Facundo et al., 2004).

During the propagation phase of lipid peroxidation, lipid hydroperoxides can accumulate. In the presence of iron (or copper), the lipid hydroperoxides can be degraded to an alkoxyl or peroxyl radicals:

$$LOOH + Fe^{2+} \rightarrow Fe^{3+} + LO^{\bullet} + OH^{-}$$

$$LOOH + Fe^{3+} \rightarrow Fe^{2+} + LOO^{\bullet} + H^{+}$$

These alkoxyl and peroxyl radicals can go on to damage membrane proteins and also propagate the lipid peroxidation further by attacking another nearby lipid molecule.

Finally, lipid hydroperoxides can also simply decompose to an aldehyde, isoprostane, hydrocarbon, cyclic peroxide or other molecules (Kohen and Nyska, 2002; Facundo et al., 2004). Aldehydes, unlike most radicals, are relatively stable and last longer in the body. Thus they can diffuse away from where they were created and attack a distant target (de Zwart et al., 1999). A specific aldehyde, malondialdehyde, is the most widely used biochemical index of lipid peroxidation and is most frequently assayed using the TBARS assay. TBARS stands for thiobarbituric acid reactive substances and is so named because of malondialdehyde and other molecules that react with thiobarbituric

acid (TBA). Overall, malondialdehyde participates in a wide range of reactions, many of which create protein and DNA adducts. Malondialdehyde is mutagenic, producing frame shift and base pair mutations and has been reported to be carcinogenic in rats (Zhang et al., 2002). Besides being endogenously produced, malondialdehyde is also found in cigarette smoke (Freeman et al., 2005) and in rotting and rancid food. The TBARS assay was first used to measure rancidity by food chemists (Esterbauer et al., 1991).

Briefly, the reaction in the TBARS assay is the condensation of two molecules of TBA with one molecule of malondialdehyde. The resulting product is colored and can be quantitated spectrophometrically at 532 nm. In the present work, the TBARS assay was chosen as a preliminary assay to detect levels of malondialdehyde to serve as evidence of lipid peroxidation because it is simple and so widely used.

Oxidative Stress in the Brain

In the brain, neurons are constantly being exposed to reactive oxygen and nitrogen species from oxidative stress. This stress can be caused by the continual exposure to endogenous and exogenous toxicants or as a normal consequence of oxidative metabolism. These species are thought to create oxidative stress and, in turn, lipid peroxidation and cause damage to the brain. The brain is highly susceptible to lipid peroxidation due to its high lipid composition, particularly of polyunsaturated fatty acids, very high concentrations of oxygen, and a relatively low concentration of antioxidant enzymes like superoxide dismutase (SOD) and catalase (Giasson et al., 2002; Irizarry and Hyman, 2003; Warner et al., 2004). The brain also contains high levels of ferrous iron which as described above can undergo Fenton chemistry to hydrogen peroxide (Kohen

and Nyska, 2002; Thomas and Jankovic, 2004). Futhermore, it is speculated that these reactive species and resulting oxidative stress attack cell's essential machinery and cause damage to the genes, whether it be a point mutation or under- or overexpression of vital genes thus beginning a cascade of degeneration and neuronal death. In fact, several genes have been implicated in increased susceptibility to Parkinson's disease (Prasad et al., 1999). Sources shown to increase intracellular free radical production and damage to DNA, proteins and mitochondria could be a result of lipid peroxidation consequent to oxidative stress. Thus, malondialdehyde from lipid peroxidation would be an intermediate step to the neurodegeneration observed in Parkinson's disease.

Indeed, necropsy studies of patients with Parkinson's disease have provided evidence of increased lipid peroxidation in the substantia nigra (Dexter et al., 1989; Jenner, 1994). Further, dopamine metabolism can lead to the formation of hydrogen peroxide (Yoritaka et al., 1996) which can then form the extremely fast acting and toxic hydroxyl radical in the presence of iron which is in high concentrations in the brain (Halliwell, 2001; Kohen and Nyska, 2002). Dexter et al. (1989, 1994) have shown an increase in ferric iron in the substantia nigra of patients with Parkinson's disease as well as an increase in the basal levels of malondialdehyde in the substantia nigra (Dexter et al., 1989; Dexter et al., 1994). Levels of lipid peroxidation are increased in the plasma of Parkinson's disease patients (Younes-Mhenni et al., 2007). Yoritaka et al. (1996) used immunohistochemical methods to detect 4-hydroxynonenal in the brains of control and Parkinson's patients and reported a significantly higher proportion of 4-HNE-modified proteins in substantia nigra neurons in patients with Parksinson's disease. 4-hydroxynonenal (4-HNE) is another aldehyde that is produced during the lipid

peroxidation of membranes and is known to cause a variety of effects on DNA, RNA, protein synthesis, enzyme activities and heat shock proteins (Yoritaka et al., 1996). Overall, glial cells were not positively immunostained, leading to the conclusion that lipid peroxidation is occurring mostly within the substantia nigra neurons of Parkinson's patients (Yoritaka et al., 1996). Also, levels of hydrogen peroxide and malondialdehyde were measured as markers of oxidative stress in patients with Parkinson's disease. Patients were subjected to an exercise treatment program that successfully reduced these levels of oxidative stress (Bloomer et al., 2008). Finally, increased levels of 4-HNE and malondialdehyde have also been found in the caudate nucleus of human methamphetamine abusers (Fitzmaurice et al., 2006).

Furthermore, lipid peroxidation is known to occur throughout the body. Malondialdehyde has been measured in many peripheral tissues such as the kidney, lung, liver and blood plasma (Esterbauer et al., 1991; Zhang et al., 2002; Niedernhofer et al., 2003; Facundo et al., 2004; Thiele et al., 2004). Overall, oxidative stress and lipid peroxidation leading to the production of malondialdehyde also comes from a wide variety of sources like mitochondria, xenobiotics, food, and the body's natural response to pathogenic invasion (Esterbauer et al., 1991; Freeman et al., 2005). Therefore, it is hypothesized that an increase in peripheral lipid peroxidation may correlate with neuronal damage consequent to toxicant-induced lipid peroxidation. As noted, exposure to endogenous and exogenous factors that cause oxidative stress in the form of lipid peroxidation may cause Parkinson's disease. Since exposure is likely to be through oral or inhalation routes, it follows that levels of lipid peroxidation will be elevated during or shortly after this exposure and damage may accumulate over time to result in

neurodegeneration. By measuring a peripheral marker of lipid peroxidation, namely liver malondialdehyde, it is predicted that these acutely raised levels of lipid peroxidation will link with the long-lasting dopaminergic neurotoxicity. Furthermore, it is also predicted that treatment with exogenous antioxidants and anti-inflammatory drugs will serve to protect against neurodegeneration.

II. AMPHETAMINE MODEL OF PARKINSON'S DISEASE: LEVELS OF MALONDIALDEHYDE AND THE EFFECTS OF PRETREATMENTS ON TOXICITY

Repeated administration of high doses of amphetamine has been used as a model of Parkinson's disease as it causes nigral neurodegeneration with dopamine depletion and decreased tyrosine hydroxylase activity. In addition, there is a correlation between amphetamine sensitivity and increased age, as well as a gender preference, with male animals being more sensitive to the drugs effects than females (Kita et al., 2003a). Amphetamine also serves as a model of an exogenous chemical that causes neurodegeneration after multiple low level exposures. In the environment, the actual exogenous low-level chemical exposure may be to toxicants like herbicides and pesticides that are also currently a widely studied potential cause of Parkinson's disease (de Rijk et al., 1997; Thiruchelvam et al., 2000).

Amphetamines exert their neurotoxicity in part via oxidative stress pathways although exact mechanisms are still unknown. Central nervous systems effects typically observed are long lasting dopamine depletion, decreases in dopamine transporter levels, decreases in tyrosine hydroxylase, and degeneration of dopamine nerve terminals. Methamphetamine has also been shown to cause loss of substantia nigra neurons (Sonsalla et al., 1996). Generally, amphetamines are thought to cause damage by means of excess dopamine release from the striatal terminals, mediated by dopamine transporters. This excess dopamine is proposed to undergo auto-oxidation forming 6-hydroxydopmamine. During this process, superoxide anion and hydrogen peroxide are

also formed leading to neurotoxicity through reactive oxygen species damage and possibly through lipid peroxidation mechanisms with the same reactive species (Kahlig et al., 2005).

Amphetamines are potent central nervous system stimulants that also have effects in the periphery. Physical and behavioral symptoms of amphetamine use or exposure are increased blood pressure, heart rate, perspiration, respiration; incidence of tremor, hyperactivity, as well as stereotypic and self-injurious behavior. These are thought to be caused by amphetamine's ability to act indirectly on the monoamine systems in the brain (Fleckenstein et al., 2007).

Long-term dopaminergic depletion has been observed in monkeys, guinea pigs, rats, and mice following repeated amphetamine exposure. Depletions have been observed up to at least six months in rhesus monkeys and in rats with no trend of recovery observed. Moreover, autopsy studies on humans that had abused amphetamines long before their death also showed reduced striatal dopamine, tyrosine hydroxylase, and dopamine transporter levels compared to control patients (McCann et al., 1998). Methamphetamine has also recently been shown to produce neuronal inclusions in both the substantia nigra and striatum of mice (Fornai et al., 2004a; Fornai et al., 2004b) and in the substantia nigra of methamphetamine abusers (Quan et al., 2005). Those found in the substantia nigra are immunopositive for a-synuclein and ubiquitin (Fornai et al., 2004b).

Peripherally, amphetamines cause a release of epinephrine and norepinephrine, thus explaining some of the physical symptoms mentioned previously. In the liver, amphetamine causes cellular glutathione depletion by oxidation of glutathione and activation by cytochrome P450 2D6. Some metabolites of amphetamines are capable of

reacting with glutathione in the liver to create a reduction of free glutathione that can then lead to large influx of calcium, lipid peroxidation and eventually cell death (Kalant, 2001).

Overall, the toxicant amphetamine is known to cause dopamine depletion through generation of reactive oxygen species. Amphetamine is an acutely acting agent and in high doses is able to induce long-lasting dopaminergic consequences. By contrast, Parkinson's disease is a slowly developing disease evidenced by permanent dopamine depletion. However, high dose amphetamine, like other models of Parkinson's disease, is used as a expedited model of this disease in order to efficiently study potential mechanisms and therapeutic interventions. Thus, amphetamine was selected as a model toxicant causing cell death through oxidative stress. The following studies will examine amphetamine's effects on striatal dopamine depletion and lipid peroxidation levels in the liver, as well the effects of antioxidants in this model of Parkinson's disease.

Hypothesis: A single high-dose of amphetamine can cause significant and long-lasting dopamine depletion comparable to the more commonly used multiple lower dose amphetamine model of Parkinson's disease.

Specific aims:

- **Characterize the acute and long-term effects on striatal dopamine levels after a single high dose of amphetamine**
- **Measure levels of MDA in the liver and striatum after amphetamine administration**

Experiment 1: The acute effects of amphetamine on striatal dopamine levels

Rationale

Dopamine release from the striatal neurons is known to be an acute effect of amphetamine. While some work has been conducted on low doses of amphetamine, virtually no studies have been conducted on the acute effects of high, toxic doses of amphetamine on dopamine and serotonin levels. A single, high dose of amphetamine has never been characterized previously and was chosen here due to its long-lasting dopamine depletion and low mortality rates. Accordingly, in Experiment 1, dopamine, serotonin and their metabolite levels were measured after acute high dose amphetamine exposure to determine the time course when dopamine levels begin to be depleted.

Animals

Eight week old, adult male BALB/c mice (Jackson Laboratories; Bar Harbor, ME) were maintained according to the NIH Guide for the Care and Use of Laboratory Animals. Mice had access to standard rodent chow and water *ad libitum* for the duration of the study. Mice were housed in a temperature controlled colony room on a 12 hour on/12 hour off light cycle beginning at 8 AM. Animals were housed individually in hanging wire cages (20 cm x 10 cm x 12 cm).

Drug treatment

Mice received one subcutaneous injection of 50.0 mg/kg amphetamine or saline and sacrificed 15, 30 or 60 minutes after injection. Mice were sacrificed by decapitation, striata were dissected and used for neurochemical analysis.

Neurochemical Analysis

Following sacrifice, brains were quickly removed and bilateral striata were immediately dissected and stored in liquid nitrogen. Tissue was homogenized in 300 μl of 0.4 N perchloric acid with 0.1 mM ethylenediamine tetraacetic acid (EDTA) added to inhibit biochemical degradation. Samples were centrifuged at 20,000 g for 20 minutes at 4^{o}C and the supernatant was assayed for dopamine (DA), serotonin (5-HT) and their metabolites dihydryoxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) by high pressure liquid chromatography (HPLC) (Bioanalytical Systems; West Lafayette, IN). 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 (5mm, 250 x 4.6 mm i.d.), and oxidized with a $^{+}$0.72 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, 5.0 mg EDTA and 14% methanol. Flow rate was 0.7 ml/min. Quantification was against external standards injected between every six samples.

Statistical Analyses

One-way ANOVAs were performed to analyze effects of drug treatment on neurotransmitter concentration or turnover ratios. For all appropriate analyses, differences between groups were determined using Fisher's PLSD post-hoc tests.

Results

Neurochemical analysis revealed an overall significant effect of sacrifice time for DA, DOPAC and HVA levels. There was no overall significant effect of sacrifice time on 5-HT or 5-HIAA levels. Furthermore, there was no difference in neurochemistry of animals treated with saline sacrificed at any time so these animals were combined into one control group for statistical analysis.

Dopamine was significantly higher at 15 minutes compared to controls ($F(3,20) = 23.484$, $p = .0065$). Dopamine was significantly lower at 30 and 60 minutes compared to controls (30 minutes: $F(3,20) = 23.484$, $p = .0051$; 60 minutes: $F(3,20) = 23.484$, $p = .0001$). Dopamine was significantly higher at 15 minutes compared to 30 minutes and 60 minutes (30 minutes: $F(3,20) = 23.484$, $p < .0001$; 60 minutes: $F(3,20) = 23.484$, $p < .0001$). DOPAC was significantly lower in amphetamine-treated animals versus controls at all time points (15 minutes: $F(3,20) = 14.337$, $p = .0023$; 30 minutes: $F(3,20) = 14.337$, $p = .0002$); 60 minutes: $F(3,20) = 14.337$, $p < .0001$). DOPAC was also significantly higher in amphetamine-treated animals at 30 minutes compared to 60 minutes ($F(3,20) = 14.337$, $p = .0098$). HVA levels were significantly higher at 60 minutes compared to controls ($F(3,20) = 10.832$, $p < .0001$) and significantly lower at 15 and 30 minutes compared to 60 minutes (15 minutes: $F(3,20) = 10.832$, $p = .0007$; 30 minutes: $F(3,20) = 10.832$, $p < .0001$). 5-HT was significantly higher at 30 minutes compared to controls ($F(3,20) = 2.424$, $p < .05$). 5-HIAA was significantly higher at 30 minutes compared to 60 minutes ($F(3,20) = 10.832$, $p < .05$). See figure 1.

Analysis of the neurotransmitter turnover ratios revealed a significant effect of sacrifice time in all three turnover ratios tested (DOPAC/DA: $F(3,20) = 7.242$, $p = .0018$;

HVA/DA: $F(3,20) = 40.589$, $p < .0001$); 5-HIAA/5-HT: $F(3,20) = 6.783$, $p = .0025$). *Post hoc* analysis showed that turnovers were significantly different for DOPAC/DA at 15 minute, 30 minute and 60 minute sacrifice times compared to controls (15 minutes: $p = .0009$; 30 minutes: $p = .0200$; 60 minutes: $p = .0005$). *Post hoc* analysis showed that turnover ratios were significantly different for HVA/DA at 30 minute and 60 minute sacrifice times compared to controls (30 minutes: $p = .0065$; 60 minutes: $p = .0001$) and compared to each other (15, 30 minutes: $p = .0004$; 15, 60 minutes: $p < .0001$; 30, 60 minutes: $p < .0001$). Finally, *post hoc* analysis showed that turnovers were significantly different for 5-HIAA/5-HT at 60 minute sacrifice compared to controls ($p = .0002$) and to each other (15, 30 minutes: $p = .0122$; 30, 60 minutes: $p = .0259$). See figure 2.

Conclusions

Treatment with amphetamine causes an immediate release of dopamine. At 15 minutes, dopamine levels were significantly higher in amphetamine-treated animals versus controls. This reflects the rapid, acute release of dopamine in the striatum and inhibition of monoamine oxidase (MAO). By 30 minutes post-injection, dopamine levels in amphetamine-treated animals had dropped significantly compared to controls. This reflects the beginning of the exhaustion of dopamine stores. Finally, at 60 minutes post-injection, dopamine levels are at their lowest in amphetamine-treated animals, significantly lower than controls. This is likely the beginning of the complete exhaustion of dopamine stores in the striatum.

The amounts of DOPAC and HVA found were also an indication of dopamine depletion. DOPAC levels were lower in amphetamine-treated animals compared to

controls at all time points. This is expected because amphetamine is known to inhibit monoamine oxidase (MAO), the enzyme that metabolizes dopamine to DOPAC. Dopamine is metabolized by catechol-O-methyltransferase (COMT) into 3-methyltyrosine (3-MT) and then to HVA. Levels of HVA were higher in both control and amphetamine-treated animals. At 60 minutes, HVA in amphetamine-treated animals was significantly elevated compared to controls. Amphetamine's half-life in the mouse is approximately 60 minutes. The initial effect was to increase dopamine with concentrations peaking at 15 minutes post-amphetamine. Following that, dopamine levels decrease as stores are depleted. Further, there was a significant difference in dopamine turnover ratios. At all time points, amphetamine-treated animals had significantly greater DOPAC/DA turnover and at 30 and 60 minutes, amphetamine-treated animals had significantly greater HVA/DA turnover. These data show that amphetamine causes an acute increase in dopamine turnover.

Figure 1

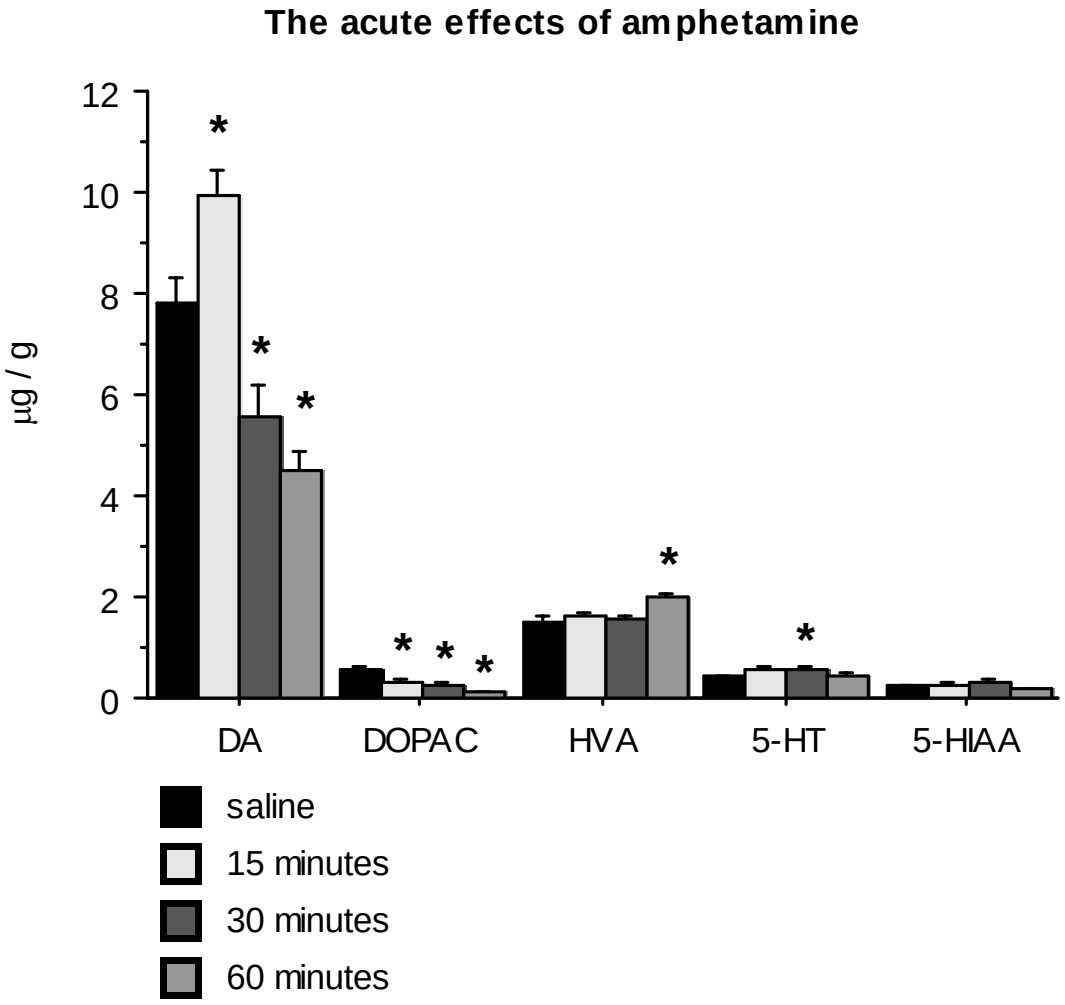


Figure 1: Concentration of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in striatum of male BALB/c mice for saline or amphetamine.

* denotes significantly different from saline; $p < .05$

Figure 2

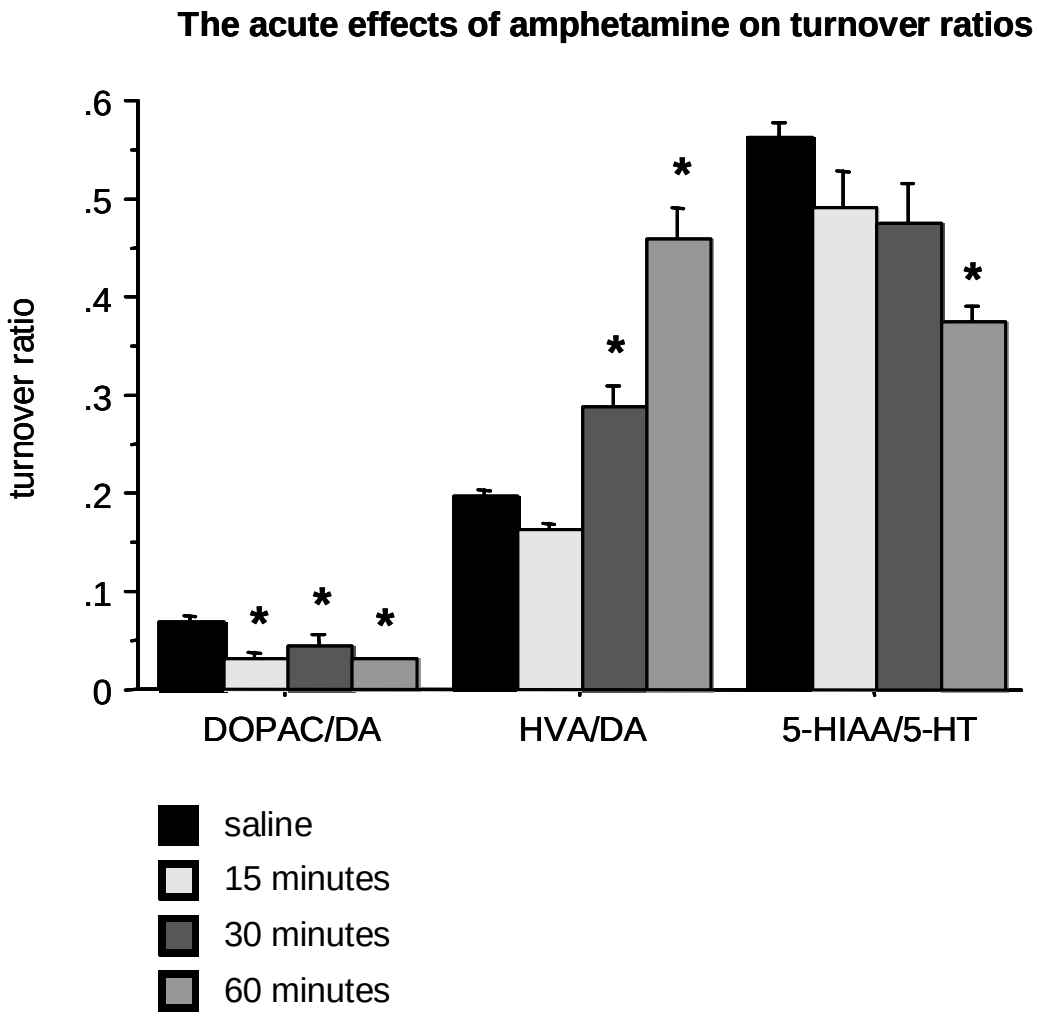


Figure 2: Turnover ratios of DOPAC/DA, HVA/DA and 5-HIAA/5-HT in the striatum of male BALB/c mice for saline or amphetamine.

* denotes significantly different from saline; $p < .05$

Experiment 2: The long-term effects of amphetamine on striatal dopamine levels

Rationale

Determine the long-term effects of a single high dose of amphetamine. Mice were sacrificed one day or more, up to two weeks after amphetamine exposure.

Animals

Eight week old adult male BALB/c mice (Jackson Laboratories; Bar Harbor, ME) were maintained according to the NIH Guide for the Care and Use of Laboratory Animals. Mice had access to food and water *ad libitum* for the duration of the study. Mice were housed in a temperature controlled colony room on a 12 hour on/12 hour off light cycle beginning at 8 AM. Animals were housed individually in hanging wire cages (20 cm x 10 cm x 12 cm).

Drug treatment

Mice received one subcutaneous injection of 50.0 mg/kg amphetamine or saline. Mice were sacrificed one day, three days or two weeks after injections. Four mice were also sacrificed without any injections.

Neurochemical Analysis

Following sacrifice, brains were quickly removed and bilateral striata were immediately dissected, stored in liquid nitrogen and analyzed as in Experiment 1.

Statistical Analyses

One-way ANOVAs were performed to analyze the effect of drug challenge on neurotransmitter concentration or turnover ratio. For all appropriate analyses, differences between groups were determined using Fisher's PLSD post-hoc tests.

Results

No significant difference in any neurotransmitters tested was found between saline-injected animals at any time point and those who were sacrificed without any injections and therefore these groups were combined in all further analyses and designated "control."

Neurochemical analysis revealed a 75% depletion of dopamine at one day sacrifice as compared to controls, an 84% depletion of dopamine at three days and a 70% depletion of dopamine at two weeks. Essentially, these data show that dopamine depletion was complete at one day post-amphetamine and this lesion remains stable over time for up to two weeks. See figure 3.

Overall, a significant effect of sacrifice time was seen in dopamine, DOPAC, HVA and 5-HIAA levels between sacrifice times (DA: $F(3,22) = 59.462$, $p < .0001$; DOPAC: $F(3,22) = 13.290$, $p < .0001$; HVA: $F(3,22) = 11.662$, $p < .0001$; 5-HIAA: $F(3,22) = 11.700$, $p < .0001$).

Post hoc analysis revealed dopamine levels of control mice were significantly higher than those of mice sacrificed at one day, three days and two weeks (1 day: $p <$

.0001; 3 days: $p < .0001$; 2 weeks: $p < .0001$). The dopamine levels of one day, three day and two week mice were all statistically similar.

Post hoc analysis revealed DOPAC levels of control mice were significantly higher than DOPAC of mice sacrificed at one day, three days and two weeks (1 day: $p = .0001$; 3 days: $p = .0014$; 2 weeks: $p < .0001$). The DOPAC levels of one day, three day and two week mice were all statistically similar. *Post hoc* analysis revealed HVA levels of control mice were significantly higher than HVA of mice sacrificed at one day, three days and two weeks (1 day: $p = .0181$; 3 days: $p = .0025$; 2 weeks: $p < .0001$). This shows that the effects of amphetamine on dopamine metabolism (inhibition of MAO) persist for up to two weeks.

Post hoc anaylsis showed that controls had significantly lower 5-HIAA compared to one day and three days (one day: $p = .0013$; three days: $p < .0001$). Also, 5-HIAA at two weeks was significantly lower that one day and three days (one day: $p = .0292$; three days: $p = .0006$). While, amphetamine increased levels of the serotonin metabolite 5-HIAA at one and three days post-amphetamine, levels returned to control levels by two weeks indicating no long-lasting effects.

Analysis showed that turnover ratios were significantly different between amphetamine-treated mice and controls (DOPAC/DA: $F(3,22) = 23.552$, $p < .0001$; HVA/DA: $F(3,22) = 24.151$, $p < .0001$; 5HIAA/5HT: $F(3,22) = 14.149$, $p < .0001$). See figure 4. *Post hoc* analysis revealed significance between specific groups: DOPAC/DA was significantly greater in amphetamine-treated animals compared to controls at one day and three days (one day: $p = .0112$; three days: $p < .0001$). DOPAC/DA was significantly higher at three days compared to both one day and two weeks as well (one

day: $p = .0001$; two weeks: $p < .0001$). HVA/DA was significantly greater in amphetamine-treated animals compared to controls at one day, three days and two weeks (one day: $p < .0001$; three days: $p < .0001$; two weeks: $p = .0256$). HVA/DA was also significantly different among the treatment groups as well (one day, three days: $p = .0264$; one day, two weeks: $p = .0077$; three days, two weeks: $p < .0001$). 5-HIAA/5-HT was significantly lower in controls compared to one day and three days (one day: $p < .0001$; three days: $p = .0002$). 5-HIAA/5-HT was also significantly lower at two weeks compared to one day and three days (one day: $p = .0002$; three days: $p = .0021$).

Dopamine turnover ratios were increased overall at all time points tested. DOPAC/DA returned to control levels by two weeks but were increased at one day and three days. This indicates that amphetamine's inhibitory effects on tyrosine hydroxylase continue for at least three days and recover by two weeks. Meanwhile, HVA/DA ratios remain elevated compared to control levels at all time points. This effect is highest at one and three days. This indicates that amphetamine's inhibitory effects on MAO also persist longer than its effects on tyrosine hydroxylase and persist for up to two weeks. In parallel to 5-HIAA levels, the turnover ratio of 5-HT was increased at one and three days but returned to control levels by two weeks.

Conclusions

Statistical analysis showed significant depletion of dopamine and its metabolites for up to two weeks after exposure to amphetamine. Turnover ratios were significantly increased at one day and three days post-amphetamine compared to controls most likely due to the pharmacological effects of the high dose of amphetamine. Amphetamine

inhibits tyrosine hydroxylase (therefore dopamine is reduced) and at the same time, inhibits monoamine oxidase (therefore metabolite levels go down), though DOPAC is more affected than HVA. The inflated turnover ratios indicate that the effect on dopamine may be greater than the effect on its metabolites. At two weeks post-amphetamine turnover ratios returned to near control levels. This is probably because dopamine levels had normalized at their now lower levels, as did the metabolism of dopamine as the amphetamine was cleared after approximately two days.

Collectively, the data from Experiment 1 and Experiment 2 indicate that amphetamine-induced striatal dopamine depletion develops as early as 30 minutes after amphetamine administration and continues for at least two weeks post-administration.

Figure 3

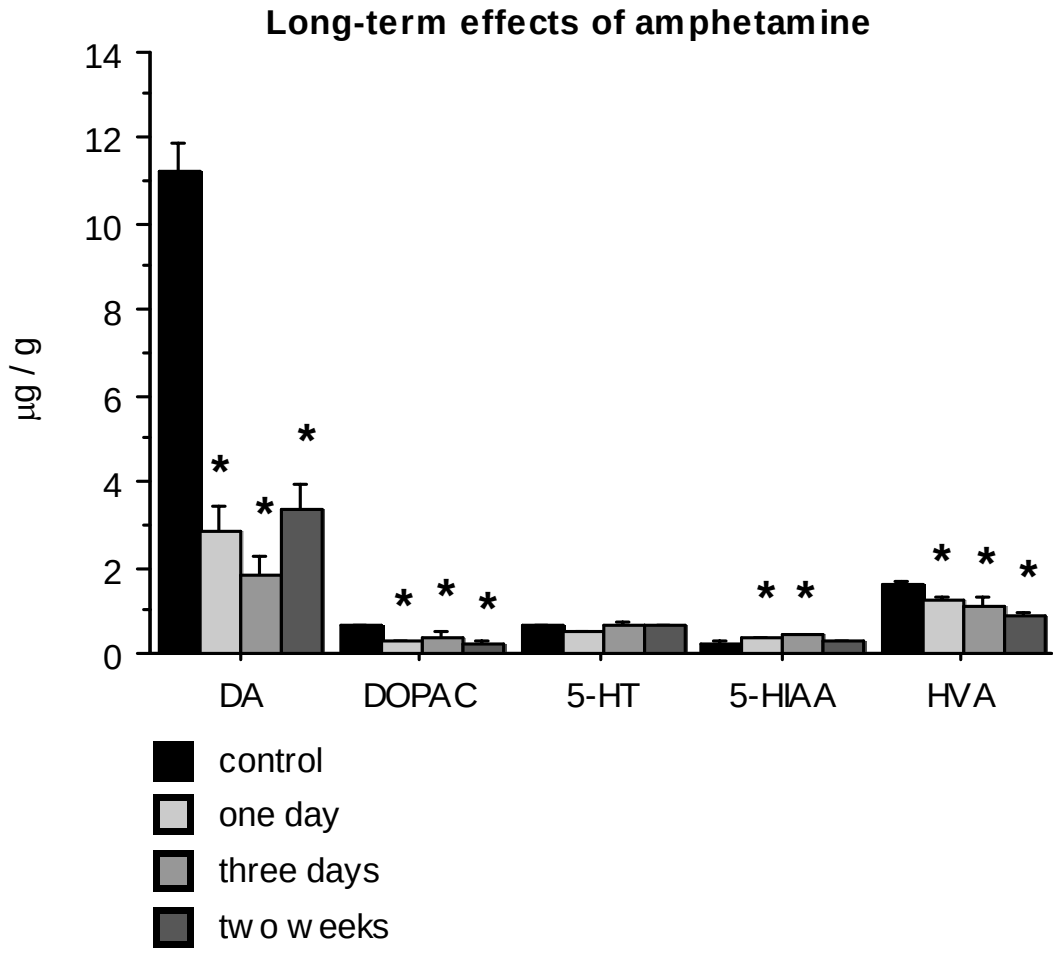


Figure 3: Concentration of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in striatum of male BALB/c mice for saline or amphetamine.

* denotes significantly different from saline; $p < .05$

Figure 4

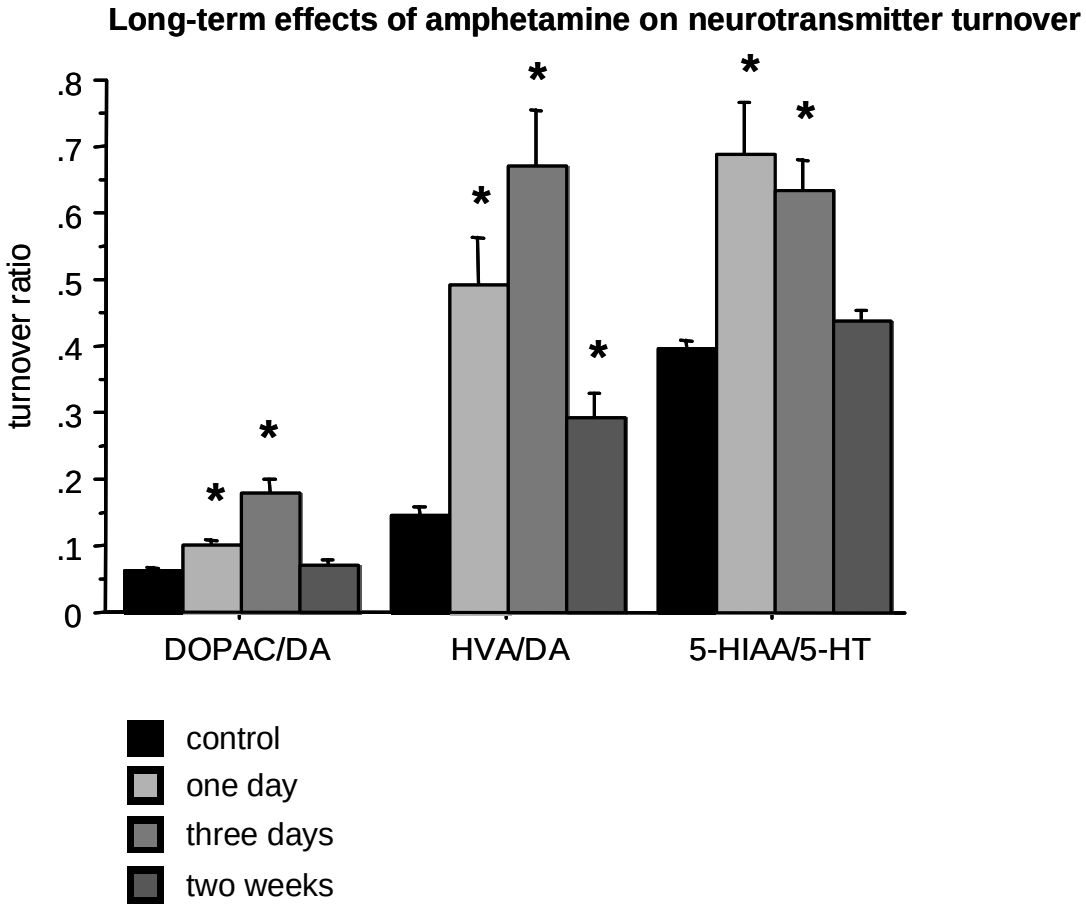


Figure 4: Turnover ratios of DOPAC/DA, HVA/DA and 5-HIAA/5-HT in the striatum of male BALB/c mice for saline or amphetamine.

* denotes significantly different from saline; $p < .05$

Experiment 3: The effects of amphetamine on striatal dopamine in a resistant mouse strain

Rationale

C57Bl/6J (C57) mice have previously been shown to be more resistant to the effects of amphetamine on striatal dopamine levels (Kita et al., 1998). It is important to compare the effects of amphetamine on both sensitive and resistant strains of mice to fully characterize this lesion, as this strain will serve as the background strain for later genetic studies.

Animals

Eight week old, adult male C57 mice (Jackson Laboratories; Bar Harbor, ME) were maintained according to the NIH Guide for the Care and Use of Laboratory Animals. Mice had access to food and water *ad libitum* for the duration of the study. Mice were housed in a temperature controlled colony room on a 12 hour on/12 hour off light cycle beginning at 8 AM. Animals were housed individually in hanging wire cages (20 cm x 10 cm x 12 cm).

Drug treatment

Mice received one subcutaneous injection of 50 mg/kg amphetamine or saline. Mice were sacrificed one day, three days or two weeks after injections. Four mice were also sacrificed without any injections.

Neurochemical Analysis

Following sacrifice, brains were quickly removed and bilateral striata were immediately dissected, stored in liquid nitrogen and analyzed as described in Experiment 1.

Statistical Analyses

One-way ANOVAs were performed to analyze the effects of drug challenge on neurotransmitter concentration or turnover ratios as main factors and drug challenge as between groups factor. For all appropriate analyses, differences between groups were determined using Fisher's PLSD post-hoc tests.

Results

Analysis with a one-way ANOVA revealed no significant difference in dopamine, DOPAC, HVA or 5-HIAA at any time point. However, there was a significant effect of sacrifice time on 5-HT levels ($F(3,30) = 7.240$, $p = .0009$) with *post hoc* analysis showing a significant difference between the three day and two week sacrifice compared to controls (3 days: $p = .0126$; 2 weeks: $p = .0332$). See figure 5.

Analysis of turnover ratios showed no significant difference in dopamine turnover but a significant difference in serotonin turnover (5-HIAA/5-HT: $F(3,30) = 3.208$, $p = .0370$), specifically serotonin turnover at two weeks was significantly lower compared to controls ($p = .0382$). See figure 6.

Conclusions

Analysis of neurochemistry showed that there was no significant difference in dopamine or its metabolites at any time after amphetamine administration. There also was no difference in dopamine turnover. This confirms that the C57BL/6J strain of mice is resistant to the dopamine depleting effects of amphetamine observed in the BALB strain. It is interesting that C57s seem to be slightly more sensitive to amphetamine's effects on the serotonin system in this experiment. This has not been found in other experiments looking at the effect of methamphetamine on the C57 strain (Kita et al., 1998).

Figure 5

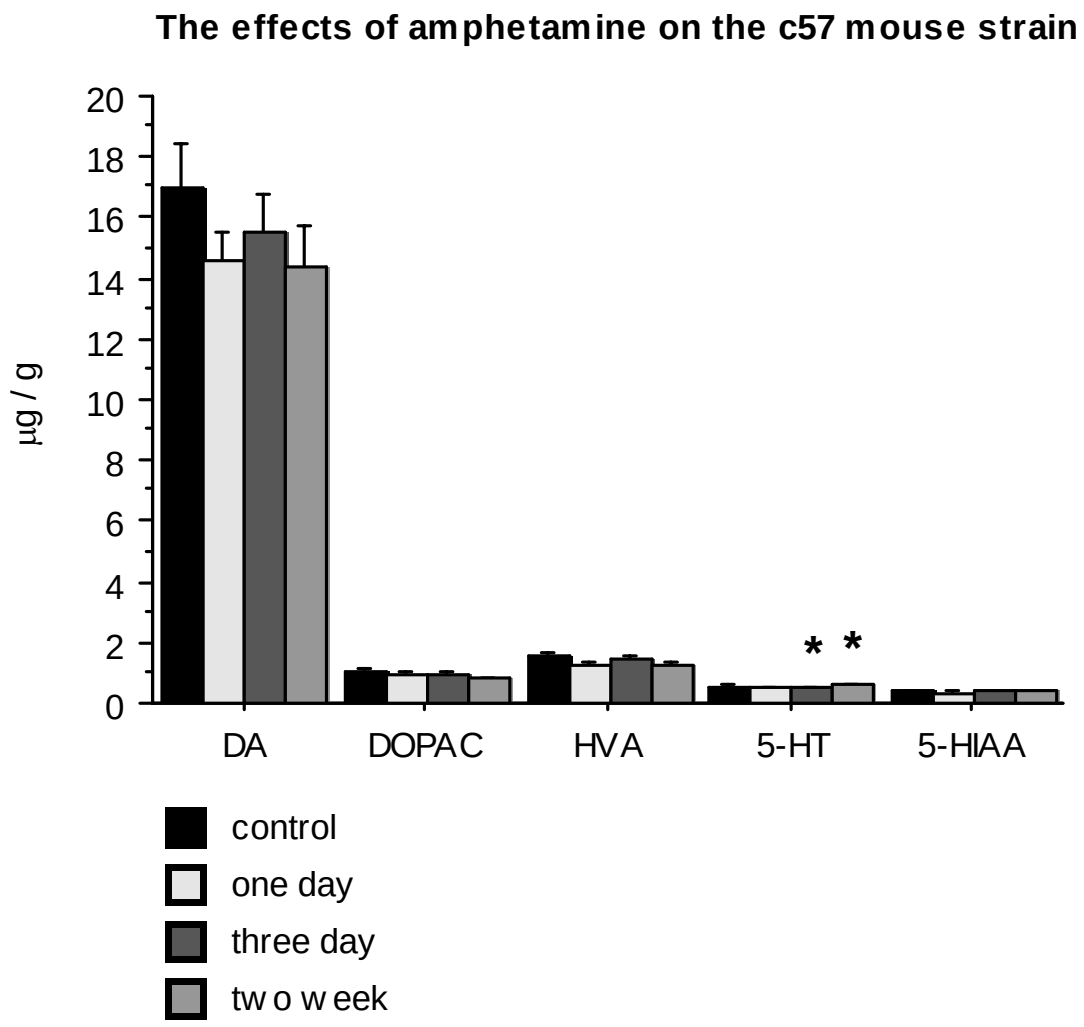


Figure 5: Concentration of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in striatum of male C57Bl6J mice for saline or amphetamine.

* denotes significantly different from saline; $p < .05$

Figure 6

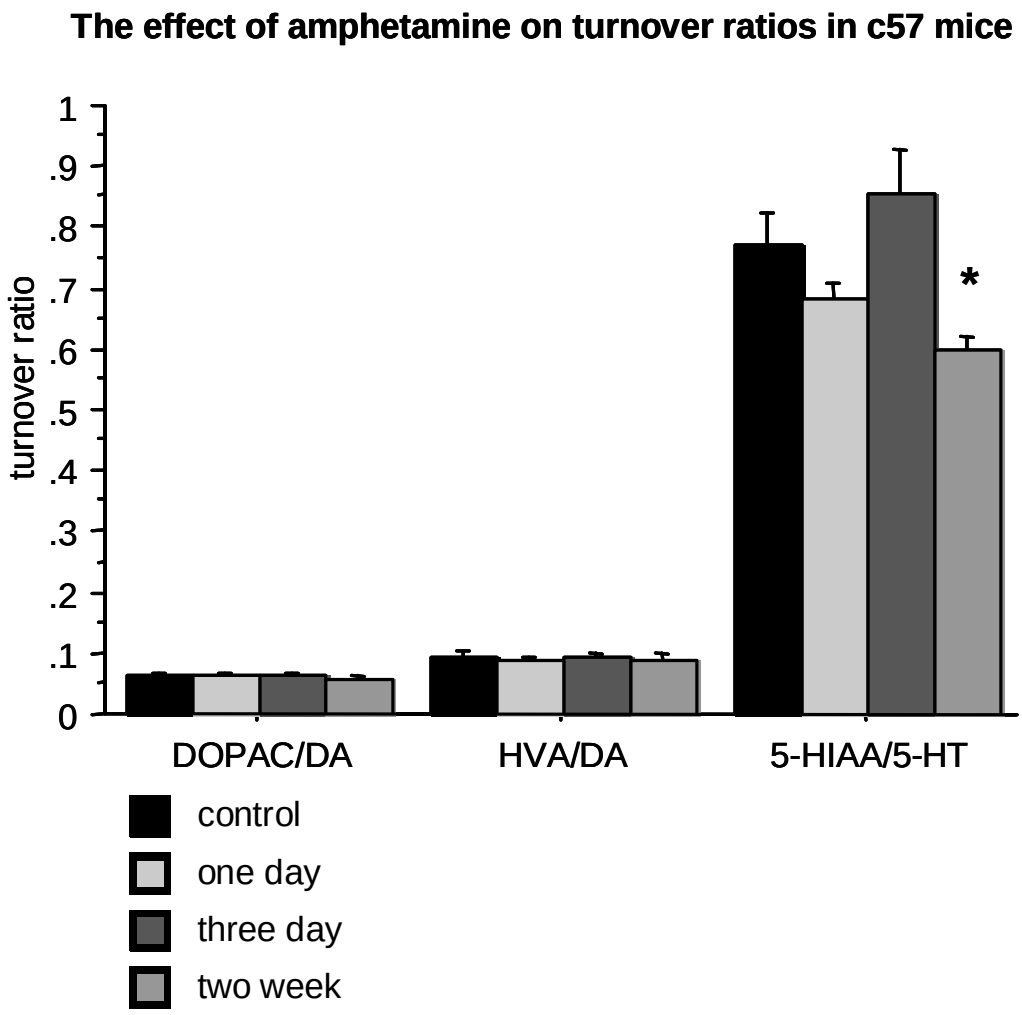


Figure 6: Turnover ratios of DOPAC/DA, HVA/DA and 5-HIAA/5-HT in the striatum of male C57Bl6J mice for saline or amphetamine.

* denotes significantly different from saline; $p < .05$

Experiment 4: The effects of amphetamine on levels of malondialdehyde, a marker of oxidative stress, in the liver

Rationale

To determine the effects of amphetamine on levels of oxidative stress in the periphery as measured by levels of malondialdehyde in the liver.

Animals

Eight week old, adult male BALB/c mice (Jackson Laboratories; Bar Harbor, ME) were maintained according to the NIH Guide for the Care and Use of Laboratory Animals. Mice had access to food and water *ad libitum* for the duration of the study. Mice were housed in a temperature controlled colony room on a 12 hour on/12 hour off light cycle beginning at 8 AM. Animals were housed individually in hanging wire cages (20 cm x 10 cm x 12 cm).

Drug treatment

Mice received one subcutaneous injection of 50.0 mg/kg amphetamine or saline. Mice were sacrificed 30 minutes, one hour, two hours, four hours, 24 hours and 72 hours after injection.

MDA analysis

Following sacrifice, livers were quickly dissected and stored in liquid nitrogen until assay. Approximately 100 mg of tissue was homogenized in 900 ml of .1M phosphate buffer and kept on ice. Then, 0.05 ml of the homogenate was added to 0.1 ml

of 8.1% sodium dodecyl sulfate solution and vortexed. Samples were incubated with 0.75 ml of 20% glacial acetic acid, pH 3.5 and 0.75 ml thiobarbituric acid (TBA). After heating at 95^{o} for 60 minutes, the samples were allowed to cool in ice water to room temperature, distilled water and a 15:1 solution of n-butanol and pyridine were added and samples were centrifuged at 2200 g for 10 minutes. The red pigment in the supernatant fractions were estimated at 532nm in a spectrophotometer. The n-butanol:pyridine mixture was used as a standard to calibrate the spectrophotometer. Samples were run in duplicate.

Statistical Analyses

One-way ANOVAs were performed with MDA level as main factors and sacrifice time as between groups factor. Differences between groups were determined using Fisher's PLSD post-hoc tests.

Results

Controls at each time point were analyzed using a one-way ANOVA. No statistical difference was found and thus these were combined into one group designated controls.

Analysis with a one-way ANOVA showed there was a significant effect of sacrifice time on levels of MDA in the liver compared to controls ($F(6,109) = 39.352$, $p < .0001$). *Post hoc* analysis revealed that all time points had significantly higher MDA compared to controls except for the 72 hour sacrifice (control, 30 min: $p = .0055$; control,

1 hour: $p < .0001$; control, 2 hours: $p < .0001$; control, 4 hours: $p < .0001$; control, 24 hours: $p < .0001$; control, 72 hours: $p < .0001$). See figure 7.

Conclusions

This dose (50 mg/kg) of amphetamine caused lipid peroxidation levels to increase in the liver. Levels increased steadily up to 24 hours post-amphetamine administration and returned to control levels by 72 hours post-amphetamine. This shows that the toxicity of amphetamine lasts and builds upon itself from its pharmacological effects. Once amphetamine was cleared from the body, lipid peroxidation levels in the liver returned to baseline levels. The brain is sensitive to oxidative damage and may show longer lasting effects of a compound that can also cause increases in lipid peroxidation and oxidative stress in the periphery. In addition, as the brain is quite adaptable, it may take a significant period of time for this damage to build up so that it is phenotypically evident. An example of this is clear in Parkinson's disease: behavioral and motor symptoms are not apparent until 80% of the dopamine neurons have been lost. As such, a toxicant may begin a cascade of events that slowly lead to extensive neuronal damage and this initial insult may be evident in peripheral levels of oxidative stress. It is possible that neuronal products of oxidative stress cross the blood brain barrier and are found in the periphery, but it is more likely that the toxicant causing the insult also increases levels of oxidative stress in peripheral tissues. These systems are also expected to recover quickly and not exhibit significantly higher levels of oxidative stress, but may show low-level increases after sustained insult.

Figure 7

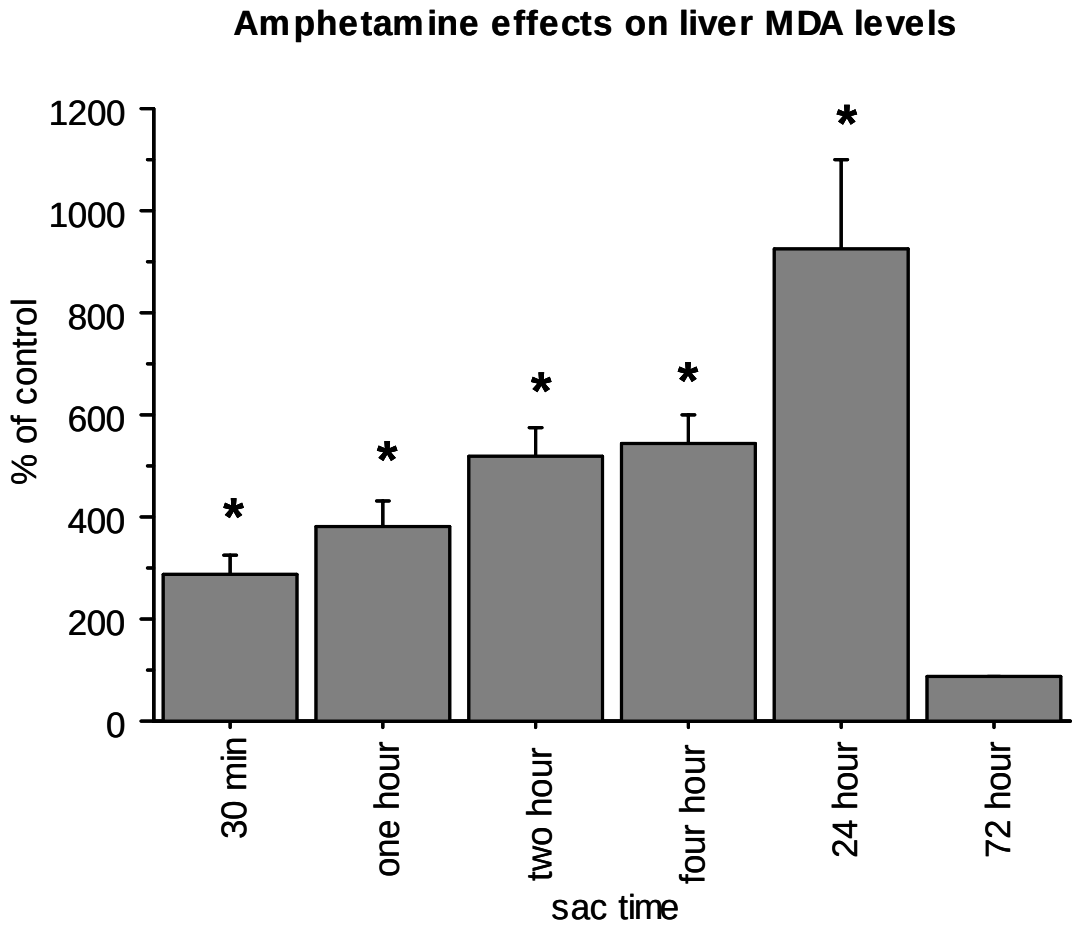


Figure 7: Levels of malondialdehyde expressed as percent of control in livers of male BALB/c mice treated with amphetamine

* denotes significantly different from saline controls; $p < .05$

Experiment 5: The effects of amphetamine on levels of malondialdehyde, a marker of oxidative stress, in the brain

Rationale

Malondialdehyde was found to peak in the liver at 24 hours after amphetamine administration in the timecourse completed in Experiement 4. Here, the levels of MDA in the striatum are determined at the same time point, 24 hours.

Animals

Adult male BALB/c mice (Jackson Laboratories; Bar Harbor, ME) were maintained according to the NIH Guide for the Care and Use of Laboratory Animals. Mice had access to food and water *ad libitum* for the duration of the study. Mice were housed in a temperature controlled colony room on a 12 hour on/12 hour off light cycle beginning at 8 AM. Animals were housed individually in hanging wire cages (20 cm x 10 cm x 12 cm).

Drug treatment

Mice received one subcutaneous injection of 50.0 mg/kg amphetamine or saline. Mice were sacrificed 24 hours after injection.

MDA analysis

Following sacrifice, bilateral striata were quickly dissected and stored in liquid nitrogen until assay. Tissue was pooled for analysis in order to have enough tissue to

assay. The striata from three to four animals was combined and homogenized in of .1M phosphate buffer and kept on ice. Samples were then analyzed as in Experiment 4.

Statistical Analyses

One-way ANOVAs were performed with MDA level as main factor and sacrifice time as between groups factor. Differences between groups were determined using Fisher's PLSD post-hoc tests.

Results

One-way ANOVA analysis revealed that MDA levels were significantly higher in amphetamine-treated animals compared to saline-treated controls ($F(1,3) = 31.043$, $p = .0114$). See figure 8.

Conclusions

As in the liver, MDA levels are significantly increased in the striatum at 24 hours post-amphetamine. This increase shows that amphetamine is capable of causing lipid peroxidation in the brain and supports the amphetamine model of Parkinson's disease, as lipid peroxidation is widely accepted to play a role in neuronal death in Parkinson's disease patients. Also, this increase of MDA corresponds to increased levels of peripheral MDA. Further studies, including a more extensive time course in both the liver and brain, are needed to determine if peripheral levels of lipid peroxidation and oxidative stress can be correlated to one another and predict later toxicity. Increased levels of lipid peroxidation, specifically MDA, have recently been found in the blood of Parkinson's patients. It is important to use this information to further characterize animal

models of the disease because these models enable researchers to observe and correlate effects in the brain that can only be seen in humans after death.

Figure 8

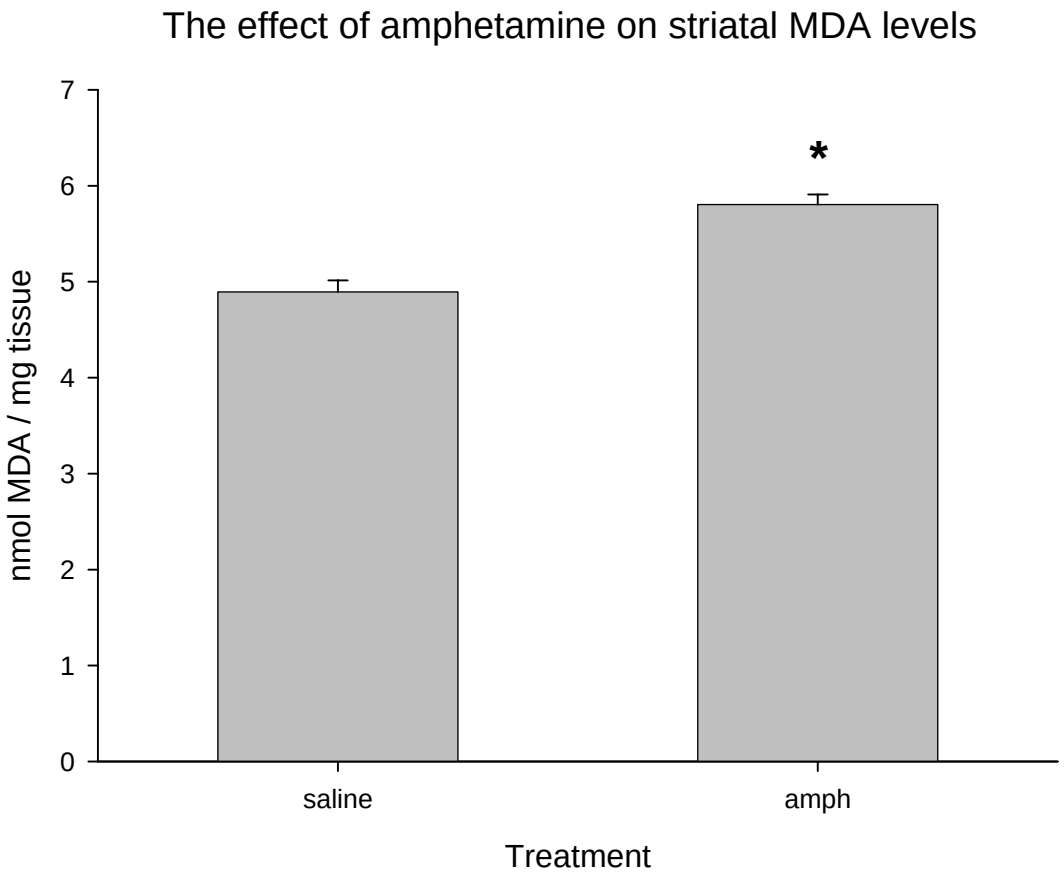


Figure 8: Levels of malondialdehyde in pooled samples of striata of male BALB/c mice treated with saline or amphetamine

* denotes significantly different from saline controls; $p < .05$

Discussion: Experiments 1 – 5

A single high dose of amphetamine was sufficient to cause an 84% depletion in dopamine of the striatum that persisted for up to two weeks. This depletion is comparable to the lesion typically observed in the most commonly used amphetamine model of Parkinson's disease (four injections of high dose of amphetamine) (Halladay et al., 2000; Carlson and Wagner, 2006). Furthermore, this single dose has a much lower rate of mortality than the four-injection regimen. On average, between zero and ten percent of mice died after one injection of 50 mg/kg of amphetamine. This is in contrast to an up to 30% mortality occurring with four injections of 18.5 mg/kg amphetamine.

