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EFFECTS OF A NOVEL TRYPTOPHAN HYDROXYLASE INHIBITOR PARA-ETHYNLPHENYLALANINE ON SURVIVAL AND GROWTH RATES IN MICE

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ABSTRACT

This study aims to investigate the effects of the novel tryptophan hydroxylase inhibitor, *p*-ethynylphenylalanine (*p*EPA) in mice specifically during the early postnatal period. A pilot study was carried out comparing *p*EPA with *p*-chlorophenylalanine (*p*CPA), which has been used previously in adult and postnatal rats and adult mice to deplete brain serotonin levels. We compared the effects of postnatal administration of *p*EPA vs. *p*CPA on growth rates and survival in two different strains of mice. Daily injections of 1 or 10 mg/kg *p*EPA, 50 or 100 mg/kg *p*CPA, or saline were administered during postnatal days 4 to 21 (P4-P21) to C57BL/6J and CD-1 mice. Pup weights were measured on P5-21 to measure early postnatal growth. Survival rates at P21 were evaluated. Brain regions including frontal cortex, hippocampus, striatum, brain stem, and hypothalamus were collected at P21 for future analysis of serotonin levels to evaluate the extent of synthesis inhibition. The current results indicate that postnatal administration of the higher 100 mg/kg *p*CPA dose resulted in significantly lower P21 weights in both sexes and strains of mice. The higher 10 mg/kg *p*EPA dose was also associated with lower P21 weights in both sexes of CD-1 mice. The CD-1 strain showed 100% survival in all cases, whereas mortality occurred across most treatment groups in the C57BL/6J strain. Based on postnatal growth and survival, CD-1 mice showed greater tolerability of these drugs and in particular, the lower 1 mg/kg dose of *p*EPA and both doses of *p*CPA. Final conclusions regarding drug, dose, and mouse strain for use in future studies will be made on the basis of these findings, in conjunction with results on the extent and selectivity of brain serotonin depletions.

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1. Chapter 1

1.1 Introduction

Depression and anxiety disorders are common mental illnesses that affect numerous individuals. Depression is estimated to be the third leading disabling condition worldwide, affecting as many as 100 million people (WHO, 2008). Depressive illness will continue to be an important health problem in the future as predictions show that this illness is expected to be one of the world's leading causes of disease in the year 2030 (Mathers and Loncar, 2006). The most common type of depression, major depressive disorder, is defined by episodes in which individuals lack interest in normally enjoyed activities and experience a low-spirited frame of mind for at least two weeks (DSM-IV-TR, 2000). Furthermore, these episodes are not the result of Substance-Induced Mood Disorder, Mood Disorder Due to a General Medical Condition, or bereavement (DSM-IV-TR, 2000). Major depressive disorder is one of the leading causes of disease burden in high/middle income countries and 8th in low-income countries worldwide (WHO, 2008).

Anxiety disorders are disorders that involve increased apprehension (DSM-IV-TR, 2000) and have been shown to affect individuals as described in an article by Kessler and colleagues (Kessler et al., 2005). Twelve-month prevalence of anxiety disorders has been estimated to occur in about 18% of American adults that are 18 years of age and over (Kessler et al., 2005). There are many subtypes of anxiety disorder including panic disorder, social phobia, and obsessive-compulsive disorder (DSM-IV-TR, 2000). One of

the more common subtypes, generalized anxiety disorder, is defined as a feeling of high and persistent uncontrollable anxiety and apprehension that takes place many more times than not in at least a six month period (DSM-IV-TR, 2000). An individual with generalized anxiety disorder may display restlessness, irritability, and concentration problems, and these issues are what brings about disturbances in their everyday functioning (DSM-IV-TR, 2000).

1.2 Serotonin

Serotonin, also known as 5-hydroxytryptamine or 5-HT, is a neurotransmitter that has been studied to understand its role in depressive and anxiety disorders in humans, as well as depressive- and anxiety-like behaviors in rodents. As illustrated in Figure **1-1**, serotonin is synthesized via a two step biological route with the first step being rate-limiting (Walther and Bader, 2003). In this rate-limiting step, tryptophan gets converted into 5-hydroxytryptophan via the enzyme tryptophan hydroxylase (TPH). Next, 5-hydroxytryptophan is converted into serotonin via the second step of this biosynthetic pathway (Walther and Bader, 2003). Serotonin that is produced in the brain is found in neurons located in the raphe nuclei of the brainstem, and these neurons are labeled as serotonergic neurons (Aghajanian and Gallager, 1975). In addition, there are two isoforms of TPH. TPH2 is associated with serotonin synthesis in the brain (Walther and Balder, 2003). Likewise, TPH1 is associated with serotonin synthesis but in the

peripheral system, as it has been shown to be present in the spleen, thymus, pineal gland, and gut (Walther and Balder, 2003).

1.3 Studies on 5-HT_{1A} Receptor Knockout Mice

There are at least 14 different receptors that bind serotonin (Raymond et al., 2001). Thirteen of the 14 serotonin receptors work via G-protein signal transduction pathways. One serotonin receptor that has been studied due to its specific effects on anxiety-related behavior is the 5-HT_{1A} receptor subtype, which is located on presynaptic and postsynaptic neurons (Pazos and Palacios, 1985; Palacios et al., 1990, Menard and Treit, 1999). Serotonin$_{1A}$ receptors that are located presynaptically are found in the midbrain raphe (Pazos and Palacios, 1985; Palacios et al., 1990, Menard and Treit, 1999). By contrast, 5-HT_{1A} receptors on postsynaptic neurons are located in limbic structures such as the amygdala, septum, hippocampus, dentate gyrus, and other regions of the brain (Pazos and Palacios, 1985; Palacios et al., 1990, Menard and Treit, 1999).

The 5-HT_{1A} receptor subtype has been studied in the context of its specific effects on anxiety-related behavior. Studies have shown that genetic inactivation of 5-HT_{1A} receptor expression (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998; Gross et al., 2002) and pharmacologic inhibition of 5-HT_{1A} receptors during the postnatal period (Vinkers et al., 2010) results in an increase in anxiety-like behavior in mice during adulthood. A study by Parks and colleagues found that mice genetically engineered to lack 5-HT_{1A} receptors exhibit increases in anxiety-like behavior compared to wildtype mice when evaluated in the open field test during adulthood (Parks et al., 1998).

Similarly, a study by Heisler et al. showed that when mice were evaluated during adulthood in the open field test and the elevated-zero maze, 5-HT_{1A} receptor knockout mice exhibited greater anxiety-like behavior (Heisler et al., 1998). A study by Ramboz and colleagues reported similar findings (Ramboz et al., 1998). The mice in these three studies were not produced on the same background strain indicating that the findings were not the result of interactions between lost 5-HT_{1A} receptor expression and specific genetic makeup. Also, these studies were performed in different laboratories. These factors strengthen the conclusion that decreased expression of 5-HT_{1A} receptors alters anxiety-like behavior in mice during adulthood.

Gross and colleagues showed that mice lacking functional postsynaptic 5-HT_{1A} receptors specifically during the postnatal period show an increase in anxiety-like behavior during adulthood (Gross et al., 2002). A further study that supports the hypothesis that 5-HT_{1A} signaling during the postnatal period is important for modulating anxiety-related behavior throughout life was published by Vinkers et al. (Vinkers et al., 2010). These authors found that postnatal pharmacologic inhibition of 5-HT_{1A} receptors in mice is associated with an increase in anxiety-like behavior during adulthood. Thus 5-HT_{1A} receptor expression and function during early postnatal development is of importance in altering anxiety-like behavior in mice during adulthood.