In Parkinson's disease, there is a loss of dopaminergic cell bodies of the substantia nigra, striatal dopamine depletion, reduced tyrosine hydroxylase activity, depletion of dopamine transporter pumps, as well as increased dopamine turnover. The effects of this amphetamine model are consistent with the primary neurochemical endpoints observed in human Parkinson's disease. Long-term depletion of striatal dopamine was achieved in levels similar to Parkinson's disease. Moreover, dopamine turnover ratios were increased which suggests persistent reduced activity of tyrosine hydroxylase. Although effects on substantia nigra cell bodies and striatal transporter pumps were not measured in this dose regimen, they have been observed in the four-injection regimen. The present data are sufficient to draw the conclusion that single, high dose amphetamine administration serves as an effective and relevant mouse model of Parkinson's disease.

This dose of amphetamine was shown to cause an increase in MDA levels in the liver, indicating increased lipid peroxidation consequent to amphetamine-mediated

oxidative stress there. Levels of MDA increase up to at least 24 hours post-amphetamine but by 72 hours post-amphetamine have returned to control levels. Striatal levels of MDA were measured at 24 hours, the peak of liver MDA levels. Striatal MDA levels were significantly elevated at this time point, indicating that oxidative stress occurred in the brain as well. However, levels of striatal MDA were not as drastically elevated compared to controls as observed in the liver. This may be because the brain's lipid peroxidation mechanism is faster or slower than the livers or that the liver is more affected by lipid peroxidation after amphetamine treatment. The latter is unlikely since the brain is known to be especially sensitive to lipid peroxidation due to its high lipid concentration and low antioxidant enzyme levels, particularly compared to the liver. At 24 hours post-amphetamine, dopamine levels are observed at their lowest. Significantly increased levels of liver and striatal MDA both occur and correspond to dopamine depletion in the striatum 24 hours after amphetamine administration. It is not known whether increased levels of liver MDA can predict later neurotoxicity but in this model, increased levels of liver MDA corresponded to increased striatal MDA as well as long-lasting dopamine depletion. If they are in fact interrelated, this would serve as a model of a toxicant that causes a systemic increase in oxidative stress while preferentially targeting the striatum and having long-lasting effects only in the brain.

III. ANTIOXIDANT DEFENSES

Components of the antioxidant defenses in the substantia nigra of the Parkinsonian brain, such as glutathione peroxidase, ascorbic acid, α-tocopherol and catalase levels appear normal. However, a study on brains of Parkinson's disease patients showed substantia nigra levels of both superoxide dismutases (Mn-SOD and Cu/ZnSOD) are elevated while the level of reduced glutathione (GSH) is low (Sian et al., 1994a). This combination would mean the delicate balance of pro-oxidants and antioxidants is disturbed in the Parkinsonian brain and could be a key to part of its mechanism. What is not known from these data is whether this perturbation of antioxidant balance is a cause of neuronal loss in the substantia nigra or is a result of the neuronal loss.

Compounds like vitamins C and E, green tea, grape seed extract and curcumin have been touted as having potent antioxidant properties that will help prevent or slow the progression of neurodegenerative diseases. These compounds scavenge free radicals and, given that Parkinson's disease is at least in part mediated by oxidative stress, can have a positive impact on individuals at high risk for Parkinson's disease (familial history of PD, brain trauma, exposure to herbicides and pesticides) (Prasad et al., 1999).

Ascorbic acid (vitamin C) is a common water-soluble antioxidant. It is a required co-factor for many biological processes and enzymes (including dopamine-β-hydroxylase) and is rapidly distributed to all of the body's tissues (Halliwell, 2001; Kohen and Nyska, 2002). It is important in humans as an essential vitamin and as a scavenger of reactive oxygen species as it can donate two of its electrons. After the first

electron is donated, an ascorbate radical molecule is left over. This radical, which can continue scavenging and donate another electron, is relatively stable. This property makes it a very powerful antioxidant in the body. Importantly, it can also directly inhibit the lipid peroxidation process and scavenge hypochlorous acid and peroxynitrous acid. On the other hand, ascorbic acid can also be a reducing agent and interact with iron to form hydroxyl radicals. Paradoxically, it follows that if ascorbic acid is present under the appropriate circumstances (in an environment with an abundance of iron or other transitional metals) it can actually serve as a pro-oxidant and thus play a role in lipid peroxidation (Halliwell, 2001; Kohen and Nyska, 2002).

α-Tocopherol, commonly known as vitamin E, is also an essential vitamin and antioxidant. All of the tocopherols, vitamin E included, have the ability to scavenge peroxyl radicals (Halliwell, 2001) and thereby hinder lipid peroxidation via the following basic equation:

α-tocopherol + $ROO^{\bullet} \rightarrow$ α-tocopherol + ROOH

During such scavenging, a tocopherolquinone is formed (Kohen and Nyska, 2002) which has a much reduced ability to propagate lipid peroxidation (Halliwell, 2001). This radical can then be recycled to its active form as seen above (Kohen and Nyska, 2002). Generally, vitamin E helps to stabilize biological membranes as it is incorporated into lipoproteins (Halliwell, 2001), but like ascorbic acid, it too can become a pro-oxidant in environments where an abundance of transition metals are present (Kohen and Nyska, 2002). Interestingly, vitamin E consumption was found to be significantly lower in patients with Parkinson's disease in a large community-based study in the Netherlands (de Rijk et al., 1997).

While vitamin E is generally only lipid soluble, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a water-soluble derivative of vitamin E. Trolox has been shown to be a scavenger of peroxyl radicals, protecting against lipid peroxidation (Castle and Perkins, 1986). Vitamin E is slowly absorbed throughout the body (Halliwell, 2001) while Trolox is able to rapidly penetrate biological membranes and as such absorbed and bioavailable faster than vitamin E.

In a previous study, De Vito and Wagner (1989) had shown that both ascorbic acid (vitamin C) and α-tocopherol (vitamin E) each attenuated methamphetamine-induced dopamine depletion in the rat brain. These animals were pretreated with the antioxidants prior to methamphetamine treatment. Furthermore, in the Wagner and De Vito (1989) study, these two antioxidants were used because of their difference in mechanisms. This was to ensure that the protection observed could be attributed only to their antioxidant properties rather than the chance that either of them actually acted by altering the metabolism of methamphetamine or amphetamine (De Vito and Wagner, 1989).

Neuroinflammation in Parkinson's disease

In addition to oxidative stress, neuroinflammation has been linked to neurodegeneration in humans and animal models (Hirsch et al., 1998; Schiess, 2003; Teismann et al., 2003) and is proposed to be a possible mechanism for Parkinson's disease (McGeer et al., 2001). There is evidence that cell death occurring in the brains of Parkinson's disease patients is caused by neuroinflammation. Neuropathological study of brains from humans and monkeys exposed to MPTP showed inflammatory reactions

several years after exposure (Langston et al., 1999; McGeer et al., 2003). In addition, non-steroidal anti-inflammatory drugs (NSAIDs) have been protective in animal models of Parkinson's disease. Furthermore, there is epidemiological evidence shows that Parkinson's disease risk is lower in individuals who take NSAIDS including ibuprofen and aspirin (Chen et al., 2005; Wahner et al., 2007).

NSAIDs act by inhibiting the proinflammatory cyclooxygenase (COX) enzymes which then lead to a reduction in inflammatory prostaglandins. COX-2, one of the two COX enzymes, has been previously implicated in Parkinson's disease and is upregulated in the dopaminergic neurons of both Parkinson's patients and mice in Parkinson's disease models (Teismann and Ferger, 2001; Hartmann et al., 2003). Kita et al. (2000) showed that COX-2 protein expression was significantly increased in the striatum 72 hours after treatment with methamphetamine. This increase in COX-2 induction corresponded to a significant loss of dopamine and an increase of thiobarbituric acid, used as a measure of oxidative damage, in the striatum of methamphetamine-treated mice (Kita et al., 2000).

Furthermore, NSAIDs might also protect against reactive oxygen species during periods of oxidative stress (Ton et al., 2006). The COX enzymes catalyze the reaction changing arachidonic acid to prostaglandin and thromboxanes. Oxidative stress from reactive oxygen species are believed to come from the peroxidase step in this COX reaction (Chan and Fishman, 1980; Armstead et al., 1988; Tsai et al., 1994). Therefore, it follows that a COX inhibitor may be able to reduce oxidative stress levels in the brain and thus protect it from cell damage and loss following exposure to an ROS causing compound.

The following studies examine the protective effects of antioxidants and an anti-inflammatory drug on amphetamine-induced dopamine depletion.

Hypothesis: Pretreatment using antioxidant and anti-inflammatory agents will protect against amphetamine-induced dopamine depletion in the striatum

Experiment 6: The effects of ascorbic acid on amphetamine toxicity

Rationale

Pretreatment with ascorbic acid has been shown to attenuate methamphetamine-induced dopamine depletion in the rat striatum. It is used here in the mouse both before and after amphetamine administration to determine its efficacy in this model. The pretreatment and post-treatment of ascorbic acid was used as an attempt to pre-load the brain's antioxidant levels and aid in amphetamine-induced oxidative stress, respectively.

Animals

Adult male BALB/c mice (Jackson Laboratories; Bar Harbor, ME) were maintained according to the NIH Guide for the Care and Use of Laboratory Animals. Mice had access to food and water *ad libitum* for the duration of the study. Mice were housed in a temperature controlled colony room on a 12 hour on/12 hour off light cycle beginning at 8 AM. Animals were housed individually in hanging wire cages (20 cm x 10 cm x 12 cm).

Drug treatment

Mice received one subcutaneous injection of 50 mg/kg amphetamine or saline and two intraperitoneal injections of 333 mg/kg ascorbic acid or saline. Both amphetamine and ascorbic acid were dissolved in saline. Animals were sacrificed 1 day, 3 days, 2 weeks and 4 weeks after injections.

Neurochemical Analysis

Following sacrifice, brains were quickly removed and bilateral striata were immediately dissected and stored in liquid nitrogen and assayed as in Experiment 1.

Statistical Analyses

Two-way ANOVAs were performed with the neurotransmitter concentration or turnover ratios as main factors and antioxidant treatment and drug challenge as between groups factors. For all appropriate analyses, differences between groups were determined using Fisher's PLSD post-hoc tests.

Experimental design

Mice were divided into 4 groups (antioxidant treatment/drug challenge) per sacrifice time: saline/saline; ascorbic acid (VC)/saline; saline/amphetamine (AMPH); VC/AMPH. Ascorbic acid or saline was administered 30 minutes before and 60 minutes after amphetamine or saline drug challenge:

- 30 min	0 min	30 min	60 min
saline	saline	saline	saline
VC	saline	VC	VC
saline	AMPH	saline	saline
VC	AMPH	VC	VC

Mice from each group were sacrificed at either 1 day, 3 days, 2 weeks or 4 weeks after injections.

Results

Saline/saline and VC/saline groups from each sacrifice time had statistically similar neurochemistry and were combined for analysis. Overall, time after amphetamine administration (referred to as "sacrifice time") had a significant effect on all neurotransmitters and metabolites measured (DA: $F(8,52) = 6.480$, $p < .0001$); DOPAC $F(8,52) = 6.803$, $p < .0001$; HVA: $F(8,52) = 4.829$, $p = .0002$; 5-HT: $F(8,52) = 2.259$, $p = .0374$; 5-HIAA: $F(8,52) = 2.378$, $p = .0290$). See figure 9. *Post hoc* analysis revealed specific significant differences between treatment groups and sacrifice times. All amphetamine-treated groups, regardless of ascorbic acid treatment, had significantly less dopamine compared to controls except VC/AMPH animals sacrificed 4 weeks post-amphetamine ($p = .0789$). However, treatment with ascorbic acid significantly attenuated dopamine loss at the 3 day sacrifice compared to animals without ascorbic acid treatment at the same time point ($p = .0423$). Dopamine levels at the 4 week sacrifice time point in ascorbic acid-treated mice approached, but did not reach, significance compared to those not receiving antioxidant treatment ($p = .0539$). The average level of dopamine in all ascorbic acid-treated animals was higher than those not receiving antioxidant treatment, although not significantly.

All amphetamine-treated groups, regardless of ascorbic acid treatment, had significantly less DOPAC compared to controls. Treatment with VC/AMPH and sacrifice at 3 days post-amphetamine produced significantly higher levels of DOPAC compared to saline/AMPH treated animals at the same time point ($p = .0137$). The difference in DOPAC levels between VC/AMPH and saline/AMPH at 1 day approached

significance (p = .0793). The average level of DOPAC in all other ascorbic acid-treated animals was higher that those not receiving ascorbic acid, although not significantly.

Except for mice sacrificed at two weeks, all mice (regardless of ascorbic acid) had levels of HVA that were statistically similar to control levels (1 day saline/AMPH; 1 day VC/AMPH; 3 day VC/AMPH; 4 week saline/AMPH; 4 week VC/AMPH). No significant difference in HVA was found between saline/AMPH and VC/AMPH groups at each time point.

Levels of 5-HT were only significantly different between mice sacrificed two weeks post-amphetamine and controls. No significant difference in 5-HT was found between saline/AMPH and VC/AMPH groups at any time point.

Only one day saline/AMPH and VC/AMPH groups were significantly different from control levels of 5-HIAA. No significant difference in 5-HIAA was found between saline/AMPH and VC/AMPH groups at each time point.

Analysis of turnover ratios showed an overall significant difference in all turnover ratios (DOPAC/DA: ($F(8,52) = 3.183$, $p = .0052$; HVA/DA: ($F(8,52) = 3.533$, $p = .0025$; 5-HIAA/5-HT: $F(8,52) = 4.527$, $p = .0003$). See figure 10. *Post hoc* analysis of DOPAC/DA turnover ratio showed that controls had significantly lower turnover ratios compared to three days ($p = .0008$), three days + VC ($p = .0187$), two weeks + VC ($p = .0125$), four weeks ($p = .0493$). *Post hoc* analysis also showed that DOPAC/DA was significantly different between one day and three days ($p = .0083$), one day and four weeks ($p = .0493$), three days and four weeks + VC ($p = .0012$), three days + VC and four weeks + VC ($p = .0241$), two weeks + VC and four weeks + VC ($p = .0171$) and four weeks and four weeks + VC ($p = .0139$). *Post hoc* analysis of HVA/DA turnover ratio

showed that controls had significantly lower turnover ratios compared to one day (p = .0345), one day + VC (p = .0006), three days (p = .0001), two weeks plus VC (p = .0027), four weeks (p = .0137). *Post hoc* analysis also showed that HVA/DA was significantly different between one day + VC and four weeks + VC (p = .0178), three days and three days + VC (p = .0239) and three days and four weeks plus VC (p = .0039). *Post hoc* analysis of 5-HIAA/5-HT turnover ratio showed that controls had significantly lower turnover ratios compared to one day (p = .0026) and one day + VC (p = .0003). *Post hoc* analysis also showed that 5-HIAA/5-HT was significantly different between one day and three days (p = .0183), one day and two weeks (p = .0012), one day and two weeks + VC (p = .0016), one day and four weeks (p = .0289), one day and four weeks + VC (p = .0008), one day + VC and three days (p = .0054),

One-way ANOVA analysis of controls versus mice sacrificed four weeks post-amphetamine without ascorbic acid treatment revealed significant results as well. See figure 11. This is an extension of the time course completed in Experiment 2. Dopamine and DOPAC were significantly higher in controls compared to 4 week post-amphetamine animals (DA: $F(1,15) = 13.399$, $p = .0023$; DOPAC: ($F(1,15) = 11.769$, $p = .0037$). Mice receiving amphetamine had 53% depletion in dopamine after four weeks compared to controls. There was no significant difference in HVA, 5-HT or 5-HIAA.

Analysis of turnover ratios with one-way ANOVA also revealed significant results. DOPAC/DA and HVA/DA turnover ratios were both significantly higher in amphetamine-treated mice compared to controls at four weeks (DOPAC/DA: $F(1,15) = 5.894$, $p = .0283$; HVA/DA: ($F(1,15) = 13.934$, $p = .0020$). Serotonin turnover ratio was not significantly affected by amphetamine after four weeks. See figure 12.

Conclusions

In general, the data show that ascorbic acid attenuated dopamine depletion in amphetamine-treated animals. At one day, three days and four weeks post-amphetamine, dopamine levels were higher in mice receiving ascorbic acid, significantly so in the three day and nearly significantly in the four week group. At four weeks, amphetamine-treated animals not receiving ascorbic acid, had significantly lower levels of dopamine compared to controls. This suggests that the loss of dopamine caused by amphetamine treatment was permanent as the low levels persisted for up to four weeks. However, amphetamine-treated mice that also received ascorbic acid, did not have dopamine levels significantly different from those of control mice. In fact, they were only 22% lower than controls, compared to 53% lower levels in mice not receiving ascorbic acid treatment. This suggests that, in the long term, treatment with ascorbic acid can help preserve the levels of dopamine in the striatum after treatment with amphetamine.

Figure 9

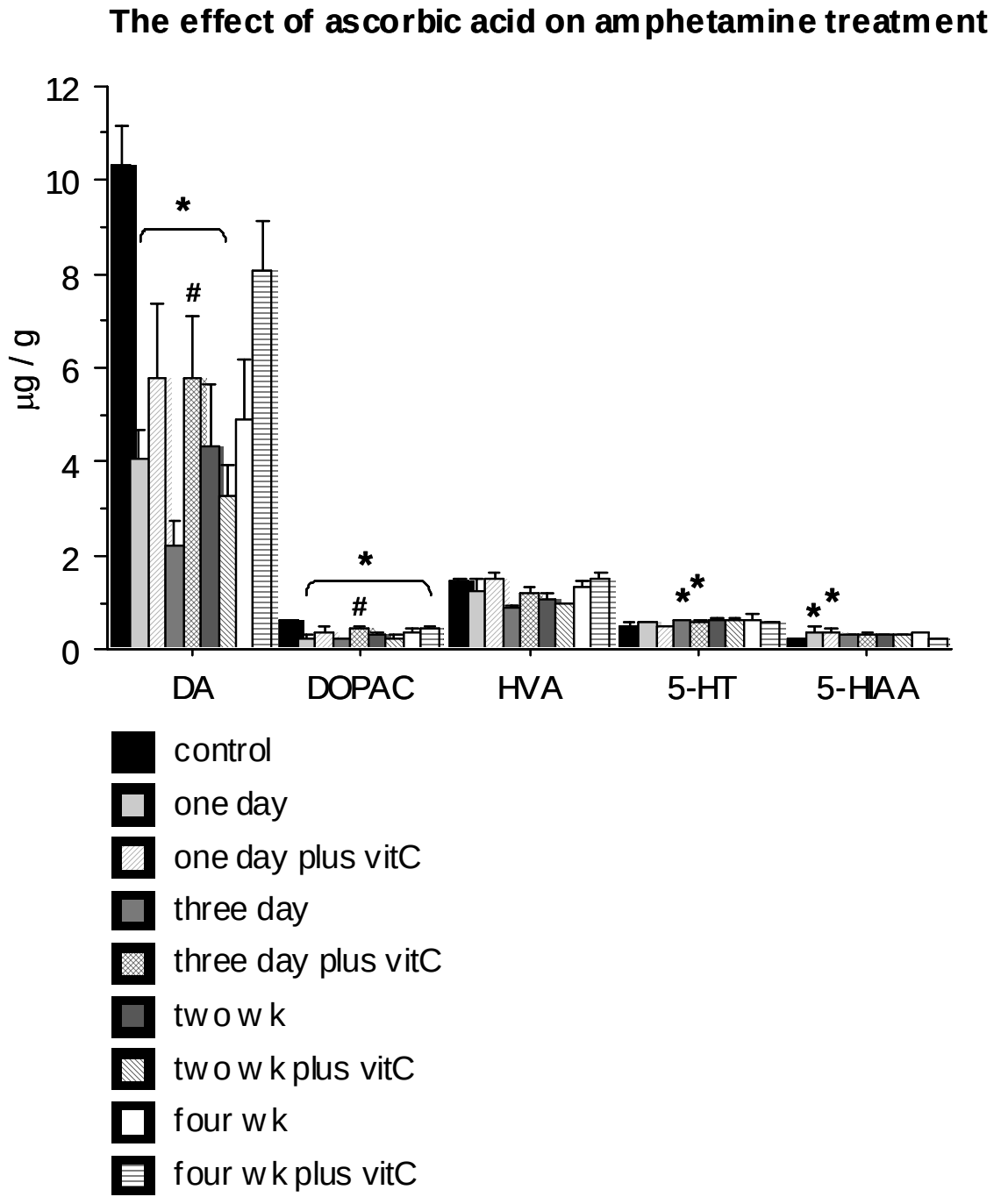

The effect of ascorbic acid on amphetamine treatment
µg / g
12
10
8
6
4
2
0
*
#
*
#
*
*
DA
DOPAC
HVA
5-HT
5-HIAA
control
one day
one day plus vitC
three day
three day plus vitC
two wk
two wk plus vitC
four wk
four wk plus vitC

Figure 9: Concentration of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in striatum of male BALB/c for controls and

* denotes significantly different from control; $p < .05$

# denotes significantly different from saline/AMPH at the same time point (3 days post-AMPH); $p < .05$

Figure 10

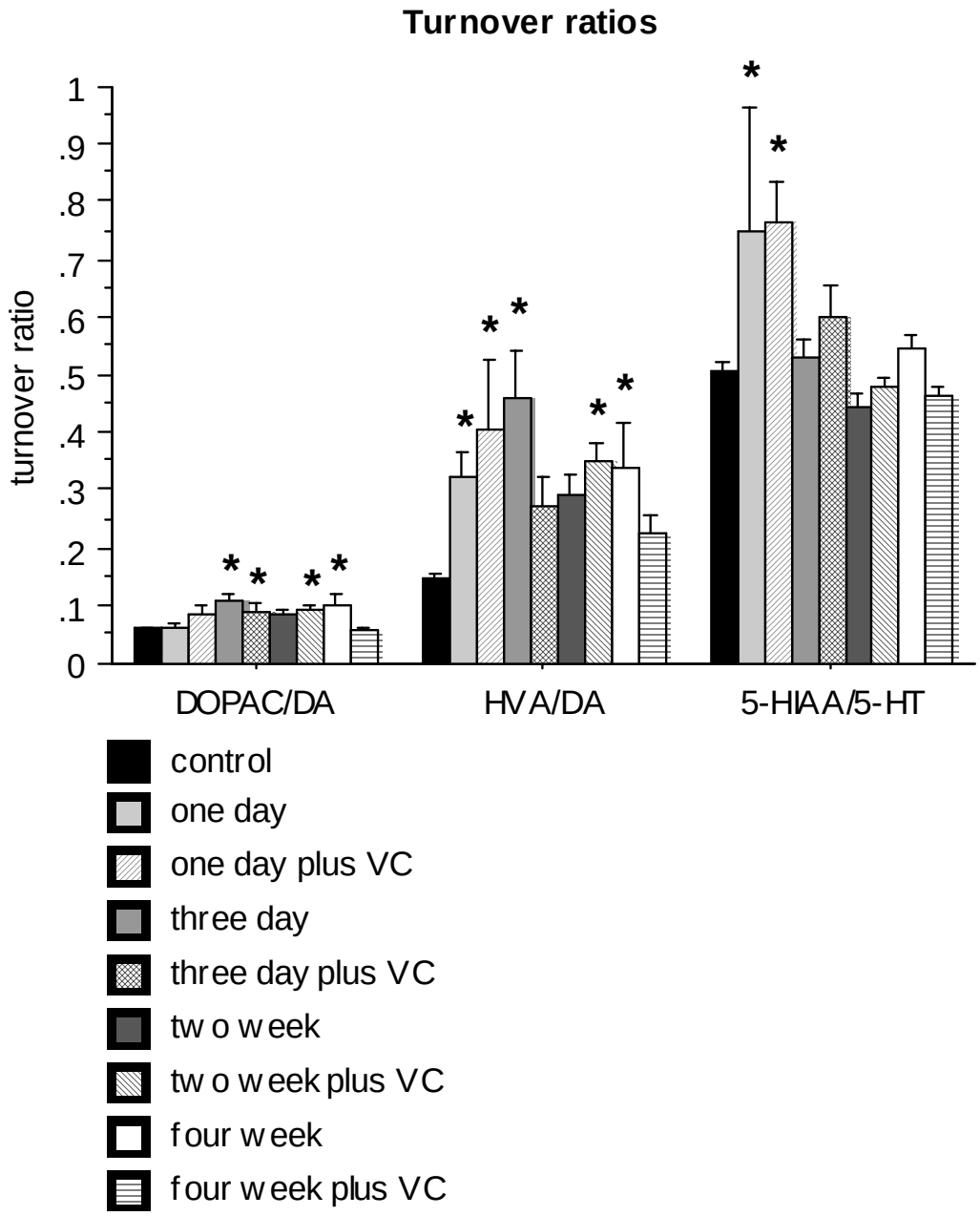


Figure 10: Turnover ratios of DOPAC/DA, HVA/DA and 5-HIAA/5-HT in striatum of male BALB/c for control, sal/AMPH and VC/AMPH treated groups

* denotes significantly different from control; $p < .05$

Figure 11

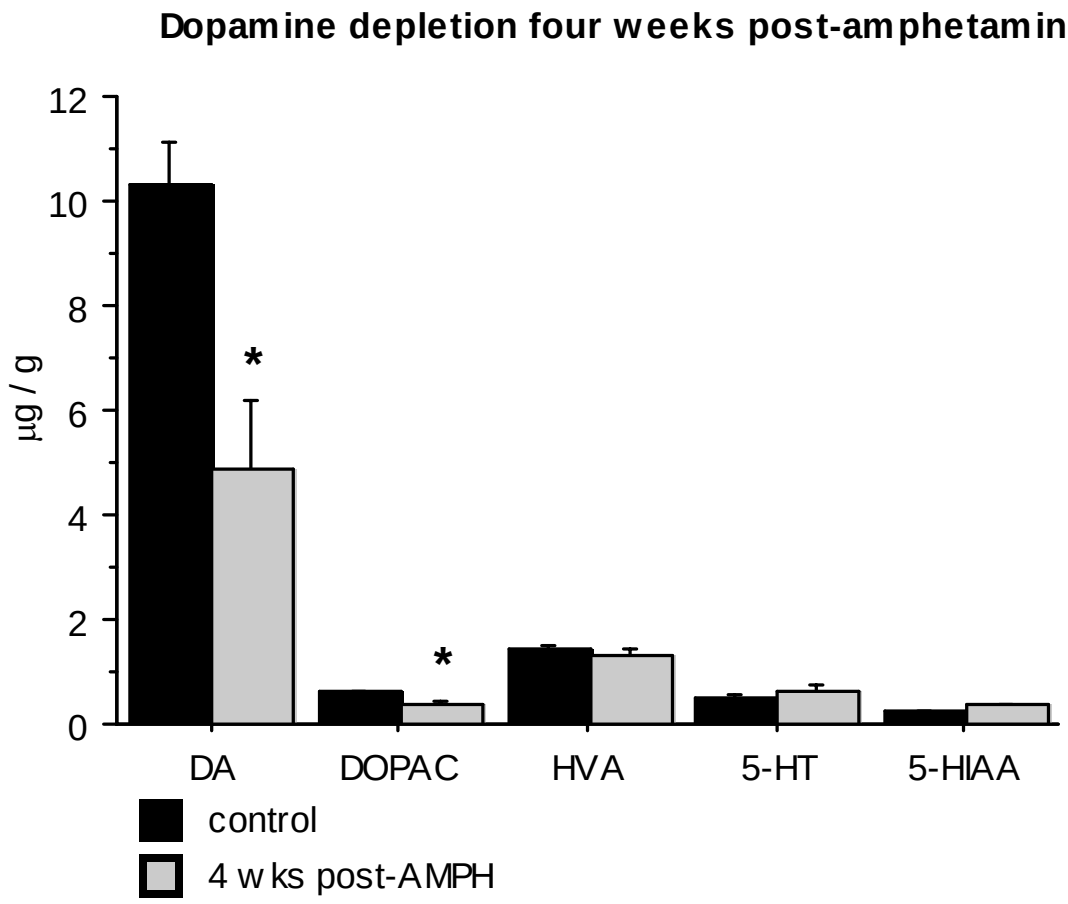


Figure 11: Concentration of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in striatum of male BALB/c for saline (control) or amphetamine (4 wks post-AMPH)

* denotes significantly different from control; $p < .05$

Figure 12

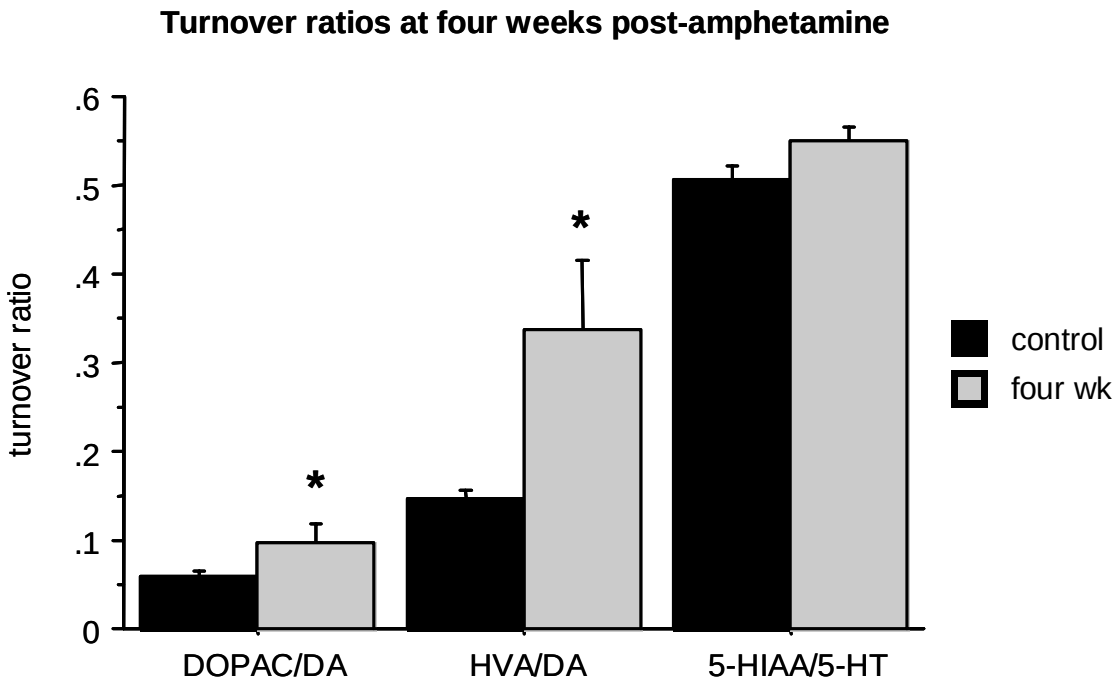


Figure 12: Turnover ratios of DOPAC/DA, HVA/DA and 5-HIAA/5-HT in striatum of male BALB/c for saline (control) or amphetamine (four wk)

* denotes significantly different from control; $p < .05$

Experiment 7: The effects of Trolox, a water-soluble derivative a vitamin E, on amphetamine toxicity

Rationale

Vitamin E has been shown to be an effective antioxidant against amphetamine toxicity previously. Trolox is a water-soluble derivative of vitamin E, making it easier to administer, more bioavailable and faster acting than lipid-soluble vitamin E.

Animals

Adult male BALB/c mice (Jackson Laboratories; Bar Harbor, ME) were maintained according to the NIH Guide for the Care and Use of Laboratory Animals. Mice had access to food and water *ad libitum* for the duration of the study. Mice were housed in a temperature controlled colony room on a 12 hour on/12 hour off light cycle beginning at 8 AM. Animals were housed individually in hanging wire cages (20 cm x 10 cm x 12 cm).

Drug treatment

Mice received one subcutaneous injection of 50 mg/kg amphetamine or saline. Mice were also given three subcutaneous injections of 2.5 mg/kg Trolox or vehicle (saline containing 1% ethanol; abbreviated "veh"). Mice were sacrificed 72 hours after injections.

Body Temperature

Body temperature was monitored using a rectal probe coupled to a BAT-10 thermometer (Physitemp, Clinton, NJ) at baseline (BT_0) and every 60 minutes at all determined time points (BT_1, BT_2, BT_3).

Neurochemical Analysis

Following sacrifice, brains were quickly removed and bilateral striata were immediately dissected and stored in liquid nitrogen and assayed as in Experiment 1.

Statistical Analyses

Two-way ANOVAs were performed with the neurotransmitter concentration or turnover ratios as main factors and antioxidant treatment and drug challenge as between groups factors. For all appropriate analyses, differences between groups were determined using Fisher's PLSD post-hoc tests.

Experimental design

Mice were divided into 4 groups (antioxidant treatment/drug challenge) per sacrifice time: veh/saline; Trolox/saline; veh/AMPH; Trolox/AMPH. Trolox or vehicle was administered 60 minutes prior to amphetamine or saline injection as well as 60 and 120 minutes after amphetamine or saline injection. Body temperature was taken just before the first injection (Trolox or vehicle) and 30 minutes after every subsequent injection. Mice were sacrificed 72 hours after amphetamine or saline injections. Injections and body temperature readings were taken as follows:

- 60 min	0 min	30 min	60 min	90 min	120 min	150 min
BT_0/vehicle	saline	BT_1	vehicle	BT_2	vehicle	BT_3
BT_0/Trolox	saline	BT_1	Trolox	BT_2	Trolox	BT_3
BT_0/vehicle	AMPH	BT_1	vehicle	BT_2	vehicle	BT_3
BT_0/Trolox	AMPH	BT_1	Trolox	BT_2	Trolox	BT_3

Results

Neurochemical analysis showed that amphetamine/vehicle-treated mice and amphetamine/Trolox-treated mice had 65% and 66% depletion of dopamine compared to saline-treated mice, respectively. These data show that treatment with Trolox did not attenuate amphetamine-induced dopamine depletion in the striatum. See figure 13.

Two-way ANOVA revealed a significant effect on DA, DOPAC, HVA and 5-HIAA levels with drug challenge only (DA: $F(1,20) = 65.791$, $p < .0001$; DOPAC: $F(1,20) = 57.627$, $p < .0001$; HVA: $F(1,20) = 32.747$, $p < .0001$; 5-HIAA: $F(1,20) = 5.198$, $p = .0337$) and showed no significant effect from Trolox treatment or the interaction of drug challenge and Trolox (drug challenge by Trolox) in any neurotransmitter or metabolite. DA, DOPAC, HVA levels were all lower in amphetamine-treated mice compared to saline-treated mice. Levels of 5-HT and 5-HIAA were similar between amphetamine- and saline-treated groups. Analysis of saline/saline-treated mice and saline/vehicle-treated mice showed no difference in any neurotransmitter or metabolite tested.

Post hoc analysis revealed a significant difference in 5-HT between vehicle and Trolox-treated groups ($p = .0497$) and saline and amphetamine-treated groups ($p = .0496$)

Two-way ANOVA analysis of turnover ratios showed a significant effect of drug treatment on HVA/DA only ($F(1,20) = 6.682$, $p = .0177$). See figure 14.

Two-way repeated measures ANOVA analysis on body temperature showed a significant effect of amphetamine ($F(1,23) = 47.348$, $p < .0001$), a significant difference in body temperature ($F(4,92) = 20.024$, $p < .0001$) and a significant interaction of body temperature and drug challenge ($F(4,92) = 29.528$, $p < .0001$). Overall, amphetamine increased body temperature as expected and Trolox did not attenuate or exacerbate this effect. See figure 15.

Conclusions

Trolox did not attenuate dopamine loss against amphetamine challenge. Metabolites DOPAC and HVA were also similarly affected by amphetamine whether treated with Trolox or vehicle. Amphetamine did not have an effect on serotonin or 5-HIAA levels although *post hoc* analysis revealed a significant effect of Trolox in amphetamine-treated mice on serotonin.

Although more water-soluble than vitamin E, Trolox did not go immediately into solution and had to be supplemented with ethanol. It is possible that it did not distribute throughout the tissues quickly enough in this dosing schedule to have proper antioxidant effects in amphetamine-treated mice. In addition, levels of Trolox in tissues were not determined so it is unknown where and at what concentrations Trolox was distributed in the body. Trolox has been shown to be protective in models of other diseases and vitamin E has been shown to be protective against methamphetamine-induced dopamine depletion. As such, the timeline of dosage with Trolox may need to be extended further

prior to amphetamine administration in order to achieve the desired protective effect. Tissue levels of Trolox would also be useful to determine distribution and at what concentration Trolox is found in the brain after systemic administration.

Figure 13

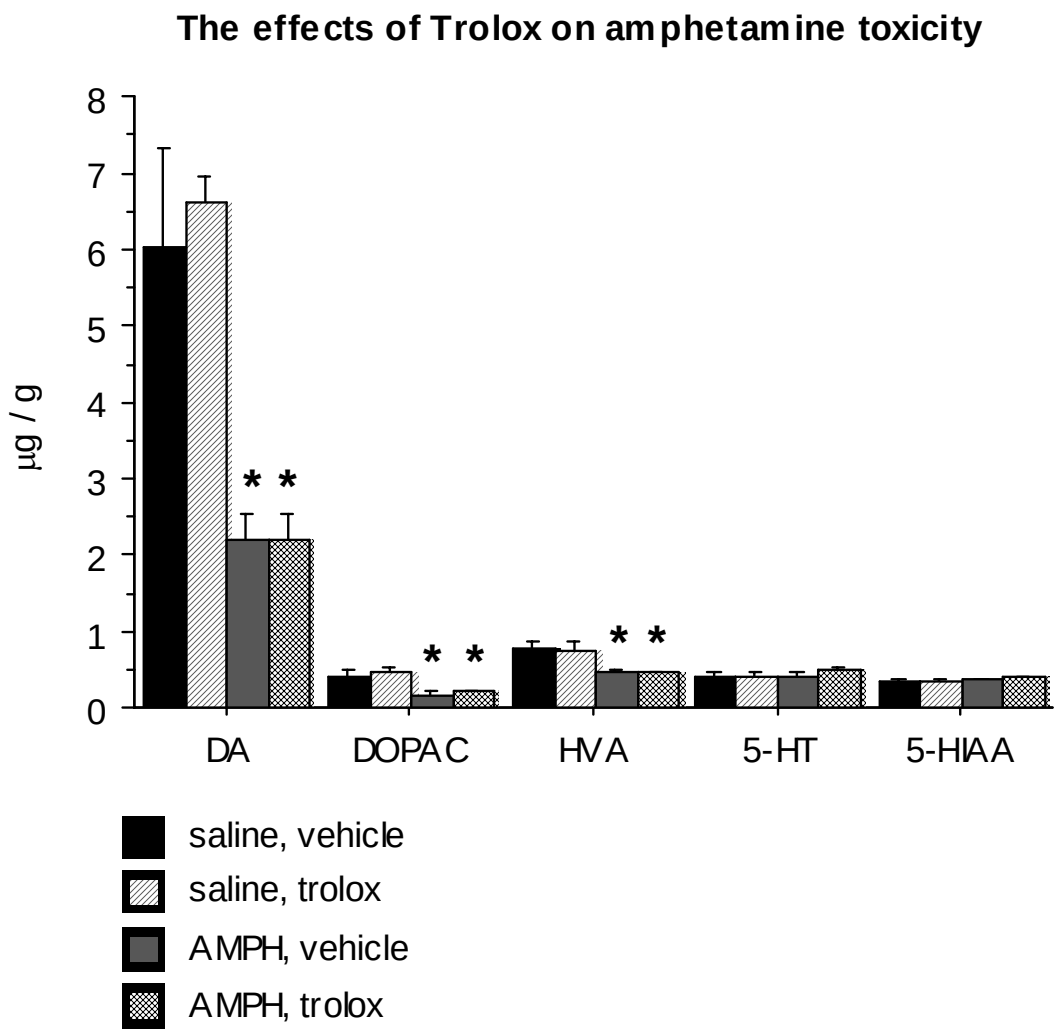


Figure 13: Concentration of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in striatum of male BALB/c for saline/vehicle, saline/Trolox, amphetamine/vehicle (AMPH,vehicle) or amphetamine/Trolox (AMPH,trolox). DA, DOPAC, HVA and 5-HIAA in saline-treated groups (saline/vehicle and saline/Trolox) were significantly different than amphetamine treated groups (AMPH/vehicle and AMPH/Trolox)

* denotes significantly different from appropriate control (either saline/vehicle control or saline/Trolox control); $p < .05$

Figure 14

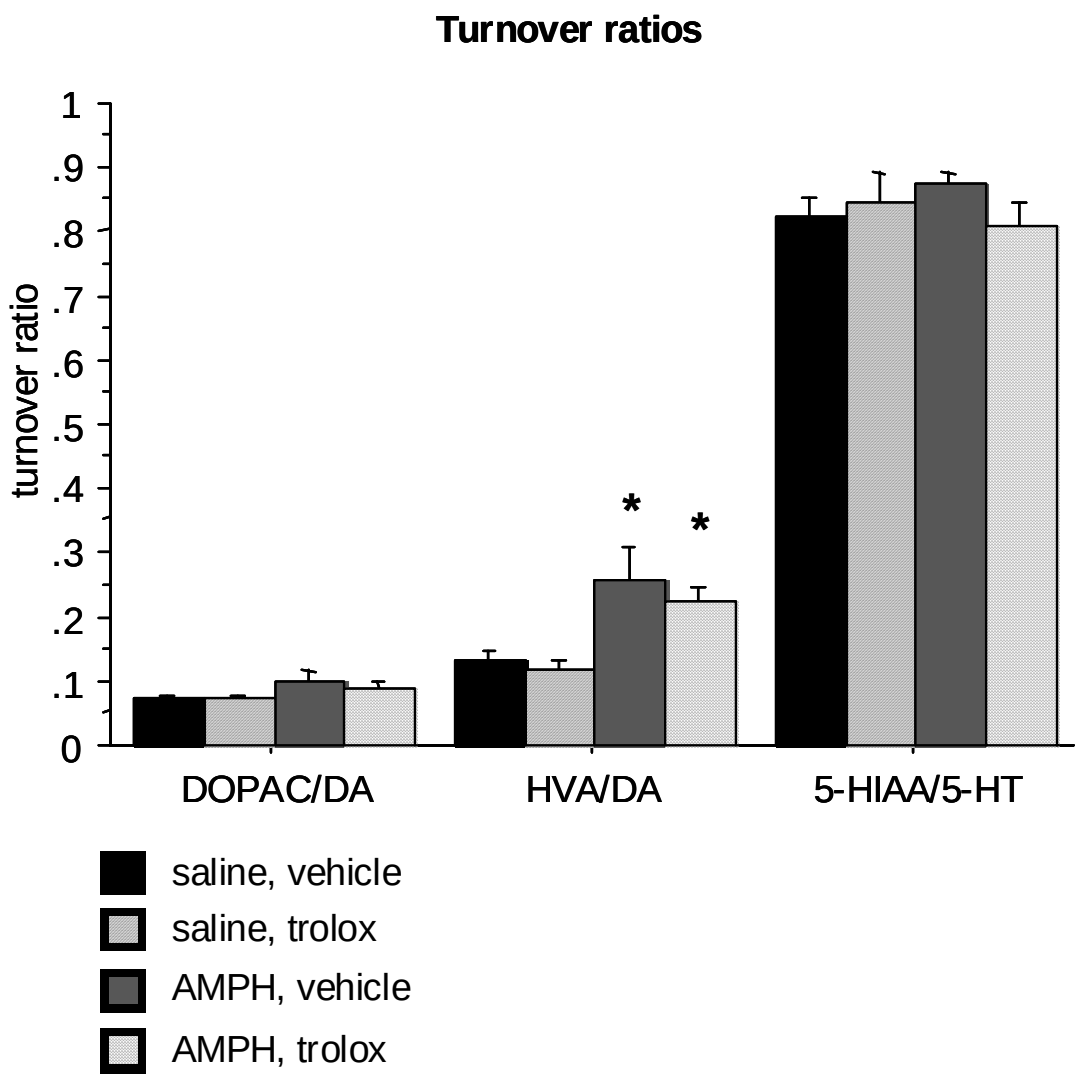


Figure 14: Turnover ratios of DOPAC/DA, HVA/DA and 5-HIAA/5-HT in striatum of male BALB/c for saline/vehicle, saline/Trolox, amphetamine/vehicle (AMPH,vehicle) or amphetamine/Trolox (AMPH,Trolox). There was a significant effect of drug treatment (saline or amphetamine) on HVA/DA turnover ratio.

* denotes significantly different from appropriate control (saline, vehicle or saline, Trolox); $p < .05$

Figure 15

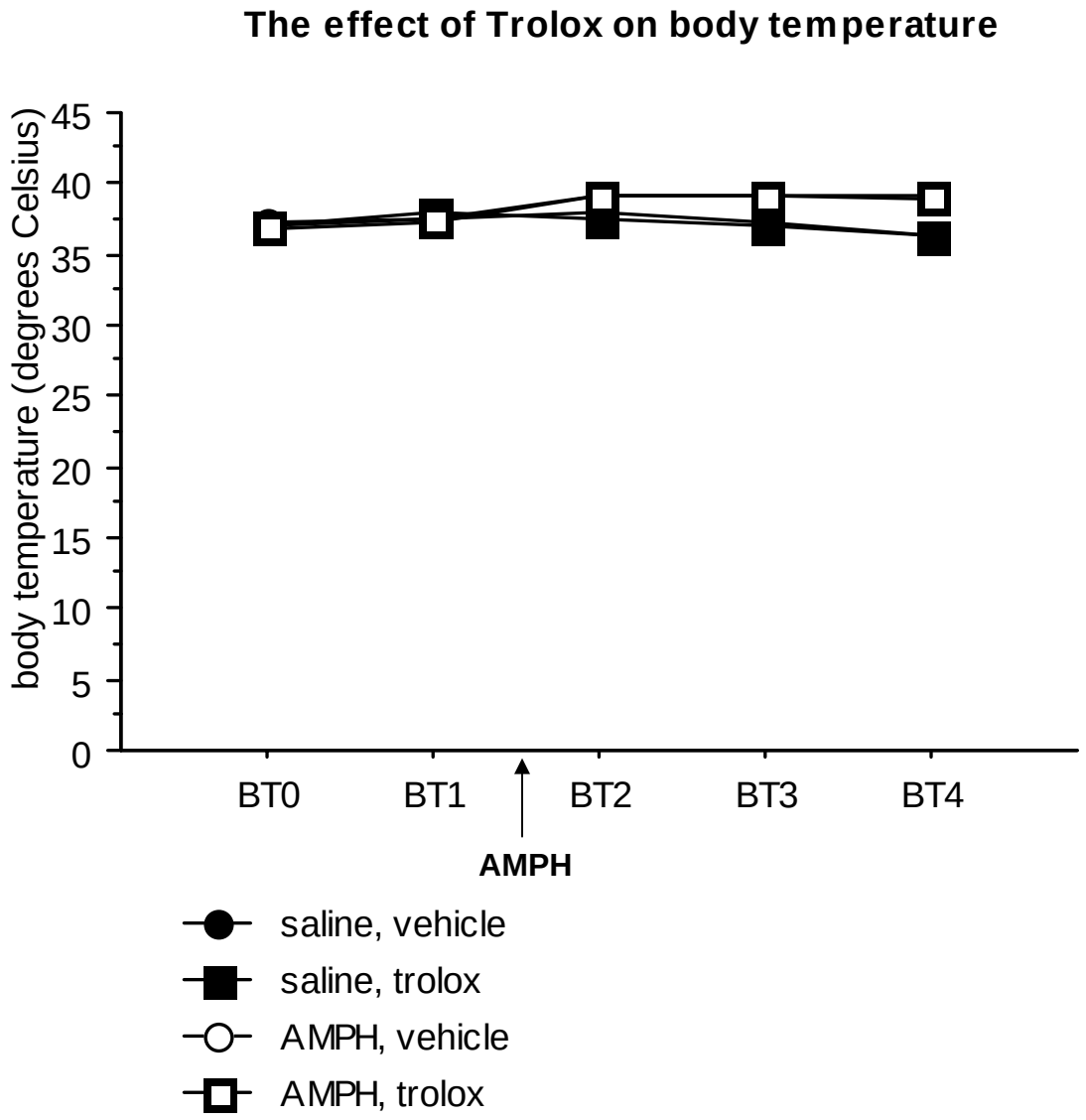


Figure 15: Body temperature (degrees Celsius) at baseline (BT0) and at 30, 90, 150 and 210 minutes. Amphetamine or saline was administered halfway between BT1 and BT2. Body temperature changed significantly over testing period ($p < .0001$). There was an overall significant effect of drug treatment (saline or amphetamine; $p < .0001$) and a significant interaction of body temperature and drug treatment ($p < .0001$). Treatment with Trolox had no significant effect on body temperature.

Experiment 7: The effects of an anti-inflammatory drug, ibuprofen, on amphetamine-induced dopamine depletion

Rationale

NSAIDS, including ibuprofen (iso-butyl-propanoic-phenolic acid), have potent anti-inflammatory actions and use of these drugs is related to decreased risk of Parkinson's disease. Also, ibuprofen has been previously shown to be effective in other animal models of Parkinson's disease. Ibuprofen is a non-steroidal anti-inflammatory drug that is a COX-1 and COX-2 inhibitor and may reduce the levels of oxidative stress occurring after amphetamine administration, sparing dopamine levels in the striatum.

Animals

Adult male BALB/c mice (Jackson Laboratories; Bar Harbor, ME) were maintained according to the NIH Guide for the Care and Use of Laboratory Animals. Mice had access to food and water *ad libitum* for the duration of the study. Mice were housed in a temperature controlled colony room on a 12 hour on/12 hour off light cycle beginning at 8 AM. Animals were housed individually in hanging wire cages (20 cm x 10 cm x 12 cm).

Drug treatment

Animals received one subcutaneous injection of 50 mg/kg amphetamine and two injections of 20 mg/kg ibuprofen (IBF) or saline: one 30 minutes before the amphetamine injection and one 30 minutes after the amphetamine injection. Both amphetamine and

ibuprofen were dissolved in saline. Animal were sacrificed 72 hours post-amphetamine injection.

Body Temperature

Body temperature was monitored using a rectal probe coupled to a BAT-10 thermometer (Physitemp, Clinton, NJ) at baseline (BT_0) and every 30 minutes at all determined time points (BT_1, BT_2, BT_3).

Neurochemical Analysis

Following sacrifice, brains were quickly removed and bilateral striata were immediately dissected, stored in liquid nitrogen and assayed as in Experiment 1.

Statistical Analyses

Two-way ANOVAs were performed with the neurotransmitter concentration or turnover ratios as main factors and NSAID treatment and drug challenge as between groups factors. Analysis of body temperature was performed by repeated measures ANOVA. For all appropriate analyses, differences between groups were determined using Fisher's PLSD post-hoc tests.

Experimental design

Mice were divided into two groups: saline/AMPH and IBF/AMPH. Body temperature was recorded at start of experiment (BT_0; prior to first injection of IBF or saline), 30 minutes (BT_1; just before amphetamine), 60 minutes (BT_2; just before second

IBF or saline injection) and 90 minutes (BT_3, 30 minutes after second and final IBF or saline injection). Mice were sacrificed 72 hours following administration of amphetamine. Drug treatment and body temperature readings were as follows:

0 min	30 min	60 min	90 min
BT_0/saline	BT_1/AMPH	BT_2/saline	BT_3
BT_0/IBF	BT_1/saline	BT_2/IBF	BT_3

Results

Neurochemical analysis revealed significant differences between saline and ibuprofen groups for dopamine and DOPAC (DA: $F(1, 13) = 5.188$, $p = .0403$; DOPAC: $F(1,13) = 8.552$, $p < .0188$). Both dopamine and DOPAC were significantly higher in ibuprofen-treated group compared to saline-treated group. There was no significant difference in HVA, 5-HT or 5-HIAA between groups. This indicates that ibuprofen was able to attenuate amphetamine-induced dopamine depletion in the striatum. See figure 16.

There were no significant differences found in the transmitter turnover ratios for DOPAC/DA, HVA/DA or 5-HIAA/5-HT indicating ibuprofen did not have any effect on turnover ratios in amphetamine-treated mice. See figure 17.

Analysis of body temperature change with a repeated measures ANOVA revealed a significant effect of treatment with ibuprofen or saline and a significant difference in body temperature (treatment: $F(1,18) = 6.345$, $p = .0214$; body temperature: $F(3,54) = 55.337$, $p < .0001$). However, there was no significant interaction between treatment and body temperature ($F(3,54) = 0.764$, $p = .5191$). One-way ANOVA to compare ibuprofen-treated mice to control mice at each time showed that body temperature was

only significantly different at BT_1 ($F(1,18) = 8.226$, $p = .0102$). Overall, ibuprofen did not affect amphetamine-induced hyperthermia. See figure 18.

Conclusions

Dopamine levels in ibuprofen-treated mice were significantly higher than in mice that did not receive ibuprofen. The data show that ibuprofen had a protective effect against dopamine depletion in the striatum caused by amphetamine. Furthermore, it has been suggested that increased body temperature contributes to amphetamine's neurotoxicity. There is conflicting evidence on the validity of this claim. Halladay et al. (2003) found that acetaminophen pretreatment was able to attenuate methamphetamine-induced hyperthermia in treated mice while methamphetamine's neurotoxicity was unaffected. In the present experiment, mice had similar body temperatures at the start of the experiment. Thirty minutes after ibuprofen administration, the body temperatures of ibuprofen-treated mice were slightly, but significantly, lower than saline-treated mice. It can be said then that ibuprofen was successful in lowering the body temperature of mice 30 minutes after administration. However, once mice were administered amphetamine, this difference was eliminated as both ibuprofen and saline-treated mice had statistically similar body temperatures 30 and 60 minutes after amphetamine treatment. Therefore, although ibuprofen was able to initially able to lower body temperature, its effects on body temperature were not seen once amphetamine was given. Moreover, ibuprofen was still able to attenuate the loss of dopamine without counteracting amphetamine's effect on body temperature.

Saline controls for amphetamine treatment were not used in this study, which was a pilot study to determine if ibuprofen would have protective effects in amphetamine-treated mice. Many studies have shown normal neurochemical levels in saline-treated animals so they were left out of this preliminary study.

Figure 16

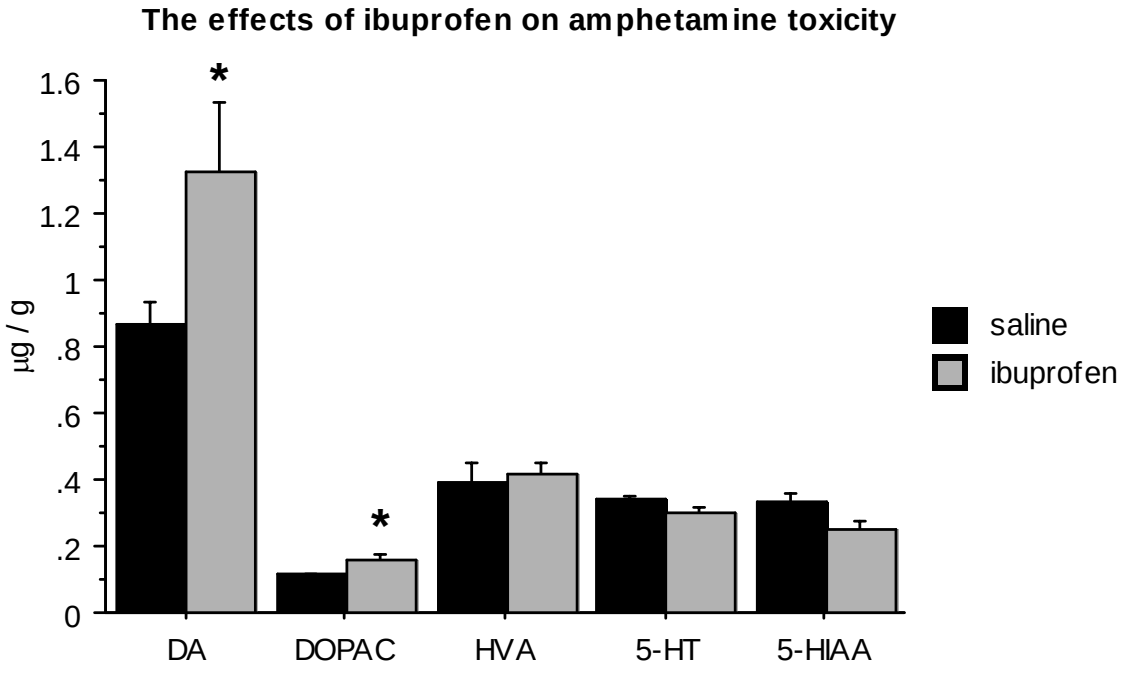


Figure 16: Concentration of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in striatum of male BALB/c treated with amphetamine and either saline or ibuprofen

* denotes significantly different from saline; $p < .05$

Figure 17

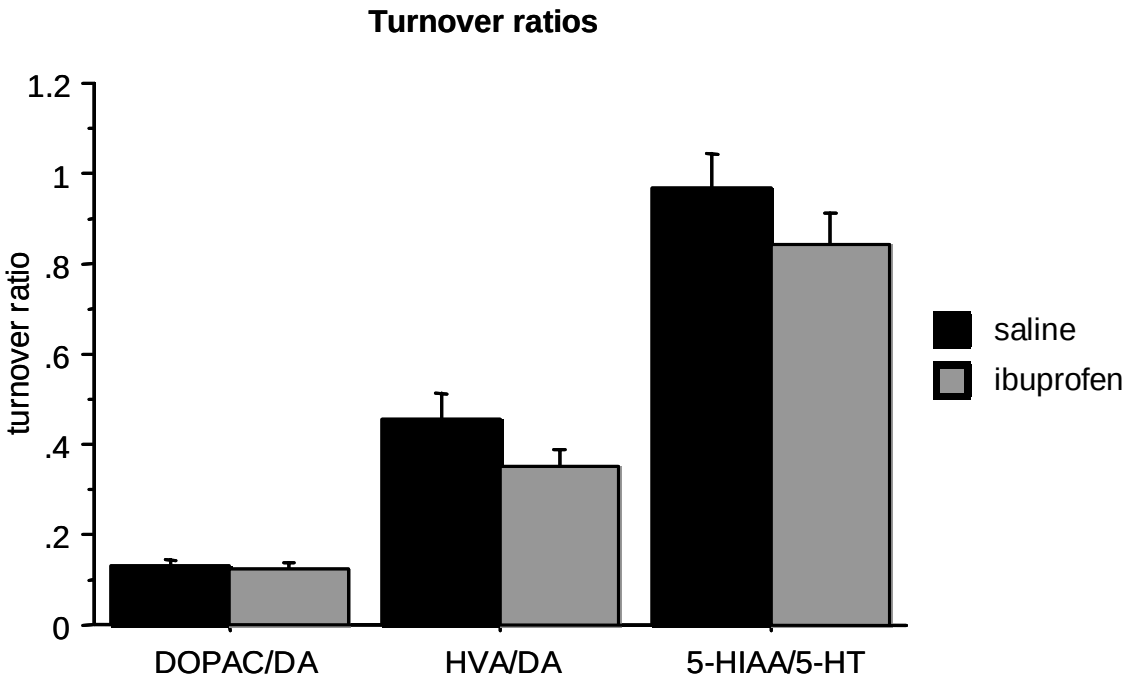


Figure 17: Turnover ratios of DOPAC/DA, HVA/DA and 5-HIAA/5-HT in striatum of male BALB/c treated with amphetamine and either saline or ibuprofen

Figure 18

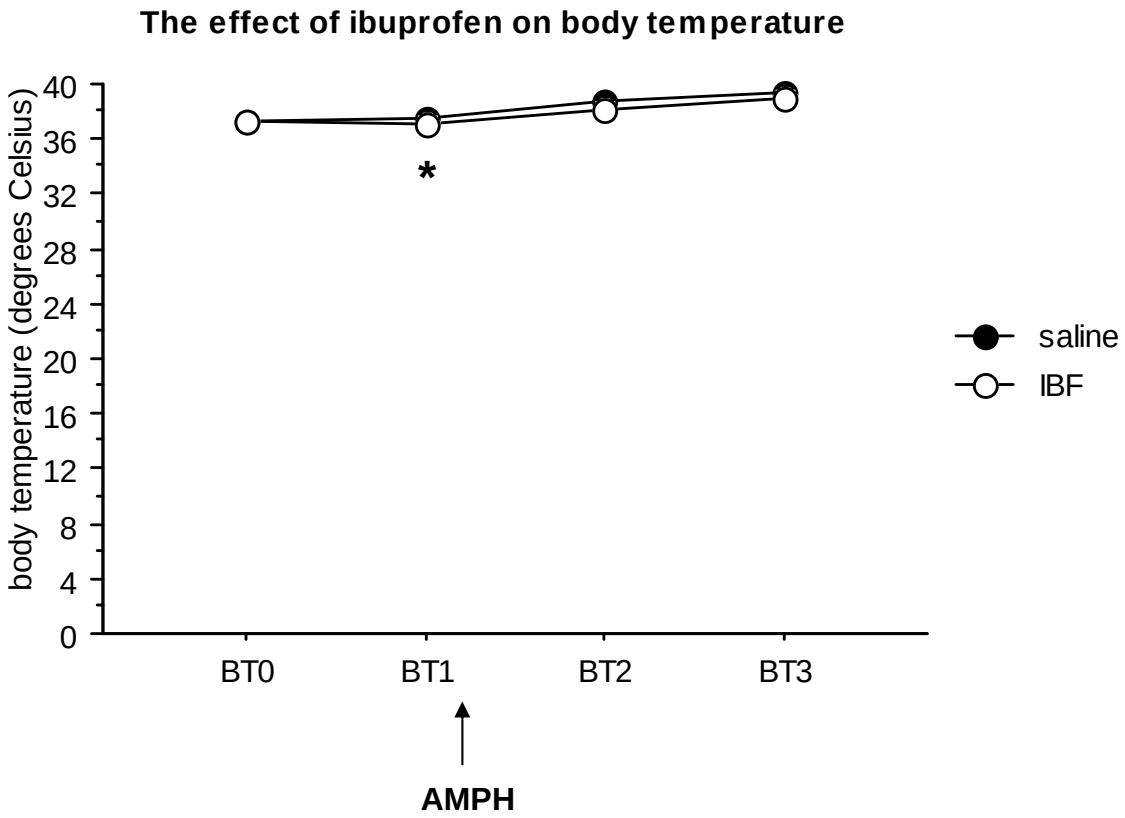


Figure 18: Body temperature (degrees Celsius) at baseline (BT0) and at 30, 60 and 90 minutes. Amphetamine was administered just after BT1 was recorded. Body temperature changed significantly over testing period ($p < .0001$). Treatment with ibuprofen significantly decreased body temperature only at BT1, just before amphetamine administration. Ibuprofen did not significantly affect body temperature after amphetamine administration.

* denotes significantly different from saline; $p < .05$

Experiment 9: The effects of major green tea components, EGCG and caffeine, on amphetamine-induced loss of striatal dopamine

Rationale

Green tea has been extensively studied and shown to be protective in models of neurodegenerative disease, including Parkinson's disease. The protective properties of tea are thought to be from their bioactive polyphenols: catechins and catechin derivatives. These have been shown to act as direct radical scavengers and metal chelators and indirectly by activating transcription factors and antioxidant enzymes this enabling them to be protective against oxidative stress (Grinberg et al., 1997; Wiseman et al., 1997; Higdon and Frei, 2003). (-)-Epigallocatechin 3-gallate (EGCG) is the most plentiful polyphenol found in tea and is attributed the protective effects reported in green tea (Jiang and Dusting, 2003). EGCG is well incorporated into the brain as well as in lung, kidney, heart, liver, spleen and pancreas (Suganuma et al., 1998).

Interestingly, there is a 5- to 10-fold lower incidence of Parkinson's disease in China and Japan than in Western countries (Zhang and Roman, 1993; Wang et al., 1996). Green tea is the most popular tea choice in Japan and China, in contrast to black tea that is favored in Western countries (Katiyar et al., 2000). In China, consumption of more than two cups of green tea per day reported to be a protective factor against Parkinson's disease (Chan et al., 1998).

In vitro studies have shown the protective effects of EGCG and tea catechins against oxidative stress. Mercer et al. (2005) investigated the effects of dietary phenols on several injury-inducing compounds and found that catechins attenuated injuries to primary rat mesencephalic cultures exposed to hydrogen peroxide, lipid peroxidation

product 4-hydroxynonenal, rotenone and 6-OHDA. The catechins were able to increase cell viability and dopamine uptake in culture (Mercer et al., 2005). Likewise, Levites et al. (2002) used EGCG to protect human neuroblastoma cells from 6-OHDA and MPP^+ damage. Intriguingly, EGCG treatment was able to promote neurite outgrowth in long-term serum-deprived PC12 cells showing a neurorescuing effect (Reznichenko et al., 2005). This is unique in that most studies examine neuroprotective effects of EGCG and other antioxidants against cell death. Instead, the authors administered EGCG once cells were compromised and found increased neurite outgrowth, a sign of improved cell welfare.

In mice, drinking tea extract and EGCG administered by oral gavage both attenuate damage caused by MPTP. Studies have shown that both the tea extract and EGCG prevented the loss of tyrosine hydroxylase-positive cells in the substantia nigra and its activity in the striatum. Furthermore, tea and EGCG treatments also attenuated loss of dopamine and its metabolites in the striatum (Levites et al., 2001; Choi et al., 2002). In another study, Levites et al. (2001) showed that green tea extract in two of the low doses they tested (0.5 and 1.0 mg/kg) prevented MPTP-induced dopamine depletion in the striatum and nigral cell loss in mice. The two higher doses (5 and 10 mg/kg) did not protect against dopamine depletion (Levites et al., 2001) suggesting that green tea extract works like true antioxidants such as ascorbic acid: protective at lower concentrations and a pro-oxidant at higher concentrations (Halliwell, 1996). They also found that EGCG protected against striatal dopamine depletion and elevated superoxide dismutase and catalase in the striatum of MPTP-treated mice. Their results led them to suggest that the protection by EGCG may be due to scavenging of reactive oxygen

species and regulation of antioxidant enzymes. In fact, an antioxidant cocktail containing tea catechins from green tea extract, sunflower seed extract and ascorbic acid was found to increase midbrain and striatal superoxide dismutase levels in rats, as well as to decrease lipid peroxidation markers in the cortex and cerebellum (Komatsu and Hiramatsu, 2000).

Green tea catechins also have been found to play a role in the signaling cascades of mitogen-activated protein kinases (MAPKs) and phosphatidylinositide 3'-OH kinase (PI3K)/AKT. Both of these pathways are integral to neuronal differentiation and cell survival as well as in neuroprotection against oxidative stress (Singer et al., 1999; Kermer et al., 2000; Gary et al., 2003). The MAPK cascade is stimulated by oxidative stress and can lead to either cell survival or cell death (Schroeter et al., 2002). Studies have shown that catechins can protect against oxidative stress by controlling ERK activity (Levites et al., 2002; Schroeter et al., 2002). MAPK pathway activation is also thought to be caused by catechins followed by the expression of stress response genes including glutathione-s-transferase (Chen et al., 2000; Owuor and Kong, 2002).

Taken together, EGCG and the tea catechins affect a variety of processes important to the normal function of the brain. They also have been shown to have protective effects in the face of neuronal injury or insult from both endogenous and exogenous sources. More specifically, they are thought to be a factor in the lower incidence of Parkinson's disease in Japan and China and are protective in the MPTP model of Parkinson's disease.

In addition, caffeine is a natural component in both black and green tea and has been suggested to be a protective factor against Parkinson's disease. Epidemiological

studies have shown lower incidence of Parkinson's disease with caffeine consumption from either tea or coffee. Animal models of Parkinson's disease have been successful in illustrating the efficacy of caffeine in protecting dopaminergic cells and transmission (Chen et al., 2001; Xu et al., 2002; Joghataie et al., 2004; Kalda et al., 2006). Case-control and prospective cohort studies have found caffeine intake to be inversely associated with Parkinson's disease risk as well (Ascherio et al., 2001; Ascherio et al., 2004).

Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid that upon ingestion, exerts general central nervous system excitation and is swiftly absorbed in the stomach and small intestine and metabolism occurs mainly in the liver by CYP1A2 (Faber et al., 2005). It is distributed to every tissue in the body, including the brain. The caffeine concentration in tea and coffee varies widely with the standard cup of coffee yielding about 75 - 100 mg on average and the standard cup of tea yielding about 20 - 30 mg on average (Barone and Roberts, 1996).

Caffeine acts as an adenosine receptor antagonist. Adenosine is an endogenous neuromodulator that when antagonized, induces stimulatory effects. The adenosine A_1 receptors are found throughout the brain and located on the presynaptic nerve terminal while the A_{2A} receptor in the brain is primarily concentrated in the striatum and located on the postsynaptic nerve terminal. A_{2A} receptors are co-localized with dopamine receptors in the striatum (Palmer and Stiles, 1995; Dixon et al., 1996). In fact, most of caffeine's biochemical and behavioral consequences have been linked to its inhibition of endogenous adenosine on dopamine transmission in the striatum. By inhibiting striatal adenosine, which normally inhibits dopamine transmission via the D_2 receptor, caffeine is

able to stimulate motor activity (Fisone et al., 2004). However, (Shiozaki et al., 1999) have shown the action of caffeine-like A_{2A} receptor antagonist is not completely reliant on dopamine transmission as MPTP-treated mice still exhibit increased motor activity with its administration. Overall, it seems caffeine's stimulatory effects, through A_{2A} receptor antagonism, utilize mechanisms that are both dopamine-dependent and dopamine-independent (Fisone et al., 2004).

As caffeine is a natural component of green tea, the effects of EGCG were examined in the amphetamine model of Parkinson's disease as well as EGCG's effects when paired with caffeine. It should be noted that the EGCG is this study was contained in a pellet diet fed to the mice, rather than given by gavage or injection to reduce stress on the mice. In this case, this pellet diet also contained a higher fat content compared to the typical chow as it was being used in this form in a concurrent study. This diet could be compared to a Western diet (with its higher fat content) and could give insight to EGCG and caffeine's effects under these conditions, as opposed to the diet that is typically consumed in Japan and China.

Animals

Eight week old, adult male BALB/c mice (Jackson Laboratories; Bar Harbor, ME) were maintained according to the NIH Guide for the Care and Use of Laboratory Animals. Mice had free access to regular chow food (chow) and tap water (tap) prior to the beginning of the study. Mice were housed in a temperature controlled colony room on a 12 hour on/12 hour off light cycle beginning at 8 AM. Animals were housed individually in hanging wire cages (20 cm x 10 cm x 12 cm).

Diet

At the start of the study, mice were given either the standard chow (chow; Purina Rodent Diet), a high fat pellet diet (HF; Modified AIN-93M Rodent Diet with 20% w/w Reddy Fat Mix, Research Diets, New Brunswick, NJ) or a high fat pellet diet containing EGCG (EGCG; Modified AIN-93M Rodent Diet with 20% w/w Reddy Fat Mix and 0.16% EGCG, Research Diets, New Brunswick, NJ**).** Mice had free access to this diet for the duration of the study, both before and after drug treatment. Some groups received regular tap water (tap) or water with caffeine (0.1 mg/ml; CAF) added. Mice also had free access to the caffeinated or tap water for the duration of the study. Body weight, food and water consumption was monitored and recorded for the entirety of the experiment.

Drug treatment

Mice were treated with one subcutaneous injection of 50 mg/kg amphetamine or saline on day 7 of diet/water treatment. Animals were sacrificed 72 hours after amphetamine/saline injection.

Neurochemical Analysis

Following sacrifice, brains were quickly removed and bilateral striata were immediately dissected and stored in liquid nitrogen and assayed as in Experiment 1.

Statistical Analyses

Three-way ANOVAs were performed with the neurotransmitter concentration and turnover ratios as main factors and diet type, water type and drug treatment as between groups factors. Animal weight, food consumption and water consumption were all analyzed using a repeated measures ANOVA. One-way ANOVAs were used to determine differences between time points. For all appropriate analyses, differences between groups were determined using Fisher's PLSD post-hoc tests.

Experimental design

Animals were divided into 10 groups (water type/diet type/drug): tap/chow/saline; tap/chow/AMPH; tap/HF/saline; tap/HF/AMPH; CAF/HF/saline; CAF/HF/AMPH; tap/EGCG/saline; tap/EGCG/AMPH; CAF/EGCG/saline; CAF/EGCG/AMPH. Mice were given the experimental food and water for one week prior to drug treatment. There were continued on this regimen post-injection until sacrifice. Mice were sacrificed 72 hours following administration of amphetamine or saline.

Results

Analysis of the neurochemistry using a three-way ANOVA showed a significant effect on dopamine with water ($F(1,45) = 7.154$, $p = .0104$), drug challenge ($F(1,45) = 29.393$, $p < .0001$) and interaction between diet and drug challenge ($F(1,45) = 6.557$, $p = .0139$) as well as an almost statistically significant effect of interaction between water, diet and drug challenge ($F(1,45) = 3.921$, $p = .0538$). *Post hoc* analysis showed that dopamine levels were significantly higher in tap water-treated groups versus caffeine-

treated groups ($p = .0074$) and in saline-treated groups versus amphetamine-treated groups ($p < .0001$). One-way ANOVA analysis that combined the water, diet, drug challenge groups showed significant differences in dopamine levels between several groups: tap/chow/saline, tap/chow/AMPH: $p = .0307$; tap/HF/saline, tap/HF/AMPH: $p < .0001$; tap/HF/saline, CAF/HF/AMPH: $p < .0001$; tap/HF/AMPH, tap/EGCG/AMPH: $p = .0295$; CAF/HF/saline, CAF/HF/AMPH: $p = .0014$; tap/EGCG/AMPH, CAF/EGCG/AMPH: $p = .0243$; CAF/EGCG/saline, CAF/EGCG/AMPH: $p = .0063$. Overall, water type and drug challenge had significant effects of dopamine levels. Mice receiving caffeinated water had lower dopamine levels overall and lower levels when treated with amphetamine as well. Amphetamine had an overall significant effect on dopamine levels, depleting dopamine in all mice treated with it. Neither water type nor food type attenuated amphetamine-induced dopamine depletion. However, there was a significant interaction of food type and drug challenge. Mice receiving EGCG diet and amphetamine had slightly higher levels of dopamine compared to mice receiving amphetamine and high fat diet without EGCG. See figure 19.

Three-way ANOVA on DOPAC levels showed a significant effect from water type, drug challenge, diet by drug challenge and water by diet by drug challenge (water type: $F(1,45) = 7.808$, $p = .0076$; drug challenge: $F(1,45) = 12.247$, $p = .0011$; diet*drug challenge: $F(1,45) = 7.042$, $p = .0110$; water*diet*drug challenge: $F(1,45) = 5.641$, $p = .0219$). Three-way ANOVA for HVA showed a significant effect from water type, drug challenge, diet by drug challenge (water type: $F(1,45) = 10.184$, $p = .0026$; drug challenge: $F(1,45) = 4.280$, $p = .0443$; diet*drug challenge: $F(1,45) = 6.244$, $p = .0162$). Mice that received caffeinated water had lower levels of DOPAC and HVA compared to

those that received tap water, an effect that also occurred with amphetamine treatment. Mice that received amphetamine had lower levels of DOPAC and HVA compared to saline-treated mice.

Three-way ANOVA for 5-HT showed a significant effect from water type, drug challenge and diet by drug challenge (water type: $F(1,45) = 14.489$, $p = .0004$; drug challenge: $F(1,45) = 3.390$, $p = .0722$; diet*drug challenge: $F(1,45) = 6.396$, $p = .0150$). Three-way ANOVA for 5-HIAA showed a significant effect from diet by drug challenge ($F(1,45) = 5.079$, $p = .0291$). Overall, mice that received caffeinated water had lower levels of 5-HT and 5-HIAA, regardless of drug challenge. Amphetamine also caused a depletion of 5-HT compared to saline-treated mice. For comparison of neurochemistry, see figure 19.

Three-way ANOVA analysis showed a significant effect of drug challenge on DOPAC/DA ($F(1,43) = 9.979$, $p = .0029$) and HVA/DA ($F(1,43) = 22.279$, $p < .0001$). *Post hoc* analysis also revealed that HVA/DA was significantly different between control high fat diet and diet containing EGCG ($p = .0329$). Overall, amphetamine caused a significant increase in DOPAC/DA and HVA/DA ratios, with HVA/DA being more affected. 5-HIAA/5-HT ratio was not affected by amphetamine treatment. See figure 20.

Analysis with a repeated measures ANOVA showed a significant change in weight over the experiment period (from start to sacrifice) ($F(3,141) = 79.332$, $p < .0001$). There was also a significant effect of amphetamine challenge on weight change ($F(3,141) = 7.427$, $p = .0001$), a significant effect of food type on weight change ($F(3,141) = 7.416$, $p = .0001$) and a significant interaction of water type, food type and amphetamine challenge on weight change ($F(3,141) = 5.628$, $p = .0011$). The weight

change between saline or amphetamine injection and sacrifice was examined to determine if amphetamine caused significant weight loss and if food or water type could influence this effect. There was a significant effect of amphetamine challenge ($F(1,46) = 11.828$, $p = .0012$), food type by amphetamine challenge ($F(1,46) = 4.859$, $p = .0325$) and water type by food type by amphetamine challenge ($F(1,46) = 10.158$, $p = .0026$). *Post hoc* analysis revealed a significant difference between drug challenge (saline or amphetamine: $p = .0003$) and food type ($p = .0043$). Prior to injection (day 0 – 7) there was no significant effect of food type, water type or interaction of food type and water type on weight change. Overall, mice receiving amphetamine lost weight/lost more weight compared to saline-treated mice. This was an expected effect since amphetamine is known to reduce appetite. See figure 21.

A one-way ANOVA on fluid consumption prior to injections, between day 0 and 6, showed a significant effect of water type ($F(1,58) = 14.063$, $p = .0004$) but not food type. Mice receiving caffeinated water drank significantly more fluid than mice that received tap water without caffeine. Analysis of fluid consumption after drug challenge (between day 6 and 10) showed a significant effect of water type ($F(1,47) = 11.920$, $p = .0012$) and a significant interaction between water type and drug challenge ($F(1,47) = 5.749$, $p = .0205$). *Post hoc* analysis showed that mice receiving caffeinated water drank significantly more fluid than mice receiving tap water ($p = .0012$). Data not shown.

One-way ANOVA analysis of food consumption prior to injections, between day 0 and 7 showed a significant effect food type ($F(1,58) = 11.871$, $p = .0011$) with mice receiving diet containing EGCG consuming significantly more food than mice receiving the control high fat diet. Analysis of food consumption after drug challenge (between

day 7 and 10) showed no significant effects of water type, food type or drug challenge. Data not shown.

Conclusions

There was no protective effect of caffeine observed in any of the comparisons. Likewise, there was no effect of EGCG in the diet observed except in one case: the dopamine levels between the groups tap/EGCG/saline and tap/EGCG/AMPH were not statistically significant. This could be interpreted as meaning that the EGCG diet without caffeinated water had a protective effect against the amphetamine challenge. However, due to animal death prior to sacrifice time, the tap/EGCG/AMPH group had only three mice in it at the conclusion of the experiment. The cause of animal death in this group is unknown. This low group "n" affected the statistical power as seen in the large standard deviation and standard error of the mean. More animals are needed in this group in order to draw firmer conclusions.

Figure 19

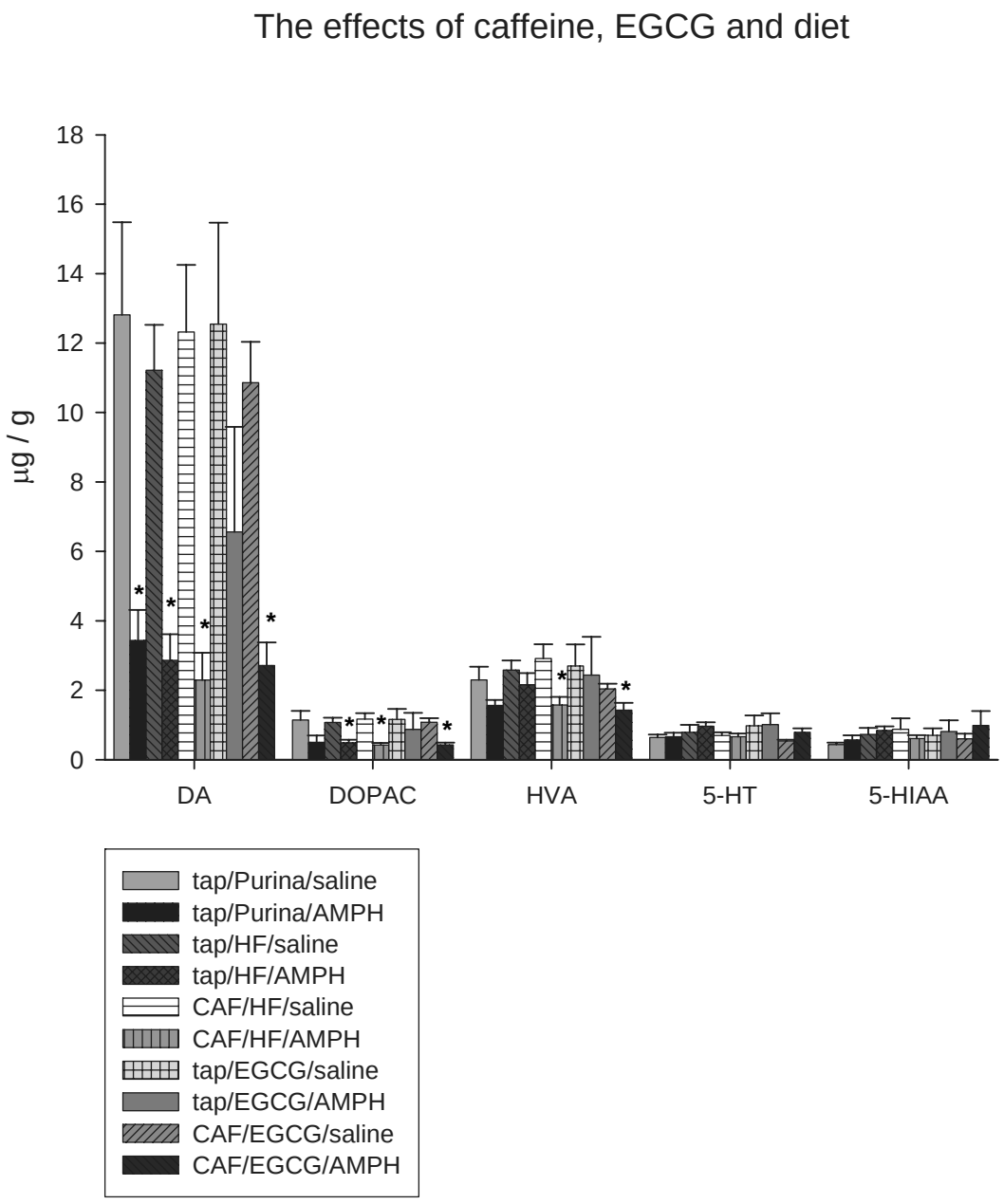

The effects of caffeine, EGCG and diet
µg / g
18
16
14
12
10
8
6
4
2
0
DA
DOPAC
HVA
5-HT
5-HIAA
tap/Purina/saline
tap/Purina/AMPH
tap/HF/saline
tap/HF/AMPH
CAF/HF/saline
CAF/HF/AMPH
tap/EGCG/saline
tap/EGCG/AMPH
CAF/EGCG/saline
CAF/EGCG/AMPH

Figure 19: Concentration of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in striatum of male BALB/c for tap/Purina/saline (n=3), tap/Purina/AMPH (n=4), tap/HF/saline (n=6), tap/HF/AMPH (n=7), CAF/HF/saline (n=6), CAF/HF/AMPH (n=9), tap/EGCG/saline (n=7), tap/EGCG/AMPH (n=3), CAF/EGCG/saline (n=7), CAF/EGCG/AMPH (n=8)

* denotes significantly different from saline treated group with matching fluid/food types; $p < .05$

Figure 20

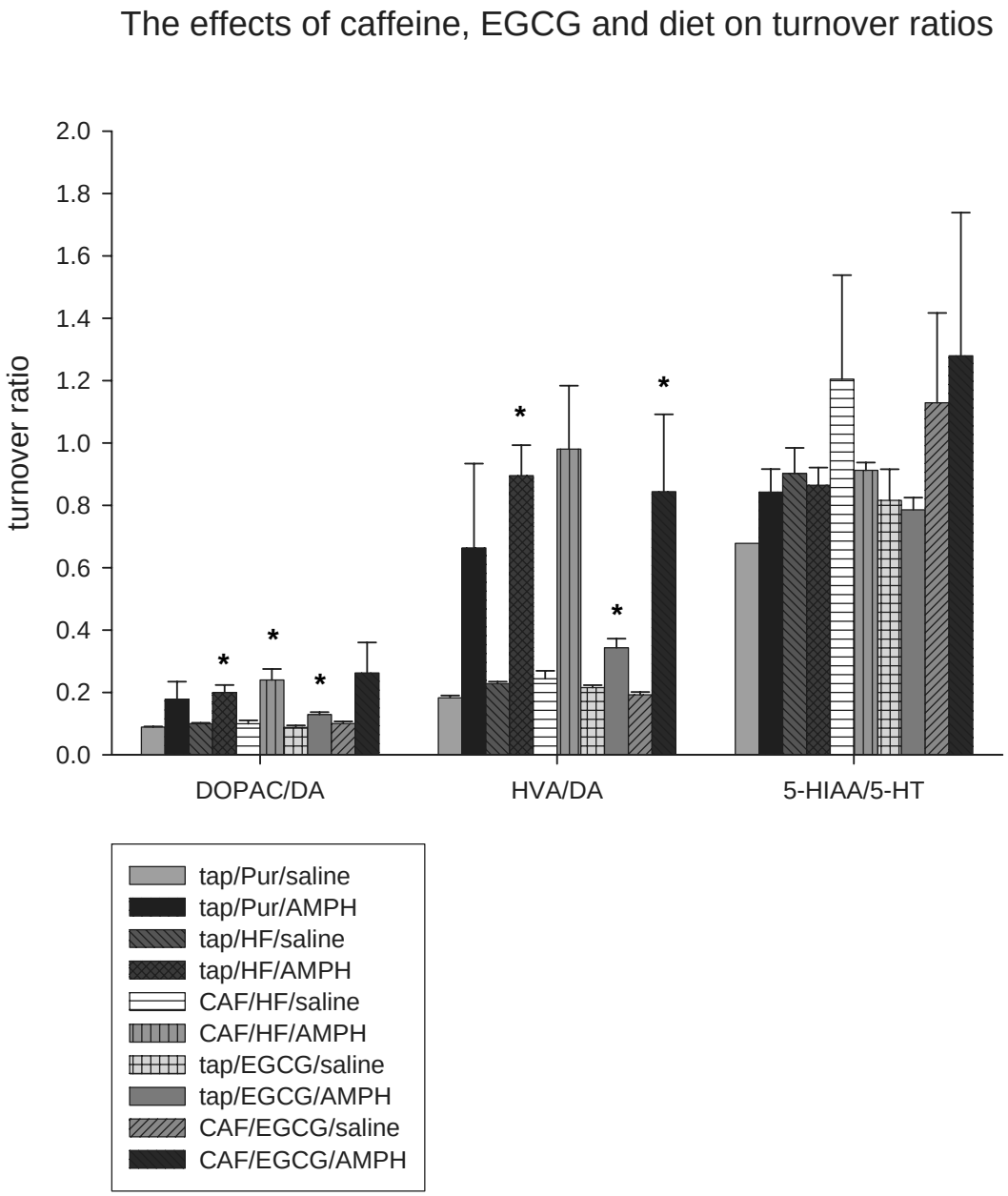

The effects of caffeine, EGCG and diet on turnover ratios
turnover ratio
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
DOPAC/DA
HVA/DA
5-HIAA/5-HT
tap/Pur/saline
tap/Pur/AMPH
tap/HF/saline
tap/HF/AMPH
CAF/HF/saline
CAF/HF/AMPH
tap/EGCG/saline
tap/EGCG/AMPH
CAF/EGCG/saline
CAF/EGCG/AMPH

Figure 18: Turnover ratios of DOPAC/DA, HVA/DA and 5-HIAA/5-HT in striatum of male BALB/c for tap/Purina/saline, tap/Purina/AMPH, tap/HF/saline, tap/HF/AMPH, CAF/HF/saline, CAF/HF/AMPH, tap/EGCG/saline, tap/EGCG/AMPH, CAF/EGCG/saline, CAF/EGCG/AMPH

* denotes significantly different from saline treated group with matching fluid/food types; $p < .05$

Figure 21

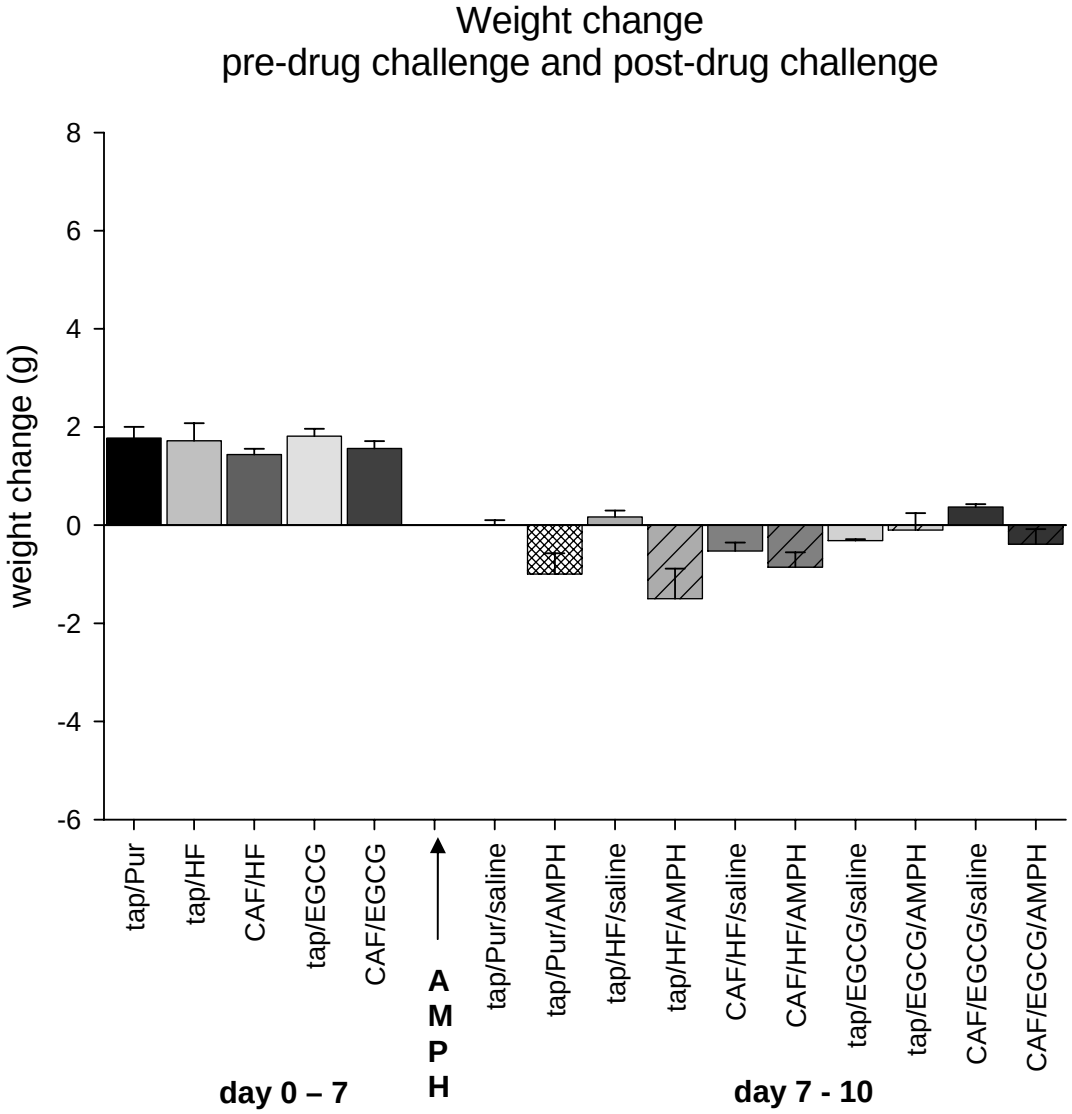


Figure 21: Average change in body weight in male BALB/c mice for tap/Pur, tap/HF, CAF/HF, tap/EGCG, CAF/EGCG prior to amphetamine or saline injection and for tap/Pur/saline, tap/Pur/AMPH, tap/HF/saline, tap/HF/AMPH, CAF/HF/saline. CAF/HF/AMPH, tap/EGCG/saline, tap/EGCG/AMPH after injection of saline or amphetamine

Discussion: Experiments 6 – 9

A portion of amphetamine's neurotoxic actions are attributed to increased oxidative stress. This increased oxidative stress can cause increases in neuroinflammation. Here, several therapeutic agents were studied to determine their effectiveness in protecting against amphetamine-induced dopamine depletion of the striatum.

Ascorbic acid was able to attenuate amphetamine-induced dopamine depletion in this model. Ascorbic acid works as an antioxidant in the brain, scavenging reactive oxygen species and inhibiting lipid peroxidation and has been shown to be protective previously in animal models of Parkinson's disease, including a rat methamphetamine model. These data show that it is effective in mediating the neurotoxicity of a single high dose of amphetamine and this protection is a long-lasting outcome.

In addition, as an extension of the time course completed in Experiments 1 and 2, animals were sacrificed four weeks after amphetamine treatment. The dopamine depletion observed at earlier time points was shown to persist for up to four weeks post-amphetamine. This suggests that this lesion is a permanent consequence of the single hose dose treatment of amphetamine.

Ibuprofen, a known anti-inflammatory agent, also showed modest but significant sparing of striatal dopamine after amphetamine challenge. Neuroinflammation has been found to be increased in the brains of Parkinson's patients and use of NSAIDs like ibuprofen have been associated with a lower risk of the disease. Neuroinflammation is most likely consequent to increased levels of oxidative stress occurring in these patients and anti-inflammatory agents work to counteract this portion of the cascade of destruction

that builds up in Parkinson's disease. Ibuprofen has been shown to be protective in other animal models of Parkinson's disease and this is confirmed here. Its effectiveness may be increased when administered in conjunction with an antioxidant such as ascorbic acid.

Trolox, a water-soluble form of vitamin E, was also tested in this model of Parkinson's disease. It has been shown to be effective in other disease models, however it had no protective effects against amphetamine-induced dopamine depletion. This may be due to ineffective distribution of Trolox to the brain. Brain and peripheral concentrations of Trolox were not measured in this study so it is unclear if Trolox had sufficiently penetrated the brain in this regimen. A longer period of time between Trolox administration and amphetamine challenge may be needed to ensure distribution to brain. Also, a higher dose of Trolox may have been needed in order to observe protective effects.

Caffeine in the drinking water and dietary EGCG were also tested in this model of dopamine depletion. Caffeine and EGCG have been shown to be protective in other models of Parkinson's disease and it is intriguing that they did not work in the model used here. This could be due to a number of factors. First, one week of dietary EGCG and/or caffeinated water may have not been enough time for these compounds to build up in the body and have a protective effect. Second, the high fat background of the diet may have altered the effectiveness of the EGCG and/or the caffeine. This is an interesting idea as the Western diet contains considerably more fat than does the diet of Japan and China, where much of the EGCG work has been performed. There is a lower incidence of Parkinson's disease in Japan and China and this has been largely attributed to the extensive consumption of green tea in these countries. However, other dietary factors

should be taken into consideration, such as dietary fat content and consumption of other antioxidants and anti-inflammatory agents as well as levels of pesticides and antibiotics found in food commonly consumed. Since green tea is not as widely consumed in Western society as it is in China and Japan, it is an intriguing possibility that incidence of Parkinson's disease may not be reduced by increased consumption of EGCG in Americans due to their diet.

In a prospective study on a range of medical history and lifestyle components, a healthy diet ("prudent dietary pattern") including high consumption of fruit, vegetables, legumes and cereals along with a low consumption of meats, showed an inverse relationship to risk of Parkinson's disease. Incidence of Parkinson's disease was also examined in those typically following a "Western dietary pattern" with no significant correlation (Gao et al., 2007). It is possible that consumption of more fruits and vegetables (hence more dietary antioxidants) and less saturated fat is a protective factor against Parkinson's disease while a less healthy diet that contains more meat, processed food and fat does not increase risk but also may obscure any positive effects of the dietary antioxidants that are consumed.

PART II. BEHAVIORAL CHARACTERIZATION AND AMPHETAMINE TOXICITY IN GENETIC KNOCKOUTS AS POTENTIAL MODELS OF PARKINSON'S DISEASE

I. GLUTATHIONE AND PARKINSON'S DISEASE

Oxidative stress is commonly thought to play a role in the etiology of Parkinson's disease. Protective mechanisms have evolved to protect against both endogenous sources of oxidative stress and oxidative stress caused by toxicants in the environment. These detoxifying mechanisms are essential to sustaining cellular homeostasis and the overall health of tissues and organisms. Alterations to and the possible differences in these mechanisms in individuals may determine susceptibility to disease caused by exposure to toxicants in the environment. It has been found that levels of both superoxide dismutases (Mn-SOD and Cu/ZnSOD) are raised in the brains of Parkinson's disease patients while the level of reduced glutathione (GSH) is low (Sian et al., 1994b). These data suggest that there is an increased level of oxidative stress taking place in the brain and that glutathione peroxidase activity may be hindered due to a reduction in normal levels of reduced glutathione (Jenner, 1994).

Glutathione is an antioxidant existing in both reduced (GSH) and oxidized (GSSG) states. The reduced state of glutathione is able to donate an electron to unstable molecules, like reactive oxygen species produced during oxidative stress. Glutathione exists mostly in the reduced state in healthy cells and an increased ratio of the oxidized form to the reduced form generally indicates increased oxidative stress.

Glutathione-S-transferases (GSTs) are a superfamily of enzymes that catalyze the conjugation of reduced glutathione and a wide variety of substrates including endogenous lipid peroxidation products, xenobiotics and environmental pollutants (Salinas and Wong, 1999). There are two distinct super families of these enzymes: one is made up of soluble, cytosolic enzymes that are involved in the biotransformation of xenobiotics and endobiotics (Hayes and Pulford, 1995; Hayes and Strange, 1995) and the other is made up of membrane-bound, microsomal enzymes that are involved in the metabolism of arachidonic acid (Jakobsson et al., 1999a; Jakobsson et al., 1999b). While these superfamilies do not contain sequence homology, they do share some functional redundancy. The cytosolic enzyme superfamily includes several genes that are divided into subclasses: alpha, kappa, mu, pi, sigma, theta, zeta and omega (Hayes and McLellan, 1999). The microsomal enzymes are grouped together and termed MA-PEG which stands for membrane-associated proteins in eicosanoid and glutathione metabolism (Jakobsson et al., 1999a).

The cytosolic GST enzymes in classes alpha, mu and pi have been found to detoxify 4-hydroxynonenal, a lipid peroxidation product (Board, 1998; Hubatsch et al., 1998), and quinone-containing products formed via oxidative stress on catecholamines (Baez et al., 1997).

The GST mu class (GSTM) of enzymes are found in the liver, brain, testis and skeletal muscle. Specifically, levels of GSTM1 are highest in the liver, testis and brain. GSTM1 has been found to be part of industrial and diet-derived aromatic amine detoxification (Clapper et al., 1995; Huber et al., 1997; Shinka et al., 1998). When GSTs are not able to conjugate glutathione with such compounds, DNA and protein damage can

occur via alternate pathways (Agostinelli et al., 1996). Aromatic amines have been found to have neurotoxic properties inhibiting mitochondrial complex I activity in the substantia nigra eventually leading to dopamine depletion as in Parkinson's disease (Singer et al., 1987; Kotake et al., 1995). Furthermore, those affected by chronic neurological problems as in Parkinson's disease and Alzheimer's disease seem to have reduced ability to detoxify exogenous compounds (Steventon et al., 1989; Waring et al., 1989; Heafield et al., 1990; Steventon et al., 1990; Peters et al., 1994).

Since GSTs function to protect against oxidative stress, the genes that encode them have been considered potential targets of mutations in Parkinson's disease. In fact, a null mutation in GSTM1 has recently been found to be significantly associated with Parkinson's disease (Stroombergen and Waring, 1999; Perez-Pastene et al., 2007). Patients with a null mutation in GSTM1 also have been found to have a significantly earlier onset of the disease (Ahmadi et al., 2000). However, other studies have failed to find an association of GSTM1 polymorphisms or null mutations and Parkinson's disease (Rahbar et al., 2000; Harada et al., 2001; Kelada et al., 2003).

Hypothesis: Mice lacking the GSTM1 gene will show a behavioral phenotype distinct from wildtype controls consequent to the gene deletion and its proposed effects on the dopaminergic system. These mice will also show an enhanced sensitivity to the neurotoxic effects of amphetamine.

Experiment 10: Behavioral characterization and drug sensitivity of GSTM1 knockout mice

Rationale

When exploring any new animal model of disease, it is important to fully characterize baseline rates of behavior and neurochemistry of the animal, especially with respect to disease-relevant tasks and drug challenges. In the following experiments, GSTμ1 knockouts and their wildtypes were subjected to a battery of behavioral tests to determine any behavioral deficits or enhancements that may related to the GSTμ1 gene, a Parkinsonian phenotype or neurochemical levels. At the completion of the behavioral experiments, mice were treated with amphetamine to observe any reduced or enhanced sensitivity to amphetamine as final step to evaluate this gene's role in the animal model used here.

Animals

All mice were on C57BL/6 genetic background and were generated in the Mirochnitchenko lab at the University of Medicine and Dentistry of New Jersey. Ten male wildtype (WT) mice and 15 male GSTμ1 knockout (KO) mice were used throughout the battery of behavioral tests. Mice were group housed in plastic shoebox cages with free access to food and water for two weeks upon arrival. They then were transferred to individual hanging wire cages (20 cm x 10 cm x 12 cm), also with free access to food and water. The animals were housed in a temperature and humidity regulated room with a 12 hr light/dark cycle. All procedures were conducted in strict

compliance with the policies on animal welfare of the National Institute of Health and the Animal Care and Use Committee at Rutgers University.

Statistical Analysis

One-way ANOVAs, two-way ANOVAs and repeated measures ANOVAs were used where appropriate. For all appropriate analyses, differences between groups were determined using Fisher's PLSD *post hoc* tests.

Activity

Locomotor activity was assessed for 30 minutes in a novel environment (42 x 22 x 14 cm Plexiglas box) with six infrared sensors placed approximately 7 cm apart and 2.5 cm above the floor. The number of beam breaks was recorded every 5 minutes for 30 minutes.

Elevated Plus Maze

Mice were placed in an elevated plus maze consisting of two open arms and two closed arms 30 cm long and 9 cm wide that cross a neutral 5 cm x 5 cm central square. Closed arms had vertical shields raised from the edges. The entire apparatus was elevated 60 cm above the floor. Each animal was placed in the center square of the maze and was given one 10-minute session to explore the maze. The number of entrances to closed arms, open arms, and jump-offs were recorded, as well as the number of fecal boli. An entrance to an arm was counted when all four feet crossed into an arm from the neutral center square.

Rotorod

Mice were assessed for balance and motor coordination on a 6.0 cm diameter rotorod rotating at 12 revolutions per minute, 60cm above a padded receptacle. The rotorod rotated in a clockwise direction and mice were placed so that they were walking forward in a counterclockwise direction. Each trial was a maximum of 60 seconds and each mouse was given three consecutive trials. The latency to fall from the rotating rod was recorded.

Social Chamber

Mice were placed in a social chamber that was 40 cm x 40 cm x 36.6 cm made of Plexiglas and covered in brown paper to reduce outside distraction while in the chamber. The chamber was interfaced to a laptop that recorded the contact data. Two cylinders made from stainless steel mesh, each 11 cm in diameter and 13 cm high, were located in opposite corners inside the chamber. Shields were added to the tops of each cylinder to prevent mice from climbing and remaining atop the cylinder. A contact was recorded when the mouse touched the cylinder with at least one paw on the cylinder and one on the floor of the chamber. Prior to the experimental sessions, the animal was placed in the middle of the chamber and was allowed to explore the chamber for 10 minutes. After the habituation period, an adult male C57BL/6 mouse was placed in the target cylinder, while the control cylinder was left empty. Each animal was allowed three 10-minute sessions across three consecutive days. The total number of contacts with the target and control cylinders during the session was recorded.

Water Maze

The water maze consisted of a circular tub measuring 71 cm in diameter and 29 cm in height. The interior of the tub was painted white and was filled ¾ full with water maintained at 23 – 26^{o}C and made opaque with white non-toxic latex paint. A starting point was determined randomly from one of four equally spaced quadrants. A hidden platform measuring 8 cm in diameter and painted white was place in one quadrant of the maze and sat 2 cm below the surface of the water. Animals received four trials per day on each of five consecutive days. Each trial lasted a maximum of 90 seconds. If a mouse failed to find the platform in 90 seconds, a score of 90 seconds was recorded and the animal was placed on the platform for 10 seconds and then removed until the next trial. The position of the platform remained constant throughout the experiment and the room was illuminated and extra-maze cues were present. Mice were placed in a random quadrant at the start of each trial. Latency to find the hidden platform was recorded.

Drug treatment

Mice were treated with one subcutaneous injection of 50 mg/kg amphetamine or saline and sacrificed 72 hours later. Bilateral striata were dissected out for neurochemical analysis.

Neurochemical Analysis

Following sacrifice, brains were quickly removed; bilateral striata were immediately dissected and stored in liquid nitrogen until assayed as in Experiment 1.

Results

Activity

Repeated measures ANOVA showed a significant change in activity over the time period spent in the activity chamber and a significant interaction between activity and genotype (activity: $F(1,5) = 27.528$, $p < .0001$; activity by genotype: $F(1,5) = 4.020$, $p = .0021$). Overall, however, *post hoc* analysis showed no significant difference between genotypes ($p = .2724$). See figure 22.

One-way ANOVA analysis was conducted to determine differences in each 5 minute time bin and total activity. Analysis showed no significant difference between genotypes at any time bin. Furthermore, there was no significant difference in total activity by either genotype.

Conclusions

Mice of both genotypes are equally active. Mice with a tendency towards a Parkinson-like phenotype may be expected to be less active as a key clinical sign of this disease in humans is akinesia and/or bradykinesia. However, these mice may not have been old enough to see a difference in motor activity if the effects of knocking out GSTM1 begin to show in aged mice. The similar motor activity between genotype eliminates that deficit as a confound in further behavior testing.

Elevated plus maze

Analysis using a one-way ANOVA showed no significant difference between genotypes in the number of entries into either the open or closed arms of the apparatus.

Also, there was no significant difference in number of fecal boli between genotypes. See figure 23.

Conclusions

Wildtypes and knockouts have similar levels of "anxiety" as evidenced by no difference in entries in the open arms of the elevated plus maze. Reduced number of entries into the open arms is thought to be indicative of greater anxiety levels where as more entries into the open arms is indicative of less anxiety. There was also no difference in close arm entries indicating normal exploratory behavior as compared to wildtypes, as well as confirming the results of activity in the open field.

Rotorod

Repeated measures ANOVA showed no significant difference overall in performance between genotypes; however, *post hoc* analysis revealed a significant effect of genotype ($p = .0056$).

On average, wildtypes performed better than knockouts on each trial. To test the performance of genotypes on each trial, one-way ANOVAs were conducted on each trial. On trial 1, wildtypes performed significantly better than knockouts ($F(1,23) = 4.510$, $p = .0047$) but on trials two and three the difference was not significant. See figure 24.

Conclusions

Rotorod performance is typically used to evaluate motor ability and coordination in mice. Overall, wildtype and knockout mice performed similarly to one another on the rotorod, indicating that knockouts do not have problems in motor coordination.

Knockouts performed significantly worse on the first trial than wildtypes. This observation requires replication at different rotorod speeds.

Performance on the rotorod is an important indicator of motor function; if motor coordination is impaired on this task, further tasks like the Morris water maze may be affected. In this case, motor coordination in both genotypes is similar and would not confound findings in the water maze task.

Also, rotorod performance could be indicative of motor learning. Knockouts performed significantly worse in the first trial compared to wildtypes but improved over trials two and three so that there was no significant difference in performance on those trials. This suggests that motor learning in knockouts is normal and comparable to that in wildtypes.

Water maze

A two-way, repeated measures ANOVA was used to analyze water maze performance over the five testing days. A significant effect was found for testing day but not for genotype or interaction of genotype and testing day ($F(4,110) = 50.327$, $p < .0001$). Mice of both genotypes improved at finding the hidden platform with each trial of each day and on each day as a whole. See figure 25.

Conclusions

The Morris water maze is used to measure spatial learning and memory attributed to hippocampal functioning. Wildtypes and knockouts performed equally well in the water maze task. As would be expected with normal spatial learning and memory, these mice improved over each trial on each testing day. By the end of testing both genotypes

performed well, finding the hidden platform quickly. Therefore, the knockouts did not exhibit any deficits in spatial learning and memory as measured by the hidden platform water maze task.

Social chamber

Repeated measures ANOVA analysis of control cup touches showed an overall significant effect of genotype and significant number of touches of the control cup over the course of experiment but no significant interaction between genotype and control cup touches (genotype: $F(1,23) = 11.496$, $p = .0025$; control cup touches: $F(2,23) = 5.992$, $p = .0049$). Repeated measures ANOVA on target cup touches showed a significant effect of target cup touches only ($F(2,23) = 15.060$, $p < .0001$).

One-way ANOVAs were conducted on each day's trial to compare behavior from a specific day between genotypes. Wildtypes contacted the control cup significantly more times than the knockouts on trial 1 only ($F(1,23) = 10.387$, $p = .0038$) with wildtypes touching the control cup an average of 38 times compared to 15 touches by the knockouts. The target cup, containing an unfamiliar mouse, was also contacted significantly more times by the wildtypes compared to the knockouts on trials 1 and 2 showing a tendency towards more social behavior (trial 1: $F(1,23) = 4.917$, $p = .0386$; trial 2: $F(1,23) = 6.322$, $p = .0194$). Wildtypes averaged 29 contacts and knockouts averaged 17.5 contacts on trial 1 and 78 and 47 contacts by wildtypes and knockouts, respectively, on trial 2. By trial 3, wildtypes and knockouts both contacted the target cup a similar number of times, 73 and 70 times for wildtypes and knockouts respectively.

A two-way ANOVA, using cup type and trial number as main factors and genotype as the between groups factor, provided further insight. In trial 1, there was significant effect of genotype but not cup type or interaction of cup type and genotype ($F(1,46) = 15.288$, $p = .0003$). In trial 2, there was significant effect of both genotype and cup type but there was no interaction between cup type and genotype (cup type*genotype) (genotype: $F(1,46) = 10.011$, $p = .0028$; cup type: $F(1,46) = 18.723$, $p < .0001$). In trial 3, there was only a significant effect with cup type ($F(1,46) = 5.127$, $p = .0283$). See figure 26.

Conclusions

Overall, wildtypes seem to exhibit more exploratory behavior as evidenced by far more contacts with the control cup and total number of cup contacts (control cup contacts plus target cup contacts). Wildtypes also seem to interact in a more social manner in the first two trials, as they have more contacts with the cylinder containing the unfamiliar mouse. However, by the third trial, both genotypes were equal in their contacts to the target cup.

Neurochemistry

Analysis with two-way ANOVA showed that there were no significant differences in levels of DA, DOPAC, HVA due to genotype, drug challenge or interaction between genotype and drug challenge. Levels of dopamine were lower in the knockouts overall compared to wildtypes, although not significantly, with saline-treated knockouts having the lowest dopamine levels. However, analysis showed a significant difference in 5-HT and its metabolite 5-HIAA overall between genotypes (5-HT: $F(1,18)$

= 6.468, p = .0204; 5-HIAA: F(1,18) = 5.824, p = .0267) with wildtypes having higher levels of both on average. See figure 27. Analysis of turnover ratios showed no significant difference in any ratios between genotypes. See figure 28.

Conclusions

The C57 strain of mice, which is the background strain for both genotypes tested here, is known to be resistant to the neurotoxic effects of amphetamine. However, it was important to learn if the knockout mice were more sensitive to amphetamine as a function of GSTM1 gene being knocked out. There was some significant difference in serotonin and 5-HIAA levels, with levels being lower in wildtypes administered amphetamine and knockouts overall. In Experiment 3, the effects of amphetamine on C57s was examined and found similar results in serotonin levels in response to amphetamine.

Figure 22

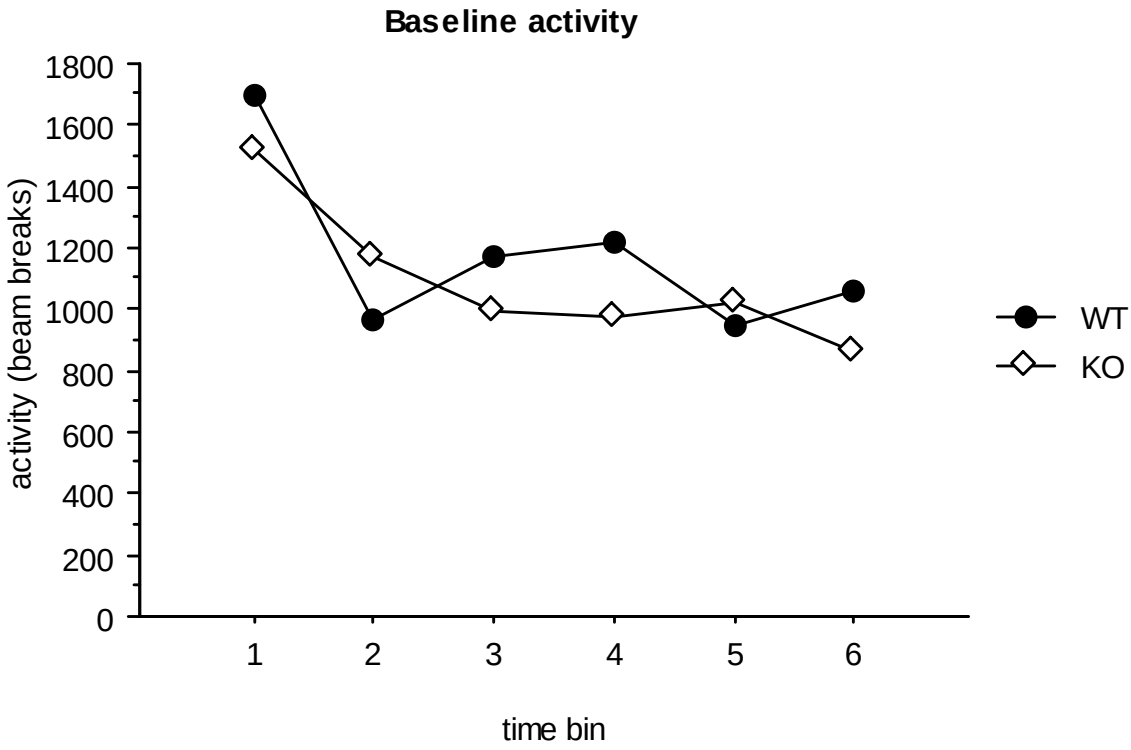


Figure 22: Average activity levels in wildtype and GSTM1 knockout mice for 30 minutes. Readings were recorded in five minute time bins. There was a significant difference in activity over the 30 minute period ($p < .0001$) and a significant interaction of activity over time and genotype ($p = .0021$). There was no significant difference in activity levels between genotypes.