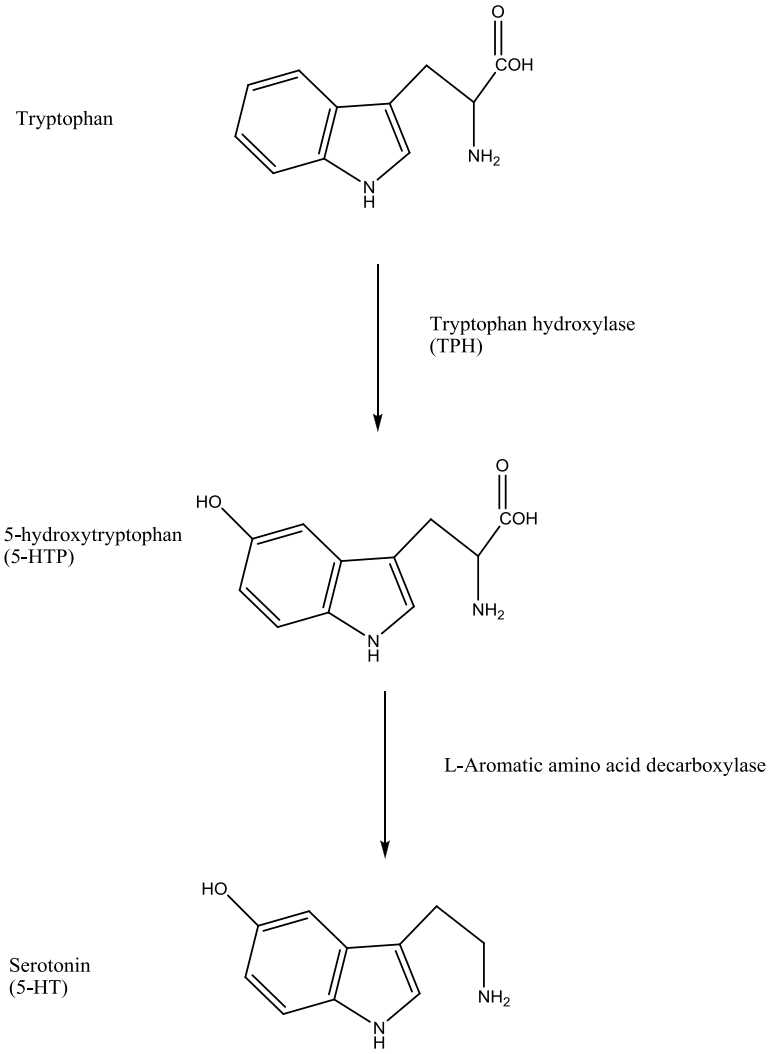

Figure **1-1** Synthesis of Serotonin.

These stuctures were created with the help of ChemDraw Std. 11.0

1.4 Studies on SERT Expression and Anxiety- and Depressive-like Behaviors in Adulthood

Serotonin not only binds to presynaptic and postsynaptic receptors but it is taken up by serotonin transporters (SERT) into presynaptic neurons after release (Blakely et al., 1994; Mathews et al., 2004; Murphy et al., 2004; Perez and Andrews, 2005). SERT plays a major role in recycling serotonin from the extracellular space (Kim et al., 2005). In a paper by Perez and Andrews, data showed that no serotonin uptake is detected in the striatum and frontal cortex of SERT knockout mice (Perez and Andrews, 2005). When there is no SERT protein available to transport serotonin from the extracellular space back into presynaptic neurons, serotonin accumulates in the extracellular space (Mathews et al., 2004). This accumulation in the extracellular space was also evident in a study by Guilloux and colleagues (Guilloux et al., 2006). They showed that higher levels of extracellular serotonin were measured in 5-HT_{1A} knockout mice treated with paroxetine, a selective serotonin reuptake inhibitor (SSRI), as compared to wildtype mice also administered an SSRI. The authors proposed that loss of presynaptic 5-HT_{1A} receptors disrupts the negative feedback loop that results in a potentiated increase in extracellular serotonin levels in 5-HT_{1A} deficient mice (Guilloux et al., 2006).

Similar to 5-HT_{1A} knockout mice, mice genetically deficient in SERT exhibit an increase in anxiety-like behavior (Holmes et al., 2003; Kalueff et al., 2007). Since serotonin reuptake and recycling does not occur in SERT knockout mice (Perez and Andrews, 2005), serotonin synthesis is increased in the brain stem, frontal cortex, striatum, hippocampus, and hypothalamus (Kim et al., 2005). A decrease in serotonin

tissue levels occurs in all of these regions compared to wildtype controls (Bengel et al., 1998; Numis et al., 2004; Kim et al., 2005).