Figure 23

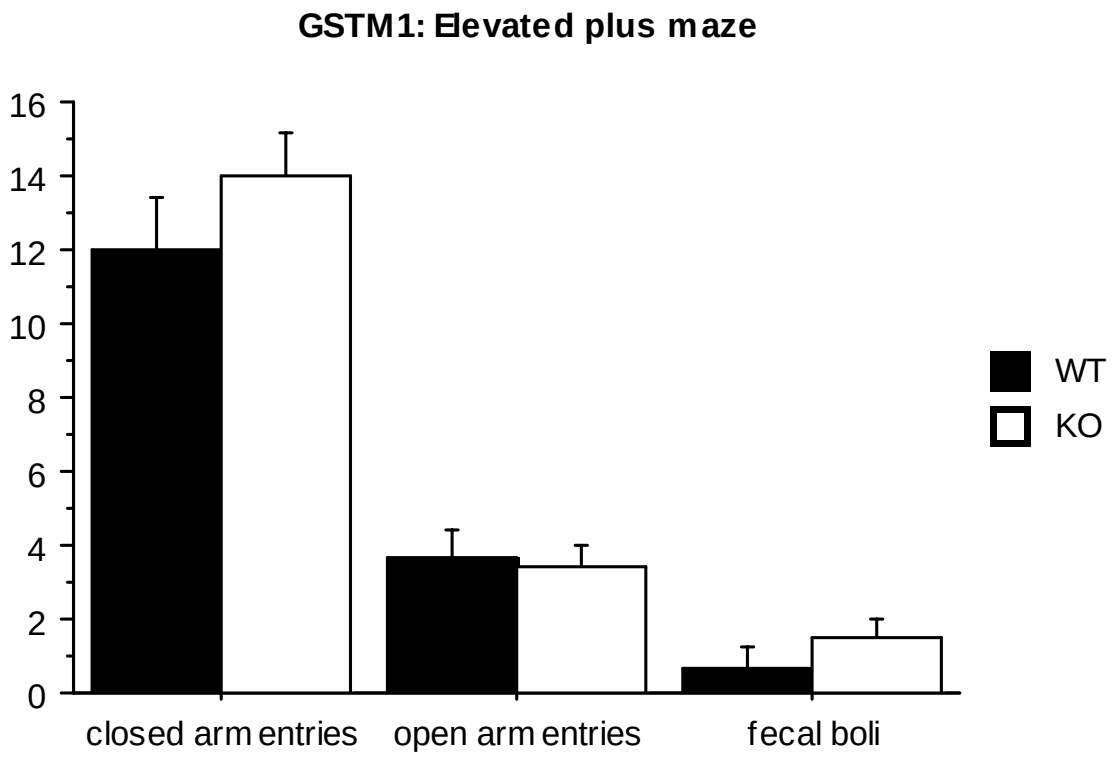


Figure 23: Performance on the elevated plus maze in wildtype and GSTM1 knockout mice: number of entries into the closed arms of the maze, number of entries into the open arms of the maze, number of fecal boli emitted during trial

Figure 24

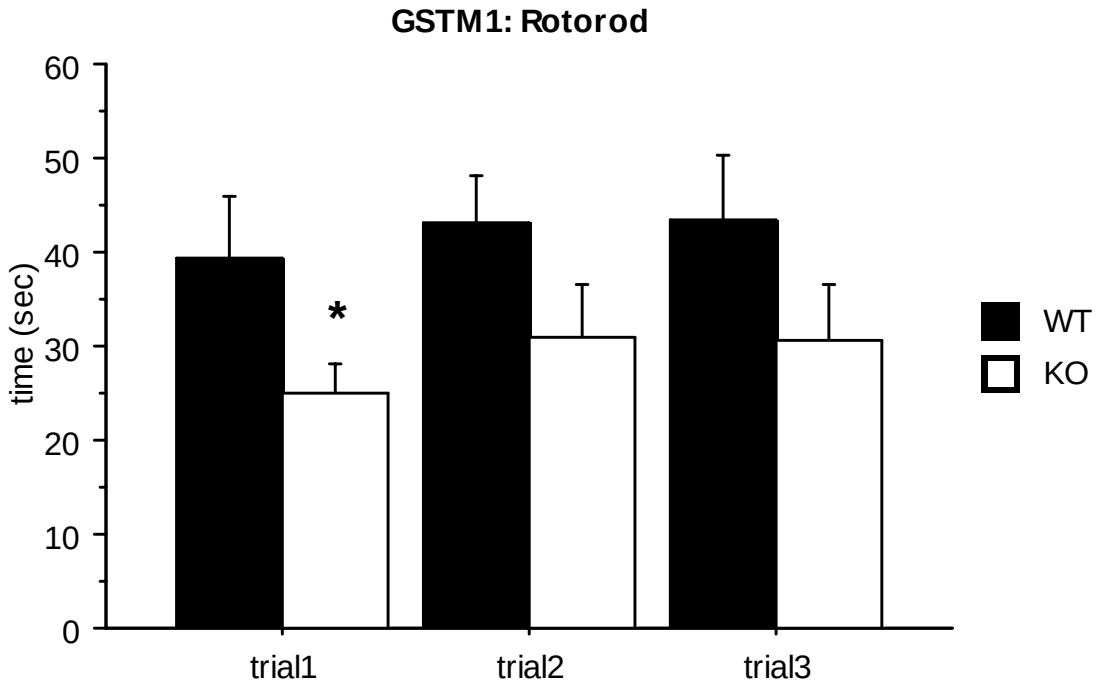


Figure 24: Performance on the rotorod by wildtype and GSTM1 knockout mice: average trial latency to fall from the rotorod rotating 12 rev/min. Maximum trial length: 60 seconds.

* denotes significant from wildtypes; $p < .05$

Figure 25

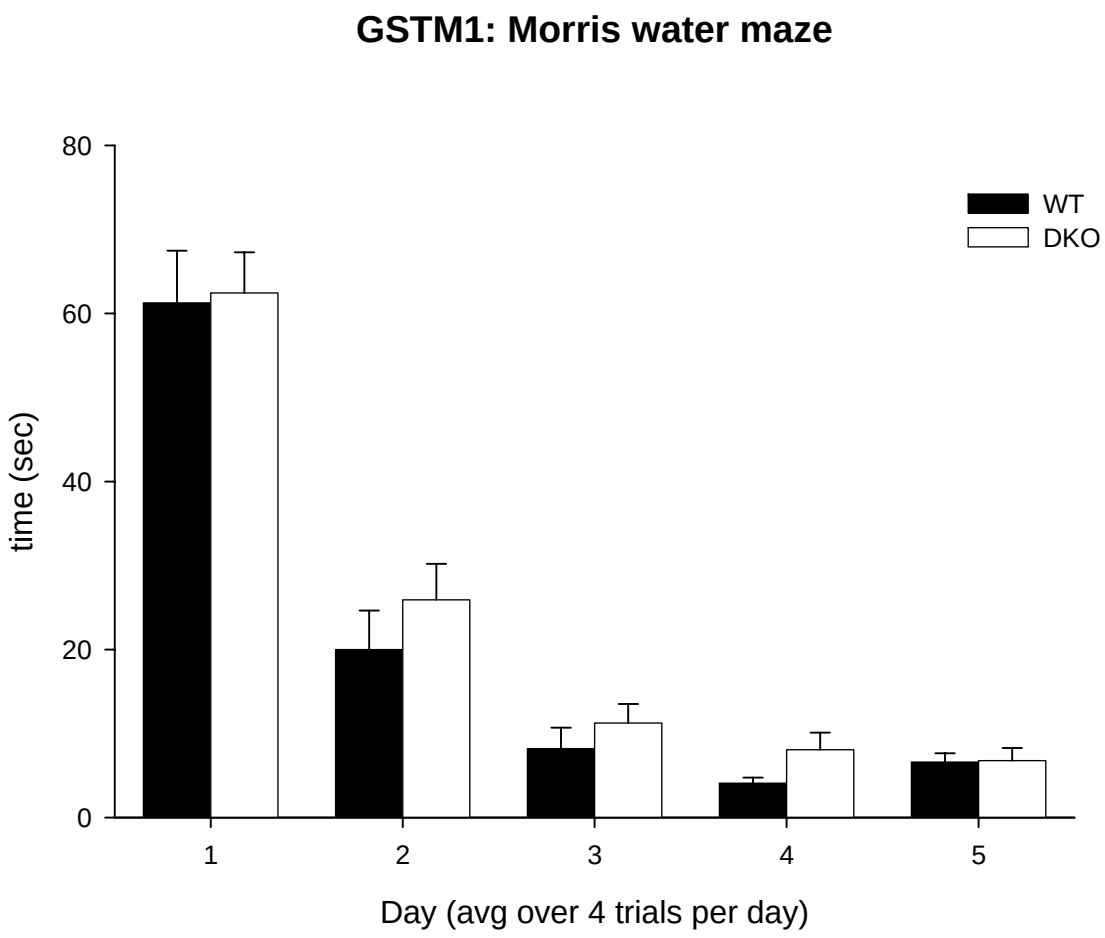


Figure 25: Performance in the Morris water maze by wildtype and GSTM1 knockout mice: average latency to find the hidden platform in four trials per day. Mice, regardless of genotype, performed significantly better over the course of testing ($p < .0001$)

Figure 26

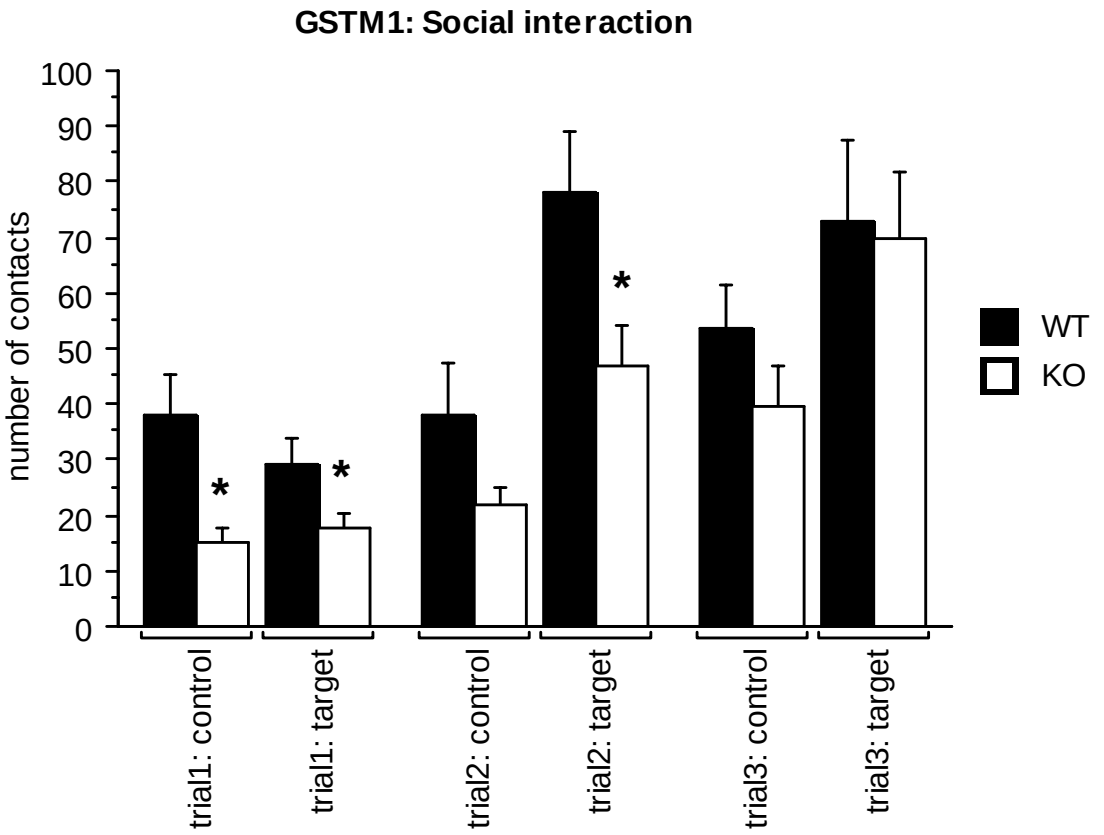


Figure 26: Social interaction in wildtype and GSTM1 knockout mice: average number of contacts with the empty control cup and the target cup containing an unfamiliar mouse

* denotes significantly different from wildtype; $p < .05$

Figure 27

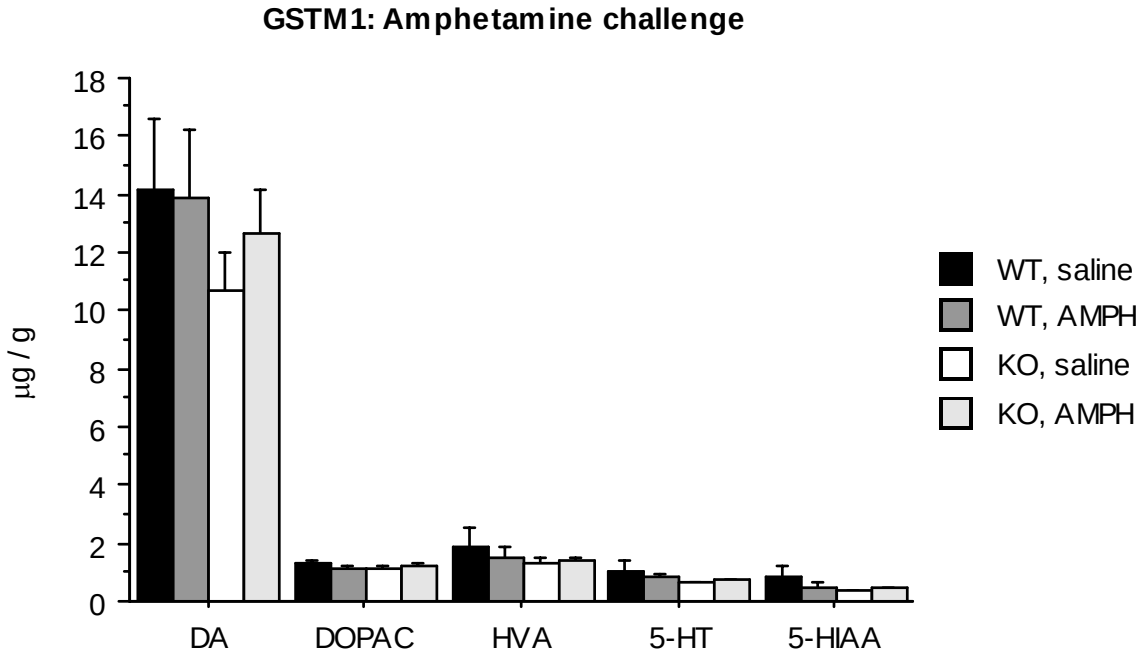


Figure 27: Concentration of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in striatum of wildtype and GSTM1 knockout mice after treatment with saline or amphetamine

Figure 28

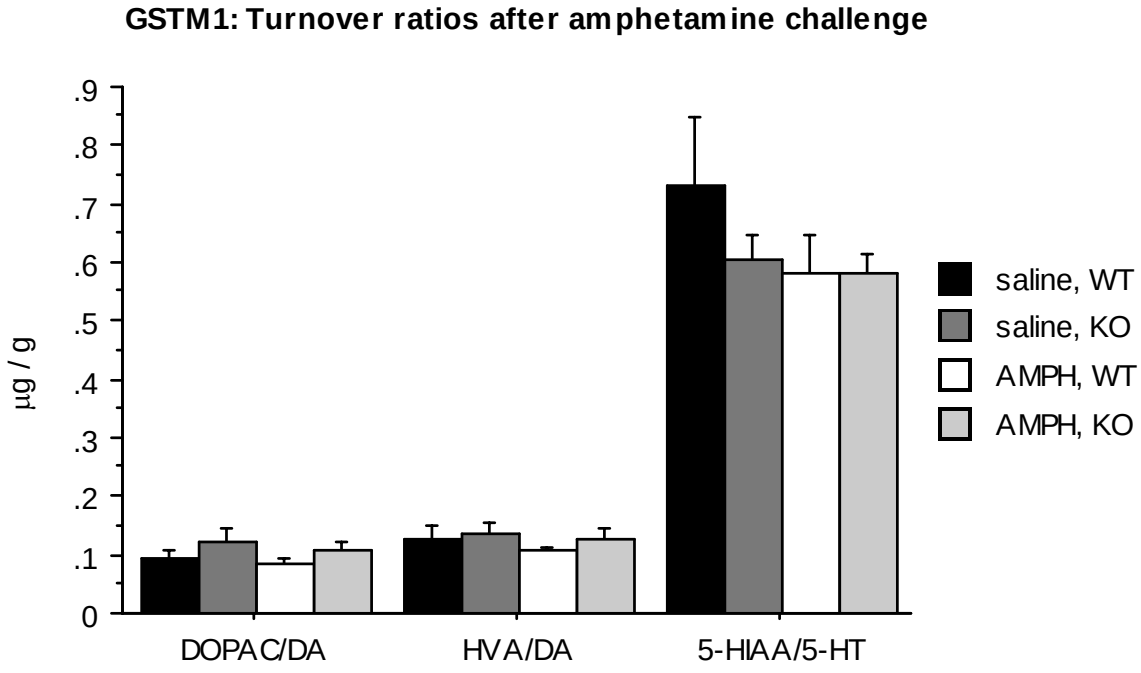


Figure 28: Turnover ratios of DOPAC/DA, HVA/DA and 5-HIAA/5-HT in striatum of wildtype and GSTM1 knockout mice after treatment with saline or amphetamine

Discussion: Experiment 10

Overall, the GSTM1 knockouts perform similarly to their wildtype counterparts. The only difference in behavior was on the rotorod and in social interactiveness. Knockouts performed worse than wildtypes on each rotorod trial, though this difference was only significant on the first trial. This may be indicative of reduced motor coordination consequent to the gene deletion.

If knockouts did have a deficit in motor coordination, it would most likely have affected water maze performance. Knockouts performed similarly to wildtypes on the Morris water maze indicating they do not have deficits in spatial learning and memory and motor coordination is not severely affected or is not affected in all types of testing.

In addition, knockouts performed worse than wildtypes in the social interaction test. Knockouts contacted both the empty control cup and the target cup containing a novel mouse less than wildtypes overall. This could indicate higher levels of anxiety if the mice simply did not explore and remained close to the edges of the chamber. However, their performance on the elevated plus maze, a good measure of anxiety, was similar to that of wildtypes. Knockouts did contact the target cup more than control cup overall, as would be expected in normal sociability.

Overall, knockouts had lower levels of dopamine compared to wildtypes, though not significantly lower. Interestingly, amphetamine-treated knockouts had slightly higher levels of dopamine compared to saline-treated knockouts. This difference was not significant and likely, not a true effect of amphetamine in these mice. However, if it were determined to be a true consequence to amphetamine-administration, it may indicate that amphetamine's effects on striatal dopamine release are delayed in GSTM1

knockouts. This could be due to a number of factors including altered amphetamine metabolism in knockouts, resistant dopamine transporter pumps, resistance to tyrosine hydroxylase or MAO inhibition. Turnover ratios were unaffected in either genotype by amphetamine-administration.

In general, it does not appear that GSTM1 knockout mice serve as a valid model of Parkinson's disease. They show relatively little difference in behavior compared to wildtypes, do not exhibit significant dopamine depletion at baseline and are not sensitive to amphetamine neurotoxicity. Other GST isoforms may have functional redundancy in the mouse brain, masking the effects of deletion of this gene. As null mutations in this gene have been associated with increased incidence in human Parkinson's disease, it follows that humans may be more sensitive to this alteration of GSTM1 function or its functional redundancy is reduced or incapacitated in affected individuals.

II. p21-ACTIVATED KINASES

p21-activated kinases (PAKs) are a highly conserved family of serine/threonine protein kinases that serve as effector molecules for the Rho GTPases Cdc42 (cell division cycle 42) and Rac (Ras-related C3 botulinum toxin substrate 1). Almost all eukaryotes encode at least one of the PAK genes, illustrating their ancient origins and critical functions (Hofmann et al., 2004). PAKs play important roles in a wide variety of cell functions including cell motility and morphology, cytoskeletal organization, apoptosis and gene transcription, though there is still much unknown about their biological and developmental roles in organisms. However, they are known to be highly expressed in the brain and are involved in diseases affecting the central nervous system. Their roles in cellular function and human diseases are discussed as potential ties to neurodegeneration and Parkinson's disease.

The mammalian PAKs are organized into two distinct families based on their amino acid sequences and structures. Group A PAKs, which consist of PAK1, PAK2 and PAK3, have very similar sequences and are distinguished by an amino-terminal regulatory domain and a carboxyl-terminal kinase domain (Sells and Chernoff, 1997; Knaus and Bokoch, 1998; Daniels and Bokoch, 1999). The regulatory domain contains a Cdc42/Rac-interactive binding (CRIB) domain that mediates PAK binding to Cdc42 and Rac. PAK4, PAK5 and PAK6 make up the group B family of PAKs (Abo et al., 1998; Yang et al., 2001; Dan et al., 2002; Jaffer and Chernoff, 2002). Unlike group A PAKs, group B PAKs do not contain an autoinhibitory domain and have a greater divergence in sequence of the GTPase binding domain and carboxyl-terminal domains of the group A

PAKs. Group B PAKs also preferentially bind to just Cdc42, unlike group A PAKs which bind to both Rac and Cdc42 (Jaffer and Chernoff, 2002).

Additionally, the expression patterns of the different PAKs vary. For group A PAKs: PAK1 is highly expressed in the brain, muscles and spleen; PAK2 is expressed ubiquitously; PAK3 is expressed only in the brain (Sells and Chernoff, 1997; Knaus and Bokoch, 1998; Bagrodia and Cerione, 1999; Vadlamudi and Kumar, 2003). For group B PAKs: PAK4 is ubiquitously expressed with highest expression levels in the prostate, testes and colon; PAK5 is expressed preferentially in the brain and to a much lesser extent in the eye, adrenal gland, pancreas, prostate and testes; PAK6 is also highly expressed in the brain, but also in the prostate, testes, thyroid, kidney and placenta (Yang et al., 2001; Jaffer and Chernoff, 2002; Pandey et al., 2002; Li and Minden, 2003). These varying expression patterns affect the viability of transgenic overexpressors and knockout mice and inevitably play important roles in the biochemical, behavioral and disease susceptibility profiles of these animals. While no knockout mice have been generated from A family members, B family members have been generated. PAK4 knockout mice die in *utero*, most likely due to cardiac complications. PAK5 and PAK6 knockout mice are viable and fertile. PAK5/PAK6 double knockout mice are also viable and fertile.

Structure:

Structurally, family A PAKs are very different from family B PAKs. This was the initial factor in classifying these kinases into different groups. However, upon further study, it was found that these differ in much more than their structures. The CRIB domain, also referred to as the GTPase binding domain (GBD) or p21 binding domain

(PBD), is located in the amino terminal region of the protein and is at least 88% identical between the family A PAKs (Jaffer and Chernoff, 2002). The CRIB domain of family B PAKs is reported to be between 60 and 75% identical among each other, however, is less than 50% similar to family A PAKs (Jaffer and Chernoff, 2002; Eswaran et al., 2007). The carboxy-terminal kinase domain is at least 93% identical among family A PAKs and at least 75% identical among family B PAKs. However, the kinase domain is only about 54% similar between family A and family B PAKs. Furthermore, the family A PAKs contain an autoinhibitory domain which has not been found in the family B PAKs (Jaffer and Chernoff, 2002). Without an autoinhibitory domain, it seems B family PAKs are constitutively active enzymes. It has been suggested that they self-regulate by autoactivating an activation segment contained in their structure via phosphorylation (Cotteret and Chernoff, 2006) and/or via an interaction with part of the amino terminal domain (Ching et al., 2003). Thus, with such varying structures, it seems the family A PAKs may be regulated differently than family B PAKs, as well as have different effectors downstream. Interestingly, both families still permit activated Rho family protein binding despite such different CRIB domains (Jaffer and Chernoff, 2002).

Activity regulation:

Most cellular functions are dependent on protein phosphorylation by kinases and phosphatases. Together these enzymes regulate the activation and inhibition of many cellular events and cascades. Consequently, the activity of protein kinases are tightly regulated as a disruption to the delicate balance needed for typical functions can have devastating results and has been linked to many human diseases.

The activity of the two PAK families differs greatly. Family A PAKs, like their name p21-activated kinases, bind to activated GTPases and subsequently exhibit an increase in their kinase activity. Family B PAKs, on the other hand, do not show an increased activity when bound to activated GTPases (Abo et al., 1998; Jaffer and Chernoff, 2002).

Family A PAKs all contain an autoinhibitory domain in their protein sequence that interacts with the CRIB domain. This conformation of the protein is an intramolecular inhibitory complex, which can be released upon binding of activated Cdc42 or Rac to the CRIB domain thus re-enabling PAK activation. The PAK protein then autophosphorylates a threonine in its activation loop which prevents refolding and inhibition. A substitution at this threonine makes PAK constitutively active.

Conversely, family B PAKs do not contain an autoinhibitory domain but a serine at a position that corresponds to the threonine autophosphorylated in family A PAKs is a predicted single autophosphorylation site and a requirement for PAK activity. One study showed that substituting another amino acid at the serine caused a more potent kinase compared to the wild type kinase (Callow et al., 2002). In addition, when PAK4 and PAK6 were truncated to contain only the CRIB domains, their activity was greater than the full length proteins (Abo et al., 1998; Yang et al., 2001). Conversely, Ching et al. (2003) found an increase in kinase activity when the N terminus (which includes the CRIB domain) was removed from PAK5. In addition, when PAK4 is coexpressed with Cdc42, PAK4 translocates to the Golgi apparatus in the cell and causes induction of filopodia. PAK5 localizes to the mitochondria. PAK6 also localizes to the mitochondria (Cotteret et al., 2003) as well as the nucleus when coexpressed with the androgen and

estrogen receptors (Lee et al., 2002). It seems that GTPases are a factor in targeting the B family members to specific locations within the cell rather than activating them (Abo et al., 1998; Dan et al., 2001).

Together, this suggests that the two families are regulated differently and family B PAKs may also have an intramolecular regulatory mechanism, but one that is not a full autoinhibitory domain controlled by activated GTPases like those in family A PAKs (Jaffer and Chernoff, 2002).

Biological function:

The biological functions and significance of family A PAKs is varied and well studied. They have been shown to be involved in gene transcription, cell cycle progression, cell survival and death signals, neurogenesis, angiogenesis, cancer metastasis and diseases of the nervous system including mental retardation and amyotrophic lateral sclerosis (Bokoch, 2003; Nadif Kasri and Van Aelst, 2008).

However, until now there has been limited information about the biological significance of the family B PAKs. Several recent studies have expanded our knowledge of their structure and speculation on their relevance. Thus far, it has been shown that B family members play roles in cytoskeletal regulation, apoptosis regulation, transformation and hormone signaling.

Cytoskeletal regulation:

Cytoskeletal organization is critical to neuronal development. Filopodia and lamellipodia are integral to growth cone guidance, associated with attractive and repulsive cues in neuronal development. Neurite extensions can occur once the filopodia

and lamellipodia are stabilized with subsequent filopodia and lamellipodia extensions occurring to continue the guidance and growth cycle (Mueller, 1999; Luo, 2000). Rho GTPases Cdc42 and Rac were first described as regulators of filopodia and lamellipodia formation and were eventually shown to be integral in all parts of neural development (Luo, 2000). PAK kinases have been shown to be effectors of these Rho GTPases. Group B PAKs have been shown to directly regulate filopodia formation (Abo et al., 1998) although the mechanism by which this happens is still unknown.

The first of the group B PAKs to be identified, PAK4, binds specifically to activated (GTP-bound) Cdc42 by its CRIB domain to promote filopodia formation and reorganization of the actin cytoskeleton. Activated PAK4 has also been shown to promote neurite outgrowth in neuroblastoma cells (Dan et al., 2002). In group B PAKs, binding of Cdc42 or Rac is not thought to activate PAK but rather affects its location within the cell. As such, PAK4 is thought to be constitutively active with or without Cdc42 but that it is the binding of activated Cdc42 with PAK4 that causes PAK4 to translocate from the nucleus to the Golgi apparatus (Abo et al., 1998). This translocation is a requirement for PAK4 to cause the formation of filopodia and reorganization of the cytoskeleton in fibroblasts and other cell types. Furthermore, the expression of activated PAK4 reduces fibroblast adhesion to the extra cellular matrix and promotes cell proliferation. This leads to anchorage-independent growth and increased cell migration and may play a role in tumorigenesis when overexpressed (Abo et al., 1998; Qu et al., 2003; Hofmann et al., 2004). Taken as a whole, PAK4 has been shown to regulate filopodia formation and the cytoskeleton, which are crucial for normal differentiation of neurons.

Activated PAK4 expression also produces a reduction in stress fibers and focal adhesions as well as a rise in actin cluster formation as seen with LIMK1 (LIM for LIM domain, an acronym from the three C. elegans gene products *lin-11, isl-1* and *mec-3*, from (Meng et al., 2002). LIMK1, a regulatory kinase with potent effects on the actin cytoskeleton, is one of the substrates for PAK4, as well as for PAK1. PAK4 binds and phosphorylates LIMK1, which stimulates LIMK1 to phosphorylate actin-depolymerization factor (ADF)/cofilin. This phosphorylation inactivates ADF/cofilin (Arber et al., 1998; Yang et al., 1998; Sumi et al., 1999; Dan et al., 2001). ADF/cofilin can directly bind and promote the disassembly of actin (Bamburg, 1999; Carlier et al., 1999).

LIMK1 and ADF/cofilin are broadly expressed in the central nervous systems of mammals (Bamburg and Bray, 1987; Mori et al., 1997; Meng et al., 2002). LIMK1 is expressed exclusively in neuronal tissues and builds up in presynaptic terminals of mature synapses (Bernard et al., 1994; Mizuno et al., 1994; Proschel et al., 1995; Wang et al., 2000). Abnormal expression of LIMK1 leads to Williams syndrome in humans (Frangiskakis et al., 1996; Bellugi et al., 1999; Boda et al., 2004). Williams syndrome is a very rare genetic disorder affecting 1 in 25,000 people that causes mild retardation, weak visual-motor integration, attention deficits, cardiovascular issues and skeletal abnormalities causing distinctive craniofacial features (Tassabehji et al., 1996).

Apoptosis regulation:

Another known substrate for PAK4 and for PAK5 is BAD, a pro-apoptotic protein (Dan et al., 2001; Gnesutta et al., 2001; Cotteret et al., 2003). BAD is a member

of the BCL-2 family that moves between the cytosol and BCL-2 or BCL-x_L on the mitochondrial membrane. BCL-2 and BCL-x_L are anti-apoptotic proteins that can bind to BAD (Gnesutta et al., 2001). When BAD translocates to the mitochondrial membrane in response to apoptotic stimuli, the newly formed complex with BCL-2 or BCL-x_L inhibits mitochondrial cytochrome *c* release which is necessary for downstream caspase protease cascade activation (Gajewski and Thompson, 1996; Golstein, 1997; Green and Reed, 1998). BAD can be rapidly phosphorylated on two serine residues in response to IL-3, a survival factor. This causes the disassociation of BCL-2 or BCL-x_L, allowing it to then block cytochrome c release from the mitochondria. This phosphorylation of BAD is thought to be related to PAK activity and acts to protect cells from apoptosis. However, it is not known whether this is catalyzed directly or indirectly by PAK4 (Tang et al., 2000; Gnesutta et al., 2001). Cotteret et al. (2003) showed that PAK5 has both direct and indirect effects on phosphorylation of BAD on two different serine residues *in vitro*. In addition, other key components of apoptosis appear to be altered with caspase 3 and PARP cleavage absent in cells that express PAK5. Furthermore, they showed that PAK5 prevents BAD from translocating to the mitochondria where it would begin the apoptotic cascade described above. Instead, PAK5 translocates to the mitochondria itself under normal circumstances, regardless of apoptotic stimuli, its own kinase activity or Cdc42 binding. On the other hand, PAK4 is typically found in the Golgi of the light microsomal fraction when coexpressed with Cdc42 and only slightly in the mitochondrial fraction otherwise (Abo et al., 1998; Cotteret et al., 2003). This difference in localization would suggest that PAK4 and PAK5 play different roles in apoptosis, although both are anti-apoptotic (Cotteret et al., 2003). PAK6 has also been found to localize to the

mitochondria but goes to the nuclear region when coexpressed with androgen and estrogen receptors. Further study needs to be done on the localization of the different family B PAKs and how this localization plays a role in their functioning.

Learning and memory:

Neural circuitry is constantly changing in response to the happenings of daily life. Learning and memory are functions of this daily remodeling of synapses. Remodeling of the cytoskeleton is the primary mediator of synaptic morphology (Matus, 2000). The PAKs are known to be an essential component in actin remodeling (Bokoch, 2003). Hayashi et al. (2004) produced transgenic mice that had a dominant-negative PAK (dnPAK) transgene that was restricted to the postnatal forebrain. The mice had fewer dendritic spines and an increased proportion of shorter, larger spines in the cortex compared to control mice. However, in the hippocampus, the spine morphology and plasticity were unchanged. Mice were tested in the Morris water maze and showed no difficulty in acquisition of spatial memories, with the transgenic mice performing similarly to the wildtype controls. However, when tested 21 days later to assess consolidation/retention of this memory, the transgenic mice performed worse than the controls suggesting an important role of PAK in cortical spine morphogenesis as it relates to long term memory (Hayashi et al., 2004).

Interestingly, mammalian PAK5 is similar to *Drosophila* MBT (mushroom body tiny) protein. PAK5 and MBT share similar kinase and CRIB domain sequences (Dan et al., 2002). The mushroom body is a neural structure in the adult *Drosophila* similar to the hippocampus in humans and plays a role in learning and memory. *Mbt* null mutants

have fewer neurons and reduced mushroom body volume leading to the conclusion that Mbt is active in cell proliferation, differentiation and/or survival of neuronal cells (Melzig et al., 1998). This, along with *in vitro* studies, suggest that PAK5 is an important moderator of Rho GTPases signaling pathways that involve cytoskeletal changes needed for neurite outgrowth promotion (Dan et al., 2002).

Role of p-21 activated kinases in human disease

Little is known about the biological functions of the p21-activated kinases, particularly those of the family B PAKs. To date, family A PAKs are known to be essential in the structure and function of the central nervous system and have been found to play a role in mental retardation, Alzheimer's disease and amyotrophic lateral sclerosis (ALS; Lou Gherig's disease) (Boda et al., 2004; Tudor et al., 2005; Zhao et al., 2006; Ma et al., 2008). Although family A and B PAKs have divergent sequences and functions, it is plausible that both play a role in human diseases. Recently, Danzer et al. (2007) reported a link between family B member PAK4 and α-synuclein in Parkinson's disease. The established role of the A family PAKs, as well as the high levels of B family PAKs in the brain and recent link to α-synuclein suggest that B family PAKs may play a crucial role in normal neural functioning. Loss of function of these B family PAK members may result in critical changes in neuronal growth and function leading to neurodegeneration. As such, PAK knockout mice may serve as relevant models of neurological diseases, including Parkinson's disease. Current links to human disease are highlighted below.

Mental retardation

Mental retardation is a broad term used to describe a wide variety of cognitive handicaps that occur in humans, including autism spectrum disorder, Down's syndrome and fragile X syndrome. Mental retardation is found in roughly 2 – 3% of children and young adults. It is characterized by reduced cognitive functioning (an IQ lower than 70) and severe deficits in basic and adaptive social skills. Mental retardation is typically divided into two major classes: syndromic mental retardation and nonsyndromic mental retardation. Syndromic mental retardation is associated with a fixed collection of other manifestations including body and brain deformities. On the other hand, nonsyndromic mental retardation manifests as only reduced cognitive function without any other clinical signs or symptoms. The causes of mental retardation are varied and numerous and include both non-genetic factors like premature birth, infectious disease, *in utero* toxicant exposure and genetic factors like chromosomal abnormalities and mutations (Nadif Kasri and Van Aelst, 2008).

Genes on the X chromosome are commonly more likely to cause mental retardation than genes on any other segment of the autosome (Mandel and Chelly, 2004; Ropers and Hamel, 2005). Numerous X-linked genes have been identified recently that are involved in genetic causes of mental retardation (Ropers et al., 2003). Out of 13 gene mutations on the X chromosome discovered so far, a mutation in PAK3 found in several afflicted families plays a role in X linked, nonsyndromic mental retardation (MRX) (Allen et al., 1998; Bienvenu et al., 2000; Chelly, 2000; Gedeon et al., 2003). While the responsible genes have been identified, the mechanisms causing the cognitive defects are still unknown.

Recently, the regulators and effectors of the Rho GTPases like PAK have become the target of interest in human neurological diseases including mental retardation. Since the Rho GTPases are known to moderate the actin cytoskeleton and dendritic spine morphogenesis, it is likely they are important in the cause of the dendritic spine structure and morphology abnormalities related to many forms of mental retardation (Nadif Kasri and Van Aelst, 2008). PAK3, one of the A family of PAK genes, has been found to be highly expressed in the brain and linked to nonsyndromic X-linked mental retardation (MRX). Three different mutations in PAK3, that either cause loss of the protein or its kinase activity, have been discovered in several lineages of MRX (Bienvenu et al., 2000).

PAK3 is found mainly in the soma, dendrites and dendritic spines of post-mitotic neurons of the cerebral cortex and hippocampus of the brain (Boda et al., 2004). There it can play an integral role in the phosphorylation and inactivation of cofilin by LIMK (Arber et al., 1998; Meng et al., 2002; Okamoto et al., 2004). This in turn causes accumulation of actin filaments needed for control of spine morphogenesis (Ramakers, 2002; Boda et al., 2004). Alterations to this schema have been suggested as contributors to the deficits in mental retardation yet it is uncertain what role PAK3 plays and exactly how its mutations affect synaptic development.

Both *in vitro* and *in vivo* models are useful exemplars of biological outcomes when this kinase is disturbed. PAK3 knockout mice, while not exhibiting any obvious structural abnormalities in their neurons, exhibited selective impairment in late phase hippocampal long-term potentiation (LTP), a long-lasting increase in synaptic strength after stimulation of a synapse that underlies the mechanism of learning and memory. These mice also demonstrated a remarkable decrease in hippocampal activated cAMP-

responsive element binding protein (CREB) with no difference in the total CREB protein (Meng et al., 2005). CREB function has been demonstrated to be important for synaptic plasticity and memory formation in mice (Kandel, 2001; Lonze and Ginty, 2002).

In an *in vitro* model, Boda et al. (2004) found that even transient suppression of either PAK3 or its dominant negative form (carrying a mutated MRX gene) in the pyramidal cells of the hippocampus causes dendritic spine shape changes, a decrease in mushroom-type or stubby spines, growth of abnormal protrusions that do not form mature synaptic contacts, and impaired synaptic transmission and plasticity.

Fragile X syndrome (FXS) is one of the most common inherited forms of mental retardation. Patients have typically have moderate to severe mental retardation and exhibit developmental delay. FXS is caused by the loss of function of the FMR1 gene and subsequent loss of the fragile X mental retardation protein (FMRP) encoded by it (O'Donnell and Warren, 2002). Problems with attention, hyperactivity, aggression, self-injurious behavior, autism, seizures, anxiety and depression are all common in children with FXS (Bailey et al., 2008).

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that results in progressive skeletal muscle weakness, muscle atrophy and death typically due to paralysis of the respiratory muscles. It is caused by preferential loss of motor neurons with death occurring from respiratory failure. Besides effected motor function, ALS shows some other similarities to Parkinson’s disease: it affects men preferentially, is

typically idiopathic with some instances of familial inheritance and average age of onset is 56 years but can range from 20 to 80 years of age (Lomen-Hoerth, 2008).

Animal models of ALS do not show any obvious clinical, histopathological or electrophysical signs of neuronal degeneration in mice lacking the Als gene (Cai et al., 2005; Devon et al., 2006; Hadano et al., 2006; Nadif Kasri and Van Aelst, 2008) which could be due to compensations during development. Alsin knockout mice exhibit a slow, but progressive loss of cerebellar Purkinje cells, together with a subclinical motor dysfunction and altered endosome trafficking (Hadano et al., 2006). Alsin is the product of ALS2, one of the genes implicated in ALS. In neurons, alsin and Rac1 co-localize in growth cones of hippocampal neurons and alsin increases neurite outgrowth in cortical neurons by stimulating the Rac1-PAK signaling pathway (Tudor et al., 2005). In addition, the human PAK6 gene is located within the locus ALS5, one of the loci identified as being involved in an inherited form of ALS (Hentati et al., 1998).

Alzheimer's disease

Alzheimer's disease is the leading cause of cognitive defects in the aged population. It is characterized by gradual cognitive impairments including deficits in memory and progressive dementia. Pathologically, it is distinguished by β-amyloid plaques and neurofibrillary tangles composed of tau protein. Like α-synuclein in Parkinson's disease, β-amyloid seems to be related to the incidence of Alzheimer's disease and is toxic in excess and when oligomerized and aggregated. It is estimated that 5 – 40% of neurons are lost in the hippocampus of those afflicted with Alzheimer's and 70 – 95% of postsynaptic proteins like actin-regulating developmentally regulated brain

protein (drebrin) are lost also (Harigaya et al., 1996; Simic et al., 1997; Hatanpaa et al., 1999; Shim and Lubec, 2002; Calon et al., 2004).

Interestingly, Alzheimer's disease shares some key features with mental retardation syndromes, namely dendritic regression and spine loss. In both Alzheimer's disease and Down syndrome, the App (amyloid precursor protein) gene is involved in tangle and plaque pathology (Wisniewski and Wen, 1985). Additionally, other genes have been implicated in mental retardation and show a grouping of proteins in the post-synaptic pathways that control assembly and disassembly of dendritic spine actin as well as dendritic spine morphogenesis. Dendritic spines are a major site of synaptic contact and need actin to function properly. As a regulator of the actin cytoskeleton and cofilin, the protein that disassembles actin, PAK kinases play a major role in dendritic maintenance including initiation, growth, branching (Ma et al., 2008). Animal models with a dominant negative PAK or knockout of PAK's downstream kinase, LIMK1, show dendritic spine defects and cognitive deficiency (Allen et al., 1998; Ong et al., 2002).

Zhao et al. (2006) report that PAK1 and PAK3 and their activity are greatly reduced in Alzheimer disease. In addition, dysregulation of cofilin, an important downstream molecule of PAK activity, due to faulty phosphorylation from PAK, may lead to actin cytoskeleton disruptions. Inclusion bodies that contain cofilin, actin rods and other actin-binding proteins are found in the brains of patients with Alzheimer disease (Mitake et al., 1997). Zhao et al. (2006) found an inverse relationship between the amount of cofilin staining and the amount of pPAK (phosphorylated PAK – or PAK in its active form).

Parkinson's disease

Besides nigral neuron loss, there is also degeneration and pathology seen in other parts of the brain, including α-synuclein aggregates in the form of Lewy bodies. This is a hallmark found in some forms of both familial and idiopathic Parkinson's disease and found concentrated in the surviving neurons of the substantia nigra.

In a normal, healthy brain, α-synuclein is abundant and exists in a native unfolded conformation that could be involved in neurotransmitter release from the vesicles. α-synuclein is prone to misfolding and aggregation that occurs more frequently when the protein is mutated or overexpressed. Aggregation can lead to proteasomal damage and more aggregation of not only α-synuclein itself but of other proteins. The overexpression of wild type α-synuclein, as well as presence of the mutant α-synuclein, have been shown to be involved in neuronal degeneration and cell death (Polymeropoulos et al., 1997; Singleton et al., 2003; Olanow, 2007). Three missense mutants of α-synuclein (A53T, A30P, E46K) have been found to increase oligomerization or fibrillation of the protein leading to its toxic form. Overexpression of these mutants in neurons of flies and mice cause alterations in behavior similar to Parkinson's disease (Polymeropoulos et al., 1997; Kruger et al., 1998; Conway et al., 2000; Zarranz et al., 2004; Greenbaum et al., 2005).

Recently, a monoamine oxidase metabolite of dopamine in the brain, 3,4-dihydroxyphenylacetaldehyde (DOPAL), was found to directly and indirectly induce rapid aggregation of α-synuclein (Burke et al., 2003; Burke et al., 2008). DOPAL is converted to 3,4-dihydroxyphenylacetic acid (DOPAC) by aldehyde dehydrogenase under normal conditions and has been shown to be highly reactive, radical forming and

neurotoxic. In neurologically normal brains, 2 -3 μM of DOPAL was found (Kristal et al., 2001). Burke et al. (2003, 2008) showed that this level of DOPAL was sufficient to cause α-synuclein aggregation *in vitro* and two times that concentration had the same effect in a cell free system. For this reason, they believe there might be defenses in place to protect against low-level DOPAL concentrations. Furthermore, they suggest that these protective factors may be depleted (Hirsch et al., 1998) and in conjunction with other known deficits may result in increased levels of DOPAL (Schapira et al., 1990; Swerdlow et al., 1998; Betarbet et al., 2000). On the other hand, dopamine and its metabolites DOPAC and HVA have not been shown to cause any aggregation of α-synuclein at physiologically relevant concentrations. This study connects two factors in Parkinson's disease etiology: the role of dopamine production and its metabolites and the presence of α-synuclein.

During a protein array aimed at determining the effects of recombinant α-synuclein on a variety of human kinases, Danzer et al. (2007) discovered that incubating globular α-synuclein oligomers with PAK4 caused a significant inhibition of PAK4's autophosphorylation. This inhibition was concentration dependent and did not occur with monomeric α-synuclein or β-synuclein (Danzer et al., 2007).

While Danzer et al. (2007) showed that α-synuclein itself is not a substrate for PAK4, they did find a difference in the phosphorylation of another substrate of PAK4, LIMK1, in α-synuclein transgenic mice. In mice that overexpress α-synuclein, there is a 74% reduction in the ratio of phospho-LIMK1 to total LIMK1. They also showed that brainstem lysates (which accumulate α-synuclein oligomers in adult transgenic mice overexpressing mutant α-synuclein) had reduced phosphorylation of LIMK1. This

suggests that the aggregated α-synuclein could also inhibit the PAK4-LIMK1 pathway *in vivo* as well. If this pathway is inhibited, the anti-apoptotic properties of PAK4 would be lost or greatly diminished (Danzer et al., 2007). Interestingly, PAK4 knockout mice die in utero around day 10.5 most likely from cardiac complications. They also show central nervous system disruptions with severe abnormalities in neuronal differentiation, development and migration to proper targets (Qu et al., 2003).

Hypothesis: PAK kinases play important roles in a variety of biological functions. Several diseases have been linked to altered PAK kinase functioning or a mutation in one of the PAK genes, including Parkinson's disease, Alzheimer's disease and ALS. The following studies characterized the behavior, drug sensitivity and neurochemistry of PAK knockout mice to determine the behavioral, neurochemical and neurotoxicity differences in PAK5 knockouts, PAK6 knockouts as well as PAK5/6 double knockouts compared to wildtype controls.

Specific aims:

- **Compare phenotypes of PAK5 knockouts and PAK6 knockouts to DKO behavioral phenotype as they relate to normal wildtype behavior**
- **Examine functional redundancy between PAK5 and PAK6 using behavioral measures**
- **Determine differences in neurochemistry of all genotypes**
- **Explore possible behavioral/neurochemical similarities to various known disease characteristics**
- **Use drug challenge to expose any other potential differences in behavioral phenotype/gene function**

Experiment 11: Behavioral characterization and drug sensitivity of PAK5/PAK6 double knockout mice

Rationale

To date, the behavior and drug sensitivity of knockout animals lacking any B family PAK genes had not been investigated and casual observation of these mice showed no obvious phenotype. However, due to the crucial role of PAK genes and protein kinases in neuronal development and function, as well as their roles in neurodegeneration, a more complete characterization and comparison to known disease phenotypes, including Parkinson's disease was needed. As such, a battery of behavior tests, as well as sensitivity to caffeine and amphetamine, was tested in PAK5/PAK6 double knockouts and their wildtype controls.

Locomotor assessment was completed to determine overall activity levels of both genotypes. The elevated plus maze was used as a measure of anxiety. The rotorod was used as an assessment of motor function and coordination. Learning and memory were assessed in the active avoidance and passive avoidance paradigms. Social interactiveness and aggression were also tested. Sensitivity to caffeine was assessed in the open field to measure activity. Finally, amphetamine was administered to evaluate sensitivity to a dopaminergic toxicant. All together, these tests give a more comprehensive picture of the behavioral and neurochemical properties as it could relate to disease phenotypes, particularly that of Parkinson's disease.

Animals

All mice were on a mixed 129/Sv x C57BL/6 genetic background and were generated in the Minden lab at Rutgers University. Eleven male double knockout (PAK5/PAK6; DKO) mice and 10 male wild type mice were used throughout the battery of behavioral tests. All mice were born within two weeks of each other and were group housed in plastic shoebox cages with free access to food and water until approximately 60 days old. They were then transferred to individual hanging wire cages (20 cm x 10 cm x 12 cm), also with free access to food and water. After a brief acclimation period, testing began at approximately three months of age. The mice were housed in a temperature and humidity regulated room with a 12 hr light/dark cycle. All of the experiments were conducted with the experimenter blind to mouse genotypes. All procedures were conducted and in strict compliance with the policies on animal welfare of the National Institute of Health and the Animal Care and Use Committee at Rutgers University.

Weight

Mice were weighed approximately once a week throughout the duration of experiments.

Activity

Locomotor activity was assessed for 30 minutes in a novel environment (42 x 22 x 14 cm Plexiglas box) with six infrared sensors placed approximately 7 cm apart and 2.5 cm above the floor. The number of beam breaks was recorded every 5 minutes.

Elevated Plus Maze

Mice were placed in an elevated plus maze consisting of two open arms and two closed arms 30 cm long and 9 cm wide that cross a neutral 5 cm x 5 cm central square. The entire apparatus was elevated 60 cm above the floor. Each animal was placed in the center square of the maze and was given one 10-minute session to explore the maze. The number of entrances to closed arms, open arms, and jump-offs were recorded, as well as the number of fecal boli. An entrance to an arm was counted when all four feet crossed into an arm from the neutral center square.

Rotorod

Mice were assessed for balance and motor coordination on a 6.0 cm diameter rotorod rotating at 12 revolutions per minute, 60cm above a padded container. Each trial was a maximum of 60 seconds and each mouse was given three consecutive trials. The latency to fall from the rotating rod was recorded.

Active avoidance

Mice were tested in an active avoidance T-maze consisting of two 20 x 11 cm chambers connected to a 40 x 10 cm corridor with 18 cm high walls made of Plexiglas. The floor was made of stainless steel bars spaced 0.75 cm apart and connected to a shock generator except in the "safe" arm of the T-maze. In each trial, a mouse was placed in the start box. After an intertrial interval of 20 seconds, a conditional stimulus tone accompanied the opening of the start box door. A correct avoidance response, moving to

the safe arm of the T-maze within 10 seconds, avoided the foot shock. Failure to make an avoidance response led to onset of a 0.8 mA foot shock that could be terminated by moving to the safe arm as a escape response. The maximum time allowed for an animal to make an escape response was also 10 seconds. The trial ended once the mouse made an avoidance or escape response to the safe chamber or at the end of 20 seconds total if no correct response was made. At the end of each trial, mice were moved back into the start box and given a 20 second intertrial interval. Each animal was given 5 sessions of 10 trials across 5 days. The type of response (avoidance or escape) and the latency for the animal to make either avoidance or escape response was recorded for each trial.

Passive Avoidance

Passive avoidance was measured in the T-maze after mice had learned the active avoidance paradigm. Conditions were identical to active avoidance testing except the safe arm of the maze was switched to the opposite arm. Mice had to inhibit their entry into the previously safe arm in order to avoid the foot shock. Each animal was given two 3 trial sessions across two consecutive days. Each trial consisted of a 20 second period when the conditioned stimulus tone was sounded. Mice began each trial in the start box as in the active avoidance trials and the lifting of the start box door with the sounding of the tone signaled the start of each trial. If the mouse entered the previously safe chamber, it received a 0.8 mA foot shock and the trial was terminated. If the mouse avoided the previously safe chamber for the 20 second period, no shock was delivered. The latency to enter the previously safe chamber was recorded.

Social Chamber

Mice were placed in a social chamber that was 40 cm x 40 cm x 36.6 cm made of Plexiglas. Two wire cylinders made of a stainless steel grid, each 11 cm in diameter and 13 cm high, were located in opposite corners inside the chamber. A contact was recorded when the subject mouse touched either cylinder with at least one paw on the cylinder and one on the floor of the chamber. Prior to the experimental sessions, the subject mouse was placed in the middle of the chamber and was allowed to explore the chamber for 10 minutes. After the habituation period, an adult male BALB/c mouse was placed in the target cylinder, while the control cylinder was left empty and the experimental mouse was placed back in the middle of the chamber. Each animal was allowed three 10-minute sessions across three consecutive days. The total number of contacts with the target and control cylinders during the session was recorded.

Aggression

At approximately 8 months of age, mice were tested for aggressive behavior using the resident-intruder test. The PAK wildtypes and knockouts (considered "resident" mice here) were housed individually in pan cages containing standard wood chip bedding for two weeks before testing began. Male C57BL/6 intruder mice were housed in cages at five mice per cage for at least two weeks prior to the start of trials. Testing began by placing an intruder in the resident's home cage for one 30-minute session per day for each of three days. The latency to attack, the number of attacks and which mouse (resident or intruder) initiated each attack was recorded.

Caffeine challenge

All mice were administered one subcutaneous injection of 12.6 mg/kg caffeine and immediately placed into the activity chambers. Locomotor activity was assessed for three hours in a non-novel environment (42 x 22 x 14 cm Plexiglas box) with six infrared sensors placed approximately 7 cm apart and 2.5 cm above the floor. The number of beam breaks was recorded every 10 minutes.

Amphetamine challenge

Mice were administered one subcutaneous injection of 50 mg/kg amphetamine or saline. Mice were sacrificed 72 hours after drug administration for neurochemical analysis.

Baseline neurochemistry

Male mice 30 (WT: n = 13; DKO: n = 13), 60 (WT: n = 10; DKO: n = 12) and 120 (WT: n = 10; DKO: n = 10) days old that were not used in any behavior tests that were also drug-naïve were sacrificed for measurement of dopamine, serotonin and their metabolites. Frontal cortex, striatum, hypothalamus, hippocampus and brain stem were dissected out, snap frozen in liquid nitrogen and stored until assay.

In addition, all mice used in the behavioral tests and drug challenges were sacrificed for measurement of dopamine, serotonin and their metabolites at the conclusion of the experiment. Bilateral striata was dissected out, snap frozen in liquid nitrogen, and stored until assayed as described in Experiment 1.

Results

Weight

Mice were weighed once a week through the duration of experiments. Statistical analysis with a repeated measures ANOVA showed that there was a significant effect of weight overall ($F(24, 504) = 55.709$, $p < .0001$) and a significant weight by genotype effect ($F(24,504) = 2.013$, $p = .0032$). Fisher's LSD *post hoc* test showed a significant difference in weight between wildtypes and double knockouts ($p < .0001$). See figure 29.

Wildtypes weighed an average of 20.0 grams at the start of measurements while double knockouts weighed an average of 23.8 grams. This difference in weight was significant at the start of experiments ($F(1,21) = 10.519$, $p = .0039$). Because of this, change in weight over time was analyzed. Analysis using a repeated measures ANOVA showed there was a significant difference in weight change over time ($F(1,20) = 4.307$, $p < .0001$) and a weight change over time between the two genotypes ($F(1,20) = 3.092$, $p < .0001$). However, Fisher's LSD *post hoc* analysis showed no significant difference in weight between genotypes ($p = .6630$) and one-way ANOVA shows that the last weight measured of the two genotypes was not significantly different ($F(1,21) = 1.922$, $p = .1802$). In addition, one-way ANOVA analysis of total change in weight over time showed no significant difference in weight gain between genotypes.

Conclusions

Overall, there was no difference in weights between the genotypes despite the double knockouts weighing more at the start of testing.

Activity

Mice were placed into the activity chamber and their activity counts were recorded for every five minutes for thirty minutes. Repeated measures ANOVA showed that overall there was a significant effect of genotype on activity levels ($F(1,19) = 13.901$, $p = .0014$) and a significant change in activity for both genotypes over the testing period ($F(1,5) = 11.201$, $p < .0001$). The interaction of genotype and activity approached, but did not reach, significance ($F(1,5) = 2.050$, $p = .0785$). However, Fisher's PLSD *post hoc* analysis revealed that there was a significant difference in overall activity between genotypes ($p < .0001$). See figure 30.

Conclusions

Both genotypes exhibited more exploratory behavior at the start of activity measurement. This decreased significantly in both genotypes by the end of activity measurement. Double knockouts were significantly less active than wildtypes overall and at each time point tested.

Elevated plus maze

Mice were placed in the center neutral square of the elevated plus maze to begin each trial. Entries in the closed and open arms were counted, as well as the number of jumps off the apparatus and fecal boli. One-way ANOVA with Fisher's LSD *post hoc* analysis was completed on each variable.

Wildtype animals entered the closed arms of the maze significantly more times than the double knockouts ($F(1,21) = 30.542$, $p < .0001$). Wildtypes also entered the

open arms of the maze significantly more times that the double knockouts ($F(1,21) = 10.466$, $p = .0040$). The ratio of open arm entries to closed arm entries is 36.2% and 42.5% for wildtypes and double knockouts, respectively. The number of fecal boli can be used as an indication of anxiety and were significantly more numerous for double knockouts compared to wildtypes ($F(1,21) = 6.678$, $p = .0173$). There was no significant difference in jump off between genotypes. See figure 31.

Conclusions

The elevated plus maze is used as a measure of anxiety. Overall, the double knockouts exhibited more anxious behavior compared to wildtypes. As the prime measure of anxiety behavior in this test, double knockouts entered the open arms of the maze significantly less wildtypes. They also had significantly more fecal boli compared to wildtypes during testing. These two findings suggest that the double knockouts are more anxious than their wildtype counterparts. However, the double knockouts also entered the closed arms less frequently compared to wildtypes. Furthermore, results from activity measurement show that double knockouts are generally less active than wildtypes. This hypoactivity could be a noteworthy factor in the performance of the double knockouts in the elevated plus maze. Still, the double knockouts entered the open arms of the maze significantly less than they entered the closed arms. The ratio of open arm entries to closed arm entries is 36.2% and 42.5% for wildtypes and double knockouts, respectively.

Rotorod

Mice were given 3 consecutive trials on the rotorod, lasting a maximum of 60 seconds. The latency to fall from the apparatus was recorded and analyzed using a repeated measures ANOVA. Analysis showed significant improvement in both genotypes over time ($F(1,2) = 5.837$, $p = .0060$) but no significant difference between genotypes over all three trials ($F(1,2) = .978$, $p = .3849$). Further *post hoc* analysis revealed a significant effect between genotypes over all three trials ($p < .001$) Also, when each trial was analyzed using a one-way ANOVA, wildtypes performed significantly better on each trial compared to double knockouts (trial 1: $F(1,20) = 10.882$, $p = .0036$; trial 2: $F(1,20) = 12.483$, $p = .0021$; trial 3: $F(1,20) = 6.633$, $p = .0181$). See figure 32.

Conclusions

The rotorod is used as a measure of motor function and coordination. It can also be looked at as a measure of motor learning. Overall, the double knockouts performed worse than wildtypes on each trial but did improve over the trials. However, the average latency to stay on the rotorod for double knockouts on trial 3 was still lower than wildtype performance on trials 2 and 3. Ultimately, the double knockouts performance on the rotorod was not significantly worse than the wildtypes performance. This shows that while worse, the double knockouts do not exhibit significantly different motor coordination and learning as measured on the rotorod. The slight deficiency in performance on the rotorod might be due to general hypoactive tendencies or a deficiency that will become increasingly pronounced as the double knockouts age.

Active Avoidance

A repeated measures ANOVA on avoidance latency revealed there were no significant differences between genotypes, avoidance latencies or genotype by avoidance latency; although avoidance latency approached significance ($F(7,77) = 1.961$, p .0713). *Post hoc* analysis showed there was a significant difference between genotypes in avoidance latency ($p = .0231$). See figure 34.