Also similar to studies on the developmental significance of 5-HT_{1A} receptor function as it pertains to adult anxiety-related behavior, postnatal (P4-P21) administration of SSRIs has been shown to increase anxiety behaviors in adult mice (Ansorge et al., 2004; Ansorge et al., 2008). Specifically, SSRI-treated postnatal mice exhibited an increase in anxiety-like behavior in the open field test and the elevated plus maze in adulthood (Ansorge et al., 2008). In addition, mice receiving postnatal fluoxetine exhibit an increase in adult anxiety-like behavior in the novelty-suppressed feeding test (Ansorge et al., 2004; Ansorge et al., 2008). Thus pharmacologically inhibiting the reuptake of serotonin during the postnatal timeframe is associated with increased anxiety-like behaviors in adulthood (Ansorge et al., 2004; Ansorge et al., 2008). When the time frame of administration of fluoxetine was shifted to 3 months of age instead of during the postnatal window, behaviors measured in the open field test and the novelty-suppressed feeding test revealed no significant changes as compared to the control mice (Ansorge et al., 2008). A study by Alexandre et al. underscores the relationship between decreased SERT expression and 5-HT_{1A} receptor function during development to influence adult anxiety-related behavior. These authors showed that inhibition of 5-HT_{1A} receptors limited to early postnatal development in SERT knockout mice was associated with decreased immobility time in the tail suspension test when compared to untreated SERT knockout mice (Alexandre et al., 2006). These data demonstrated that blocking 5-HT_{1A} receptors postnatally in SERT deficient mice reverses increases in depressive-like behavior that occur in these mice in adulthood.

There is additional evidence showing that depressive-like behavior is altered in mice during adulthood when SERT is postnatally inhibited. Wildtype mice receiving escitalopram postnatally exhibited an increase in depressive-like behavior in adulthood when evaluated in the tail-suspension and forced swim tests (Popa et al., 2008). Thus, inhibition of SERT genetically throughout life or pharmacologically during P4-P21 leads to an increase in anxiety- (Ansorge et al., 2004; Ansorge et al., 2008) and depressive-like behavior in mice during adulthood (Popa et al., 2008). When considered together, all of these studies strongly implicate alterations in serotonin neurotransmission during the early postnatal timeframe as being important for shaping anxiety- and depressive-like behavior in mice during adulthood.

1.5 Genetics of the Serotonin Transporter

Since SERT is the primary target for SSRIs, it is a major research focus in psychiatric genetics. While a single gene codes for the human serotonin transporter in the central nervous system and the periphery, there is evidence for genetic variability in SERT (Lesch et al., 1996). A polymorphic region termed the serotonin transporter-linked polymorphic region (5-HTTLPR) is located in the promoter upstream of the coding region in the SERT gene (Lesch et al., 1996). This polymorphism is characterized by a 43-base pair insertion/deletion polymorphism (Wendland et al., 2006), which has two variations -- a long or "*l*" variant and a short variant or "*s*" variant (Lesch et al., 1996). In addition, research has linked depression to the 5-HTTLPR (Clarke et al., 2010).

1.6 Studies on *p*CPA

Here, we describe a study in which we investigated the effects of two different compounds that act as inhibitors of tryptophan hydroxylase (TPH), the rate-limiting enzyme in serotonin synthesis. We were specifically interested in designing a dosing paradigm in mice that would result in transient but substantial decreases in serotonin levels limited to the postnatal period and that would be associated with limited effects on postnatal growth and survival. One method of inhibiting TPH is by using the chemical agent, *p*-chlorophenylalanine (*p*CPA) (Koe and Weissman, 1966). In a study on the effects of postnatal administration of *p*CPA, rats that were injected with *p*CPA during a narrow postnatal period (P8-P16) exhibited reductions in anxiety-like behavior during adulthood compared to control rats (Farabollini et al., 1988).

However, there are potential problems associated with the use of *p*CPA during development. Injection of *p*CPA to prenatal and neonatal-juvenile rats causes the formation of cataracts and fatalities - some of the juvenile treatment rats, or rats that were administered *p*CPA at 100 mg/kg/day during P14–P18 or 200 mg/kg/day during P19–P40, died shortly after weaning (Ogawa et al., 1999). Another issue that arises with postnatal administration of *p*CPA is that phenylketonuria occurs. Tryptophan hydroxylase and phenylalanine hydroxylase are both inhibited by *p*CPA (Kilbey and Harris, 1971). Thus, changes in anxiety-like behavior resulting from the administration of *p*CPA might be due to cognitive impairment that results from the inhibition of phenylalanine hydroxylase and/or to inhibition of tryptophan hydroxylase and associated decreases in developmental serotonin levels. (Kilbey and Harris, 1971).

1.7 Tryptophan hydroxylase is rapidly inhibited by *p*EPA

We hypothesized that the relatively new pharmacological agent *p*-ethynylphenylalanine (*p*EPA) might be useful as an alternative to *p*CPA to decrease serotonin synthesis during the postnatal period while avoiding potential confounds associated with phenylketonuria and postnatal toxicity. Experiments by Stokes et al. demonstrated that incubation of 100 μM *p*EPA with recombinant TPH diminishes enzyme activity by ~60%, whereas only an 8% decrease in TPH activity levels was detected using the same concentration of *p*CPA (Stokes et al., 2000). In another study, 24 h after a 5 mg/kg injection of *p*EPA, an 80% decrease in serotonin was measured in the raphe of male rats (Zimmer et al., 2002). Furthermore, 30, 40, and 90 min after administration of 5 mg/kg of *p*EPA, reductions in extracellular serotonin levels in frontal cortex, hippocampus, and striatum were detected (Zimmer et al., 2002). A study by Stokes et al. showed that 30 mg/kg *p*EPA administered to rats quickly decreased midbrain serotonin and 5-hydroxyindoleacetic acid (5-HIAA), the major metabolite of serotonin (Stokes et al., 2000). Specifically, within 4 h after administration, the former was decreased by 56% and the latter by 65% (Stokes et al., 2000).

1.8 Study design and hypotheses

The experiments carried out for this thesis are designed to show the effects of administration of the serotonin synthesis inhibitors, *p*EPA and *p*CPA, on two different strains of postnatal mice with regard to pup weights and survival rates. Two different

doses each of *p*EPA and *p*CPA were administered to C57BL/6J (inbred) and CD-1 (outbred) mice during P4-P21. Future experiments will be performed to analyze brain tissue serotonin levels to determine the drug and dosage associated with at least a 60-70% decrease in brain serotonin levels. If the lowest dose of either *p*EPA or *p*CPA decreases brain serotonin levels by 60-70% but also causes more than a 20% decrease in survival, we anticipate we will investigate lower doses of *p*EPA or *p*CPA. Determining the most advantageous drug, dose, and mouse strain will set the stage for future studies in which SERT knockout mice having the preferred background strain will be administered the most efficacious and least toxic drug dose during the postnatal time frame. These mice will be allowed to mature, after which they will undergo behavior testing. We hypothesize that postnatal administration of a serotonin synthesis inhibitor to SERT knockout mice will reverse increases in anxiety- and depressive-like behaviors associated with constitutive loss of SERT expression.

The reasoning behind this hypothesis is as follows. Studies have shown that when wildtype mice are injected with an SSRI (fluoxetine) postnatally (P4-P21), they exhibit an increase in adult anxiety-like behaviors compared to control mice (Ansorge et al., 2004; Ansorge et al., 2008). Furthermore, when wildtype mice are postnatally administered a different SSRI (escitalopram), they show increases in depressive-like behavior during adulthood (Popa et al., 2008). Because SSRIs inhibit SERT, thus preventing SERT from taking up serotonin into presynaptic neurons, serotonin is believed to accumulate in the extracellular space (Mathews et al., 2004; Kim et al., 2005). An increase in anxiety- (Ansorge et al., 2004; Ansorge et al., 2008) and depressive-like behaviors (Popa et al., 2008) in adult mice is hypothesized to occur because of high