A repeated measures ANOVA showed there were significant differences between genotypes ($F(1,19) = 9.754$, $p = .0056$), percentage of avoidances ($F(7,133) = 37.121$, $p < .0001$) and a significant interaction between genotype and percentage of avoidances ($F(7,133) = 2.759$, $p = .0104$). Wildtypes consistently performed better than the double knockouts and successfully avoided 90% of the time beginning on day two of testing and continuing until day eight of testing. Double knockouts, on the other hand, gradually improved their responses over the testing days but only reached about 70% avoidance by day eight. A one-way ANOVA of percentage of avoidances on the last day of testing showed that wildtypes avoided significantly more than double knockouts ($F(1,19) = 5.099$, $p = .0359$. In fact, wildtypes performed significantly better than double knockouts on most days of testing (day two: $F(1,19) = 13.523$, $p = .0016$; day three: $F(1,19) = 12.769$, $p = .0020$; day five: $F(1,19) = 6.102$, $p = .0213$; day six: $F(1,19) = 5.966$, $p = .0245$). See figure 36.

A repeated measures ANOVA could not be run on the escape latencies due to missing values. There are missing values because as animals improved on the task, there were instances of zero escapes since the animals avoided 100% of the time. Instead, a one-way ANOVA was run on the results of each day in order to compare the

performance of the wildtypes to the double knockouts. One-way ANOVAs on each day of testing showed no significant difference in escape latencies between genotypes. In general wildtypes had lower latencies than double knockouts. See figure 33.

Paralleling the number of avoidances, a repeated measures ANOVA on percentage of escapes showed there were significant differences between genotypes ($F(1,19) = 9.754$, $p = .0056$), percentage of escapes ($F(7,133) = 37.121$, $p < .0001$) and a significant interaction between genotype and percentage of escapes ($F(7,133) = 2.759$, $p = .0104$). See figure 35.

Conclusions

Overall, wildtype mice performed significantly better than double knockouts in the active avoidance paradigm. They rapidly reached optimal performance by day two of testing, successfully avoiding the foot shock an average of 90% of the time. This performance remained steady for the duration of testing. Their latency to exhibit an avoidance response remained steady over testing, with a tendency towards faster times by end of testing. In instances where wildtypes failed to avoid the foot shock and had to escape it, the latency to escape improved over testing days.

The performance of double knockout mice improved over testing as well, although much slower than wildtypes. Peak performance occurred on day four of testing with double knockouts successfully avoiding the foot shock an average of 80%. This level of performance then dropped to about 70% on the next day of testing and remained there for the duration of testing without any more improvement. Wildtypes successfully avoided significantly more times than double knockouts on the last day of testing. Furthermore, latency to avoid slightly increased over the days of testing but latency to

escape decreased over the days of testing. The performance of the double knockouts may be attributed to two possible mechanisms. First, these mice are significantly less active than wildtypes at baseline. This could account for greater latencies to respond and less avoidance responses. They may not move in time to exhibit an avoidance response but once the foot shock began, the mice were provoked to move and escape. Overall, their latency to escape was not different than that of the wildtype mice. This could show that double knockout mice have similar levels of sensitivity to the foot shock, are capable of responding and learned what the correct response is, but do not demonstrate this until provoked. In fact, in casual interactions with the mice, double knockouts were noticeably negatively thigmotaxic. In a typical mouse, approaching their body with a hand or other object generally provokes them to move away to avoid contact. Double knockouts could be prodded with little to no reaction and it took considerable force (compared to what is needed for a typical mouse) to cause the mice to move. This behavior is akin to freezing observed in animals models of Parkinson's disease as well as in human afflicted with the disease. It could be indicative of dysfunction of the nigrostriatal pathway or motor neuron dysfunction as in ALS.

Passive Avoidance

Repeated measures ANOVAs were run on the trials of each day. Analysis of day one and day two of testing showed a significant increase in latencies overall (day one: $F(2,42) = 5.202$, $p = .0096$; day two: $F(2,42) = 3.513$, $p = .0388$) and *post hoc* analysis revealed a significant difference between genotypes over the day (day one: $p = .0068$; day two: $p = .0474$).

One-way ANOVA analysis was used on the trials of each day to determine differences between genotypes on each trial. On trial one and two of day one, there was no significant difference in latencies between genotypes. On trial three of day one, double knockouts had significantly greater latency compared to wildtypes ($F(1,21) = 6.565$, $p = .0182$). See figure 37. On trial one and two of day two, there was no significant difference in latencies between genotypes. On trial three of day two, double knockouts had significantly greater latency compared to wildtypes ($F(1,21) = 4.647$, $p = .0429$). See figure 38.

Conclusions

In passive avoidance, the objective is for mice to learn to avoid the previously safe chamber of the T-maze, thus passively avoiding a foot shock that would occur if they enter that chamber. In optimal performance, a mouse will enter the old safe chamber on the first trial because this is what it learned during active avoidance trials. During passive avoidance testing, this results in a foot shock which causes a mouse to avoid this chamber on all subsequent trials. Based on this rationale, the data appear to show that double knockouts perform the passive avoidance task better than wildtype mice, learning to avoid the new instance of foot shock more quickly. Double knockouts may indeed have learned this new task, however, there are other factors that complicate this conclusions. First, double knockout mice never reached optimal performance on the active avoidance task. In fact, they only exhibited an avoidance response an average of 7 out of 10 times a trial on the last day of testing. Wildtypes, on the other hand, avoided the foot shock an average of 9 out 10 times a trial on the last day of testing. This discrepancy allows for the greater possibility of a double knockout mouse to give the correct response during

passive avoidance testing (avoid the previously safe chamber) unintentionally. In this passive avoidance run, mice that successfully avoided the foot shock (having a maximum latency of 20 seconds) were not run in remaining trials for that day. This may have skewed the data as some mice were not tested on further trials to see if they exhibited the same correct response repeatedly (which could have better illustrated intentional passive avoidance rather than an artifact of poor active avoidance learning). In addition, double knockout mice are less active overall compared to wildtype mice. This hypoactivity could have played a role in poor active avoidance learning and again in apparently accurate passive avoidance responding. If the double knockouts simply took longer to leave the start box (as evidenced by generally greater latencies in active avoidance) they could unintentionally exhibit a passive avoidance response.

As wildtypes performed well in the active avoidance paradigm, their performance in passive avoidance could be assessed more accurately. On day one, most wildtypes returned to the previously safe chamber on each trial, but latencies increased for each trial showing the mice were beginning to learn to avoid this chamber. On day two of testing, almost all wildtypes succeeded in passively avoiding the foot shock on the first trial and were not run in successive trials on that day. They were given scores of 20 seconds as this is the maximum latency per trial. The wildtype mice that did not passively avoid on the first trial were tested in subsequent trials and their latencies increased appropriately for those trials. Together, this shows that wildtypes successfully learned to passively avoid a foot shock. However, it is unclear the mechanism behind the performance of double knockouts.

Social activity

Mice were placed in the social chambers and allowed to explore for a total of 30 minutes, once a day for three days. An adult male unfamiliar mouse was placed into the "target cup" while the "control cup" was left empty. Each mouse being tested was given a new unfamiliar mouse in the target cup each day. The number of times the mouse being tested touched either cup was recorded. See figure 39. One-way ANOVA analysis was conducted on each of the three test day's results. On the first day of testing, there was no significant difference in how many times either cup was touched and no significant difference between genotypes. On days two and three of testing, the target cup containing the unknown mouse was contacted significantly more than the control cup by both genotypes (day 2: $F(1,1) = 10.043$, $p = .0029$; day 3: $F(1,1) = 8.930$, $p = .0048$). On day two, the wildtypes contacted the target cup significantly more times than the control cup ($F(1,20) = 8.918$, $p = .0073$). On day three, double knockouts contacted the target cup significantly more times than the control cup ($F(1,20) = 8.401$, $p = .0089$). One-way ANOVA analysis showed there was no significant difference in contacts overall between genotypes on test days two and three. However, *post hoc* analysis did reveal a significant difference between genotypes for day three ($p = .0458$).

In all trials, wildtype mice contacted the target cup containing the unfamiliar mouse more than the double knockouts did, although not significantly more. In all trials, wildtype mice also contacted the empty control cup more than the double knockouts, significantly more on day 3 ($F(1,20) = 7.928$, $p = .0107$).

Conclusions

Normal levels of sociability in mice should manifest as more contacts to the target cup that contains a mouse as compared to the empty control cup. Here, both genotypes displayed normal levels of contacts with each type of cup. Both wildtypes and double knockouts contacted the target cup more than the control cup on two of three testing days. However, the double knockouts contacted each cup less than the wildtypes contacted each type, respectively. This could again be a manifestation of the general hypoactivity found in these mice.

Aggression

A repeated measures ANOVA on the latency to the first attack (not taking into account which type of mouse – resident or intruder – that initiated it) showed a significant effect of genotype ($F(1,17) = 7.106$, $p = .0163$) and latency over the trials ($F(2,34) = 7.956$, $p = .0015$). One-way ANOVA analysis of each trial also showed no significant difference between genotypes for trials one and three, but the wildtypes had a significantly lower latency to attack on trial two ($F(1,17) = 7.399$, $p = .0145$). See figure 40.

A repeated measures ANOVA on the number of times an attack was initiated by an intruder mouse showed a significant difference in the number of attacks across trials ($F(2,34) = 8.680$, $p = .0009$). A one-way ANOVA on each trial showed no difference between genotypes on any trial. See figure 41.

A repeated measures ANOVA on the number of times an attack was initiated by the resident PAK mouse showed a significant difference between genotypes ($F(1,17) =$

5.272, p = .0347). A one-way ANOVA analysis on each trial showed the difference between genotypes. Analysis showed no significant differences between genotypes on trials 1 and 3. On trial 2, there was a significant difference between genotypes ($F(1,17) = 9.530$, $p = .0067$). See figure 42.

Conclusions

Wildtype PAK mice exhibit far more aggression than double knockouts. Their latency to attack is significantly shorter and they initiate significantly more attacks on the intruder mice to protect their territory (resident PAK mouse home cage). Overall, double knockouts are attacked more than wildtypes and do not generally exhibit offensive aggression towards an intruder.

Caffeine challenge

All mice were given caffeine and then immediately placed into the activity chambers. Activity counts were recorded every ten minutes for three hours. A repeated measures ANOVA showed that there was s significant change in activity over time for both genotypes ($F(1,16) = 10.808$, $p < .0001$) but there was no significant difference in activity between genotypes over the entire testing period. Therefore, the activity of the double knockouts was similar to that of the controls. See figure 43.

One-way ANOVA analysis was conducted to look at specific time periods during testing. Activity was significantly different between genotypes at only time bin 10 ($F(1,18) = 4.459$, $p = .0490$).

Conclusions

Initial measurement of activity levels showed that double knockouts were significantly less active than wildtypes. Interestingly, caffeine was able to completely eliminate this observation as DKO and wildtype activity in response to caffeine was statistically similar. In addition, caffeine administration did not affect the activity levels in wildtypes compared to their baseline measurements.

The peak effect of caffeine was observed between 30 and 50 minutes for both genotypes. The stimulatory effects of caffeine wore off wildtypes faster than it did in the double knockouts. Although, by the end of the experiment, the activity levels of both genotypes were nearly identical. Caffeine could have a longer half-life in double knockouts than in wildtypes. The half-life of caffeine has been shown to be increased in a variety of conditions and genetic backgrounds due to polymorphisms in the cytochrome P450 isoform that metabolizes caffeine. It is possible that p21-activated kinases could have an effect on the activity of other enzymes throughout the body or on the phosphorylation state of their respective substrates.

Caffeine is thought to have indirect effects on dopamine transmission. The wildtypes may be resistant to the effect on adenosine receptors, and thus dopamine transmission, in the striatum. The C57 strain of mice has already been shown to be resistant to amphetamine's effects on the dopaminergic system so it would not be unexpected that they are also resistant to caffeine's effects there. Double knockouts, however, show increased activity compared to baseline levels and therefore likely to be more sensitive to the adenosine antagonism characteristic of caffeine.

Amphetamine challenge

Mice were given either amphetamine or saline and sacrificed 72 hours after the injections. Neurochemistry was measured using HPLC and analyzed using two-way ANOVA. No significant difference was found in any neurotransmitter tested between genotypes, drug challenge (saline or amphetamine), or interaction of genotype and drug challenge. See figure 44. Analysis of turnover ratios with a two-way ANOVA showed no significant differences in DOPAC/DA and HVA/DA turnover ratios. However, the 5-HIAA/5-HT ratio approached significance and *post hoc* analysis showed there was a significant difference between genotypes ($p = .0344$). See figure 45.

Conclusions

The C57 strain of mice are known to be resistant to the neurotoxic effects of amphetamine. Here, amphetamine is used as a model of Parkinson's disease and it was hypothesized that the double knockouts may be more sensitive to amphetamine due to the knocking out of two genes highly concentrated in the brain that play crucial roles in neuronal function and structure. However, no significant difference was found in the neurochemistry in amphetamine- versus saline-treated mice.

More animals in each group would have helped improve the power of these analyses and possibly show significant results between genotypes or with drug challenge as these were close to the .05 level of significance. The p value for genotype comparison of dopamine levels ($p = .0599$) was close to being significant and suggests that data from more mice might show that double knockouts have lower dopamine than wildtypes, regardless of drug challenge.

Baseline Neurochemistry

A two-way ANOVA was used to analyze neurotransmitter and metabolite levels in the striatum, frontal cortex, hippocampus, hypothalamus and brain stem across age and genotype. One-way ANOVAs were also conducted on the neurochemistry of each age group separately to compare wildtypes to double knockouts. Mice were 30 days (30), 60 days (60) or 120 days old (120).

In the striatum, a two-way ANOVA revealed dopamine levels were significantly different overall between ages ($F(2,62) = 70.667$, $p < .0001$) but not between genotype. Post hoc analysis showed that dopamine levels were significant between each age (30, 60: $p < .0001$; 30, 120: $p < .0001$; 60, 120: $p < .0001$). HVA levels were also significantly different across ages but not genotypes ($F(2,62) = 22.007$, $p < .0001$). Post hoc analysis showed that HVA levels were significant between each age (30, 60: $p < .0001$; 30, 120: $p < .0001$; 60, 120: $p = .0396$). 5-HT levels were significantly different overall between age groups ($F(2,62) = 71.035$, $p < .0001$), genotype ($F(1,62) = 5.087$, $p = .0276$), and there was a significant interaction between age and genotype ($F(2,62) = 9.554$, $p = .0002$). Post hoc analysis showed that 5-HT levels were significantly different between each age group (30, 60: $p = .0003$; 30, 120: $p < .0001$; 60, 120: $p < .0001$) and between genotypes ($p = .0355$). For 5-HIAA, there was a significant interaction between age group and genotype ($F(2,62) = 7.264$, $p = .0015$). Post hoc analysis revealed that 5-HIAA in 30 day old animals was significantly different than in 120 day old animals ($p = .0218$).

A one-way ANOVA on the striatum of 30 day old mice revealed significantly higher levels of dopamine in wildtypes compared to double knockouts ($F(1,24) = 8.418$, $p = .0078$). Also, levels of 5-HT and 5-HIAA were significantly lower in wildtypes compared to double knockouts (5-HT: $F(1,24) = 8.448$, $p = .0077$; 5-HIAA: ($F(1,24) = 21.231$, $p = .0001$). A one-way ANOVA on the striatum of 60 day old mice revealed no significant differences in any neurotransmitter or metabolite between genotypes. A one-way ANOVA on the striatum of 120 day old mice revealed only significantly lower levels of 5-HT in wildtypes compared to double knockouts ($F(1,18) = 10.526$, $p = .0045$). See figure 46.

A two-way ANOVA on turnover ratios in the striatum revealed that there was a significant effect of age on turnover ratios: DOPAC/DA ($F(2,62) = 18.584$, $p < .0001$), HVA/DA ($F(2,62) = 10.886$, $p < .0001$) and 5-HIAA/5-HT ($F(2,62) = 10.821$, $p < .0001$). There was also a significant effect of genotype on HVA/DA levels ($F(1,62) = 5.564$, $p = .0215$). *Post hoc* analysis showed that there was significantly increased HVA/DA and 5-HIAA/5-HT in 30 day and 60 day old mice compared to 120 day old mice (HVA/DA: 30,120: $p < .0001$; 60,120: $p = .0006$; 5-HIAA/5-HT: 30,120: $p < .0001$; 60, 120: $p = .0013$). One-way ANOVA on the striatum of 30 day old mice showed a significant difference between genotype in HVA/DA ($F(1,24) = 7.204$, $p = .0130$) and 5-HIAA/5-HT ($F(1,24) = 8.484$, $p = .0076$). One-way ANOVA on the striatum of 60 day old mice showed no significant difference between genotypes in any turnover ratio tested. One-way ANOVA on the striatum of 30 day old mice showed a significant difference between genotype in HVA/DA ($F(1,24) = 7.204$, $p = .0130$) and 5-HIAA/5-HT ($F(1,24) = 8.484$, $p = .0076$). See figure 47.

In the frontal cortex, a two-way ANOVA revealed dopamine levels were significantly different overall between ages ($F(2,62) = 34.980$, $p < .0001$) but not between genotype. Post hoc analysis showed that dopamine levels were significant between each age (30, 60: $p < .0001$; 30, 120: $p < .0001$; 60, 120: $p < .0001$). DOPAC levels were significantly different between age groups ($F(2,62) = 18.779$, $p < .0001$) and there was a significant interaction between age group and genotype ($F(2,62) = 4.868$, $p = .0109$). Post hoc analysis revealed that dopamine levels were significantly different between mice 30 and 60 days old ($p < .0001$) and 30 and 120 days old ($p < .0001$). HVA levels were also significantly different across ages but not genotypes ($F(2,62) = 22.838$, $p < .0001$). Post hoc analysis showed that HVA levels were significant between each age (30, 60: $p < .0001$; 30, 120: $p < .0001$; 60, 120: $p = .0249$). 5-HT levels were significantly different overall between age groups ($F(2,62) = 33.700$, $p < .0001$). Post hoc analysis showed that 5-HT levels were significantly different between each age group (30, 60: $p < .0001$; 30, 120: $p < .0001$; 60, 120: $p < .0373$). For 5-HIAA, there was a significant difference between age groups ($F(2,62) = 8.819$, $p = .0004$). Post hoc analysis revealed that 5-HIAA in 30 day old animals was significantly different than in 60 and 120 day old animals (30, 60: $p = .0007$; 30, 120: $p = .0006$).

A one-way ANOVA on the frontal cortex of 30 day old mice revealed significantly lower levels of DOPAC and HVA in wildtypes compared to double knockouts (DOPAC: $F(1,24) = 5.469$, $p = .0280$; HVA: $F(1,24) = 6.083$, $p = .0212$). In addition, levels of 5-HT and 5-HIAA were significantly lower in wildtypes compared to double knockouts (5-HT: $F(1,24) = 6.557$, $p = .0172$; 5-HIAA: ($F(1,24) = 20.649$, $p = .0001$). A one-way ANOVA on the frontal cortex of 60 day old mice revealed no

significant differences in any neurotransmitter or metabolite between genotypes. A one-way ANOVA on the frontal cortex of 120 day old mice revealed only significantly higher levels of DOPAC in wildtypes compared to double knockouts ($F(1,18) = 4.888$, $p = .0402$). See figure 48.

A two-way ANOVA on turnover ratios in the frontal cortex revealed that there was a significant effect of age on turnover ratios: DOPAC/DA ($F(2,62) = 11.595$, $p < .0001$), HVA/DA ($F(2,62) = 5.230$, $p = .0080$) and 5-HIAA/5-HT ($F(2,62) = 5.655$, $p < .0055$). There was also a significant effect of genotype on HVA/DA levels ($F(1,62) = 4.782$, $p = .0325$). *Post hoc* analysis showed that there was significantly increased DOPAC/DA and HVA/DA in 30 day and 60 day old mice compared to 120 day old mice (DOPAC/DA: 30,120: $p < .0001$; 60,120: $p = .0001$; HVA/DA: 30,120: $p < .0045$; 60, 120: $p = .0049$) and a significant difference in 5-HIAA/5-HT between 30 day old and 120 day old mice ($p = .0013$). *Post hoc* analysis also showed that HVA/DA was significantly different between genotypes ($p = .0312$). One-way ANOVA on the frontal cortex of 30 day old mice showed a significant difference between genotype in 5-HIAA/5-HT ($F(1,24) = 14.208$, $p = .0009$). One-way ANOVAs on the frontal cortex of 60 day old mice and 120 day old mice showed no significant difference between genotypes in any turnover ratio tested. See figure 49.

In the hippocampus, a two-way ANOVA revealed no significant difference in dopamine or DOPAC levels. HVA levels were significantly different across ages but not genotypes ($F(2,62) = 8.071$, $p = .0008$). Post hoc analysis showed that HVA levels were significant between mice 30 days old and 60 and 120 days old (30, 60: $p = .0008$; 30, 120: $p < .0018$). 5-HT levels were significantly different overall between age groups

($F(2,62) = 34.304$, $p < .0001$). Post hoc analysis showed that 5-HT levels were significantly different between mice 30 days old and 60 and 120 days old (30, 60: $p < .0001$; 30, 120: $p < .0001$). For 5-HIAA, there was a significant interaction between age groups overall as well ($F(2,62) = 11.669$, $p < .0001$). Post hoc analysis revealed that 5-HIAA in 30 day old animals was significantly different than in 60 and 120 day old animals (30, 60: $p < .0001$; 30, 120: $p = .0006$).

A separate one-way ANOVA on the neurochemistry of the hippocampus from 30, 60 and 120 day old mice showed no significant difference between genotypes in each respective age group. See figure 50. In addition, a two-way ANOVA on turnover ratios revealed that there were no significant effect of age or genotype on turnover ratios. Data not shown.

In the hypothalamus, a two-way ANOVA revealed dopamine levels were significantly different overall between ages ($F(2,61) = 8.695$, $p = .0005$) but not between genotype. Post hoc analysis showed that dopamine levels in 120 day old mice were significant different than levels in 30 and 60 day old mice (120, 30: $p < .0001$; 120, 60: $p = .0208$). Levels of DOPAC were significantly different between age groups ($F(2,61) = 9.200$, $p = .0003$) and between genotypes ($F2,61) = 16.163$, $p = .0002$). Post hoc analysis showed that DOPAC levels of mice 120 days old were significantly different compared to 30 and 60 day old mice (30, 120: $p < .0001$; 60, 120: $p = .0200$) and between genotypes ($p = .0001$). Levels of HVA were significantly different between age groups ($F(2,61) = 5.825$, $p = .0048$) and genotypes ($F(2,61) = 18.895$, $p < .0001$). Post hoc analysis showed that HVA levels of mice 120 days old were significantly different compared to 30 and 60 day old mice (30, 120: $p = .0011$; 60, 120: $p = .0218$) and between

genotypes ($p < .0001$). Levels of 5-HT were significantly different overall between age groups ($F(2,61) = 42.010$, $p < .0001$) and there was a significant interaction between age and genotype ($F(2,61) = 7.219$, $p = .0015$). Post hoc analysis showed that 5-HT levels were significantly different between each age group (30, 60: $p < .0001$; 30, 120: $p < .0001$; 60, 120: $p = .0018$). For 5-HIAA, there was a significant interaction between age group and genotype ($F(2,61) = 7.877$, $p = .0009$). Post hoc analysis revealed that 5-HIAA in 30 day old animals was significantly different than in 60 and 120 day old animals (30, 60: $p = .0003$; 30, 120: $p = .0087$).

A one-way ANOVA on the hypothalamus of 30 day old mice revealed significantly lower levels of DOPAC and HVA in wildtypes compared to double knockouts (DOPAC: $F(1,23) = 14.241$, $p = .0010$; HVA: $F(1,23) = 23.180$, $p < .0001$). Also, levels of 5-HIAA were significantly different in wildtypes compared to double knockouts (5-HIAA: ($F(1,23) = 9.941$, $p = .0045$). A one-way ANOVA on the hypothalamus of 60 day old mice revealed no significant differences in any neurotransmitter or metabolite between genotypes except for 5-HT ($F(1,20) = 5.592$, $p = .0283$). A one-way ANOVA on the hypothalamus of 120 day old mice revealed significantly different levels of DOPAC ($F(1,18) = 5.682$, $p = .0284$), HVA ($F(1,18) = 4.997$, $p = .0383$), 5-HT ($F(1,18) = 4.427$, $p = .0497$) between genotypes. See figure 51.

A two-way ANOVA on turnover ratios in the hypothalamus revealed that there was a significant effect of genotype on DOPAC/DA levels ($F(1,61) = 4.347$, $p = .0413$) and HVA/DA levels ($F(1,61) = 9.424$, $p = .0032$). For 5-HIAA/5-HT, there was also a significant effect of age ($F(2,61) = 4.812$, $p = .0115$) and a significant interaction of age and genotype ($F(2,61) = 3.623$, $p = .0326$). *Post hoc* analysis showed that there was

significantly difference in DOPAC/DA and HVA/DA between 30 day old mice compared to 120 day old mice (DOPAC/DA: $p = .0170$; HVA/DA: $p = .0480$) and between genotypes (DOPAC/DA: $p = .0425$; HVA/DA: $p = .0033$). In addition, *post hoc* analysis showed a significant difference in 5-HIAA/5-HT in 120 day old mice compared to 30 day old mice ($p = .0054$) and 60 day old mice ($p = .0187$). One-way ANOVA on the hypothalamus of 30 day old mice showed a significant difference between genotypes in HVA/DA ($F(1,23) = 6.400$, $p = .0187$) and 5-HIAA/5-HT ($F(1,23) = 25.546$, $p < .0001$). One-way ANOVAs on the frontal cortex of 60 day old mice and 120 day old mice showed no significant difference between genotypes in any turnover ratio tested. See figure 52.

In the brain stem, a two-way ANOVA revealed dopamine levels were not significantly different overall between ages or genotypes but, there was significant interaction of age and genotype ($F(2,61) = 4.444$, $p = .0158$). There was a significant interaction of age and genotype for DOPAC levels ($F(2,61) = 14.061$, $p < .0001$). Post hoc analysis revealed that DOPAC levels were significantly different between genotypes ($p = .0462$). HVA levels were also significantly different across ages ($F(2,61) = 5.100$, $p = .0090$) and there was a significant interaction between age and genotype ($F(2,61) = 7.900$, $p = .0009$). Post hoc analysis showed that HVA levels of 60 day old mice were significantly different than 30 and 120 day old mice (30, 60: $p = .0117$; 60, 120: $p = .0028$). For 5-HIAA, there was a significant interaction between age and genotype ($F(2,61) = 6.614$, $p = .0025$).

A one-way ANOVA on the brain stem of 30 day old mice revealed significantly lower levels of dopamine ($F(1,24) = 10.123$, $p = .0040$), DOPAC ($F(1,24) = 16.921$, $p =$

.0004) and HVA (F(1,24) = 7.833, p = .0100) in wildtypes compared to double knockouts. Also, levels of 5-HIAA were significantly lower in wildtypes compared to double knockouts (F(1,24) = 10.162, p = .0040). A one-way ANOVA on the brain stem of 60 day old mice revealed no significant differences in any neurotransmitter or metabolite between genotypes. A one-way ANOVA on the brain stem of 120 day old mice revealed significantly higher levels of DOPAC (F(1,17) = 12.199, p = .0028 and HVA (F(1,17) = 21.063, p = .0003) in wildtypes compared to double knockouts. See figure 53.

A two-way ANOVA on turnover ratios in the brain stem revealed that there was a significant interaction of age and genotype on DOPAC/DA turnover ratio (F(2,61) = 5.775, p = .0050). There was also a significant effect of age on HVA/DA levels (F(2,61) = 3.375, p = .0407). *Post hoc* analysis showed that there was significantly different HVA/DA turnover ratios in 30 day old mice compared to 60 day old mice (p = .0407) and in 60 day old mice compared to 120 day old mice (p = .0160). One-way ANOVA analysis on the brain stem of 30 day old mice revealed a significant difference in DOPAC/DA (F(1,25) = 9.941, p = .0042). One-way ANOVA analysis of the brain stem showed that there was significant difference in 5-HIAA/5-HT levels between genotypes in 60 day old mice (F(1,20) = 4.666, p = .0431) and 120 day old mice (F(1,17) = 4.655, p = .0456). See figure 54.

Conclusions

Although there were some significant differences in neurotransmitter, metabolite and turnover ratios in the various brain parts across ages, there were no remarkable findings. Differences were not drastic and generally evened out in older animals. Ages

where transmitter levels were significantly different may indicate potential sensitive ages of double knockouts to dopaminergic toxins or toxicants. The two drug challenges utilized in these studies – caffeine and amphetamine – were not given at any of the ages analyzed for baseline neurochemistry so it is not known if this could be true.

Figure 29

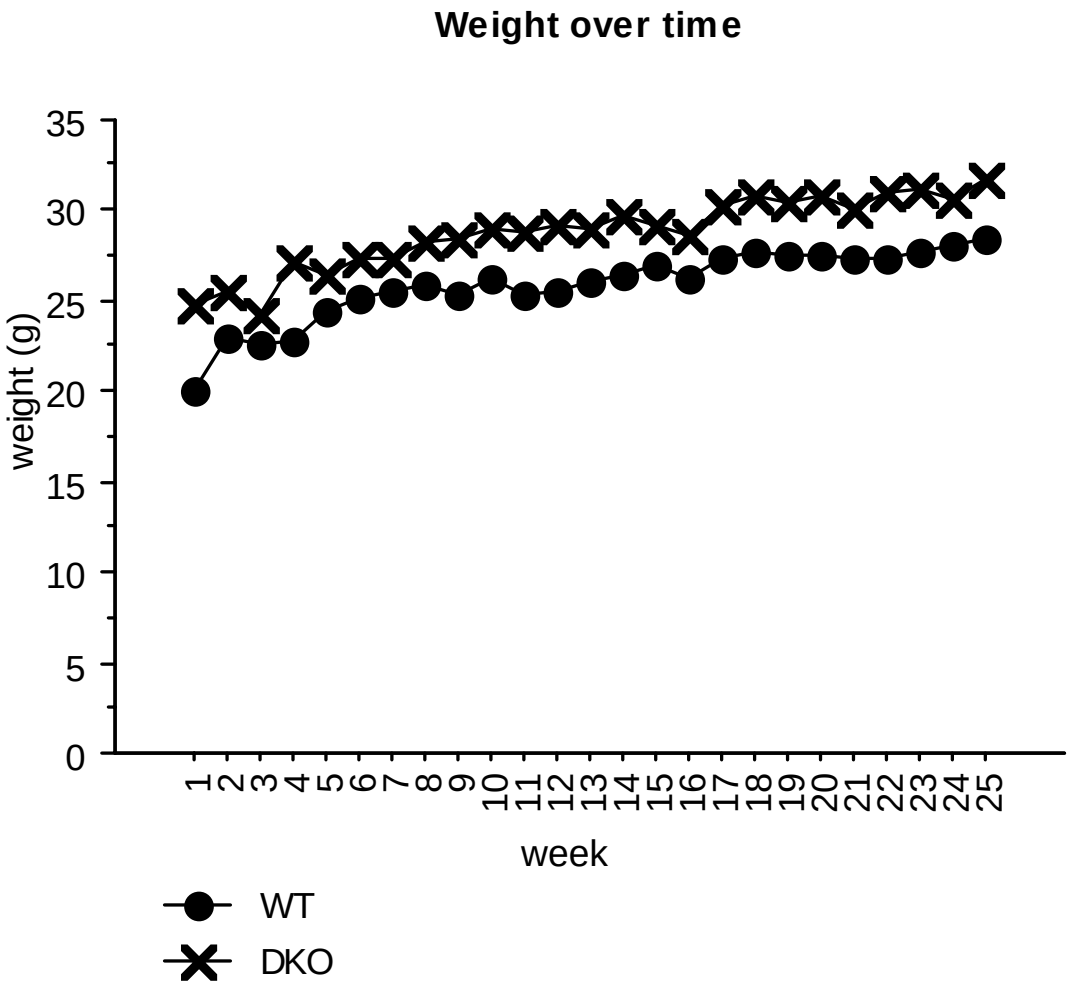


Figure 29: Weight of wildtype and PAK double knockout mice over testing period. Both genotypes gained a significantly amount of weight over testing ($p < .0001$), but this gain was not significant between genotypes.

Figure 30

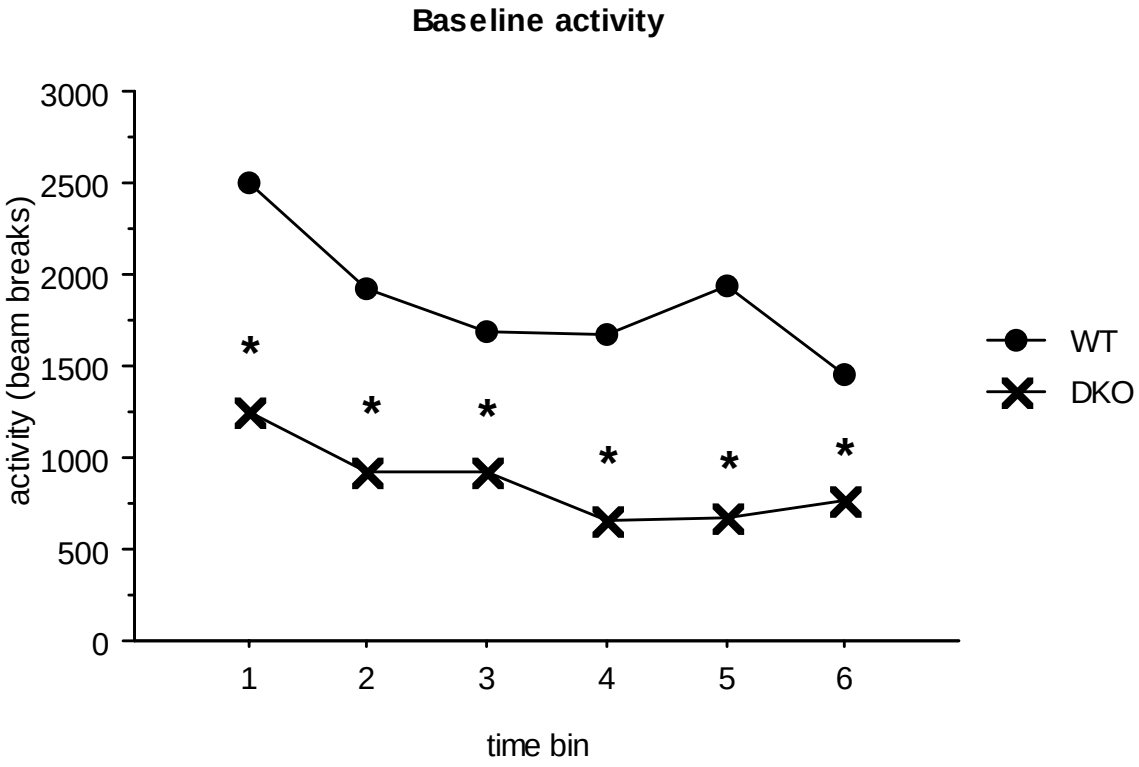


Figure 30: Average activity levels in wildtype and PAK double knockout mice for 30 minutes. Readings were recorded in five minute time bins. There was a significant difference in activity over the 30 minute period ($p < .0001$) and a significant effect of genotype ($p < .0001$). There was significant difference in activity levels between genotypes for each time bin.

* denotes significantly different than wildtype activity during the same time bin; $p < .05$

Figure 31

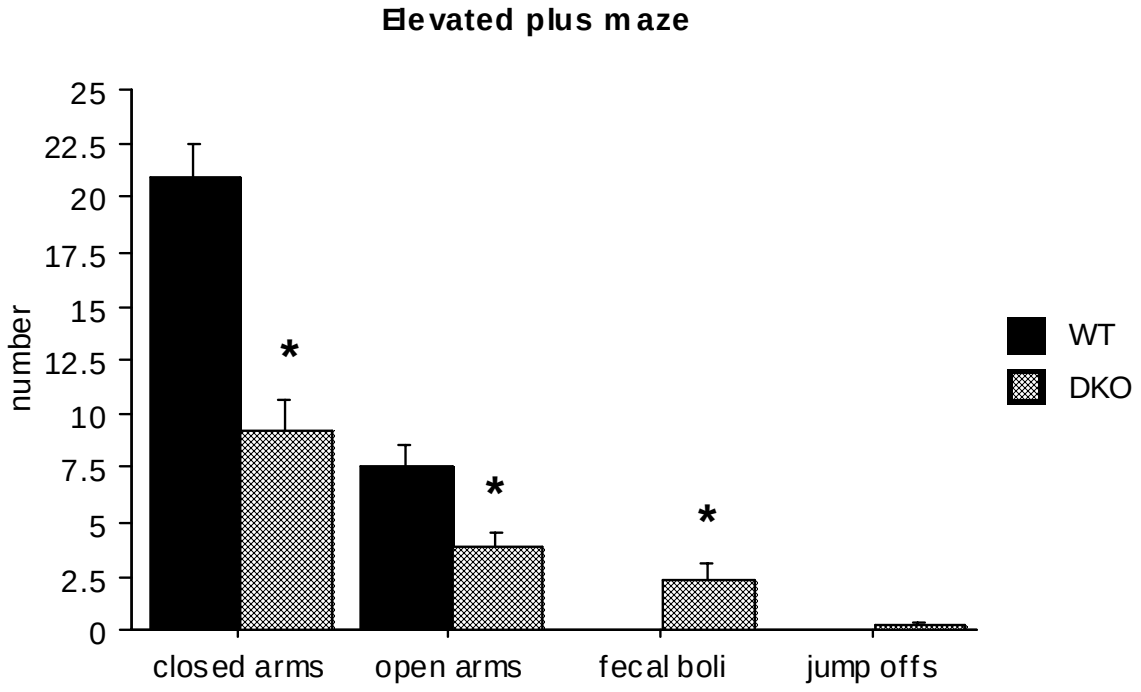


Figure 31: Performance on the elevated plus maze in wildtype and PAK double knockout mice: number of entries into the closed arms of the maze, number of entries into the open arms of the maze, number of fecal boli emitted over trial, and number of jump offs the maze

* denotes significantly different from wildtype performance on the same measurement; $p < .05$

Figure 32

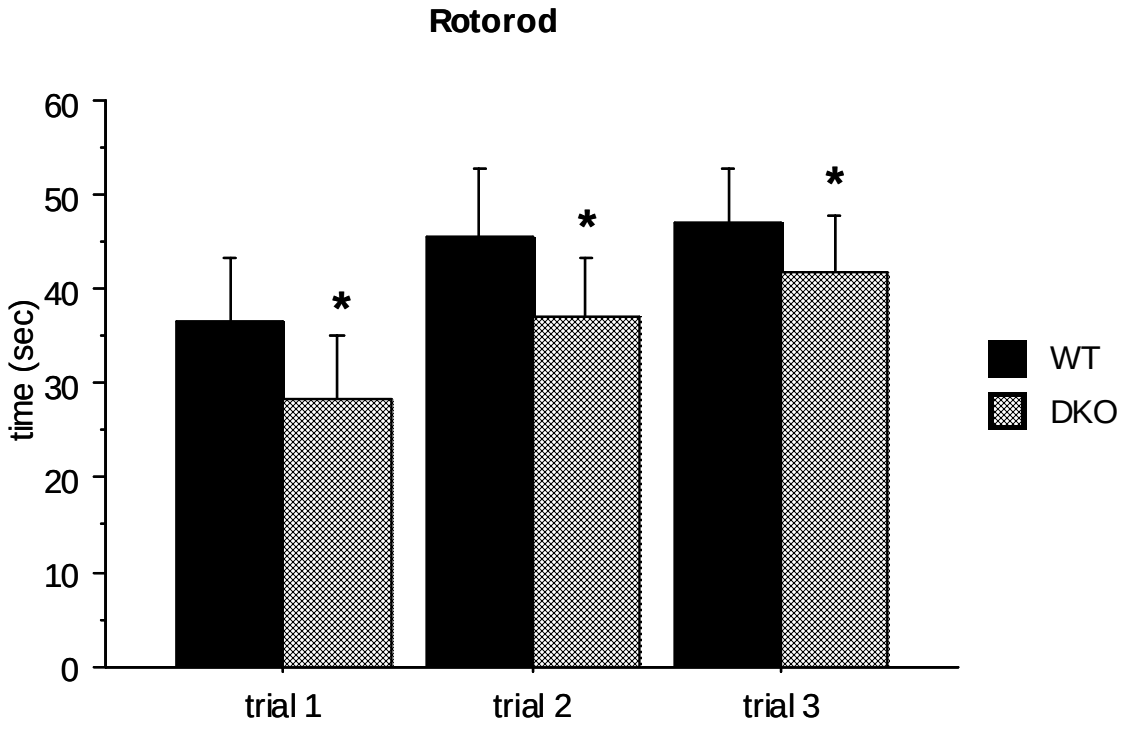


Figure 32: Performance on the rotorod by wildtype and PAK double knockout mice: average trial latency to fall from the rotorod rotating 12 rev/min. Maximum trial length: 60 seconds. Mice performed significantly better over the trials ($p = .0060$) and *post hoc* analysis showed a significant effect of genotype over the trials ($p < .001$).

* denotes significant from wildtype on the same trial; $p < .05$

Figure 33

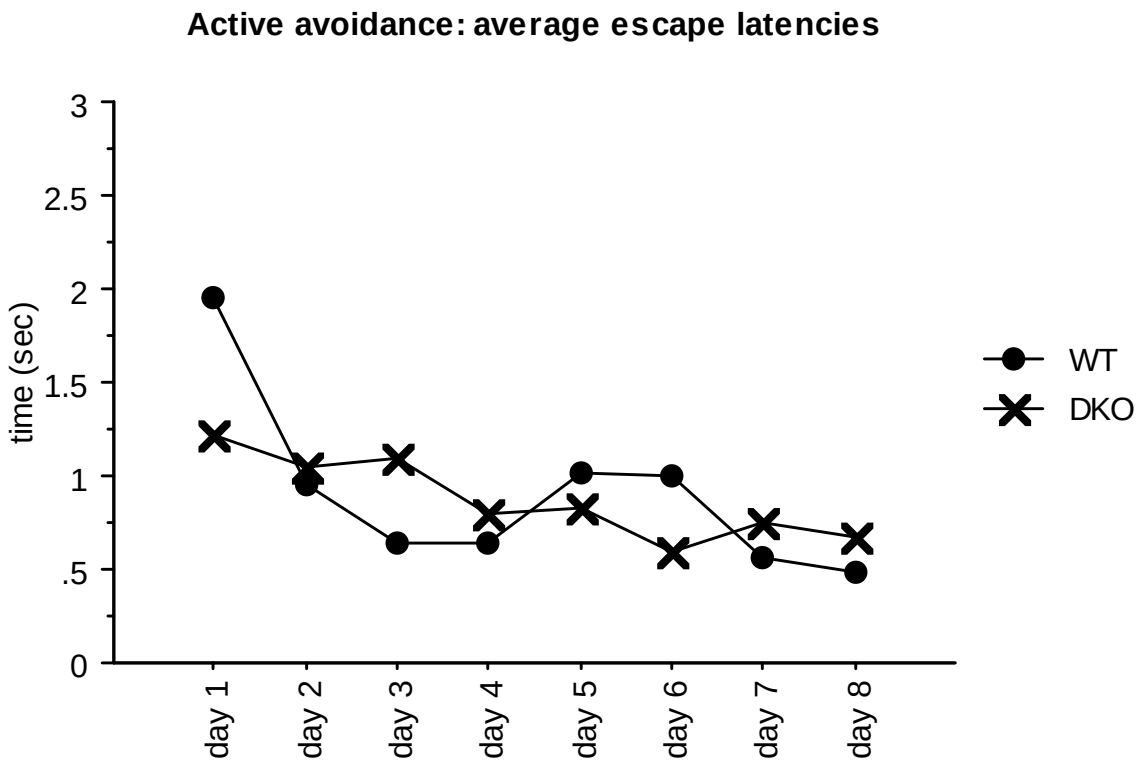


Figure 33: Latency to escape a 0.8mA foot shock in wildtype and PAK double knockout mice, averaged over ten trials per day. No significant difference in escape latencies.

Figure 34

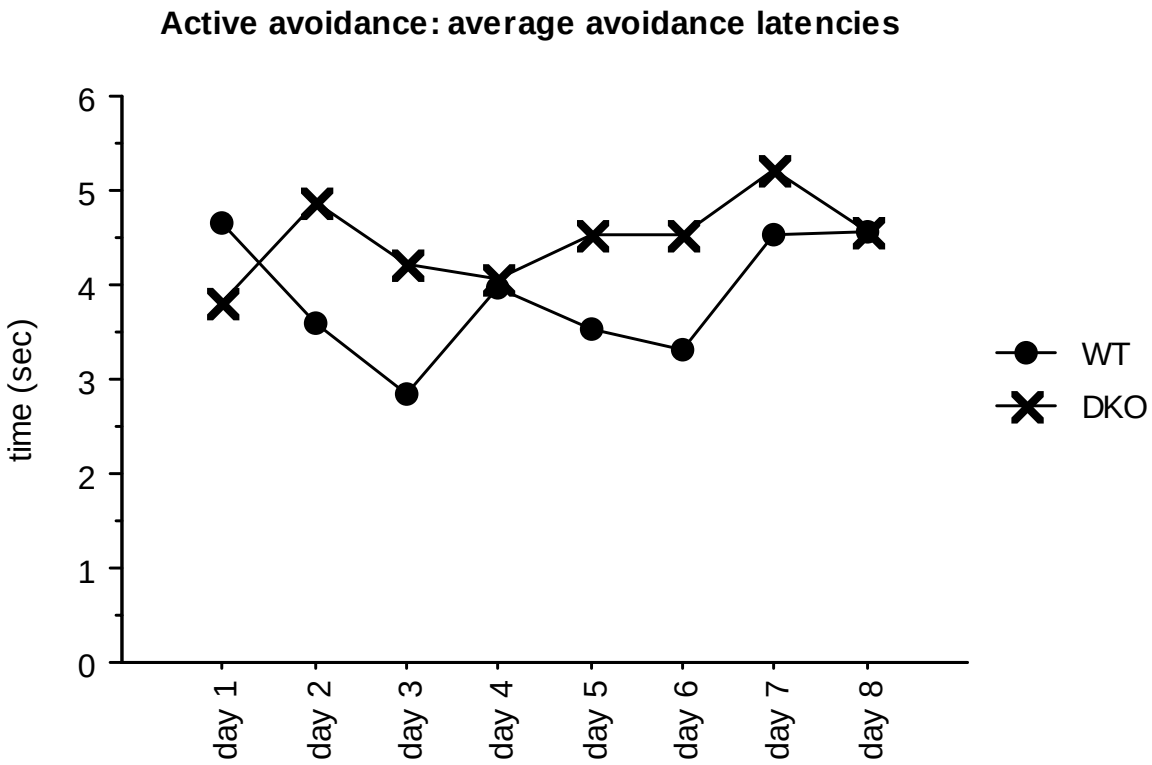


Figure 34: Latency to avoid a 0.8mA foot shock in wildtype and PAK double knockout mice, averaged over ten trials per day. *Post hoc* analysis showed a significant difference in avoidance latency between genotypes overall ($p = .0231$)

Figure 35

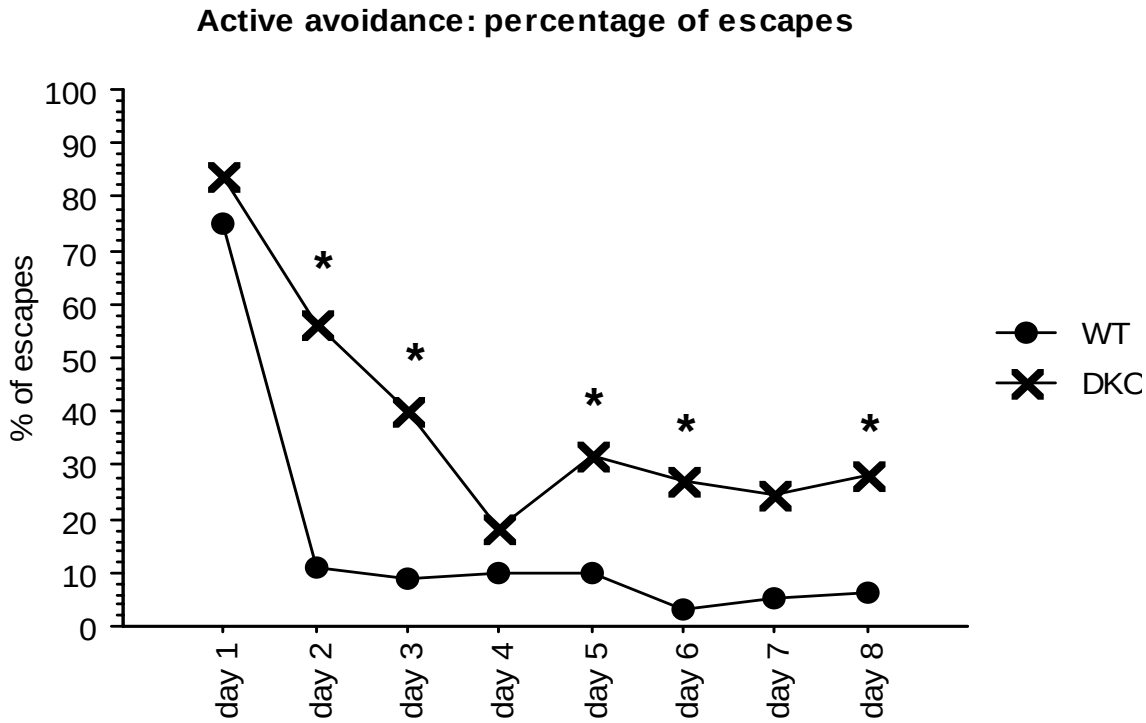


Figure 35: Percent escape responses in wildtype and PAK double knockout mice, averaged over ten trials per day. There was a significant difference in escapes between genotypes ($p = .0056$), total number of escape across days ($p < .0001$), and a significant interaction between genotype and escapes ($p = .0104$)

* denotes significantly different compared to wildtype; $p < .05$

Figure 36

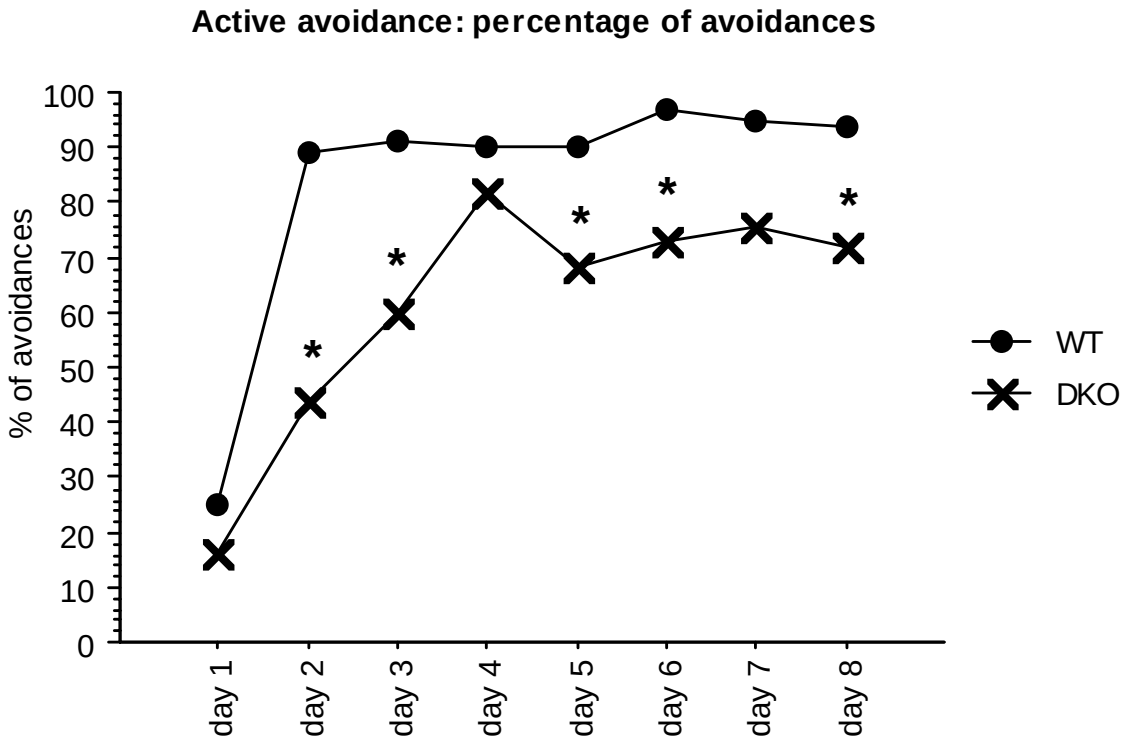


Figure 36: Percent avoidance responses in wildtype and PAK double knockout mice, averaged over ten trials per day. There was a significant difference in avoidances between genotypes ($p = .0056$), total number of avoidances across days ($p < .0001$), and a significant interaction between genotype and avoidances ($p = .0104$)

* denotes significantly different from wildtype; $p < .05$

Figure 37

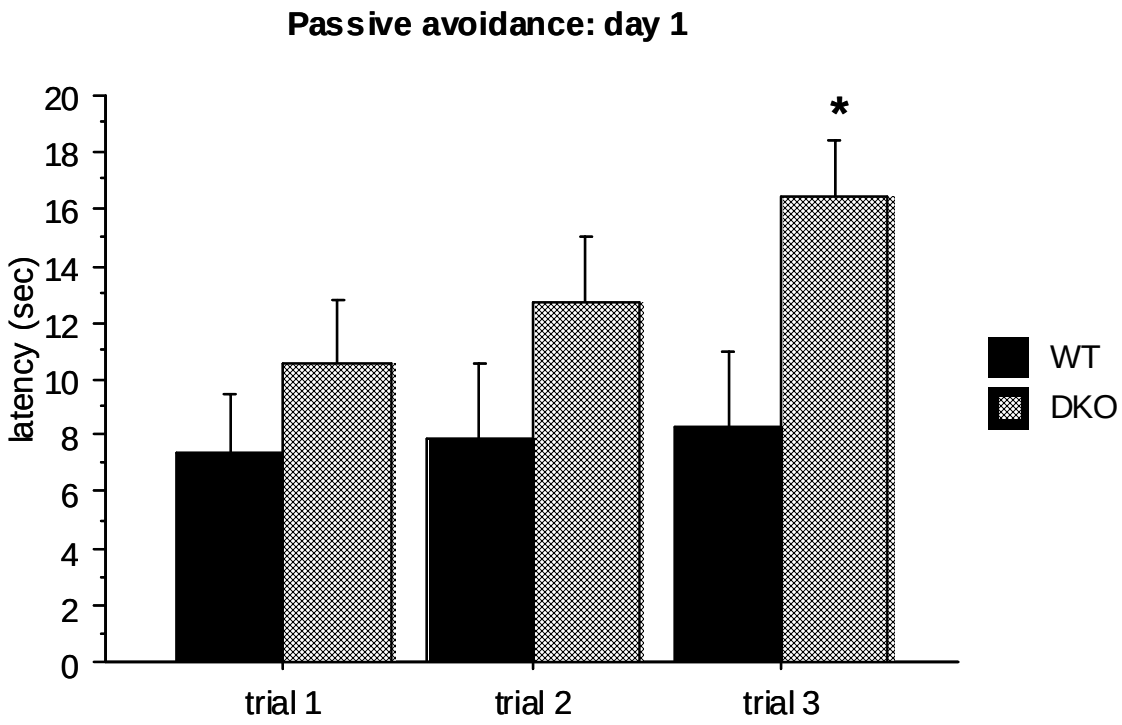


Figure 37: Day one average latency to passively avoid a 0.8mA foot shock in wildtype and PAK double knockout mice

* denotes significantly different from wildtype; $p < .05$

Figure 38

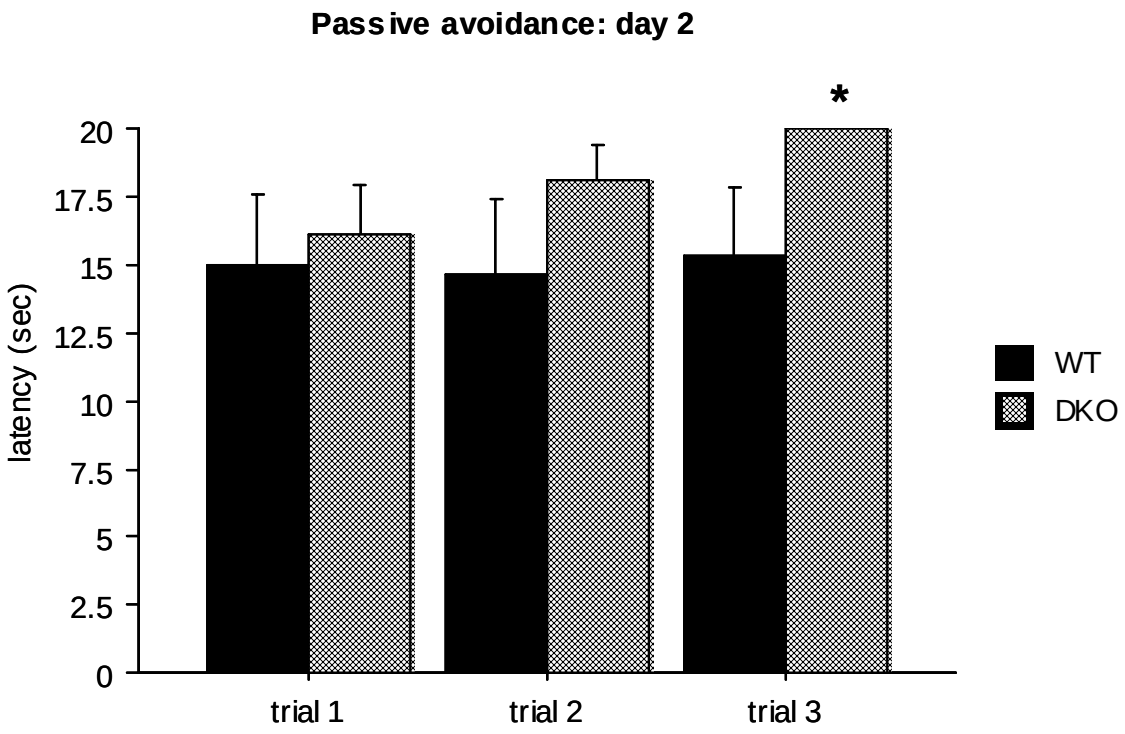


Figure 38: Day two average latency to passively avoid a 0.8mA foot shock in wildtype and PAK double knockout mice

* denotes significantly different from wildtype; $p < .05$

Figure 39

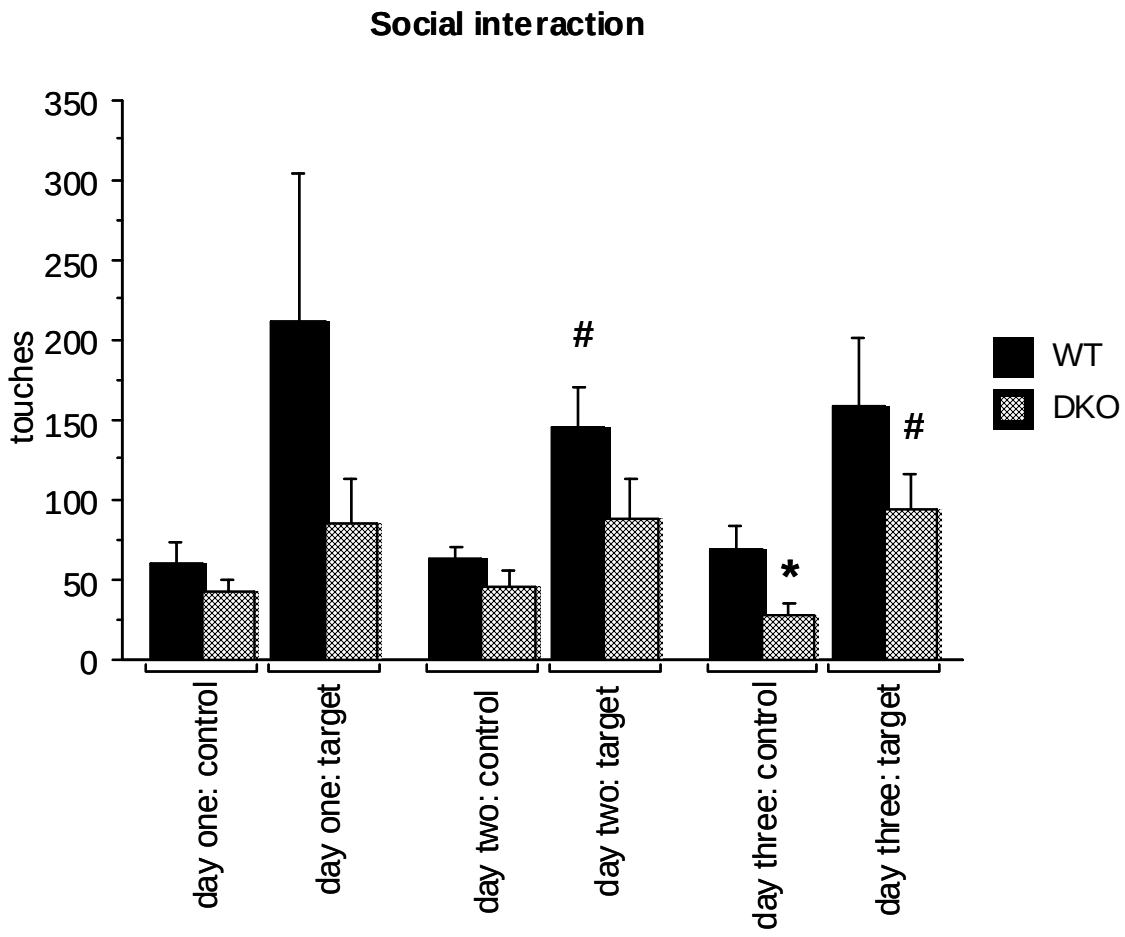


Figure 39: Social interaction in wildtype and PAK double knockout mice: average number of contacts with the empty control cup and the target cup containing an unfamiliar mouse

* denotes significantly different from wildtype; $p < .05$

# denotes significantly different from control cup; $p < .05$

Figure 40

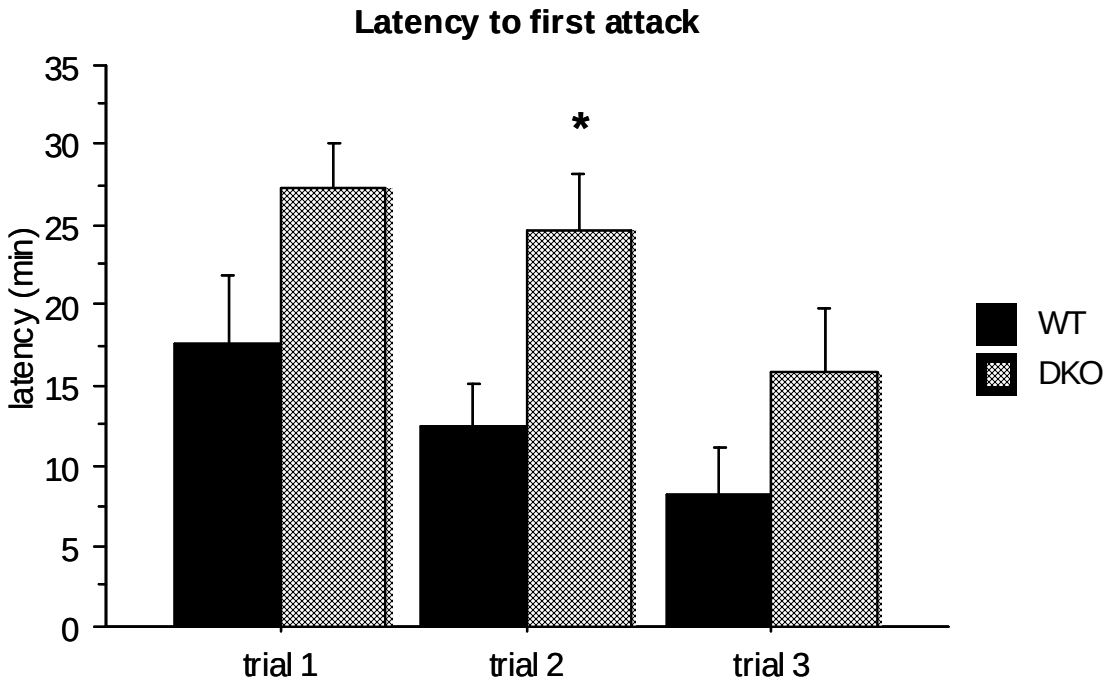


Figure 41: Latency to the first attack (regardless of which mouse initiated it) to occur once the intruder mouse was introduced into the home cage of the resident PAK mouse. * denotes significantly different from wildtype; $p < .05$

Figure 41

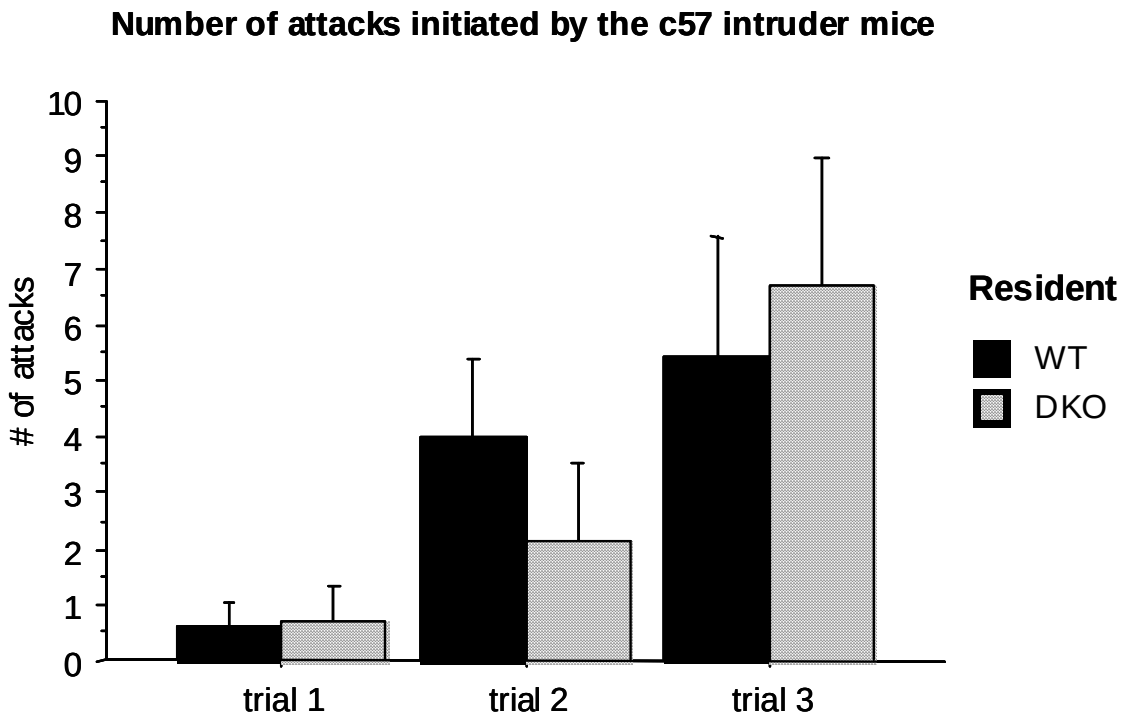


Figure 41: The average number of attacks initiated by C57 intruder mice on resident PAK mice in their home cages.

Figure 42

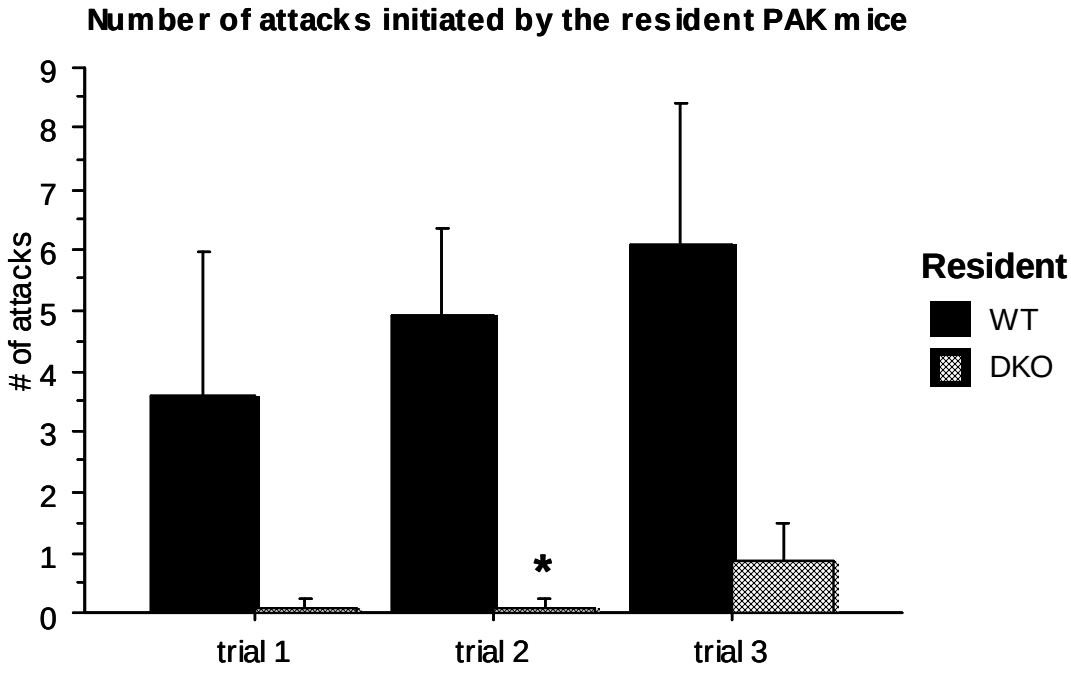


Figure 42: The average number of attacks initiated by resident PAK mice on C57 intruder mice introduced into the residents' home cages.

* denotes significantly different from wildtype; $p < .05$

Figure 43

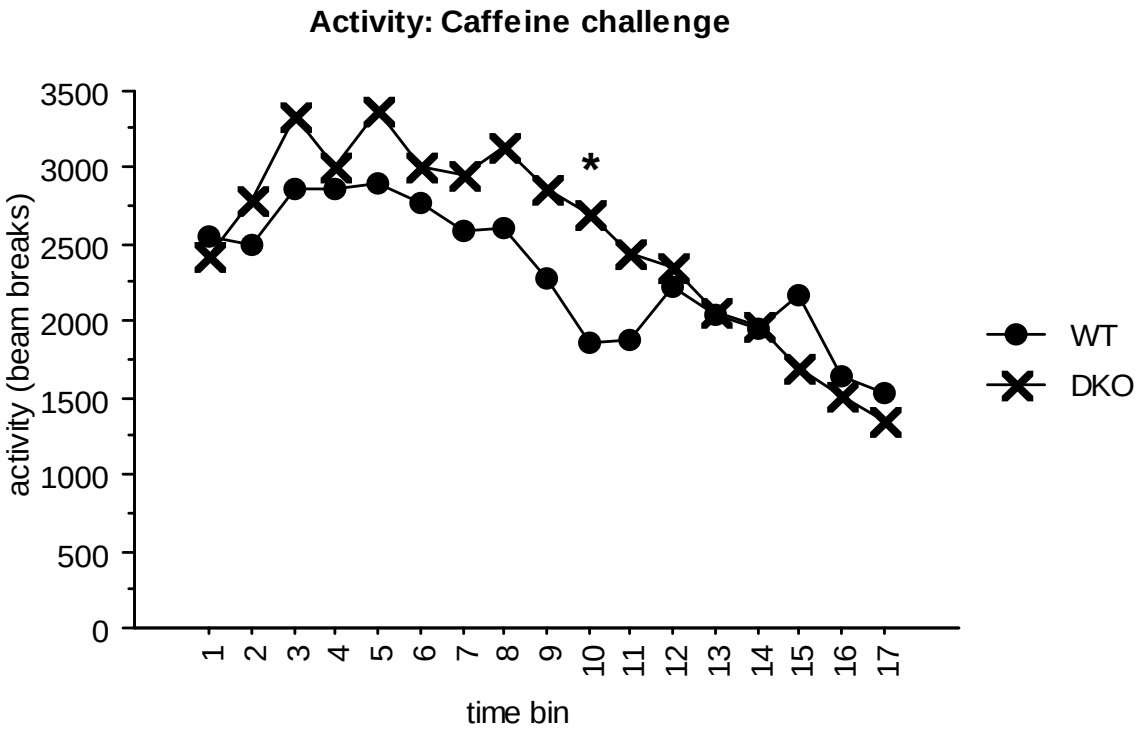


Figure 43: Activity levels in wildtype and double knockout mice after caffeine administration. Readings were recorded in ten minute time bins. Activity in both genotypes decreased significantly over testing ($p < .0001$) but there was no significant difference between genotypes

* denotes significantly different from wildtype; $p < .01$

Figure 44

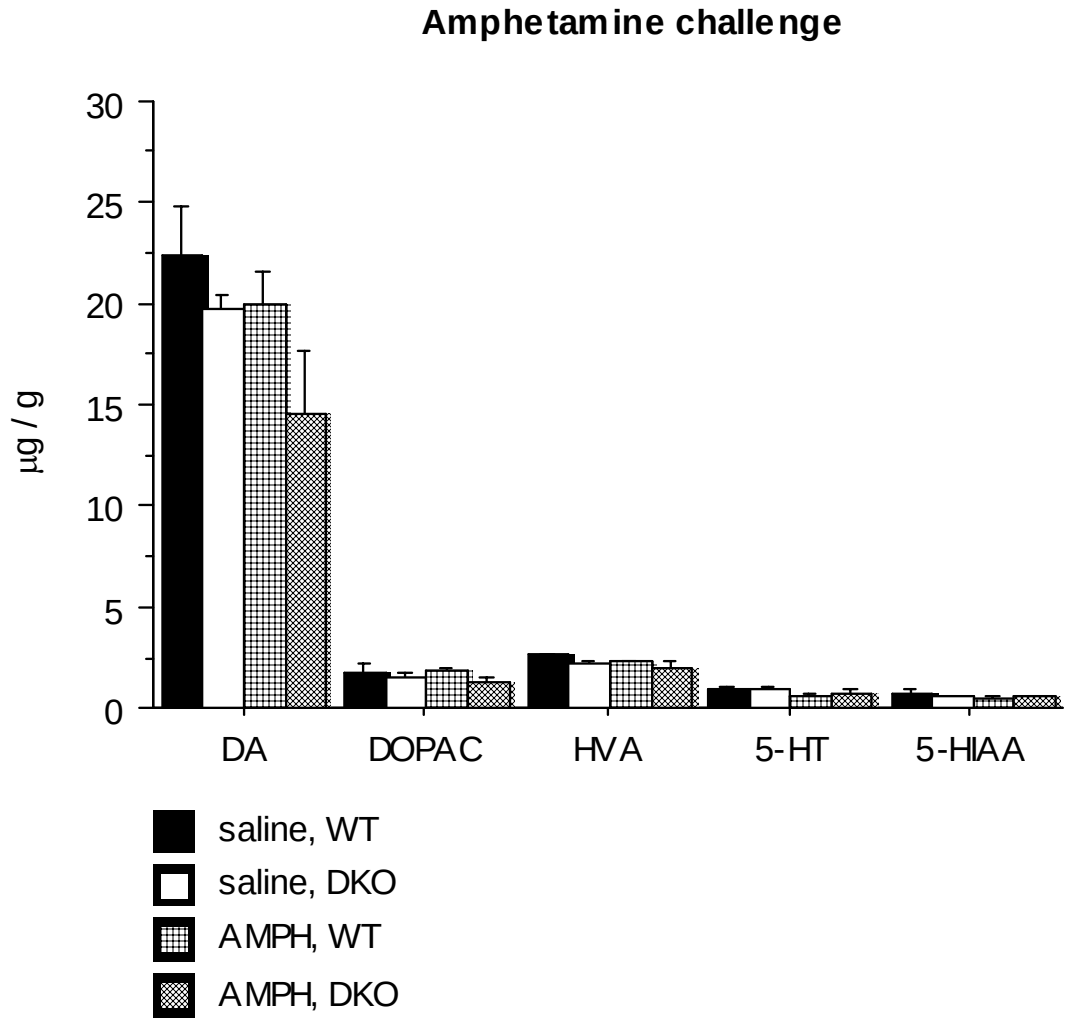


Figure 44: Concentration of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in striatum of wildtype and PAK double knockout mice after treatment with saline or amphetamine. No significant difference in neurotransmitters or metabolites was found.

Figure 45

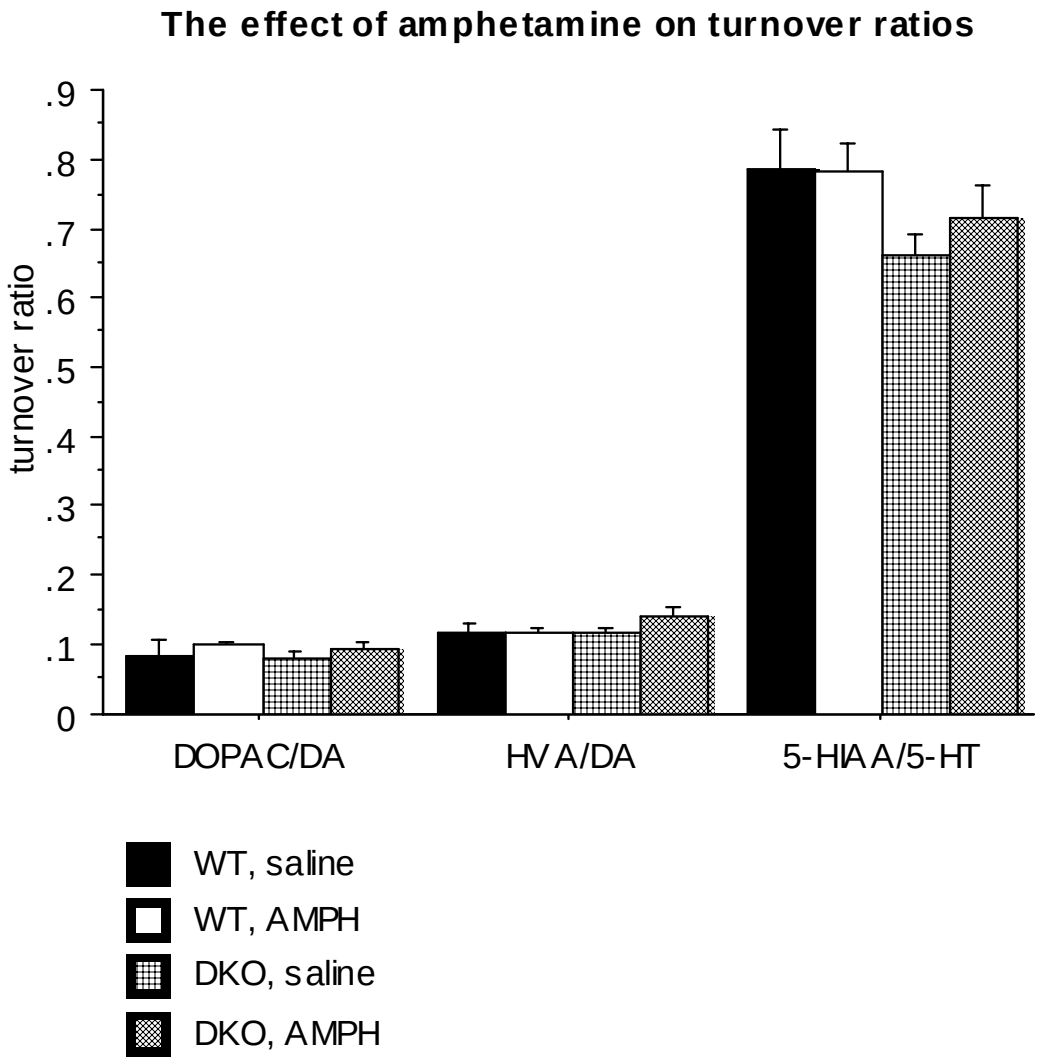


Figure 45: Turnover ratios of DOPAC/DA, HVA/DA and 5-HIAA/5-HT in striatum of wildtype and PAK double knockout mice after treatment with saline or amphetamine **.** No significant difference in turnover ratios was found.

Figure 46

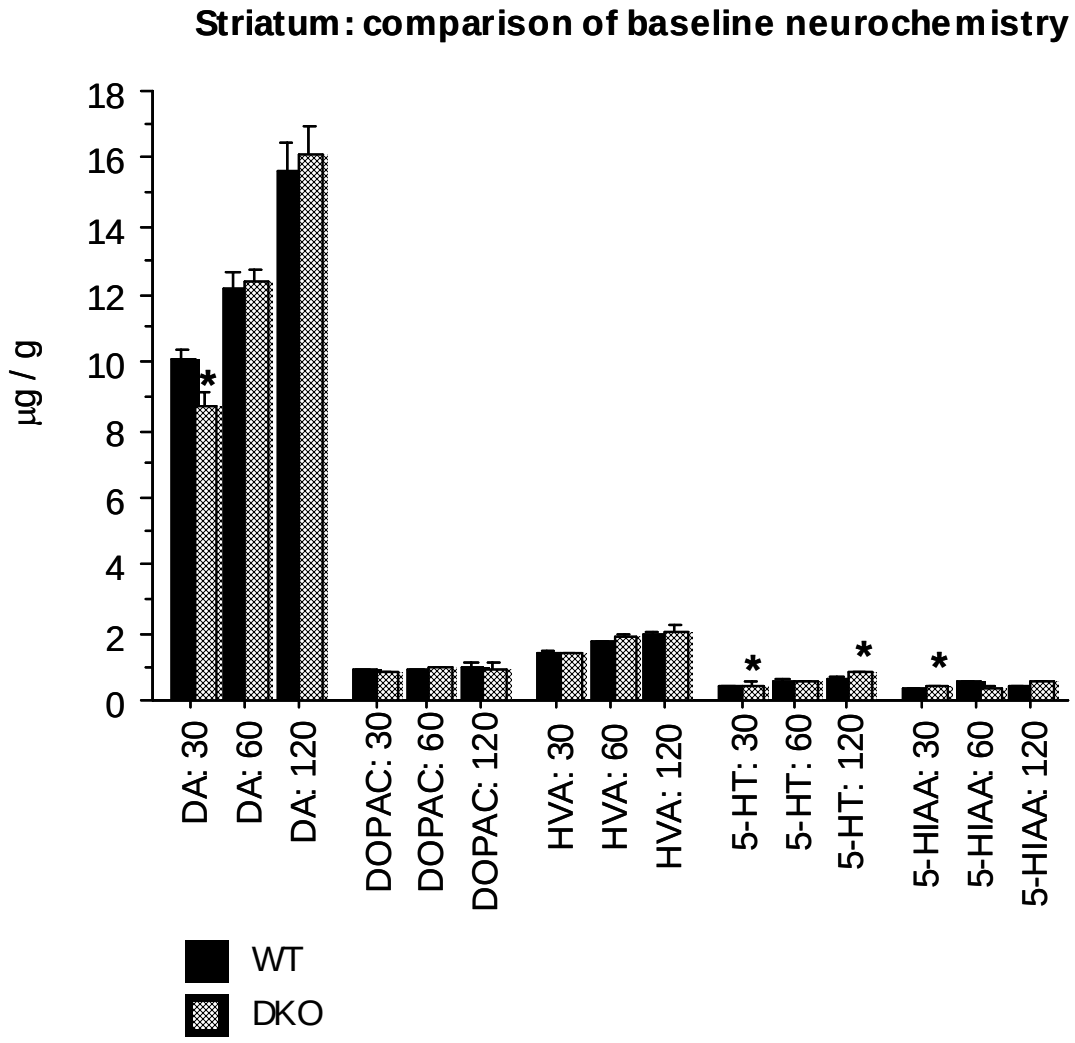


Figure 46: Concentration of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in striatum of wildtype and PAK double knockout mice 30, 60 and 120 days old

* denotes significantly different from wildtype; $p < .05$

Figure 47

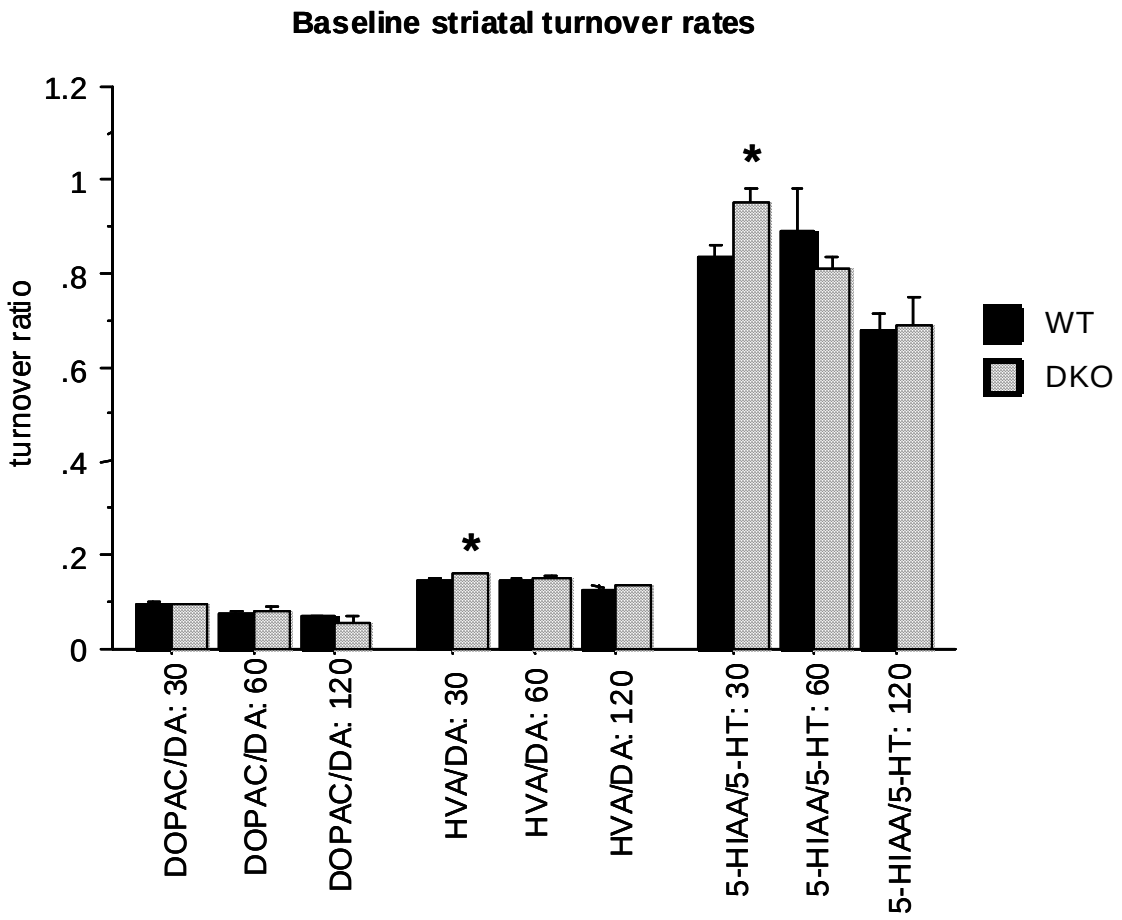


Figure 47: Turnover ratios of DOPAC/DA, HVA/DA and 5-HIAA/5-HT in striatum of wildtype and PAK double knockout mice 30, 60, and 120 days old

* denotes significantly different from wildtype; $p < .05$

Figure 48

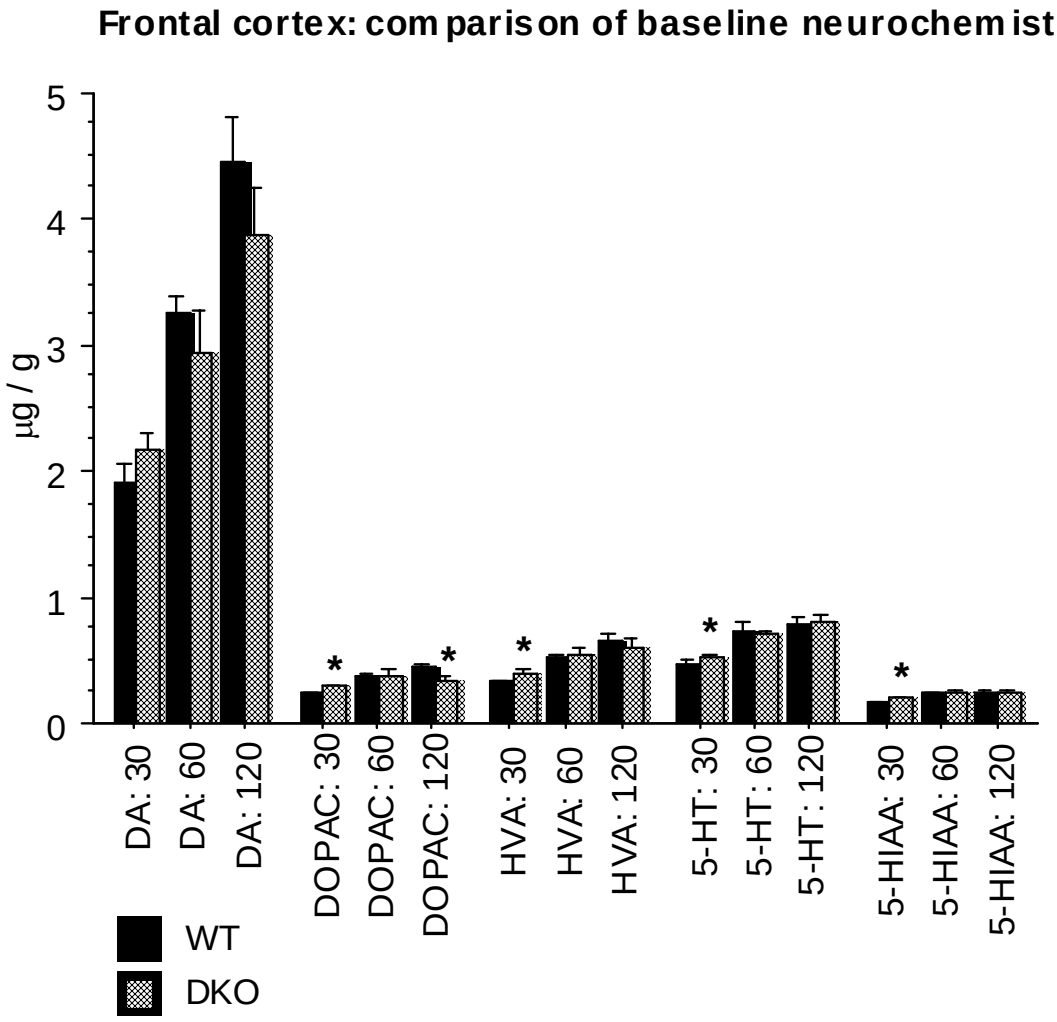


Figure 48: Concentration of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in frontal cortex of wildtype and PAK double knockout mice 30, 60 and 120 days old

* denotes significantly different from wildtype; $p < .05$

Figure 49

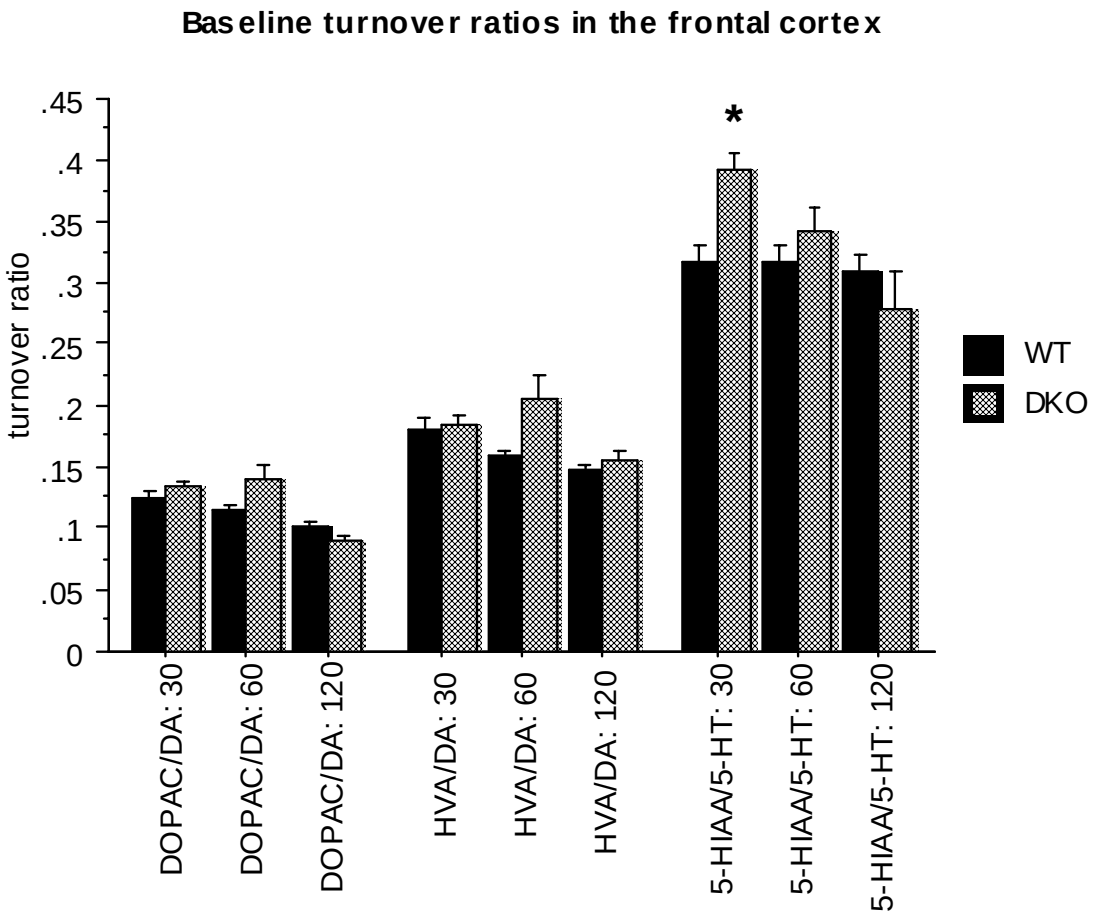


Figure 49: Turnover ratios of DOPAC/DA, HVA/DA and 5-HIAA/5-HT in frontal cortex of wildtype and PAK double knockout mice 30, 60, and 120 days old

* denotes significantly different from wildtype; $p < .05$

Figure 50

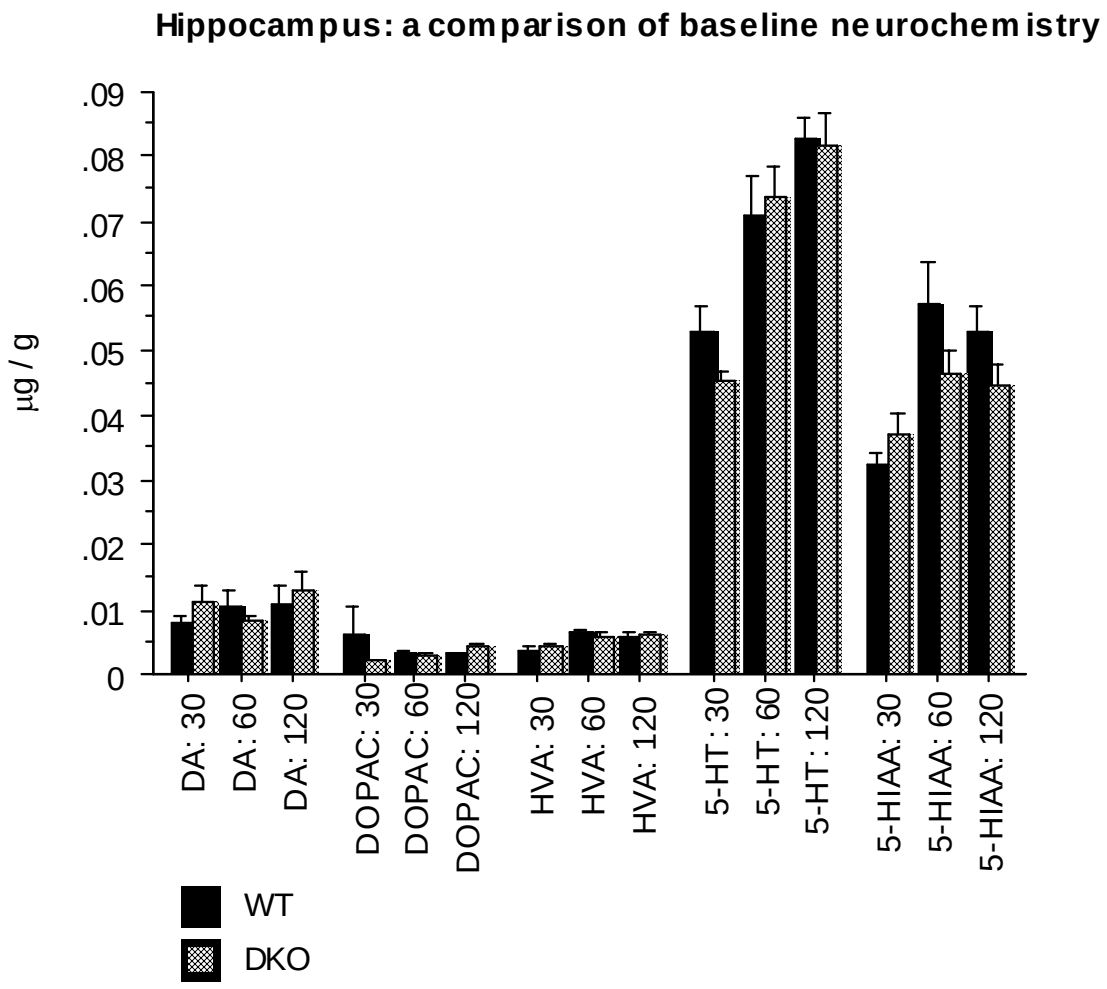


Figure 50: Concentration of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in hippocampus of wildtype and PAK double knockout mice 30, 60 and 120 days old

Figure 51

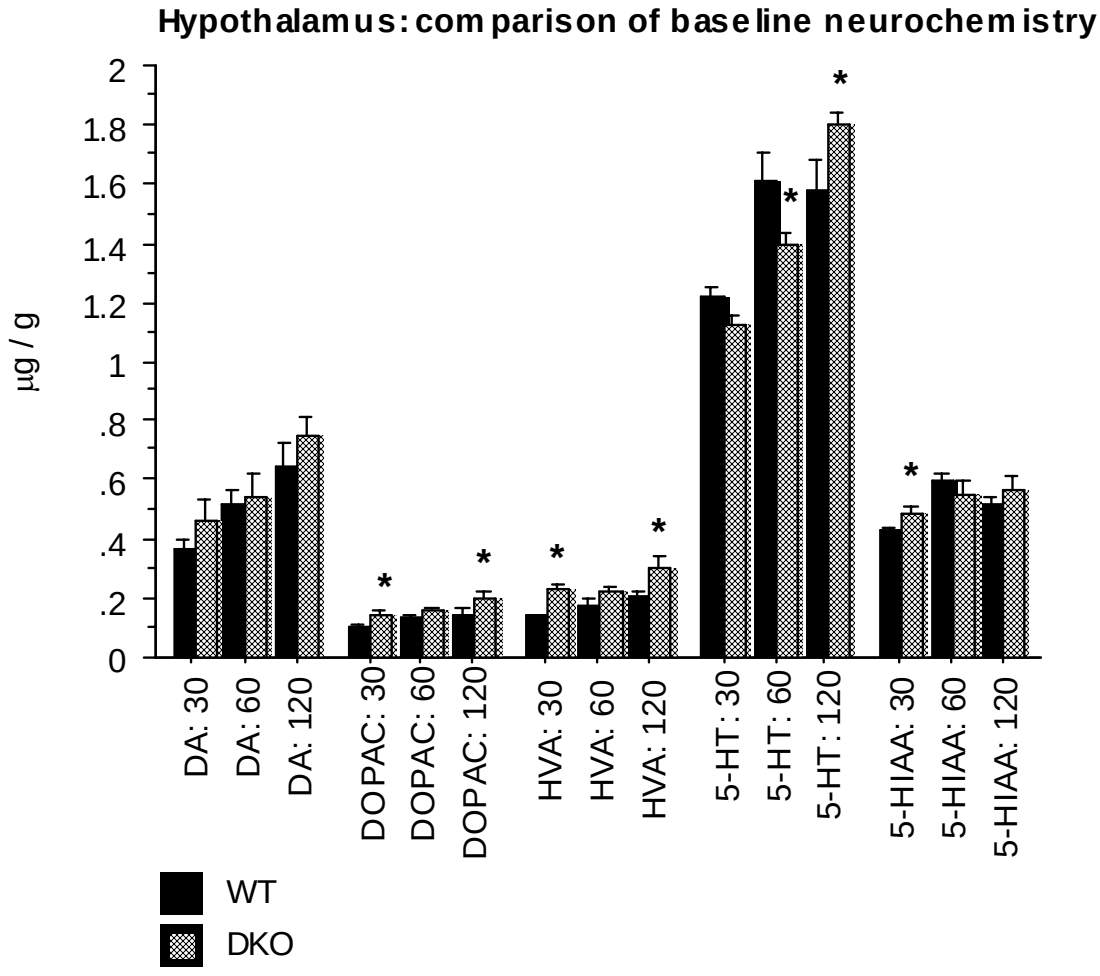


Figure 51: Concentration of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in hippocampus of wildtype and PAK double knockout mice 30, 60 and 120 days old

* denotes significantly different from wildtype; $p < .05$

Figure 52

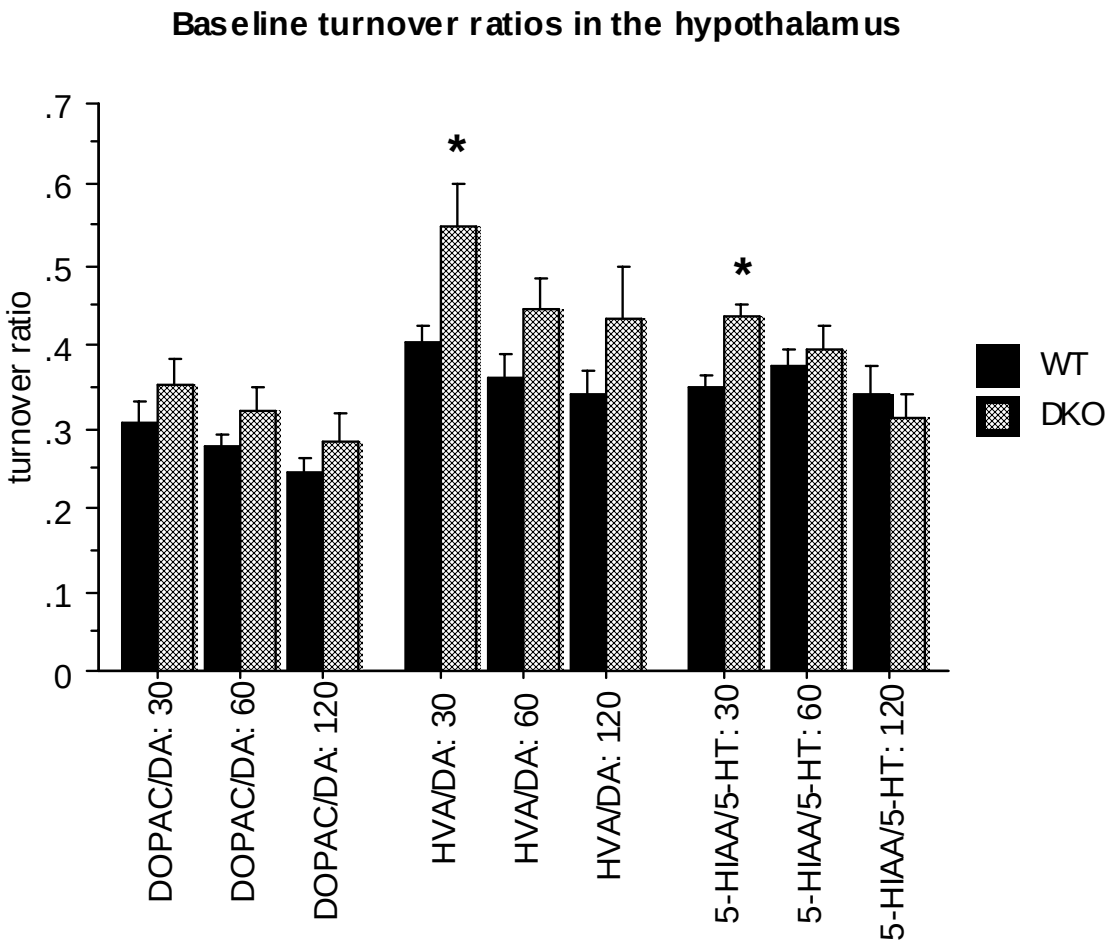


Figure 52: Turnover ratios of DOPAC/DA, HVA/DA and 5-HIAA/5-HT in hypothalamus of wildtype and PAK double knockout mice 30, 60, and 120 days old

* denotes significantly different from wildtype; $p < .05$

Figure 53

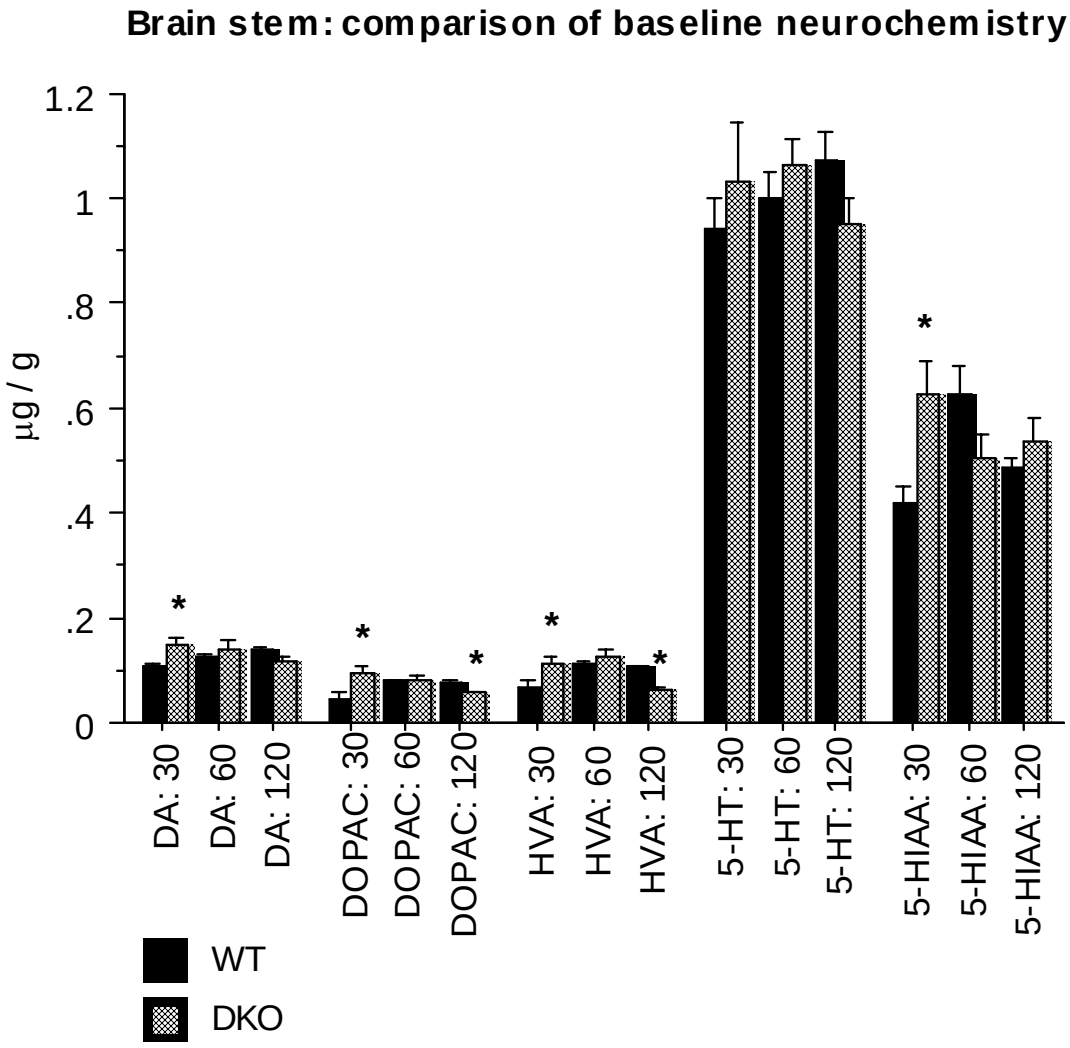


Figure 53: Concentration of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in brain stem of wildtype and PAK double knockout mice 30, 60 and 120 days old

* denotes significantly different from wildtype; $p < .05$

Figure 54

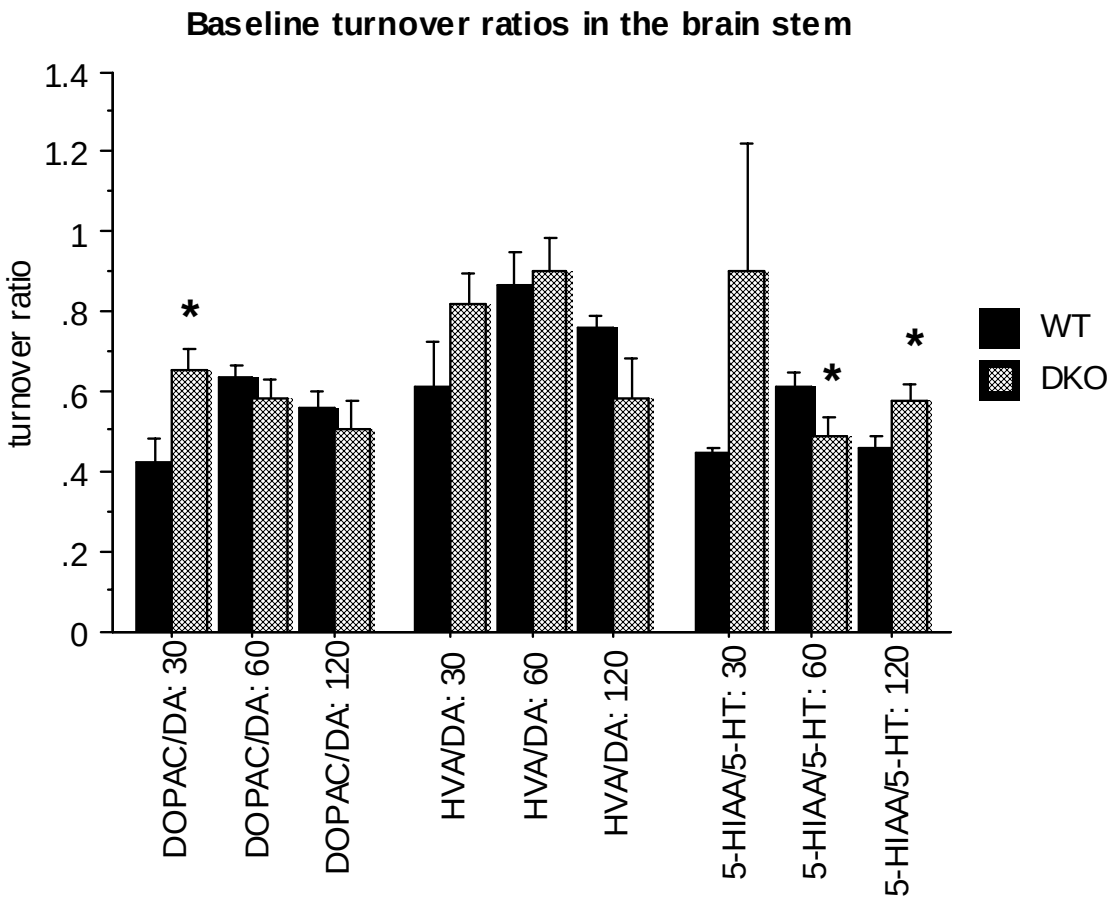


Figure 54: Turnover ratios of DOPAC/DA, HVA/DA and 5-HIAA/5-HT in brain stem of wildtype and PAK double knockout mice 30, 60, and 120 days old

* denotes significantly different from wildtype; $p < .05$

Experiment 12: Behavioral characterization and drug sensitivity of single knockouts PAK5 and PAK6 in addition to PAK5/PAK6 double knockout mice

Rationale

In Experiment 11, double knockouts showed significant hypoactivity compared to wildtypes. They also showed increased anxiety behavior, reduced social interactiveness, poor motor coordination and deficits in learning and memory. Finally, double knockouts showed increased activity in response to caffeine and some depletion in response to amphetamine.

To determine if one gene or the other (PAK5 or PAK6) contributed to the behaviors observed in the double knockouts versus the wildtypes in Experiment 11, single knockout mice lacking the PAK5 or PAK6 genes were generated. A battery of behavioral tests and neurochemical analysis was completed on all four genotypes to determine if either or both genes contributed or if there is functional redundancy between these two B family members.

Locomotor assessment was completed to determine overall activity levels of the genotypes. The elevated plus maze was used as a measure of anxiety. The rotorod was used as an assessment of motor function and coordination. Learning and memory was assessed in the active avoidance T-maze. Mice were also tested on a forced treadmill test as a second form of active avoidance as activity was forced and constant. Social interactiveness and aggression were also recorded. Sensitivity to caffeine was assessed in the open field to measure activity. Finally, amphetamine was administered to evaluate dopamine depletion in response to amphetamine's neurotoxic effects. All together, these

tests give a broad picture of the behavioral and neurochemical properties as it could relate to a disease phenotype.

Animals

All mice were on mixed 129/Sv x C57BL/6 genetic background and were generated in the Minden lab at Rutgers University. Five male double knockout mice, five male Pak5 knockout mice, eight male Pak6 knockout and eight male wild type mice were used in this set of tests. All mice were group housed in plastic shoebox cages with free access to food and water before being transferred to individual hanging wire cages (20 cm x 10 cm x 12 cm), also with free access to food and water, one week prior to testing began. Mice were housed in these cages during all tests unless otherwise noted. The mice were housed in a temperature and humidity regulated room with a 12 hr light/dark cycle. All of the experiments were conducted with the experimenter blind to mouse genotypes.

Body weight

Mice were weighed approximately once a week for the duration of studies.

Activity

Locomotor activity was assessed for 30 minutes in a novel environment (42 x 22 x 14 cm Plexiglas box) with six infrared sensors placed approximately 7 cm apart and 2.5 cm above the floor. The number of beam breaks was recorded every 5 minutes.

Elevated Plus Maze

Mice were placed in an elevated plus maze consisting of two open arms and two closed arms 30 cm long and 9 cm wide that cross a neutral 5 cm x 5 cm central square. The entire apparatus was elevated 60 cm above the floor. Each animal was placed in the center square of the maze and was given one 10-minute session to explore the maze. The number of entrances to closed arms, open arms, and jump-offs were recorded, as well as the number of fecal boli. An entrance to an arm was counted when all four feet crossed into an arm from the neutral center square.

Rotorod

Mice were assessed for balance and motor coordination on a 6.0 cm diameter rotorod rotating at 12 revolutions per minute, 60cm above a padded receptacle. Each trial was a maximum of 60 seconds and each mouse was given three consecutive trials. The latency to fall from the rotating rod was recorded.

Active avoidance

Mice were tested on two separate occasions (an acquisition phase and a retention phase) two months apart in an active avoidance T-maze consisting of two 20 x 11 cm chambers connected to a 40 x 10 cm corridor with 18 cm high walls made of Plexiglas. The floor was made of stainless steel bars spaced 0.75 cm apart and connected to a shock generator except in the "safe" arm of the T-maze. In each trial, a mouse was placed in the start box. After an intertribal interval of 20 seconds, a conditional stimulus tone accompanied the opening of the start box door. A correct avoidance response, moving to

the safe arm of the T-maze within 10 seconds, avoided the foot shock. Failure to make an avoidance response led to onset of a 0.8 mA foot shock that could be terminated by moving to the safe arm as a escape response. The maximum time allowed for an animal to make an escape response was also 10 seconds. The trial ended once the mouse made an avoidance or escape response to the safe chamber or at the end of 20 seconds total if no correct response was made. At the end of each trial, mice were moved back into the start box and given a 20 second intertrial interval. Each animal was given 5 sessions of 10 trials across 5 days. The type of response (avoidance or escape) and the latency for the animal to make either avoidance or escape response was recorded for each trial.

Active Avoidance – Treadmill

Mice were placed on a six lane rodent treadmill (Exer 3R Treadmill, Columbus Instruments, Columbus, OH) at the start of each trial. The treadmill was turned on and operated at a speed of 120 m/min. The running surface for each mouse was 17" L x 2.375" W x 5" H and the shock grid was 4.5" L x 2.375" W. Six mice were run at a time. If a mouse did not run and keep up with the treadmill speed, it would be shocked with a 0.8 mA shock. Mice were given one 8 minute trial per day for five days. The number of shocks received was recorded.

Social Chamber

Mice were placed in a social chamber that was 40 cm x 40 cm x 36.6 cm made of Plexiglas. Two wire cylinders made of a stainless steel grid, each 11 cm in diameter and 13 cm high, were located in opposite corners inside the chamber. A contact was recorded

when the subject mouse touched either cylinder with at least one paw on the cylinder and one on the floor of the chamber. Prior to the experimental sessions, the subject mouse was placed in the middle of the chamber and was allowed to explore the chamber for 10 minutes. After the habituation period, an adult male BALB/c mouse was placed in the target cylinder, while the control cylinder was left empty and the experimental mouse was placed back in the middle of the chamber. Each animal was allowed three 10-minute sessions across three consecutive days. The total number of contacts with the target and control cylinders during the session was recorded.