postnatal levels of extracellular serotonin. On the contrary, *p*EPA decreases serotonin synthesis in neurons and thus, serotonin levels are decreased in the extracellular space (Zimmer et al., 2002). Because excess serotonin in the extracellular space is believed to occur when SERT function is genetically (SERT knockout mice) or pharmacologically (SSRIs) inhibited during the postnatal time frame, and because postnatal SERT inhibition is associated with an increase in anxiety- and depressive-like behavior in mice during adulthood, we hypothesize that diminished levels of serotonin in the extracellular space during the postnatal time frame will be associated with decreases in anxiety- and depressive-like behavior in adulthood, particularly in animals having high developmental extracellular serotonin, e.g., SERT deficient mice.

In this thesis, we hypothesize that the drug *p*EPA will be better for use in the proposed studies than *p*CPA because of scientific literature illustrating the negative effects the latter has on rats (Ogawa et al., 1999). Unlike *p*CPA, *p*EPA does not significantly inhibit phenylalanine hydroxylase and *p*EPA reduces serotonin levels at doses 10 times lower than *p*CPA (Stokes et al., 2000). We hypothesize that the 1 mg/kg dose of *p*EPA will be the most advantageous because like *p*CPA (Jequier et al., 1967), *p*EPA is an irreversible TPH inhibitor (Zimmer et al., 2002) whose effects will accumulate with successive injections. Zimmer et al. used a single injection of 5 mg/kg of *p*EPA (Zimmer et al., 2002) and observed a decrease in serotonin levels. We hypothesize that repeated 1 mg/kg injections will be sufficient to cumulatively inhibit TPH to a large extent without causing significant effects on postnatal growth and survival. We further theorize that repeated injections of 10 mg/kg *p*EPA will be associated with toxicity in postnatal mice as evidenced by decreased postnatal weights and survival.

The results presented in this thesis are the first to report on the use of *p*EPA in mice and in postnatal rodents in general. We anticipate this study will provide new knowledge regarding the role of high levels of serotonin during development on the manifestation of increased depressive and anxiety-related behaviors in adulthood as they relate to depression and anxiety disorders.

2. Chapter 2

2.1 Materials and Methods

Animals: Two strains of mice were investigated – C57BL/6J and CD-1 mice. Male and female mice from the C57BL/6J strain were obtained from Jackson Laboratories (Bar Harbor, ME), while male and female CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA). We chose these strains because SERT knockout mice are available on either background strain. Breeding pairs were housed in vented cages on a 12:12 h light:dark cycle (lights on at 0600 h). Food and water were provided *ad libitum*. The Pennsylvania State University Institutional Animal Care and Use Committee approved all procedures that animals underwent during testing and the National Institutes of Health Animal Care Guidelines were strictly abided during experiments involving animal.

Drugs: Two separate groups of mice were injected daily during postnatal days P4-P21 with either 1 mg/kg *p*-ethynylphenylalanine (*p*EPA) or 10 mg/kg *p*EPA. Two additional groups of mice were injected with either 50 mg/kg 4-chloro-DL-phenylalanine methyl ester hydrochloride (*p*CPA) or 100 mg/kg *p*CPA. Two control groups were included in the study. One received sterile filtered saline (vehicle; 0.9%) and the second was uninjected and unhandled during the postnatal period except for routine cage changes. Uninjected mice were included to determine the potential effects of handling, injection, and brief maternal separation experienced by mice that were drug or saline

injected. The *p*EPA was kindly synthesized and provided by the laboratory of Dr. Kent Vrana at the Penn State College of Medicine (Stokes et al., 2000). The *p*CPA was obtained from Sigma Aldrich (St. Louis, MO).