Aggression

At approximately 8 months of age, mice were tested for aggressive behavior using the resident-intruder test. The PAK wildtypes and knockouts (considered "resident" mice here) were housed individually in pan cages containing standard wood chip bedding for two weeks before testing began and for the remainder of behavioral and drug testing. Male C57BL/6 intruder mice were housed in cages at five mice per cage for at least two weeks prior to the start of trials. Testing began by placing an intruder in the resident's home cage for one 30-minute session per day for each of three days. The latency to the first attack as well as the number of attacks and which mouse (resident or intruder) initiated each attack was recorded.

Caffeine challenge

All mice were administered one subcutaneous injection of 12.6 mg/kg caffeine and immediately placed into the activity chambers. Locomotor activity was assessed for

three hours in a non-novel environment (42 x 22 x 14 cm Plexiglas box) with six infrared sensors placed approximately 7 cm apart and 2.5 cm above the floor. The number of beam breaks was recorded every 10 minutes.

Amphetamine challenge

Mice, in their home cage, were placed in the activity chambers with six infrared sensors placed approximately 7 cm apart and 2.5 cm above the floor. The number of beam breaks was recorded every 10 minutes for 30 minutes as a measure of baseline activity. After 30 minutes, mice were administered one subcutaneous injection of 50 mg/kg amphetamine or saline and then placed back into their home cage. Activity (number of beam breaks) was recorded every ten minutes for 90 minutes post-injection. Incidence of self-injurious or stereotyped behavior was recorded every 15 minutes beginning after injection. Mice were sacrificed 72 hours after drug administration for neurochemical analysis.

Neurochemistry

Mice were sacrificed for measurement of dopamine, serotonin and their metabolites. Bilateral striata were dissected out, snap frozen in liquid nitrogen and stored until assayed as in Experiment 1.

Weight

Mice were weighed approximately once a week for the duration of experiments. Statistical analysis with a repeated measures ANOVA showed that there was a significant

effect of genotype ($F(3,18) = 6.827$, $p = .0029$), weight ($F(29,522) = 80.179$, $p < .0001$) and a significant weight by genotype effect ($F(87, 522) = 6.324$, $p < .0001$). Fisher's LSD *post hoc* test showed a significant difference in weight between wildtypes and PAK5 knockouts ($p < .0001$), wildtypes and PAK6 knockouts ($p < .0001$), wildtypes and double knockouts ($p < .0001$), PAK5 and PAK6 knockouts ($p < .0001$), and PAK6 knockouts and double knockouts ($p < .0001$). Overall, PAK5 knockouts and double knockouts did not significantly differ in weight. See figure 55.

Wildtypes weighed an average of 30.8 grams at the start of measurements while PAK5 knockouts weighed an average of 24.3, PAK6 knockouts weighed an average of 28.6 and double knockouts weighed an average of 23.8 grams. This difference in weight was significant at the start of experiments ($F(3,22) = 7.907$, $p = .0010$). At week one, wildtypes weighed significantly more than PAK5 knockouts and double knockouts (WT, PAK5: $p = .0011$; WT, DKO: $p = .0006$). In addition, PAK6 knockouts weighed significantly more than PAK5 knockouts and double knockouts at week one of testing (PAK5, PAK6: $p = .0179$; PAK6, DKO: $p = .0095$). PAK5 knockouts and double knockouts weighed similarly as did wildtypes and PAK6 knockouts.

By the end of the experiments, one-way ANOVA analysis of the last weight (before amphetamine challenge) showed there was a still a significant difference in weights ($F(3,19) = 17.943$, $p < .0001$). However, *post hoc* analysis showed that only the PAK6 knockouts were significantly different in weight compared to the other genotypes (WT, PAK6: $p < .0001$; PAK5, PAK6: $p < .0001$; PAK6, DKO: $p < .0001$). Although the genotypes weighed significantly different at the start of experimentation, the weights of wildtypes, PAK5 knockouts and double knockouts evened out by completion of the

studies. PAK6 knockouts, on the other hand, gained more weight than all other genotypes and were significantly heavier at the completion of the study. Analysis of overall weight change showed that there was a significant difference in weights ($F(3,19) = 26.736$, $p < .0001$). See figure 56.

Conclusions

PAK6 knockouts gained a significant amount of weight during the course of testing, despite receiving the same diet and water type and amounts as the other genotypes. Actual food and water consumption was not measured during these tests and could lend insight into this significant weight gain. This increased weight gain could be caused by a number of factors including hypothyroidism, excess cortisol, fluid retention, reduced levels of leptin or reduced levels of α-melanocyte stimulating hormone, all of which have been shown to play a role in weight gain. Interestingly, PAK6 is expressed in low levels in the thyroid gland. The knockout of PAK6 may lead to hypothyroidism and be the cause of the significant difference in weight of PAK6 knockouts compared to the other genotypes.

Activity

Mice were placed into the activity chamber and their activity counts were recorded for every five minutes for thirty minutes. Repeated measures ANOVA showed that overall there was a significant effect of genotype ($F(3,22) = 5.256$, $p = .0069$), a significant difference in activity for all mice over the testing period ($F(5,110) = 4.993$, $p = .0004$) and a significant interaction between genotypes and activity over the testing period ($F(3,22) = 2.048$, $p = .0179$). *Post hoc* analysis showed that wildtypes and PAK5

knockouts had statistically similar levels of activity over the entire testing period, as did wildtypes and PAK6 knockouts (WT, PAK5: $p = .0692$; WT, PAK6: $p = .1814$). Moreover, activity of wildtype, PAK5 knockout and PAK6 knockouts was statistically different from double knockout activity over testing (WT, DKO: $p < .0001$; PAK5, DKO: $p < .0001$; PAK6, DKO: $p < .0001$), as were PAK5 knockouts and PAK6 knockouts ($p = .0031$). Overall, the activity levels of wildtypes, PAK5 knockouts and PAK6 knockouts were comparable over the session. See figure 57.

Conclusions

As in Experiment 11, double knockouts were significantly less active than wildtypes. Activity levels of wildtypes and double knockouts were similar in Experiment 11 to those observed here. Interestingly, activity of PAK5 single knockouts (PAK5 KOs) and PAK6 single knockouts (PAK6 KOs) were statistically similar to wildtypes and statistically different from double knockouts. This shows that there may be a functional redundancy in the PAK5 and PAK6 genes as it relates to general activity levels. It is only when both of these genes are missing that the deficit is uncovered as seen in the activity levels of the double knockouts.

Elevated plus maze

Mice were placed in the center neutral square of the elevated plus maze to begin each trial. Entries in the closed and open arms were counted, as well as the number of jumps off the apparatus and fecal boli. See figure 58. One-way ANOVA with Fisher's LSD *post hoc* analysis was completed on each variable.

Overall, analysis shows that there is a significant difference in closed arm entries, open arm entries and jump offs between all four genotypes. PAK5 knockouts entered the closed arms of the maze significantly more times than all the other genotypes (WT, PAK5: $p = .0086$; PAK5, PAK6: $p = .0223$; PAK5, DKO: $p < .0001$). Double knockouts entered the closed arms significantly less than all other genotypes (WT, DKO: $p = .0004$; PAK5, DKO: $p < .0001$; PAK6, DKO: $p = .0001$). Wildtypes and PAK6 knockouts entered the closed arms the same amount on average.

Overall, there was a significant difference in open arm entries across genotypes. *Post hoc* testing revealed the specific differences between each genotype. PAK5 knockouts also entered the open arms of the maze on average more times than all other genotypes and significantly more than PAK6 knockouts and double knockouts (PAK5, PAK6: $p = .0388$; PAK5, DKO: $p = .0012$). Again, double knockouts entered the open arms less than all other genotypes on average, significantly less than wildtypes and PAK6 knockouts (WT, DKO: $p = .0116$; PAK6, DKO: $p = .0012$) and close to significantly less than PAK5 knockouts ($p = .0679$). Double knockouts entered the open arms on average only 0.8 times during the session compared to 5.75, 8.20, and 4.25 entries of the wildtypes, PAK5 knockouts and PAK6 knockouts, respectively.

There was no significant difference in the number of fecal boli.

Conclusions

The elevated plus maze is used as a measure of anxiety in behavioral models. Here, PAK5 KOs entered the closed and open arms of the maze more than other genotypes. This indicates a possible higher level of overall activity, although one was not observed in the open field activity measurements, but more likely is a sign of reduced

anxiety compared to the other genotypes. This effect was significant for closed arm entries compared to all three other genotypes. Although, PAK5 KOs entered the open arms more on average than wildtypes, they did not so significantly more.

PAK6 KOs performed similarly to wildtypes. They entered both the closed and open arms about the same number of times as wildtypes, thus exhibiting normal anxiety levels by comparison. PAK6 KOs performed the most like wildtypes on all measures of the elevated plus maze.

As in Experiment 11, double knockouts entered both open and closed arms significantly less times than wildtypes. This time, double knockouts only entered the open arms on average 0.8 times whereas the wildtypes entered the open arms 5.75 times on average. Again, this could be due to general hypoactivity but the ratio of open arm entries to closed arm entries was of greater deficit in this Experiment. Wildtypes had a ratio of 50% while double knockouts had a ratio of only 22%. Therefore, while a reduced activity level played a role in the number of entries, the ratio shows that double knockouts exhibited more anxiety based behavior compared to wildtypes as in Experiment 1.

Rotorod

Mice were given 3 consecutive trials on the rotorod, lasting a maximum of 60 seconds. The latency to fall from the apparatus was recorded and analyzed using a repeated measures ANOVA. Analysis showed significant improvement in genotypes over time ($F(3,22) = 5.801$, $p = .0044$) but no significant difference between genotypes over all three trials ($F(2,6) = .645$, $p = .6936$). Further *post hoc* analysis revealed that

PAK5 and double knockouts did significantly worse than wildtypes (PAK5: $p = .0005$; DKO: $p < .0001$) and PAK6 KOs (PAK5: $p = .0033$; DKO: $p = .0002$). PAK6 and wildtypes performed similarly across the trials ($p = .4940$) while PAK5 and double knockouts also performed similarly to each other over the trials ($p = .4108$). See figure 59.

In addition, one-way ANOVAs were used to determine the performance of the genotypes on each trial. In trial one, there was a significant effect of genotype ($F(3,22) = 6.074$, $p = .0036$) with *post hoc* tests confirming that PAK6 KOs and wildtypes performed similarly, as did PAK5 KOs and double knockouts. PAK5 KOs and double knockouts performed significantly worse than wildtypes and PAK6 KOs. Similar results were found in trial two. On trial three, however, there was no longer a significant effect of genotype and *post hoc* analysis showed that the performance of PAK5 KOs had caught up to PAK6 KOs and wildtypes. double knockouts still performed significantly worse than PAK6 KOs and wildtypes.

Conclusions

The rotorod is used as a measure of motor function and coordination. It can also be used as an indicator of motor learning. As in Experiment 11, double knockouts performed worse than wildtypes. In this Experiment, they performed much worse than the double knockouts tested in Experiment 11, averaging latencies of only 8.6, 7.6 and 9.1 seconds on trials 1, 2 and 3, respectively. They did not improve over the trials demonstrating reduced motor learning as well as reduced motor coordination.

The PAK5 KOs performed similarly to the double knockouts over trials 1 and 2 but greatly improved on trial 3. This could show that PAK5 KOs have better motor learning abilities compared to double knockouts.

The differences in performance suggest the deficit in rotorod performance can be attributed to PAK5 since PAK5 KOs performed similarly to double knockouts. PAK6 KOs did not have any difficulties performing on the rotorod and improved on each trial, as did the wildtypes.

Active Avoidance

First run (acquisition phase):

Mice were given 10 trials per day for five days. The number of and latency for each type of response (escape or avoidance) was recorded and averaged over the 10 trials giving a daily average latency or daily average responses for each mouse. These daily average latencies and response numbers for each mouse were used in all statistical tests. A repeated measures ANOVA was ran without significant results, however, because the statistical power was low due to missing values. Values were missing due to lack of escape responses, as animals improved over the trials/days, escape responses lessened and avoidance responses increased. Single one-way ANOVAs on each day were conducted to determine differences in genotypes by day of testing. On days one and two, one-way ANOVA analysis showed no significant results between genotypes. However, on day two, *post hoc* analysis showed that double knockouts had significantly lower escape latencies compared to PAK6 KOs ($p = .0273$). On day three of testing, one-way ANOVA analysis showed a significant difference between genotypes ($F(3,14) = 3.345$, p

= .0499). PAK6 KOs had the highest latency of all genotypes, significantly higher than PAK5 KOs and double knockouts (PAK5, PAK6: p = .0270; PAK6, DKO: p = .0124). One-way ANOVA analysis of day four showed no significant difference between genotypes. *Post hoc* analysis revealed that PAK6 KOs had a significantly higher latency compared to double knockouts and near significantly higher latency compared to wildtypes (PAK6, DKO: p = .0185; WT, PAK6: p = .0544). Day 5 of testing showed no significant differences in latency in any of the genotypes. See figure 60.

A repeated measures ANOVA was used to analyze the avoidance latencies across days. Overall, there was no significant differences between genotypes or interaction between genotype and avoidance latency but there was significant difference in avoidance latency across days ($F(4,52) = 7.077$, $p = .0001$). In general, avoidance latencies decreased over the testing days, except in double knockouts. *Post hoc* analysis showed that PAK5 KOs had significantly higher avoidance latencies compared to wildtypes (p = .0259). Overall, wildtypes had the lowest avoidance latencies. Single one-way ANOVAs on each day were conducted to determine differences in genotypes by day of testing. On all days except day five, one-way ANOVA analysis showed no significant difference between genotypes. However, on day five, there was a significant difference between genotypes ($F(3,13) = 4.621$, $p = .0207$) with *post hoc* analysis showed that double knockouts had significantly higher escape latencies compared to wildtypes (p = .0276) and PAK6 knockouts (p = .0026). See figure 61.

Analysis with a repeated measures ANOVA revealed a significant difference in numbers of escape responses over the testing period ($F(4,88) = 49.830$, $p < .0001$) and a significant interaction between genotype and escape responses ($F(12,88) = 1.970$, p =

.0365). Single one-way ANOVAs on each day were conducted to determine differences in genotypes by day of testing. On all days except day five, one-way ANOVA analysis showed no significant results between genotypes. However, *post hoc* analysis on day three showed that wildtypes had significantly lower number of escape responses compared to PAK5 knockouts ($p = .0291$) and double knockouts ($p = .0291$). On day five, there was a significant difference in number of escape responses between genotypes overall. *Post hoc* analysis showed that wildtypes had significantly less escape responses compared to PAK5 knockouts ($p = .0411$) and double knockouts ($p = .0014$). In addition, PAK6 knockouts had significantly less escape responses compared to PAK5 knockouts ($p = .0054$) and double knockouts ($p = .0002$) as well. See figure 62.

Similarly, analysis with a repeated measures ANOVA also revealed a significant difference in number of avoidance response over the testing period ($F(4,88) = 58.104$, $p < .0001$) and a significant interaction between genotype and avoidance responses ($F(12,88) = 2.232$, $p = .0165$). Overall, wildtypes and PAK6 KOs had higher numbers of avoidances compared to PAK5 KOs and double knockouts. On all days except day five, one-way ANOVA analysis showed no significant results between genotypes. However, *post hoc* analysis on day three showed that wildtypes had significantly higher number of avoidance responses compared to PAK5 knockouts ($p = .0291$) and double knockouts ($p = .0291$). On day five, there was a significant difference in number of avoidance responses between genotypes overall. *Post hoc* analysis showed that wildtypes had significantly more avoidance responses compared to PAK5 knockouts ($p = .0411$) and double knockouts ($p = .0014$). In addition, PAK6 knockouts had significantly more

avoidance responses compared to PAK5 knockouts (p = .0054) and double knockouts (p = .0002) as well. See figure 63.

Second run (retention phase):

Mice were given 10 trials per day for eight days. The number of and latency for each type of response (escape or avoidance) was recorded and averaged over the 10 trials giving a daily average latency or daily average responses for each mouse. These daily average latencies and response numbers for each mouse were used in all statistical tests. Repeated measures ANOVA analysis could not be used on escape latency over the eight days due to missing values. Values were missing due to lack of escape responses, as animals improved over the trials/days, escape responses lessened and avoidance responses increased. Single one-way ANOVAs on each day were conducted to determine differences in genotypes by day of testing. No significant differences were found on any day of testing. However, on day 7 only, *post hoc* analysis showed a significant difference between PAK6 KOs and double knockouts (p = .0254). See figure 64.

A repeated measures ANOVA was used to analyze the average avoidance latencies and showed a significant interaction between genotype and latencies ($F(3.21) = 1.658$, p = .0455). *Post hoc* analysis revealed that wildtypes, PAK5 KOs and PAK6 KOs had significantly lower avoidance latencies overall compared to double knockouts (WT, DKO: p = .0004; PAK5, DKO: p = .0245; p = .0001). One-way ANOVA analysis was used to determine the difference in latencies between genotypes on each day. There was a significant difference between genotypes on day one ($F(3,19) = 4.291$, p = .0180) with

post hoc analysis showing a significant difference between PAK5 knockouts and wildtypes ($p = .0211$) as well as PAK6 knockouts ($p = .0033$). Analysis of day six showed a significant difference between genotypes ($F(3,19) = 4.821$, $p = .0116$) with *post hoc* analysis revealing that double knockouts were slower than the other three genotypes (WT, DKO: $p = .0031$; PAK5, DKO: $p = .0048$; PAK6, DKO: $p = .0045$). Analysis of days two, three, four, five, seven and eight showed no significant differences between genotypes overall. However, *post hoc* analysis on day three, double knockouts were significantly slower that PAK5 knockouts ($p = .0388$) and on day seven, PAK6 knockouts were significantly slower than PAK5 knockouts ($p = .0488$). See figure 65.

A repeated measures ANOVA on percentage of escape responses and avoidance responses showed a significant difference between genotypes ($F(3,21) = 4.212$, $p = .0176$), number of escapes ($F(7,147) = 2.425$, $p = .0222$) and a significant interaction between genotypes and number of escapes ($F(7,21) = 2.178$, $p = .0038$). The statistical analysis results are identical because these responses exact opposites of each other, out of a possible 10 responses (i.e. when a mouse exhibited 3 escape responses, then the other 7 responses were avoidances). *Post hoc* analysis showed that double knockouts had a significantly higher amount of escapes/lower amount of avoidances compared to all other genotypes (WT, DKO: $p < .0001$; PAK5, DKO: $p < .0001$; PAK6, DKO: $p < .0001$). PAK5 KOs also had a significantly higher number of escape responses/lower number of avoidances compared to wildtypes ($p = .0334$). One-way ANOVA analysis was used on each day to determine the difference in percentage of responses between genotypes. There were significant differences between genotypes on day one ($F(3,21) = 4.557$, $p = .0131$), day five ($F(3,21) = 3.947$, $p = .0223$), day six ($F(3,21) = 6.071$, $p = .0038$) and

day seven ($F(3,21) = 3.658$, $p = .0289$). *Post hoc* analysis showed the specific differences on each day. On day one, double knockouts had significantly more escapes/less avoidances than wildtypes ($p = .0203$), PAK5 knockouts ($p = .0476$) and PAK6 knockouts ($p = .0014$). On day two, double knockouts had significantly more escapes/less avoidances than wildtypes ($p = .0198$). On day three, double knockouts had significantly more escapes/less avoidances than PAK5 knockouts ($p = .0298$). On day four, double knockouts had significantly more escapes/less avoidances than wildtypes ($p = .0074$). On day five, double knockouts had significantly more escapes/less avoidances than wildtypes ($p = .0057$) and PAK6 knockouts ($p = .0141$). On day six, double knockouts had significantly more escapes/less avoidances than wildtypes ($p = .0008$), PAK5 knockouts ($p = .0034$) and PAK6 knockouts ($p = .0022$). On day seven, double knockouts had significantly more escapes/less avoidances than wildtypes ($p = .0063$) and PAK6 knockouts ($p = .0109$). See figure 66 and 67.

Conclusions

Overall, wildtype and PAK6 knockout mice performed significantly better than PAK5 knockouts and double knockouts in the active avoidance paradigm. In the acquisition phase, wildtypes reached optimal performance by day three of testing, successfully avoiding the foot shock greater than 90% of the time. This performance remained steady for the duration of testing. Their latency to exhibit an avoidance response remained steady over testing. In instances where wildtypes failed to avoid the foot shock and had to escape it, the latency to escape improved over testing days. PAK6 knockouts steadily improved avoidance responding over testing and also successfully avoided greater than 90% of the time by the end of testing. Avoidance latencies were

consistent with wildtype latencies throughout. Escape latencies of PAK6 knockouts were consistent, although slightly higher, with wildtype latencies throughout.

The performance of PAK 5 knockouts and double knockout mice improved over testing as well, although both avoided the shock less than 75% of the time and the end of testing. The avoidance latency of PAK5 knockouts and double knockouts was similar to latencies of wildtypes and PAK6 knockouts. Escape latency in double knockouts was highest on day one but was similar to all other genotypes by day two.

In the retention phase (second run) of active avoidance, PAK6 knockouts began at optimal performance, avoiding the shock over 90% of the time on average. Wildtypes and PAK5 knockouts also performed well, averaging 75% avoidance on day one of testing. PAK5 knockouts, PAK6 knockouts and wildtypes performed similarly on each of the rest of the days. On the other hand, double knockouts avoided the shock less than 45% of the time on day one. Their performance improved slowly over subsequent days but still had significantly lower number of avoidances compared to each other genotype. The escape and avoidance latencies on the second run of active avoidance were lower than in active avoidance I. The escape latencies of all genotypes were statistically similar and the avoidance latencies were similar between wildtypes, PAK5 knockouts and PAK6 knockouts. The avoidance latency of double knockouts was significantly higher compared to the other genotype. These data indicate that PAK5 knockouts, PAK6 knockouts and wildtypes effectively recalled the T-maze paradigm and were able to perform very well on the task from the beginning of testing. Double knockouts did not perform well at the start and this may be indicative of poor memory function. Also, their

performance never reached the levels of the other genotypes, indicating poorer motor learning.

Active Avoidance: Treadmill

A repeated measures ANOVA showed that there was significant difference in shocks on the treadmill over the testing period ($F(4,84) = 15.672$, $p < .0001$) but no significant differences between genotypes or a significant interaction between genotype and treadmill shocks. Although, the difference between genotypes approached significance. However, *post hoc* analysis revealed that double knockouts received more shocks during testing than any other genotype (WT, DKO: $p = .0084$; PAK5, DKO: $p = .0109$; PAK6, DKO: $p = .0364$). See figure 68.

Conclusions

Double knockouts performed worse than any other genotype on this test, although by the last session performed as well as the other genotypes. Generally, wildtypes, PAK5 KOs and PAK6 KOs all performed similarly to one another. This is in contrast to performance on the T-maze active avoidance paradigm. Double knockouts never reached the performance level of the other genotypes in T-maze active avoidance but do in this paradigm. This could indicate normal abilities in motor learning but deficits in motor ability. In the T-maze, mice are placed in the start box and left inactive for 30 seconds between each trial. They are free to explore the small area or be still until the trial starts. Double knockouts had the slowest avoidance latencies, indicating potentially poor motor learning or reluctance or inability to initiate movement. Here, on the treadmill, mice are forced to move continually or they will receive a foot shock. Although double knockouts

received more shocks overall, they did steadily improve and performed at normal levels by the end of testing. This may indicate that once they are moving, they can continue (possibly with difficulty) and therefore are able to improve their performance to normal levels. This can be compared to motor symptoms in Parkinson's disease. Patients have difficulty initiating movement and bradykinesia, or slow movement. Double knockout mice show signs of this in their reduced baseline activity levels as well as in their poor/ low performance in the elevated plus maze, rotorod and both active avoidance paradigms.

Social activity

Mice were placed in the social chambers and allowed to explore for a total of 30 minutes, once a day for three days. An adult male unfamiliar mouse was placed into the "target cup" while the "control cup" was left empty. Each mouse being tested was given a new unfamiliar mouse in the target cup each day. The number of times the mouse being tested touched either cup was recorded. See figure 69.

A repeated measures ANOVA showed there was a significant difference in touches to the two cups ($F(3,1) = 9.271$, $p = .0042$) but no other significant differences in genotype effect or interaction of cup type and genotypes. *Post hoc* analysis showed that PAK5 and DKO performed statistically different from each other ($p = .0399$)

One-way ANOVA analysis was conducted on each of the three test day's results. On the first day of testing, there was a significant effect of genotype ($F(3,39) = 10.641$, $p < .0001$), cup type ($F(1,39) = 17.877$, $p = .0001$) and interaction of genotype and cup type ($F(3, 39) = 4.331$, $p = .0100$). *Post hoc* analysis showed that wildtypes performed significantly different compared to all three other genotypes (PAK5: $p = .0069$, PAK6: p

< .0001; DKO: p < .0001). Wildtypes, PAK5 KOs and PAK6 KOs all contacted the target cup more than the control cup. On the other hand, double knockouts contacted both cups about equally. Trial 2 results were similar to trial 1 with the exception that both cups were contacted less on average. By trial 3, the control cup was contacted very little by all genotypes.

A one-way ANOVA was also conducted on each genotype to determine differences between contacts with the cups. Wildtypes contacted the target cup significantly more that the control cup on trial one only ($F(1,12) = 14.443$, $p = .0025$). PAK5 knockouts contacted both cups similarly on all trials. PAK6 knockouts contact the target cup significantly more times than the control cup on trial one and three (trial one: $F(1,12) = 6.454$, $p = .0259$; trial three: $F(1,12) = 5.569$, $p = .0361$). Double knockouts contacted both cups similarly on all trials.

Conclusions

Overall, PAK5 knockouts were the most active in the social chambers as evidenced by more contacts with both cups overall. This is in parallel to their slightly higher overall activity levels measured in the open field. All genotypes showed generally normal social behavior in that they contacted the target cup more than the control cup although double knockouts did so with the smallest margin. Their overall hypoactivity compared to the other genotypes most likely plays a significant role in this observation.

Aggression

A repeated measures ANOVA on the latency to the first attack (not taking into account which type of mouse – resident or intruder – that initiated it) showed no

significant effect of genotype, latency or interaction of genotype and latency. See figure 70.

A repeated measures ANOVA on the number of times an attack was initiated by an intruder mouse showed a significant effect of genotype ($F(3,20) = 3.708$, $p = .0286$) but no significant difference in number of attacks overall or interaction between genotype and overall attacks. *Post hoc* analysis showed that wildtypes were attacked significantly less times than double knockouts ($p = .0033$). PAK5 knockouts were attacked more than wildtypes as well but the difference did not reach significance ($p = .0507$). In addition, PAK6 knockouts were attacked significantly less than double knockouts, but the difference did not reach significance ($p = .0565$). A one-way ANOVA on each trial showed the difference between genotypes. Analysis showed no significant difference overall between genotypes on any of the three trials. However, *post hoc* analysis revealed a significant difference between wildtypes and PAK5 knockouts on trial 2 ($p = .0426$) and on trial 3 between wildtypes and double knockouts ($p = .0157$) and between PAK5 knockouts and double knockouts ($p = .0384$). See figure 71.

A repeated measures ANOVA on the number of times an attack was initiated by the resident PAK mouse showed a significant difference of numbers of attacks over the trials ($F(2,40) = 4.004$, $p = .0260$) but no significant difference of genotype, number of attacks across trials or interaction of genotype and attacks across trials. However, *post hoc* analysis showed that wildtypes initiated significantly more attacks than all of the knockouts (WT, PAK5: $p = .0007$; WT, PAK6: $p = .0131$; WT, DKO: $p = .0005$). A one-way ANOVA analysis on each trial showed the difference between genotypes. Analysis showed no significant differences between genotypes on trials 1 and 3. On trial 2, there

was a significant difference between genotypes ($F(3,20) = 3.548$, $p = .0330$) with *post hoc* analysis showing that wildtypes initiated significantly more attacks on intruder mice than did any other genotype (WT, PAK5: $p = .0230$; WT, PAK6: $p = .0226$; WT, DKO: $p = .0116$). See figure 72.

Conclusions

Wildtype mice protected their territory – their home cage – more than the knockout mice, evidenced by significantly more attacks towards the intruder mice. They were also attacked less by intruder mice than the knockouts. Double knockouts were attacked the most overall. PAK5 KOs, PAK6 KOs and double knockouts exhibited the least amount of offensive aggression, evidenced by their much lower levels of attacks on intruder mice.

Caffeine challenge

All mice were given caffeine and then immediately placed into the activity chambers. Activity counts were recorded every ten minutes for three hours. A repeated measures ANOVA revealed that there was a significant change in activity over the recording period ($F(18,342) = 19.666$, $p < .0001$) but no difference in genotypes. See figure 73.

Conclusions

In contrast to baseline activity levels, which differ significantly between genotypes, all genotypes have similar activity levels after receiving caffeine. Caffeine administration is able to offset intrinsically lower levels of activity observed in the double knockouts. Caffeine is known to be an adenosine antagonist acting as a competitive

inhibitor on the adenosine receptor. Adenosine acts as a retrograde neurotransmitter and can effectively inhibit neurotransmitter release from the presynaptic neuron. By antagonizing adenosine, caffeine in doses typical of human daily consumption increases dopamine levels in the prefrontal cortex and caudate nucleus. This can increase locomotor activity. The increase in activity of the double knockouts shows that they are sensitive to caffeine's effects on adenosine and dopamine transmission. In fact, the activity levels on double knockouts were greater than activity levels of wildtypes for the first 60 minutes after caffeine administration. The baseline activity of wildtypes was about 3000 (measured as beam breaks) per ten minutes. After caffeine administration, the average activity level of wildtypes was only 2500 per ten minutes while the double knockouts averaged 2700 per ten minutes. The total average activity count for wildtypes was 47756 whereas it was 52074 for double knockouts. Taken together, it seems as though double knockouts are more sensitive to caffeine's stimulatory effects.

Amphetamine challenge

Amphetamine treatment had a significant effect on weight. Two-way ANOVA analysis of the change in weight by genotype after saline or amphetamine treatment showed there was a significant difference between genotypes ($F(3,12) = 14.267$, $p = .0003$), a significant difference between drug ($F(1,12) = 18.390$, $p = .0011$) and a significant interaction between genotype and drug ($F(3,12) = 7.472$, $p = .0044$). PAK6 knockouts lost the most weight after amphetamine injection. One-way ANOVA of amphetamine-treated mice showed a significant difference between genotypes ($F(3,7) = 15.941$, $p = .0016$) with *post hoc* analysis showing that PAK6 knockouts lost significantly

more weight compared to all other genotypes (WT, PAK6: p = .0010; PAK5, PAK6: p = .0050; PAK6, DKO: p = .0004). After amphetamine, wildtypes lost an average of 0.4 grams, PAK5 knockouts lost an average of 0.95 grams, PAK6 knockouts lost an average of 3.633 grams and double knockouts gained an average of .167 grams. This translated into a significantly greater percentage of body weight lost in PAK6 knockouts: 8.038% of their body weight. Wildtypes and PAK5 knockouts lost 1.113% and 2.713% of their body weight, respectively. Double knockouts gained 0.6% of their body weight. It is not know whether this loss of body came from a reduction of fat, muscle mass or water content. Data not shown.

There was also a significant change in weight in saline-treated mice ($F(3,5) = 6.002$, $p = .0412$). *Post hoc* analysis showed significant differences between wildtypes and double knockouts ($p = .0142$), as well as between wildtypes and double knockouts ($p = .0240$). After saline injection, wildtype mice gained an average of 1.2 grams, PAK5 knockouts gained an average of 0.1 grams, PAK6 knockouts lost an average of 0.267 grams and double knockouts lost an average of 0.6 grams. Interestingly, PAK5 knockouts and double knockouts lost weight after treatment with saline, possibly from the stress of the injection. Data not shown.

Two-way repeated measures ANOVA showed a significant difference in activity ($F(23, 345) = 8.105$, $p < .0001$) and a significant interaction between activity levels and drug challenge ($F(23, 345) = 1.739$, $p = .0199$). In general, activity levels decreased over the time period and saline animals were much less active by the end of testing compared to amphetamine-treated mice.

One-way repeated measures ANOVA of amphetamine treated mice showed a significant difference in activity over testing ($F(23,230) = 5.711$, $p < .0001$). *Post hoc* analysis revealed that PAK5 knockouts were significantly more active after amphetamine treatment compared to wildtypes ($p < .0001$) and double knockouts ($p < .0001$). PAK6 knockouts were similarly active (although slightly less) to PAK5 knockouts and also significantly more active than wildtypes ($p = .0006$) and double knockouts ($p < .0001$). This showed that PAK5 and PAK6 knockouts were more sensitive to the hyperactivity effects of amphetamine compared to wildtypes and double knockouts.

A one-way ANOVA was also used to analyze the effects of the saline injection on the activity. Overall activity significantly decreased over testing ($F(23,115) = 4.104$, $p < .0001$). This was expected as the injection itself would increase activity levels since animals are handled and injected. By the end of testing, saline-treated animals were all relatively inactive after the initial stress of the handling and injection wore off. *Post hoc* analysis showed that all genotypes were similarly active, although double knockouts were the most hypoactive.

A one-way ANOVA was used to analyze each genotypes behavior in response to drug treatment. Wildtypes were not significantly effected by amphetamine treatment. The activity levels of the saline-treated mice were comparable to those of the amphetamine-treated mice. The activity of PAK5 knockouts was significantly impacted by drug challenge ($F(1,3) = 17.835$, $p = .0243$). Activity levels of amphetamine-treated mice around 70 minutes post-injection and remained steady for the duration of recording. The activity of PAK6 knockouts was also not significantly affected by amphetamine, although it was increased ($p = .0784$). Activity was highest immediately after injection,

decreased slightly and then remained steady for the duration of recording. Finally, double knockouts were also not significantly effected by amphetamine treatment. Their activity levels were near identical at all time points in both saline- and amphetamine-treated mice. These data show that wildtypes and double knockouts were the least sensitive to the hyperactivity effects of amphetamine. PAK5 and PAK6 knockouts, on the other hand, were more hyperactive after amphetamine treatment compared to controls with PAK5 knockouts being the most affected. See figure 75.

Two-way ANOVA analysis of the neurochemistry after amphetamine challenge showed a significant effect of drug challenge on dopamine levels ($F(1, 11) = 7.389$, $p = .0200$). Overall, dopamine was lower in mice receiving amphetamine compared to saline-treated controls. DOPAC, HVA, 5-HT and 5-HIAA levels were unaffected by amphetamine treatment. One-way ANOVA analysis of each genotype showed no significant difference in any neurotransmitter or metabolite between saline- and amphetamine-treated groups in wildtypes, PAK5 knockouts and double knockouts. However, in PAK6 knockouts, dopamine was significantly lower in amphetamine-treated mice compared to controls ($F(1,3) = 18.361$, $p = .0233$). See figure 76.

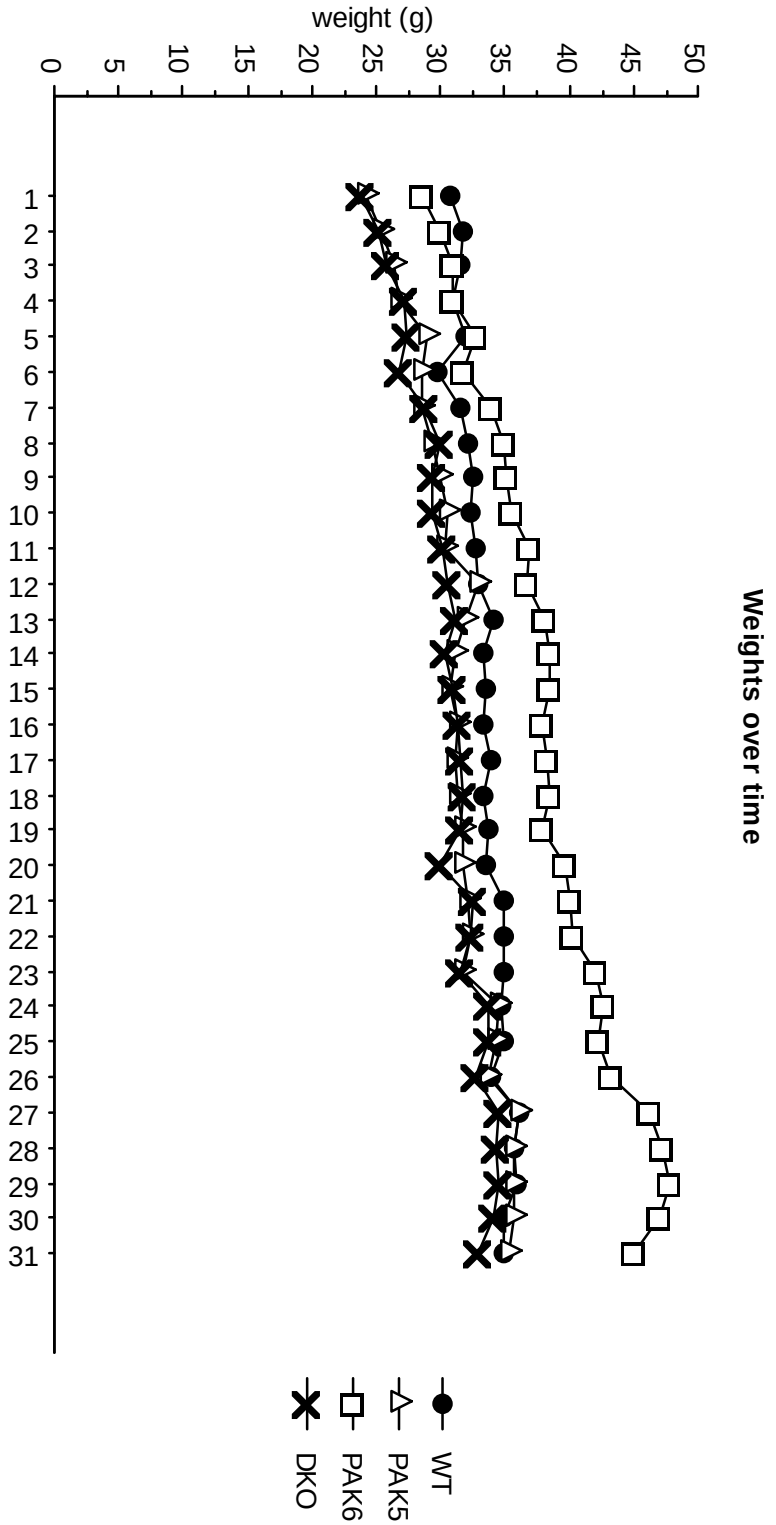
Conclusions

PAK6 knockouts appear to be the most sensitive to the neurotoxic effects of amphetamine. In addition, their activity levels were markedly increased and they lost a significant amount of body weight in response to amphetamine treatment. The increased activity levels show that dopamine release was increased in the striatum of these mice after amphetamine administration and possibly significantly enough to enable the neurotoxic effects of amphetamine. In addition, the drastic weight loss in the three days

between amphetamine injection and sacrifice could be linked to increased neurotoxicity compared to other genotypes. It is possible that PAK6 knockouts were considerably affected by amphetamine's effects on appetite and/or were too sick to eat or drink following amphetamine. These outcomes (increased activity and drastic weight loss) may serve as effective indicators of significant neurotoxicity.

Double knockouts showed enhanced sensitivity to amphetamine neurotoxicity similar to that observed in Experiment 11. However, the depletion did not reach statistical significance, although was close. Further studies with larger numbers of mice need to be conducted to further investigate the sensitivity of double knockout mice to amphetamine.

Figure 55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Weights over time
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PAK6
DKO

Figure 55: Weight of wildtype, PAK5 knockouts, PAK6 knockouts and PAK double knockout mice over testing period. At arrival, wildtypes and PAK6 knockouts weighed significantly more than PAK5 knockouts and double knockouts. By the end of testing, PAK6 knockouts weighed significantly more than other genotypes

Figure 56

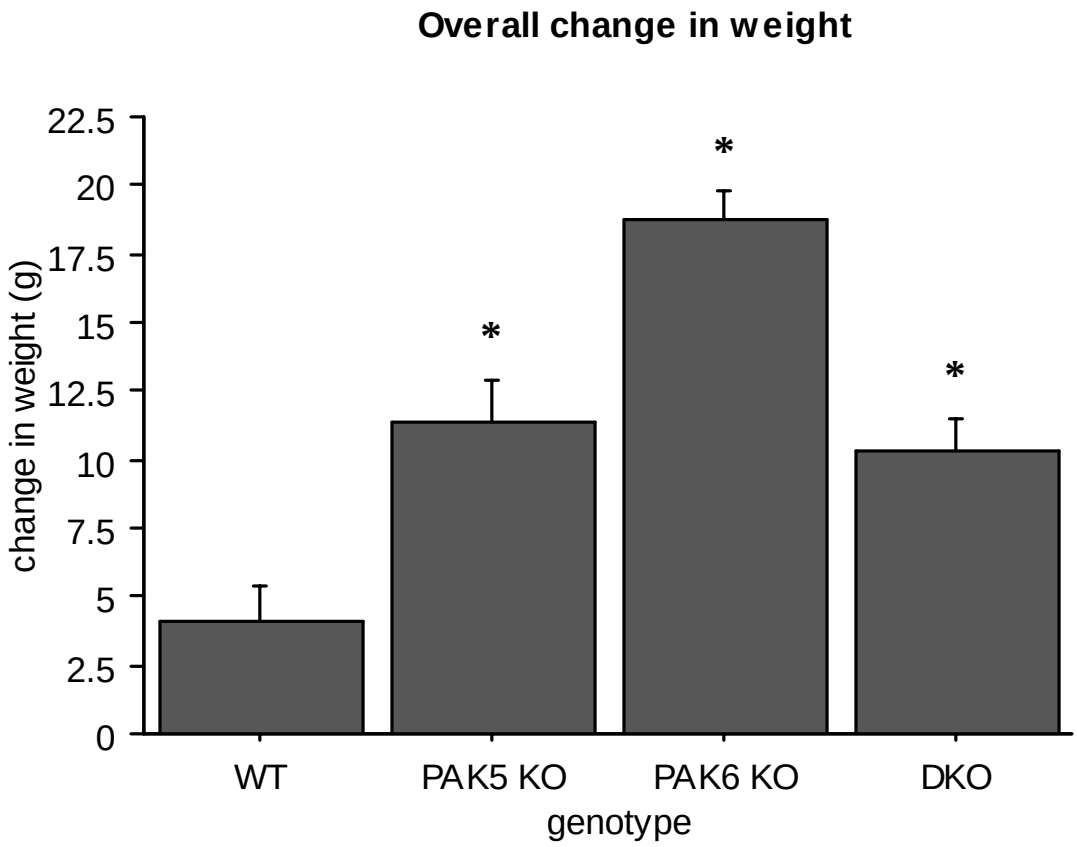

Figure 56: Overall change in weight in wildtype, PAK5 knockouts, PAK6 knockouts and double knockouts. PAK6 knockouts began and ended the study weighing more, but they also gained the most weight.

* denotes significantly different from wildtype; $p < .05$

Figure 57

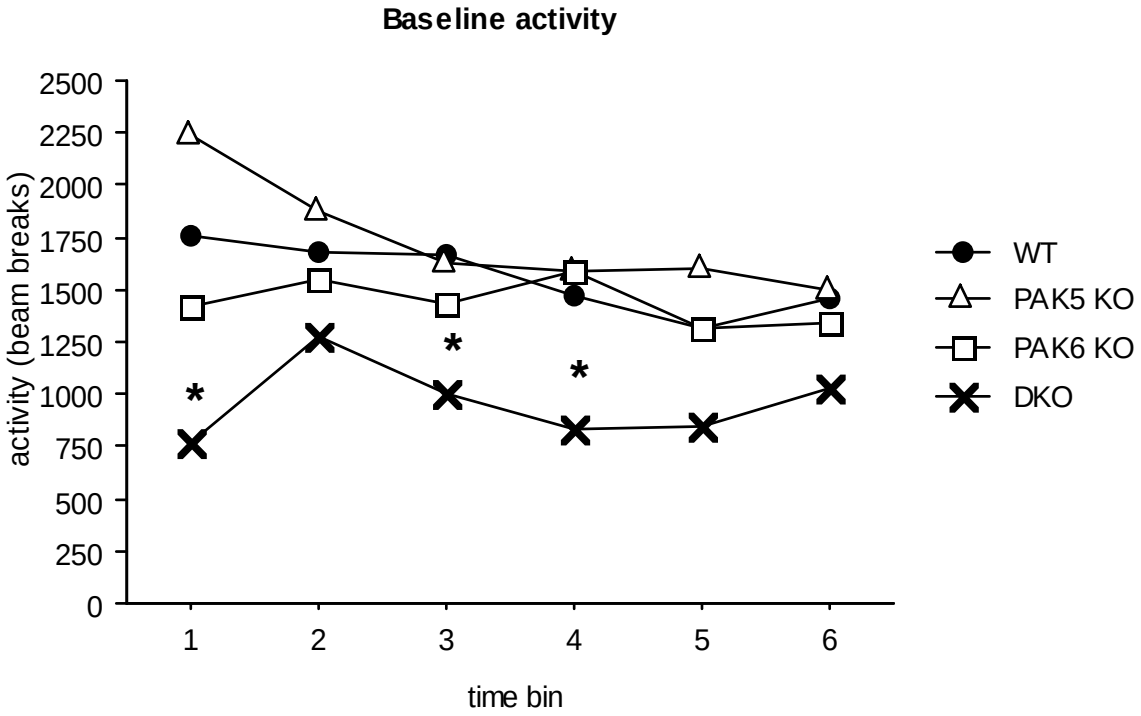

Figure 57: Average activity levels in wildtype, PAK5 knockouts, PAK6 knockouts and double knockout mice for 30 minutes. Readings were recorded in five minute time bins. There was a significant difference in activity over the 30 minute period ($p = .0004$), a significant effect of genotype ($p = .0069$) and a significant interaction of genotype and overall activity ($p = .0179$). Overall, double knockout activity was significantly lower compared to each of the other three genotypes ($p < .0001$)

* denotes significantly different than wildtypes; $p < .05$

Figure 58

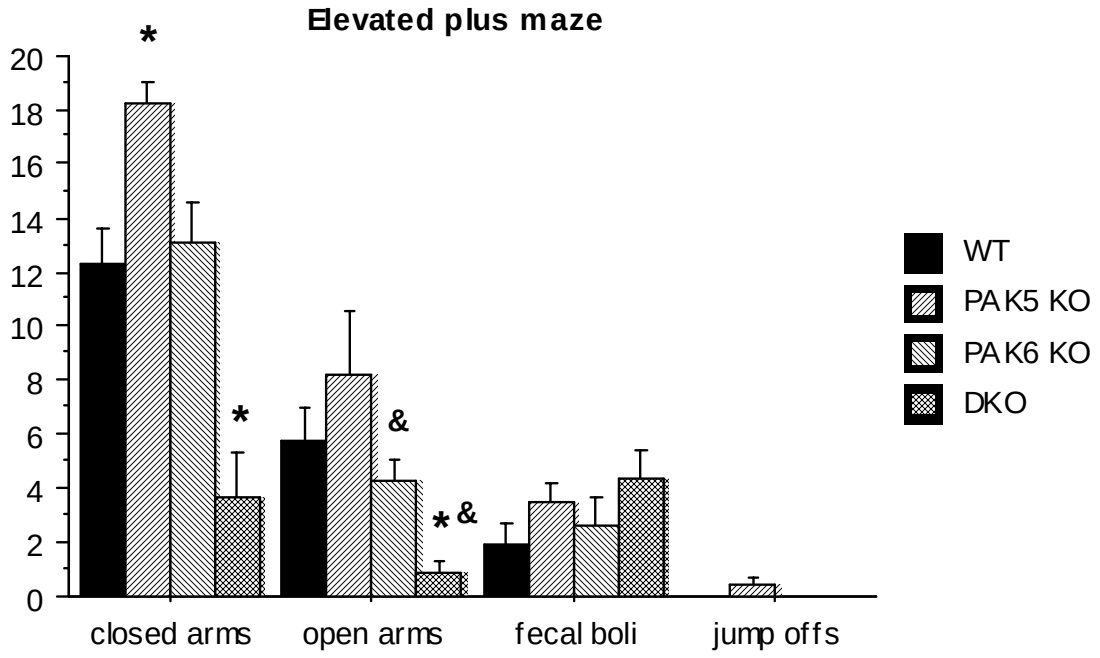

Figure 58: Performance on the elevated plus maze in wildtype and PAK double knockout mice: number of entries into the closed arms of the maze, number of entries into the open arms of the maze, number of fecal boli emitted over trial, and number of jump offs the maze

* denotes significantly different from wildtype performance on the same measurement; $p < .05$

& denotes significantly different from PAK5 knockouts

Figure 59

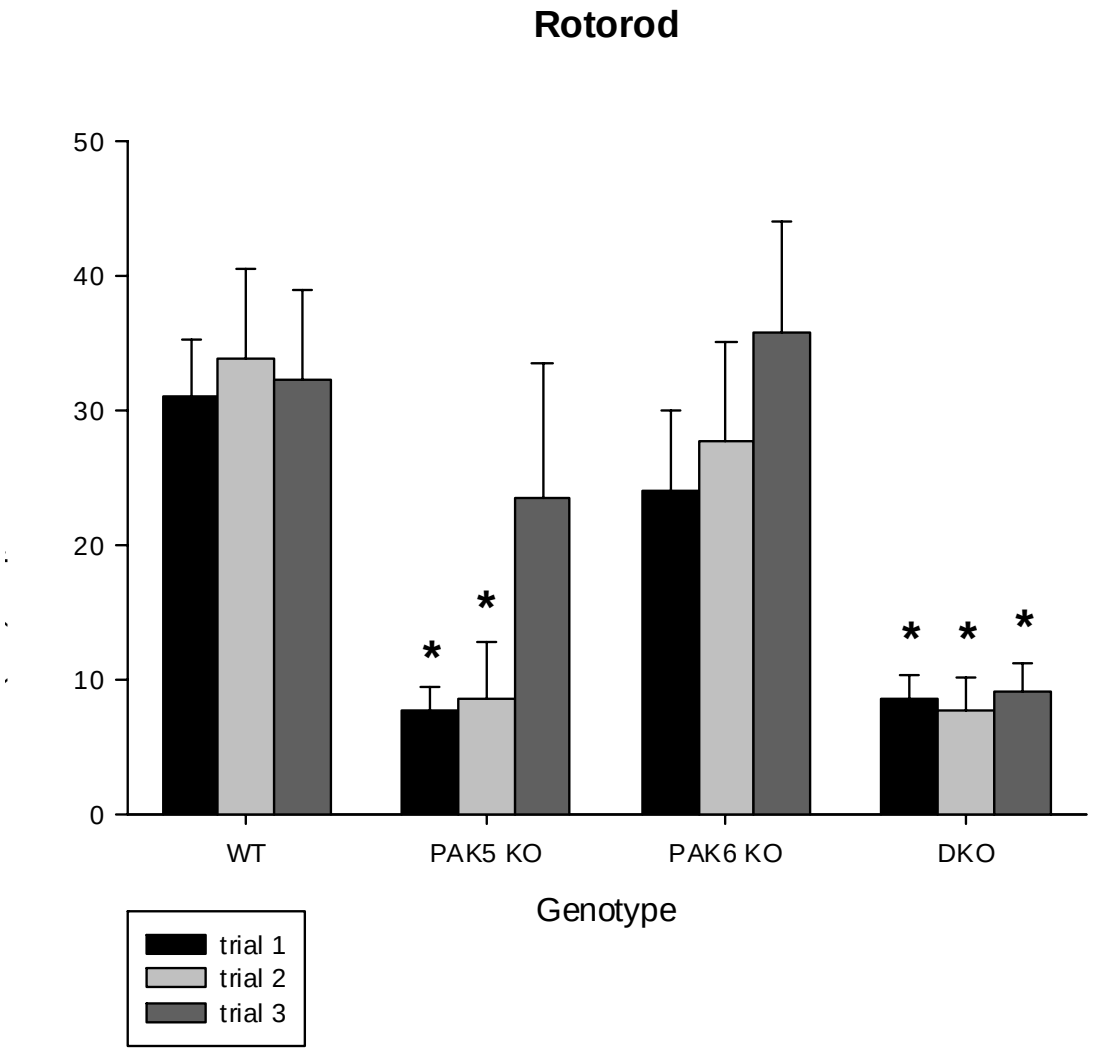

Figure 59: Performance on the rotorod by wildtype, PAK5 knockouts, PAK6 knockouts and double knockout mice: average trial latency to fall from the rotorod rotating 12 rev/min. Maximum trial length: 60 seconds. Mice performed significantly better over the trials ($p = .0044$).

* denotes significant from wildtype on the same trial; $p < .05$

Figure 60

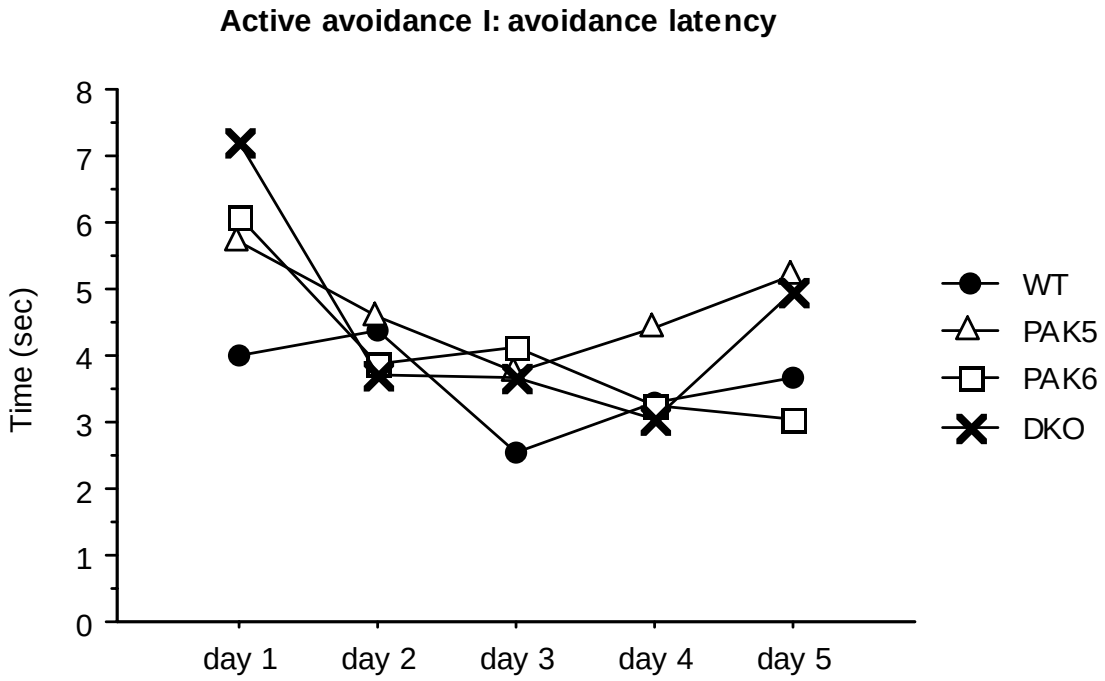

Figure 60: Latency to avoid a 0.8mA foot shock in wildtype, PAK5 knockouts, PAK6 knockouts and double knockout mice, averaged over ten trials per day. Overall, latencies significantly improved over testing days ($p = .0001$).

Figure 61

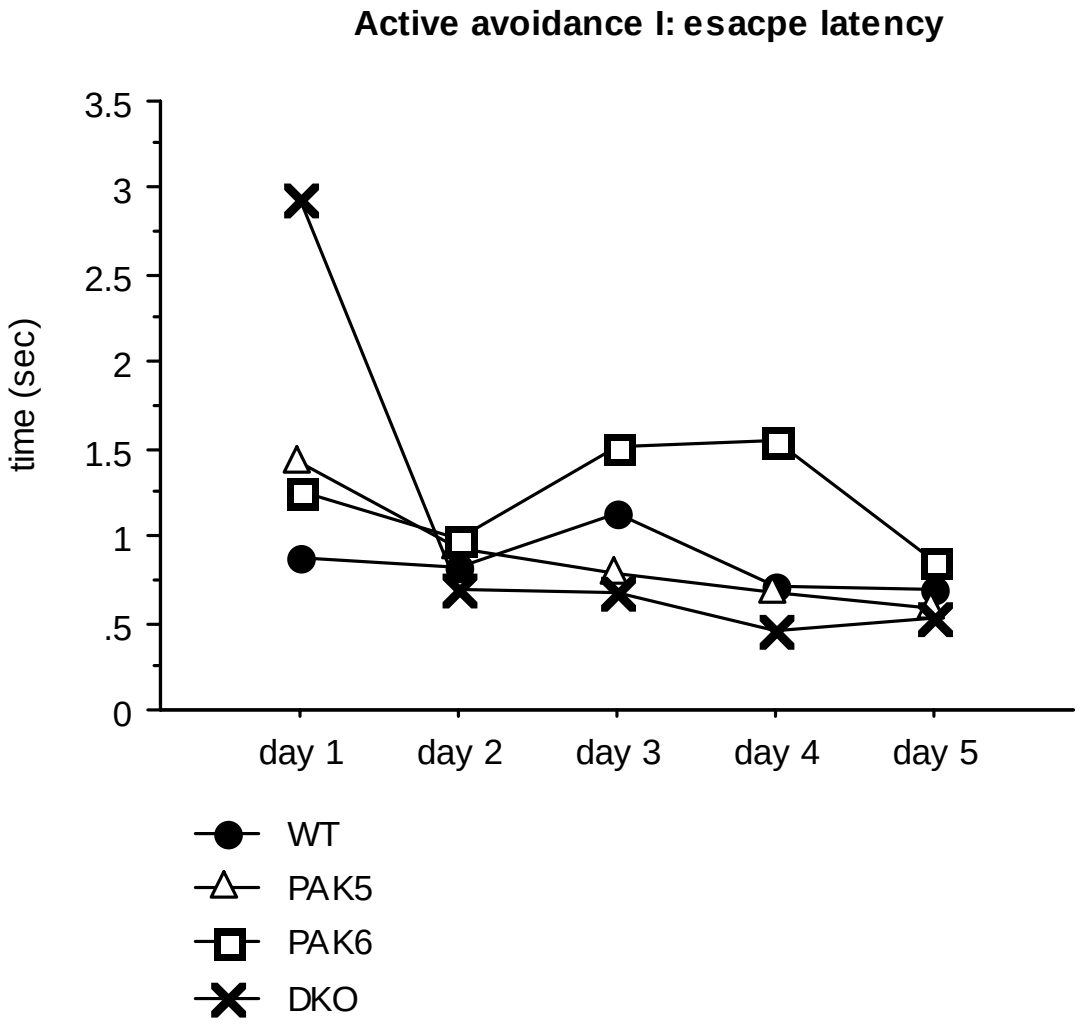

Figure 61: Latency to escape a 0.8mA foot shock in wildtype, PAK5 knockouts, PAK6 knockouts and double knockout mice, averaged over ten trials per day.

Figure 62

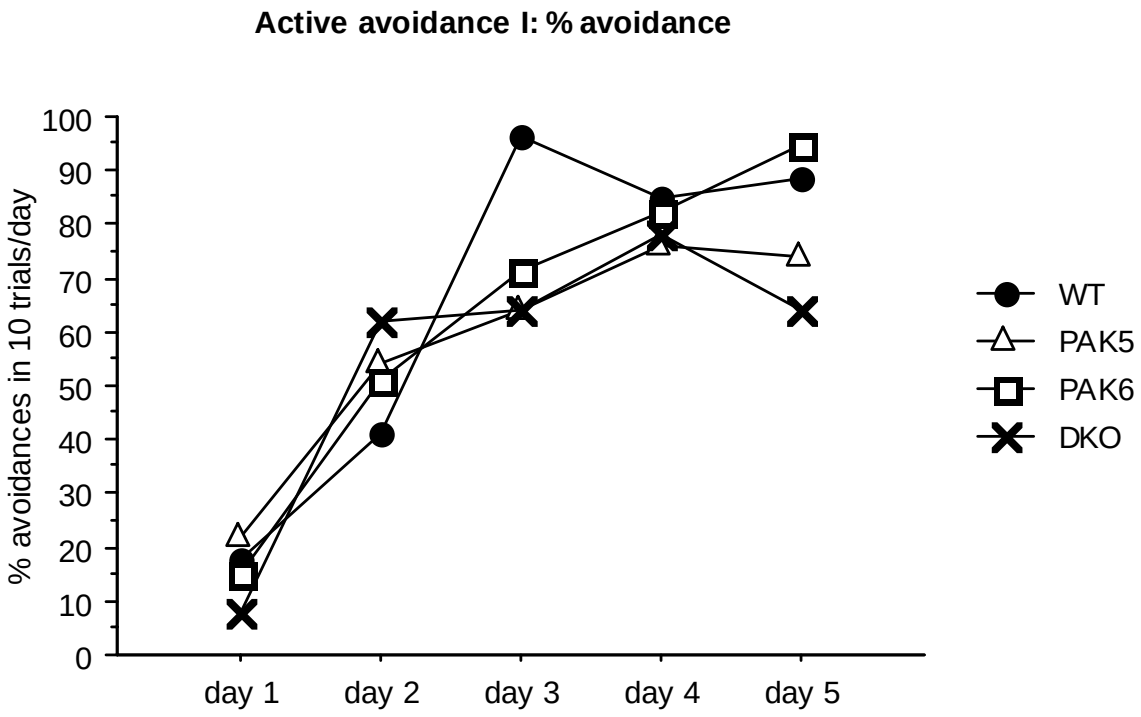

Figure 62: Percent avoidance responses in wildtype, PAK5 knockouts, PAK6 knockouts and double knockout mice, averaged over ten trials per day. There was a significant difference in total number of avoidances across days ($p < .0001$), and a significant interaction between genotype and avoidances ($p = .0165$).

Figure 63

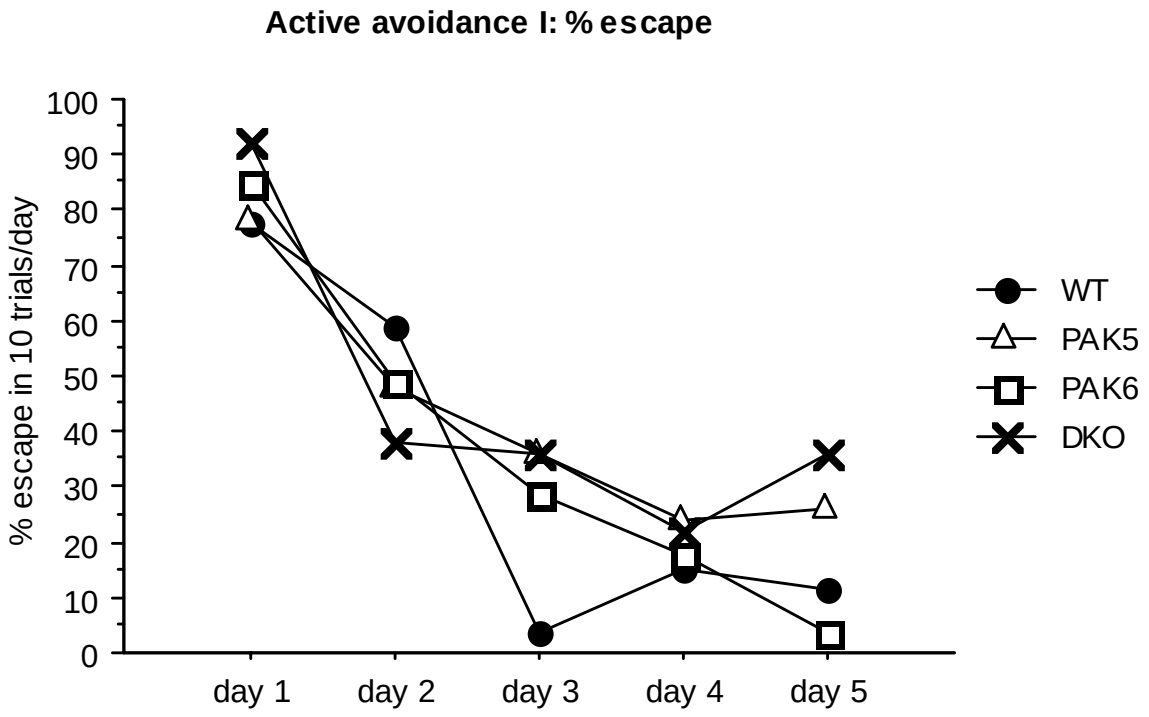

Figure 63: Percent escape responses in wildtype, PAK5 knockouts, PAK6 knockouts and double knockout mice, averaged over ten trials per day. There was a significant difference in total number of avoidances across days ($p < .0001$), and a significant interaction between genotype and avoidances ($p = .0365$).

Figure 64

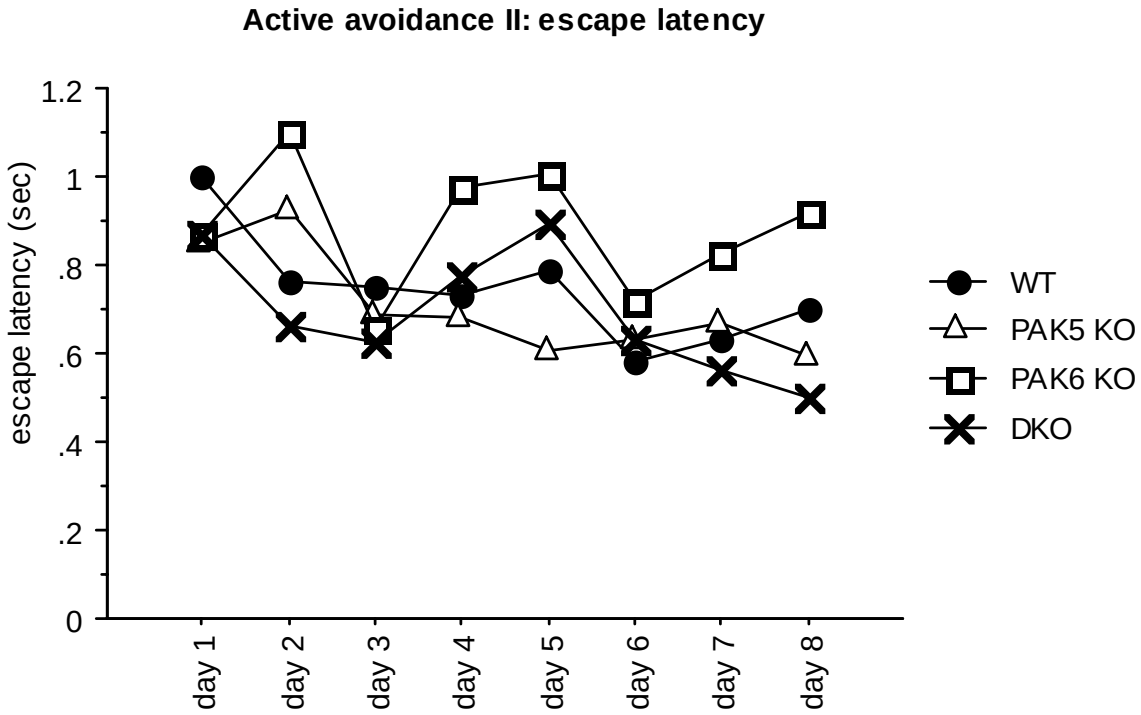

Figure 64: Latency to escape a 0.8mA foot shock in wildtype, PAK5 knockouts, PAK6 knockouts and double knockout mice, averaged over ten trials per day.

Figure 65

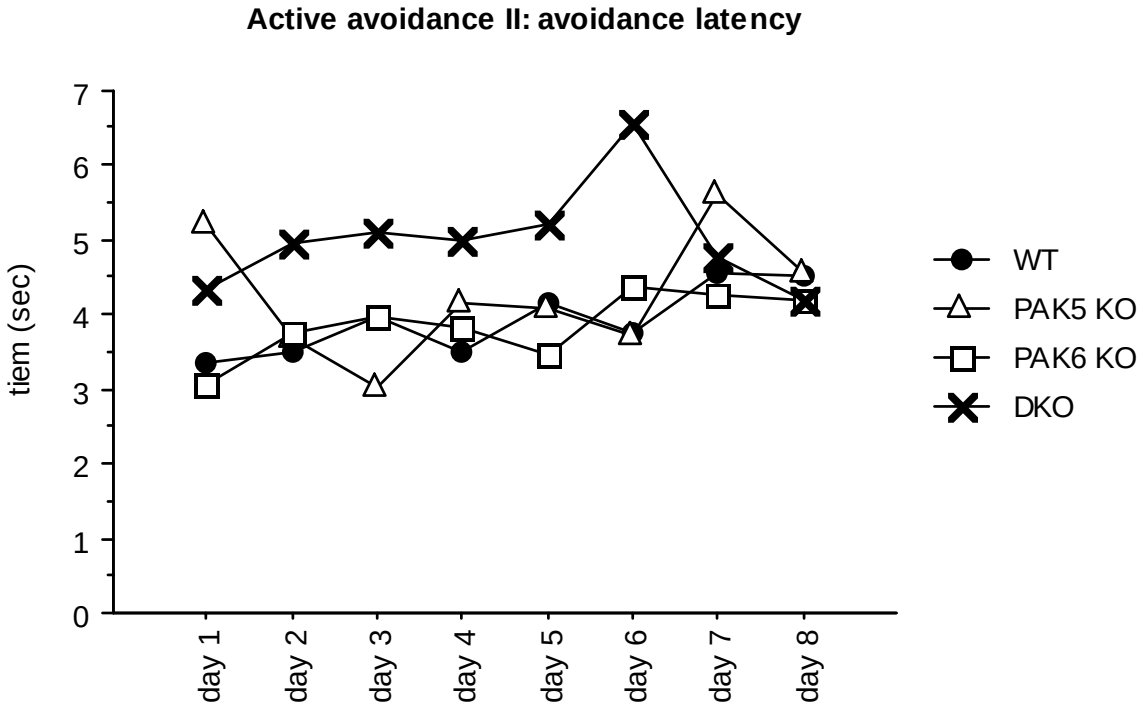

Figure 65: Latency to avoid a 0.8mA foot shock in wildtype, PAK5 knockouts, PAK6 knockouts and double knockout mice, averaged over ten trials per day, during the second run of active avoidance. Overall, there was a significant interaction of latency and genotype ($p = .0455$). Double knockouts had significantly higher latencies overall compared to wildtypes, PAK5 knockouts and PAK6 knockouts.

Figure 66

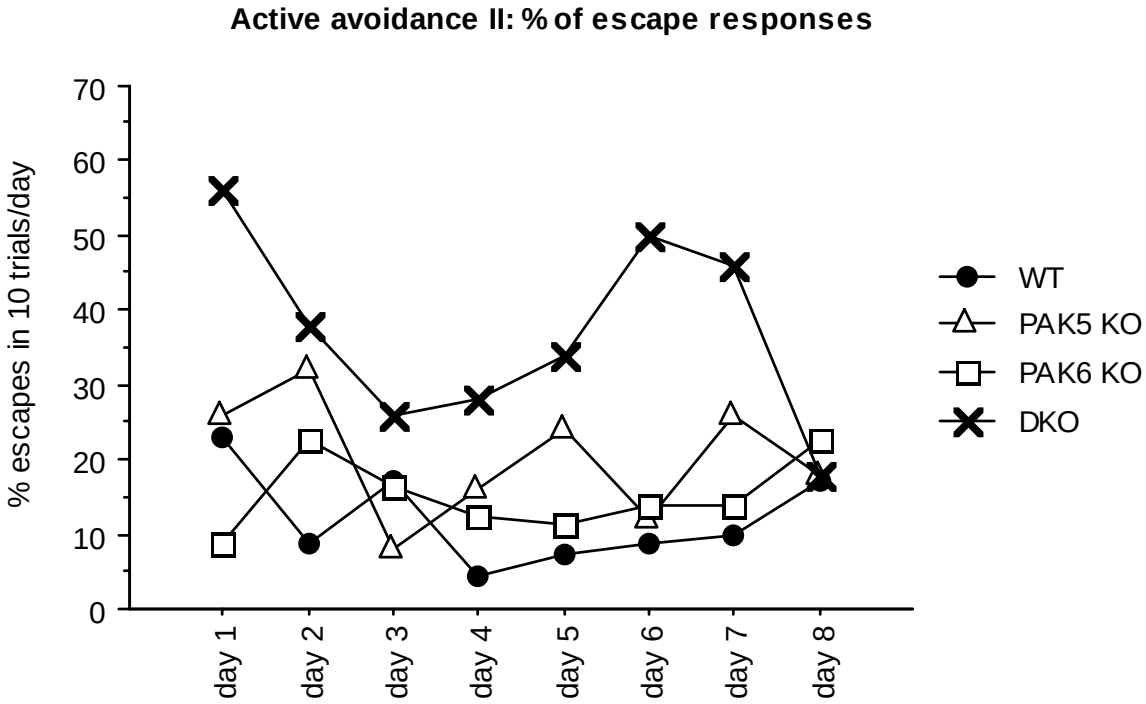

Figure 66: Percent escape responses in wildtype, PAK5 knockouts, PAK6 knockouts and double knockout mice, averaged over ten trials per day, in the second run of active avoidance. There was a significant difference in total number of escapes across days ($p = .0222$), genotypes ($p = .0176$) and a significant interaction between genotype and escapes ($p = .0038$). Overall, double knockouts had significantly higher number of escapes compared to each other genotype and PAK5 knockouts had significantly higher escape responses compared to wildtypes.

Figure 67

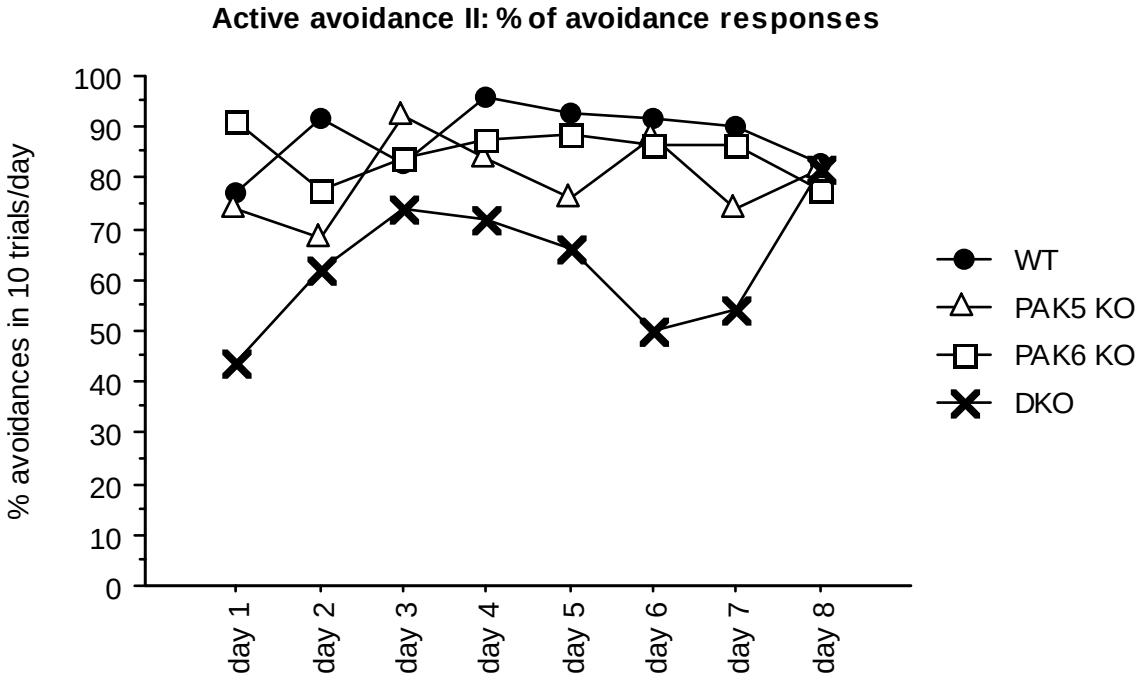

Figure 67: Percent avoidance responses in wildtype, PAK5 knockouts, PAK6 knockouts and double knockout mice, averaged over ten trials per day, in the second run of active avoidance. There was a significant difference in total number of avoidances across days ($p = .0222$), genotypes ($p = .0176$) and a significant interaction between genotype and avoidances ($p = .0038$). Overall, double knockouts had significantly lower number of avoidances compared to each other genotype and PAK5 knockouts had significantly lower avoidance responses compared to wildtypes.

Figure 68

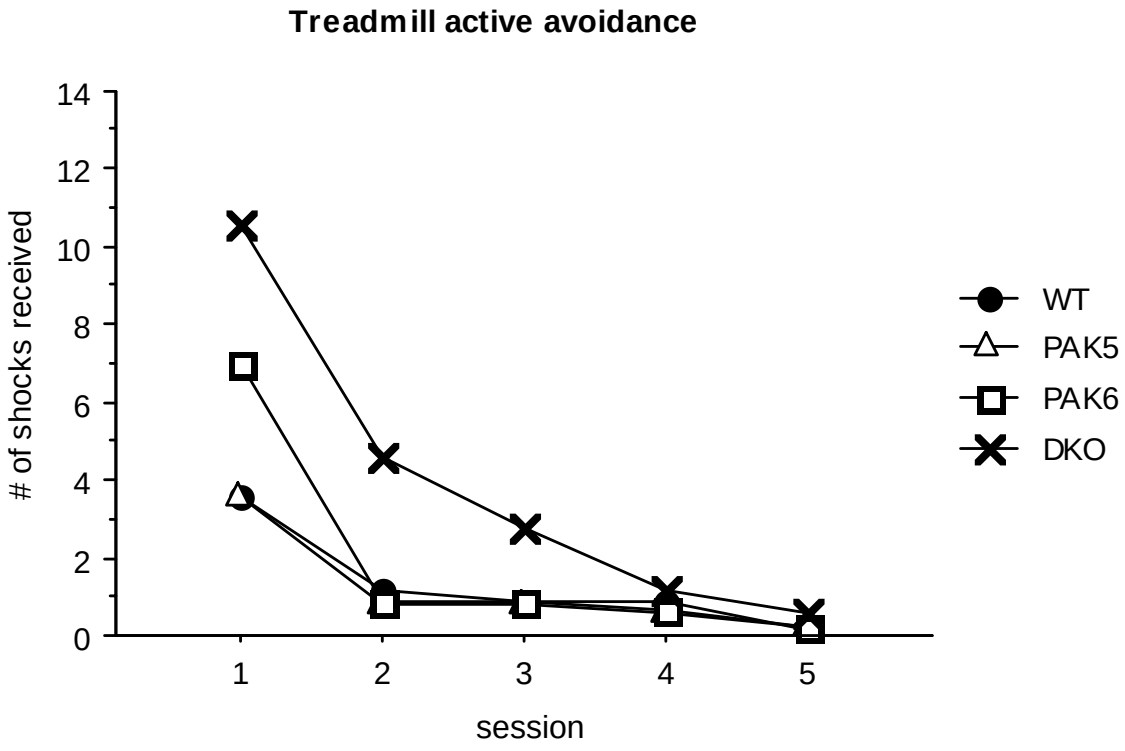

Figure 68: Average number of shocks received during each session of treadmill active avoidance. There was a significant reduction in number of shocks received over testing ($p < .0001$). Double knockouts received significantly more shocks compared to each other genotype.

Figure 69

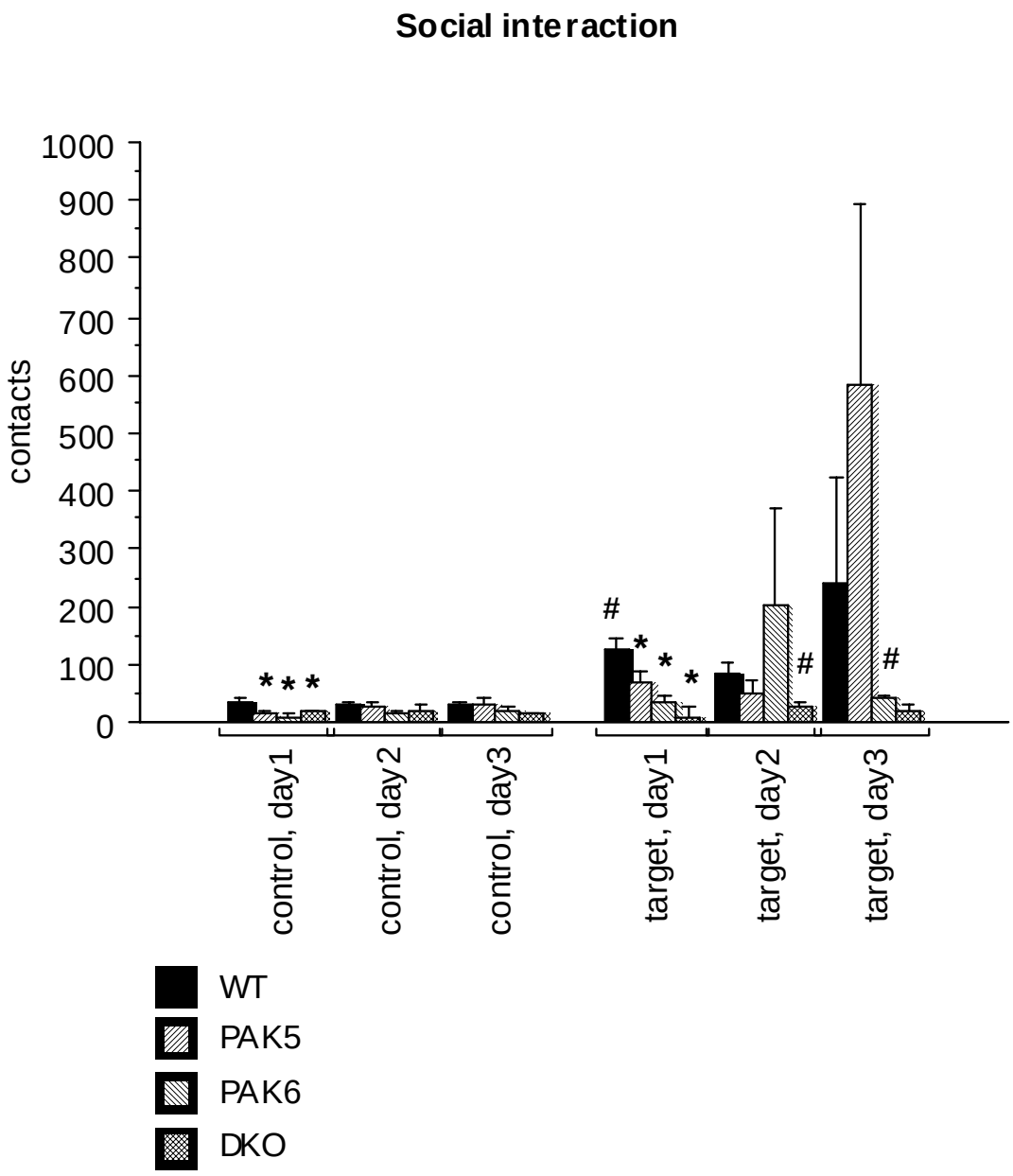

Figure 69: Social interaction in wildtype, PAK5 knockouts, PAK6 knockouts and double knockout mice: average number of contacts with the empty control cup and the target cup containing an unfamiliar mouse.

* denotes significantly different from wildtype; $p < .05$

# denotes significantly different from control cup; $p < .05$

Figure 70

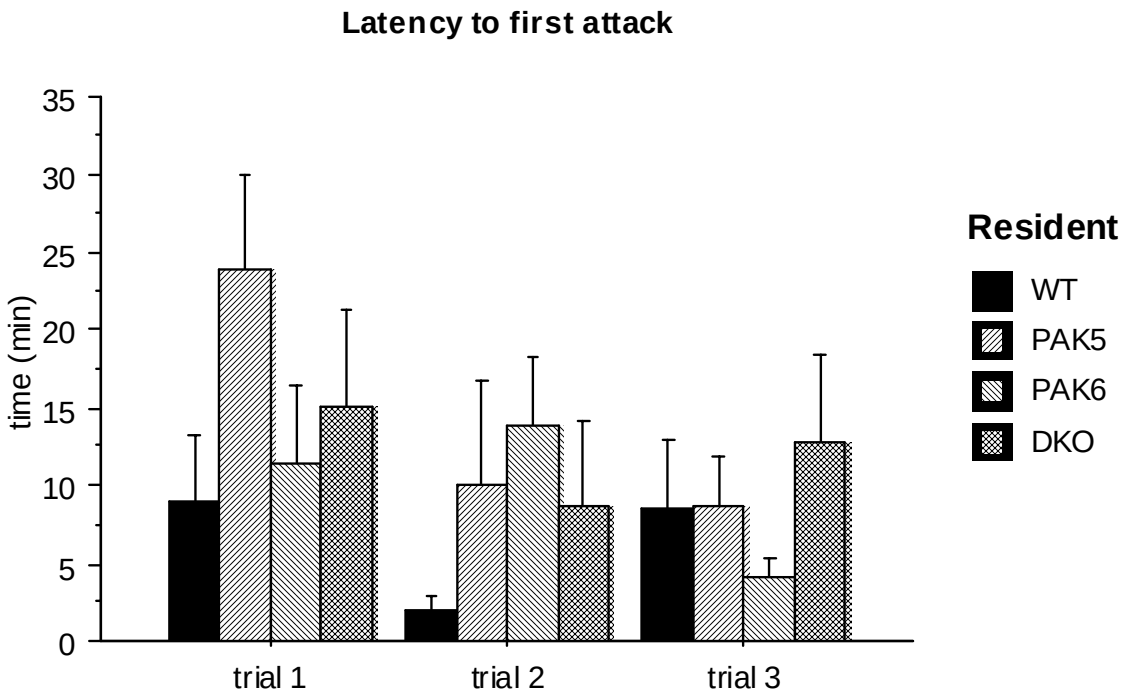

Figure 70: The latency for the first attack (regardless of which mouse initiated it) to occur once the intruder mouse was introduced into the home cage of the resident PAK mouse. Overall, there was no significant difference between genotypes or latency to the first attack.

Figure 71

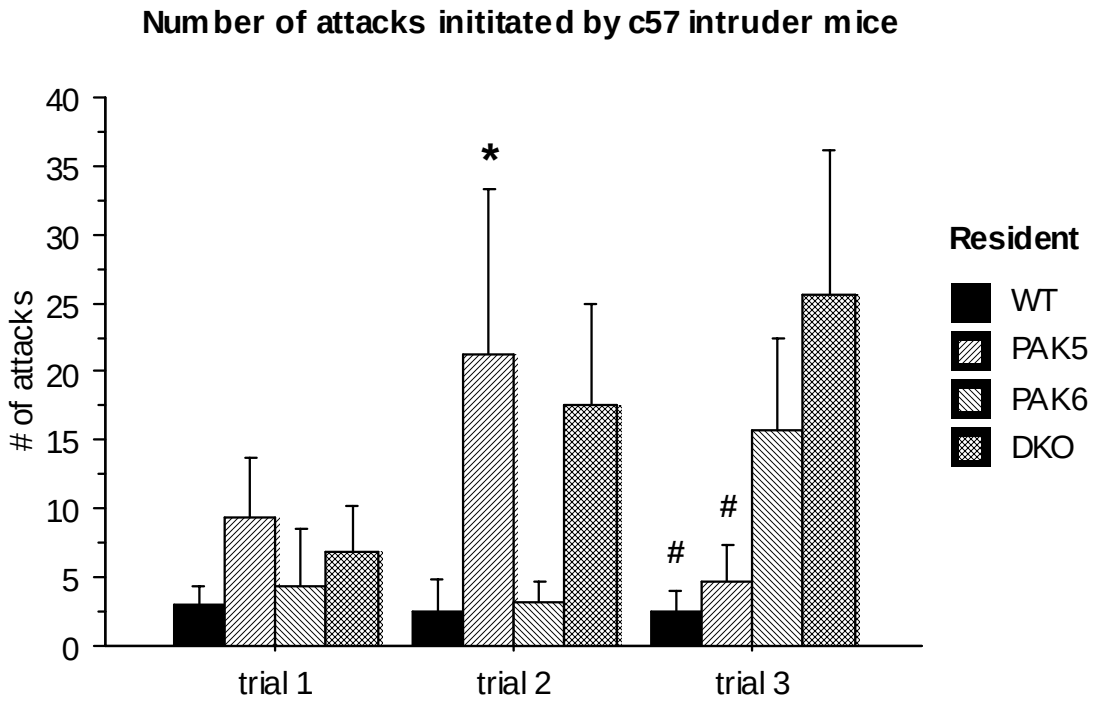

Figure 71: The average number of attacks initiated by C57 intruder mice on resident PAK mice in their home cages.

* denotes significantly different from wildtype; $p < .05$

# denotes significantly different from double knockout; $p < .05$

Figure 72

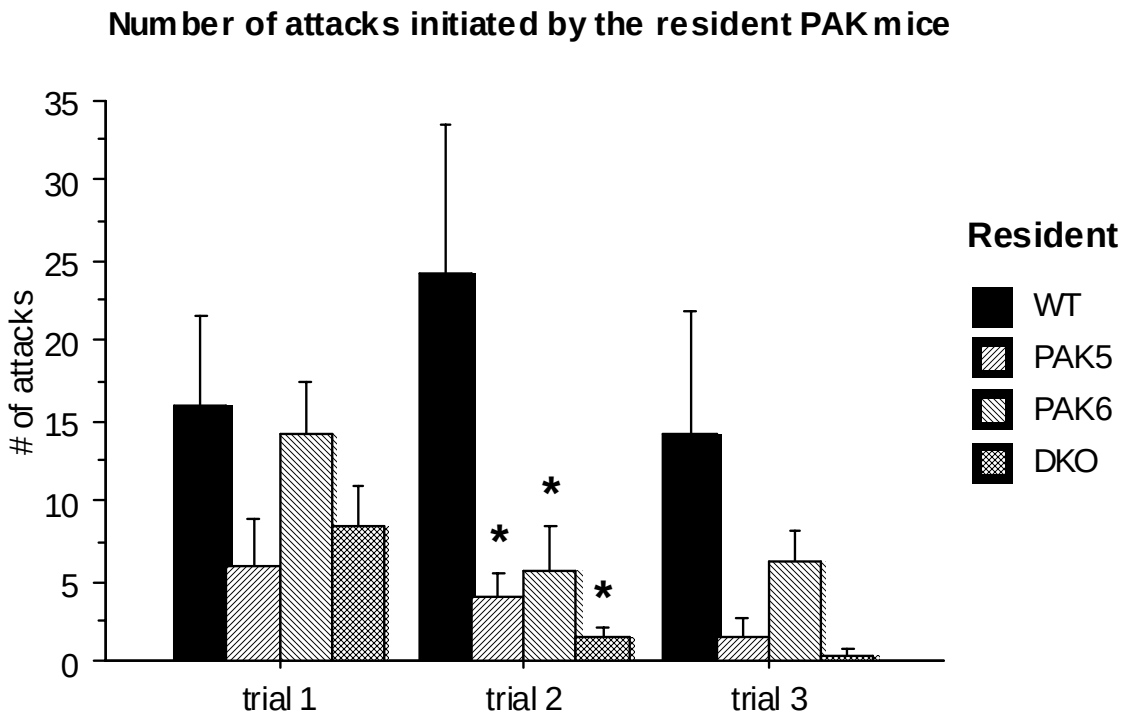

Figure 72: The average number of attacks initiated by resident PAK mice on C57 intruder mice introduced into the residents' home cages. Wildtypes initiated significantly more attacks overall compared to each other genotype.

* denotes significantly different from wildtype; $p < .05$

Figure 73

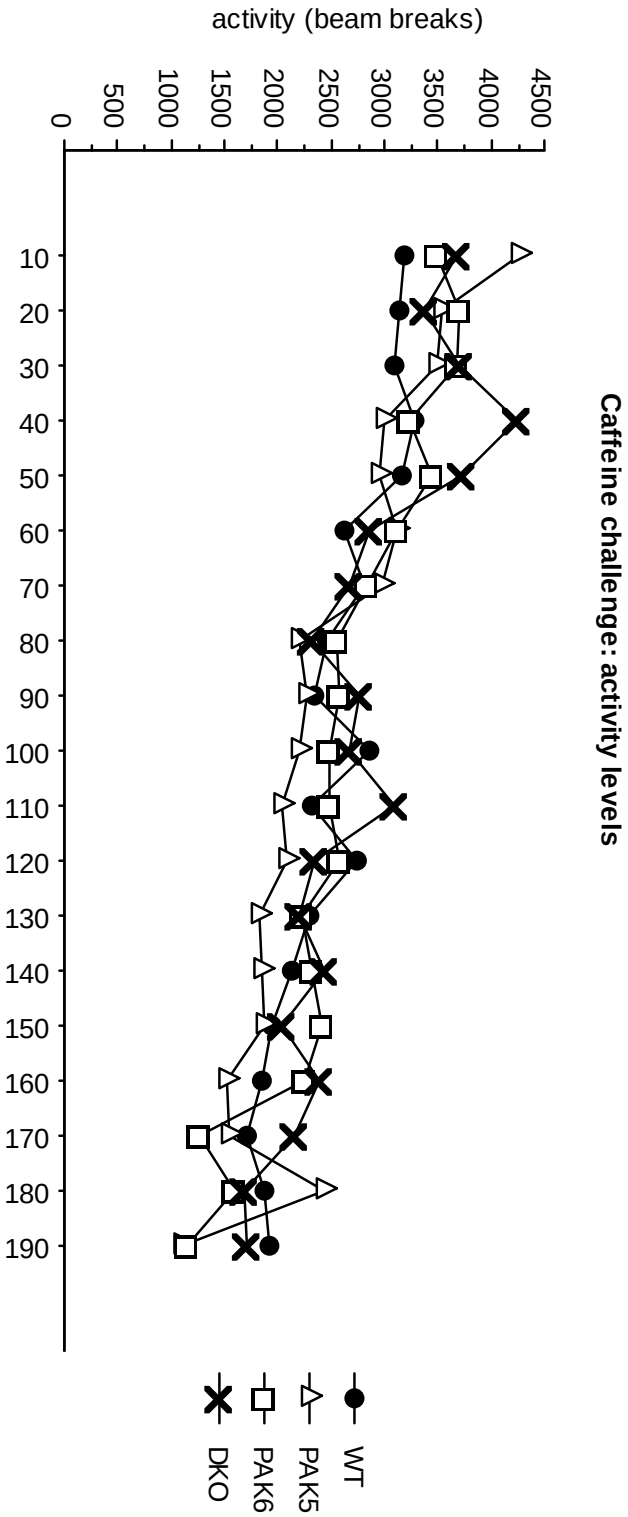
activity (beam breaks)
0
500
1000
1500
2000
2500
3000
3500
4000
4500
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
Caffeine challenge: activity levels
DKO
PAK6
PAK5
WT

Figure 73: Activity levels in wildtype, PAK5 knockouts, PAK6 knockouts and double knockout mice after caffeine administration. Readings were recorded in ten minute time bins. Activity in both genotypes decreased significantly over testing ($p < .0001$) but there was no significant difference between genotypes

Figure 74

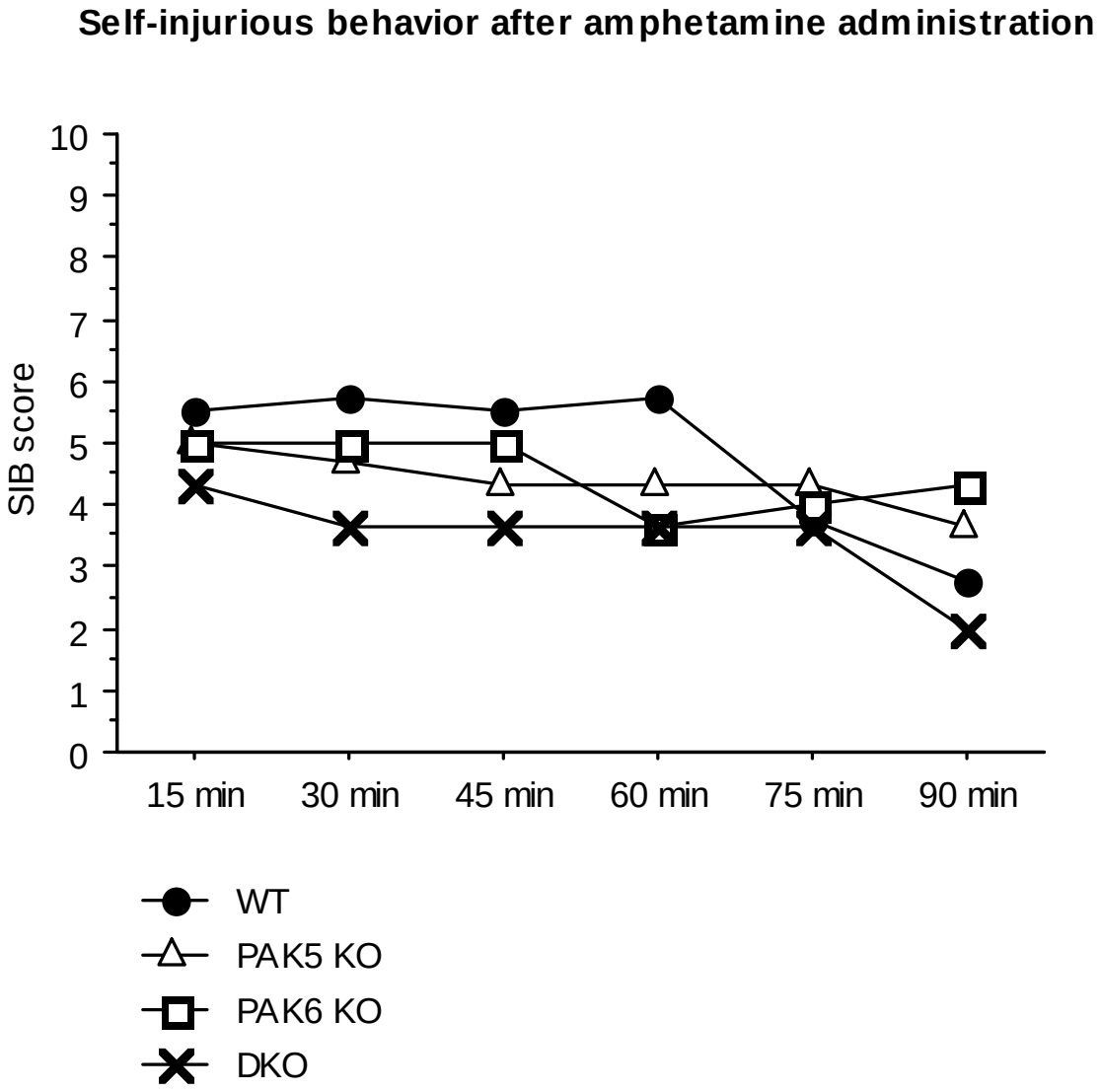

Figure 74: Incidence of self injurious behavior in wildtypes, PAK5 knockouts, PAK6 knockouts and double knockouts treated with amphetamine

Figure 75

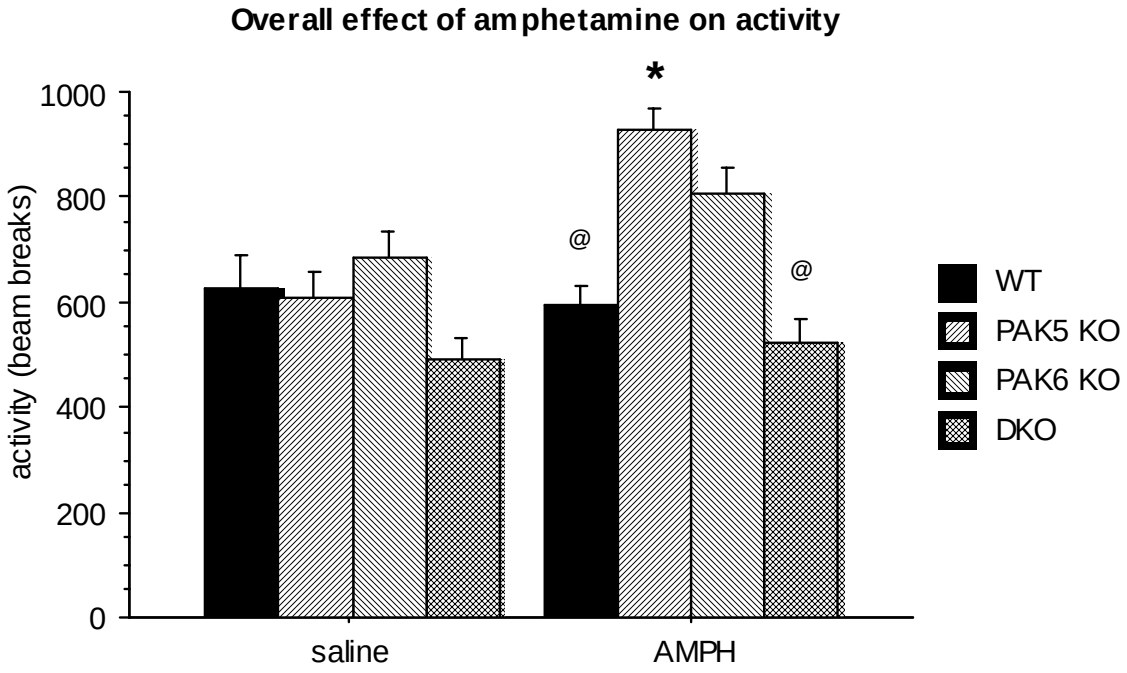

Figure 75: The overall effect of amphetamine on activity levels over 90 minutes after saline or amphetamine injection

* denotes significantly different from saline; $p < .05$

@ denotes significantly different from amphetamine-treated PAK5 and PAK6 knockout activity levels; $p < .05$

Figure 76

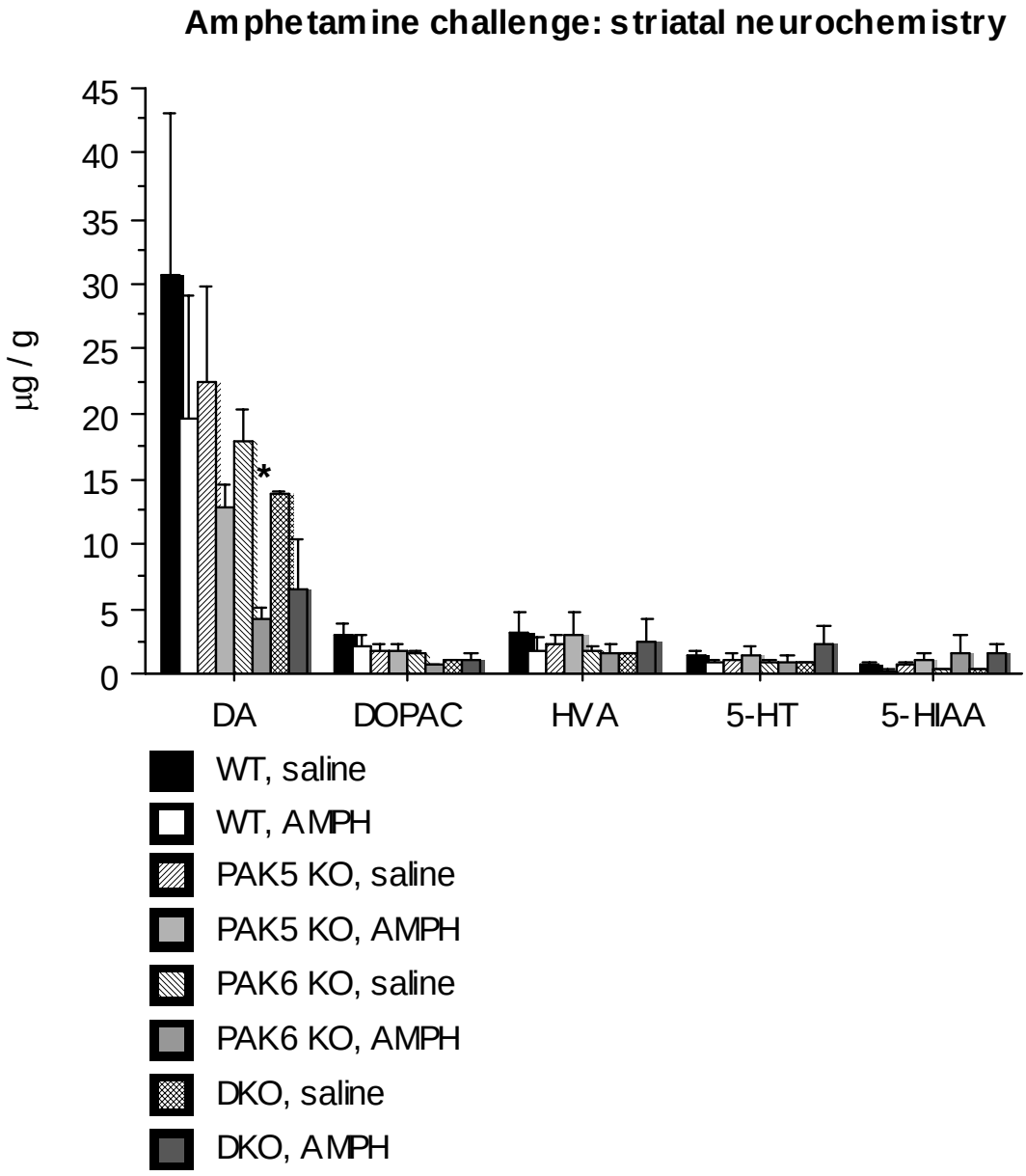

Figure 76: Concentration of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in striatum of wildtype, PAK5 knockouts, PAK6 knockouts and double knockout mice after treatment with saline or amphetamine.

Discussion: Experiments 11 and 12

Overall, there are distinct differences between the genotypes tested in these studies. PAK6 knockouts are significantly heavier than wildtypes, PAK5 knockouts and double knockouts. They had the greatest change in weight as well. This could be due to a number of factors. As PAK6 is normally expressed in the thyroid gland in low levels, PAK6 knockouts may be affected by hypothyroidism, a cause of weight gain in humans. A test of their thyroid function (measuring levels of thyrotropin stimulating hormone, triiodothyronine or levothyroxine) would be useful in determining if this is the cause of significant weight gain in these mice. Individuals with hypothyroidism also tend to have lower basal body temperatures so routine body temperature measurements in these mice compared to controls may also help determine if these mice exhibit hypothyroidism. On the other hand, PAK6 knockouts are not hypoactive compared to the other genotypes and hypoactivity is also generally associated with hypothyroidism.

It is also possible that these mice have lower levels of leptin compared to the other genotypes. Leptin is a hormone produced by adipose tissue that is integral to regulating appetite and metabolism. Typically, leptin binds to receptors in the hypothalamus and signals satiety. In individuals with abnormally low levels of leptin, this pathway is disrupted and food craving is increased. Mutations in the leptin gene are not common but are known to be one cause of obesity in humans. If PAK6 plays a role in leptin levels, the PAK6 knockouts may serve as a new model of abnormal leptin-induced obesity.

Double knockouts were shown to be deficient in several behavioral tests in Experiment 11. Experiment 12 aimed to determine if one gene, PAK5 or PAK6 played a

greater role than the other in these results. Both PAK5 and PAK6 knockouts were normally active compared to wildtypes. In fact, PAK5 knockouts were actually more active than wildtypes. These data suggest that there is functional redundancy between PAK5 and PAK6 concerning activity levels. It is not until both are knockout out that a significant hypoactivity is found, as in double knockouts.

Interestingly, PAK5 knockouts generally perform worse than wildtypes and PAK6 knockouts on the rotorod test for motor coordination. However, their performance did improve to near control levels by trial three. Double knockouts performed poorly on all three trials, while PAK6 knockouts performed similarly to wildtypes. Thus, poor performance in double knockouts may be attributed more to the function of PAK5 in the brain.

Active avoidance is a measure of learning and memory. Here, PAK6 knockouts performed most like wildtypes and showed almost 100% avoidance in both runs of T-maze active avoidance. PAK5 knockouts and double knockouts only avoided the foot shock about 70% of the time by the end of the first run and improved to control levels by the end of the second run. In the second run, PAK5 knockouts, PAK6 knockouts and wildtypes all re-acquired active avoidance almost immediately. Double knockouts were much slower to re-acquire this behavior and also had the greatest avoidance latencies. However, when making an escape response double knockouts had the fastest latencies compared to the other genotypes. These data suggest that PAK5 may play a role in the poor performance of double knockouts in active avoidance but when both PAK5 and PAK6 are knocked out the deficit becomes greater. Poor performance in an active avoidance paradigm is characteristic of dopamine depletion and has been used in animal

models of Parkinson's disease (Halladay et al., 2000; Kita et al., 2003a; Kita et al., 2003b). These deficits in avoidance responding can be considered parallel to the freezing and akinesia symptoms of Parkinson's disease. Indeed, in observing the double knockouts during active avoidance testing, they appear to have difficulty in initiating movement and do so much slower than the other genotypes. There are also negatively thigmotaxic. Once the foot shock is occurring, they are able to quickly move to the safe chamber and escape further shock. This suggests the mice have learned the correct response in the active avoidance paradigm and yet have difficulty or slowness in executing it.

The double knockouts also performed worse on the treadmill version of active avoidance, getting significantly more shocks than other genotypes. This is an interesting variation of testing motor function while also testing motor learning as mice were forced to continually walk on the treadmill as soon as the trial started. In the T-maze, mice were given a wait period between trials and had the ability to choose whether to respond and how fast to respond before getting a foot shock. Despite receiving the most shocks over all of the testing days, double knockout performance improved over the trials and received very few shocks on the last day of testing. This supports the results on the T-maze version of active avoidance. Double knockouts initially show more difficulty on this task but learn to continue moving on the treadmill in order to avoid getting a foot shock. It follows that they may have difficulty initiating movement but once moving could do so for the trial periods.

Measurement of aggression also showed intriguing results. Overall, all the knockouts showed reduced offensive aggression and were much less likely to initiate an

attack on an intruder mouse. Double knockouts were the least likely to initiate an attack, followed by PAK5 knockouts and then PAK6 knockouts. PAK5 knockouts and double knockouts were also the most likely to be attacked by intruder mice. Increased aggression is most often attributed to low serotonin levels in the brain. Analysis of baseline serotonin levels in wildtype and double knockout mice showed significantly more serotonin in the striatum and hypothalamus of 120 day old double knockout mice compared to 120 day old wildtype mice. The lower levels of serotonin in the wildtype mice may be the reason for greater aggression displayed by them against intruder mice. Moreover, these baseline neurochemical measurements were in 120 day old mice whereas aggression was tested in mice at least 8 months (240 days) old (see figures 46 - 54). It is possible that this difference in serotonin levels became greater as the mice aged. It is not known what the levels of serotonin are in either of the single knockout mice. However, PAK5 knockouts have similar levels of aggression to double knockouts and thus PAK5 may be contributing to the lower aggression levels. PAK6 knockouts are also not as aggressive as wildtypes but still exhibit greater aggression compared to PAK5 knockouts and double knockouts. In addition, PAK6 is known to interact with the androgen receptor and inhibit its activity. It does not appear that knocking out this gene has an effect on aggression levels in PAK6 knockouts as their levels of aggression were similar to (although a bit lower than) wildtypes. Therefore, it may be concluded that both PAK5 and PAK6 likely contribute to the lower levels of aggression observed in double knockouts.

As in Experiment 11, caffeine was able to ameliorate the hypoactivity observed in double knockouts. Here, caffeine caused all four genotypes to exhibit similar levels of

activity. Caffeine has been shown to increase dopamine transmission by inhibiting the adenosine receptor. However, dopamine levels after caffeine administration were not measured in any of the mice in these studies so the mechanism of action is not known. With regards to Parkinson's disease, it is interesting that the significant hypoactivity of double knockouts is reversed with caffeine administration. Caffeine has been shown to be protective in animal models of Parkinson's disease and increased human consumption has been linked to reduced risk of Parkinson's disease. If double knockouts have reduced baseline levels of dopamine compared to wildtypes and are particularly sensitive to caffeine's effects on adenosine receptors and dopamine transmission, this could be the basis for improvement in activity levels. At 120 days old, there was no difference in dopamine levels. But, in the amphetamine challenges of Experiments 9 and 10, all mice were older (at least 9 months of age at the time of sacrifice after saline or amphetamine challenge) and double knockouts (in the saline-treated group) had lower levels of dopamine (about 18 μg/g) compared to wildtypes (about 26 μg/g). Double knockouts also exhibited sensitivity to the neurotoxic effects of amphetamine, although the difference in dopamine levels between amphetamine- and saline-treated mice was not significant.

PAK5 and PAK6 knockouts were the most sensitive to amphetamine's effects on activity, although they did not exhibit different levels of stereotyped behavior. The increase in overall activity could be indicative of increased dopamine transmission nonetheless. Moreover, PAK6 knockouts were the only genotype to show significantly lower levels of dopamine after amphetamine challenge compared to saline-treated PAK6 knockouts. This is interesting because the C57 strain of mice are generally not sensitive

to the neurotoxic effects of amphetamine and PAK wildtypes (on a mixed C57/129Sv background) were also not sensitive to its effects. This suggests that PAK6 may play a unique role in amphetamine's toxicity and removal of this gene imparts neuronal changes that result in increased sensitivity.

Overall, PAK double knockouts are an intriguing possibility as a new model of Parkinson's disease. They show a distinct behavioral phenotype that can be likened to behavior observed in other animal models of Parkinson's disease as well as in human signs of the disease. In addition, the double knockouts appear to have reduced levels of dopamine in aged mice compared to age-matched wildtypes and may be more sensitive to amphetamine's neurotoxicity. Interestingly, PAK5 and PAK6 genes are both expressed later in development and levels increase with age. If PAK5 and PAK6 are involved in the progression of neurodegeneration, mice with deletions or mutations in both of these genes may show worsening of symptoms as they age. Neurochemical deficits would also likely increase with age. Future studies on mice older than studied here would reveal whether this is the case. In addition, studies on levels of tyrosine hydroxylase, dopamine transporter pump and substantial nigra neurons would show if double knockout mice also have characteristic differences in these key markers of Parkinson's disease.

Conclusions and Future Directions

Repeated administration of high doses of amphetamine-like compounds results in all of the neuropathological hallmarks of Parkinson's disease including nigral cell body loss, striatal terminal degeneration with significant loss of striatal dopamine and tyrosine hydroxylase function as well as increases in dopamine turnover and proliferation of postsynaptic receptors. The specific treatment used here, one 50 mg/kg subcutaneous dose of amphetamine appears to create a similar neuropathology but has not been fully characterized before. This single dose caused an 80% depletion of striatal dopamine three days after exposure which is comparable to depletions consistently observed in the multiple dose regimes of amphetamine and methamphetamine but with almost no animal mortality. Furthermore, dopamine turnover ratios were increased in amphetamine-treated animals for up to four weeks post-treatment. Together, these findings provide strong evidence that this single high dose of amphetamine can serve as a model of Parkinson's disease. Future studies are needed to determine its effects on cell bodies in the substantia nigra pars compacta, tyrosine hydroxylase activity and postsynaptic dopamine receptors in the striatum. Results of these studies will help confirm the validity of this model.

Oxidative stress, as well as lipid peroxidation and neuroinflammation consequent to oxidative stress, is considered a major factor in the etiology of Parkinson's disease today. Studies have shown that various antioxidants and anti-inflammatory agents are protective both in human Parkinson's disease patients as well as in animal models of Parkinson's disease. Here, several of these agents were tested in the single dose amphetamine model. Both ascorbic acid and ibuprofen were protective against

amphetamine-induced dopaminergic toxicity. Trolox, a water-soluble derivative of vitamin E, was tested also without positive results. This may be the result of insufficient amounts of Trolox and time given prior to amphetamine administration. Further studies should include a lengthier pre-treatment period in order for Trolox to have enough time to distribute to the brain. In addition, brain and other tissue levels of Trolox should be measured to determine its time course with in the body in order to best predict its efficacy.

Dietary EGCG and caffeine have also been shown to be protective in animal models of Parkinson's disease and are associated with lower incidence of Parkinson's disease in Chinese and Japanese populations. Here, EGCG and caffeine were tested in combination with a high fat diet. Neither compound showed protective effects against amphetamine-induced dopamine depletion. This may be due to altered pharmacokinetics of the amphetamine, EGCG and/or caffeine due to the high fat diet. This study should be repeated with addition of standard chow in combination with EGCG and caffeine treatments.

Besides oxidative stress, genetic components are widely studied causative factors in both familial and idiopathic Parkinson's disease. In some cases, the two ideas have been thought to work in conjunction: oxidative stress causing epigenetic changes in genes that can confer susceptibility to Parkinson's disease when mutated. Here, the p21-activated kinases and their genes PAK5 and PAK6 were investigated. Initially, the behavior of PAK5/PAK6 double knockout mice was examined to determine if the deletion of these genes caused any discernable phenotype in mice due to their high expression levels in the brain without regard for any specific disease or disorder.

However, it was during this initial testing that the significant deficits in active avoidance as well as marked negative thigmotaxia was observed and sparked the interest in these genes possibly being related to a Parkinsonian phenotype. Further testing revealed that these double knockout mice exhibited all the hallmark behaviors of Parkinson's disease including hypoactivity, reduced motor coordination and balance, poor active avoidance, subsequent "enhanced" passive avoidance and sensitivity to the dopaminergic toxicant amphetamine. These results suggest further investigation into the relationship of the B family p21-activated kinases and their potential role in neurodegeneration, specifically as it relates to Parkinson's disease.

Finally, the PAK6 single knockouts were significantly heavier than the wildtypes, PAK5 knockouts and PAK5/PAK6 double knockouts. This difference in weight did not seem to have an effect on their performance in the behavioral tests utilized here. The reason for this weight gain is so far unknown and these knockout mice may serve as a new model of obesity. PAK6 is expressed in the thyroid gland and the deletion of this gene may have had significant effects on the functioning of the thyroid gland. Interestingly, the double knockout mice weighed about the same as the wildtype mice. As such, the deletion of PAK6 in these mice was not able to cause weight gain and it appears that PAK5 expression is necessary for these results in the PAK6 single knockouts. Future studies are needed to determine the cause of this difference in weight and weight gain. These should include testing of the thyroid function, baseline body temperature, food and fluid intake, leptin levels, insulin levels and glucose sensitivity.

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