Drug administration: Beginning on P4, litters were removed from the home cage approximately 2 h after the onset of the light cycle (0800 h) once a day. All pups from each litter were placed together in a container for weighing. For at least the first 6 days of dosing, pups were placed in a warmed container because they were still growing fur. The tails of the pups were labeled with different colored non-toxic markers to distinguish individual pups and to indicate the treatment each received. Each pup was weighed daily prior to injecting and the weights were recorded. Drug solutions were given in a volume of 10 mL/kg. All but one litter (a litter of three 1 mg/kg pEPA treated mice) included at least one saline control. Mice were gently restrained and injected subcutaneously under the loose skin of the neck. After each mouse was injected, it was immediately returned to the weighing container. When all of the mice from a single litter had been injected, they were simultaneously returned to their home cage. All efforts were made to minimize the time the pups were away from the home cage and their mothers. In addition, all mice were removed from and returned to the home cage together, and they remained in the weighing container together while mice were individually injected. On P21, pups were humanely sacrificed by cervical dislocation two h after the last injection. Untreated mice were sacrificed approximately 4 h after the onset of the light cycle.

HPLC analysis: As a future part of this study, high performance liquid chromatography (HPLC) will be used to analyze levels of serotonin, its major metabolite, 5-HIAA, the catecholamine neurotransmitters, norepinephrine and dopamine, and the

dopamine metabolites, 3,4-dihydroxyphenylacetic acid and homovanilic acid, in the brain stem, frontal cortex, striatum, hippocampus, and hypothalamus. Internal standard (*N*ω-methyl-serotonin; 10,000 nM) will be diluted with 0.1 M perchloric acid to 250 nM and added to brain tissue samples (50 μl) from the hypothalamus, brain stem, frontal cortex, hippocampus, and striatum. Tissue samples will be homogenized using sonication. Aliquots of each sample (15 μL) will be reserved for Lowry protein analysis and the remainder of each sample will be centrifuged at 7,200g for 20 min. Supernatants will be analyzed using either a CMA/ESA HPLC system with coulometric detection or an Eicom HTEC HPLC system with amperometric detection. Mobile phase will be prepared by dissolving 5.5 mM EDTA, 5-9% acetonitrile, 0.10 M monochloracetic acid, 0.01% triethylamine, and 0.3-0.5 g/L octanesulfonic acid in DH_2O. It will be flowed at a rate of 0.25-1.0 ml/min though an analytical column (10 cm × 3.2 mm) packed with 3 μm Spherisorb ODS-II to perform reversed phase chromatography. Protein will be measured by the method of Lowry et al. (Lowry et al., 1951).

Data and Statistics: GraphPad Prism 4 was used for graphing data and ChemDraw Std. 11.0 was used to prepare the structures in Figure **1**. Statistical analyses were carried out using SAS® statistical software and GraphPad Prism. Two-way analysis of variance (ANOVA) was performed to analyze the effects of treatment and sex on pup weights. Tukey's *post-hoc* tests were used for individual group comparisons. Survival data were analyzed by chi-squared statistics. Statistical significance was set at the level of α=0.05.

3. Chapter 3

3.1 Results

To analyze the gross developmental effects of administration of the serotonin synthesis inhibitors *p*EPA and *p*CPA to postnatal mice, two-way ANOVA and Tukey's *post-hoc* tests were performed on postnatal pup weights. Each strain of mice was analyzed separately. There was a significant effect of treatment but no effect of sex on C57BL/6J pup weights at P21 ($F(5,68) = 10.9$, $P < 0.001$). P*ost-hoc* analysis indicated the following. As illustrated in Figure **3-1**, female C57BL/6J mice treated with the higher 100 mg/kg *p*CPA dose had significantly lower P21 weights compared to female C57BL/6J mice in the saline-treated group. Moreover, the 100 mg/kg *p*CPA group had significantly lower P21 weights compared to the 50 mg/kg *p*CPA treatment group for female C57BL/6J mice. There was no significant difference between the female C57BL/6J untreated group and the female C57BL/6J saline-treated group indicating that the injection procedure itself did not impact postnatal growth. In contrast to *p*CPA treatment, both 1 mg/kg *p*EPA and 10 mg/kg *p*EPA had no statistically significant effects of P21 pup weights in female C57BL/6J mice (Fig. **3-1**).

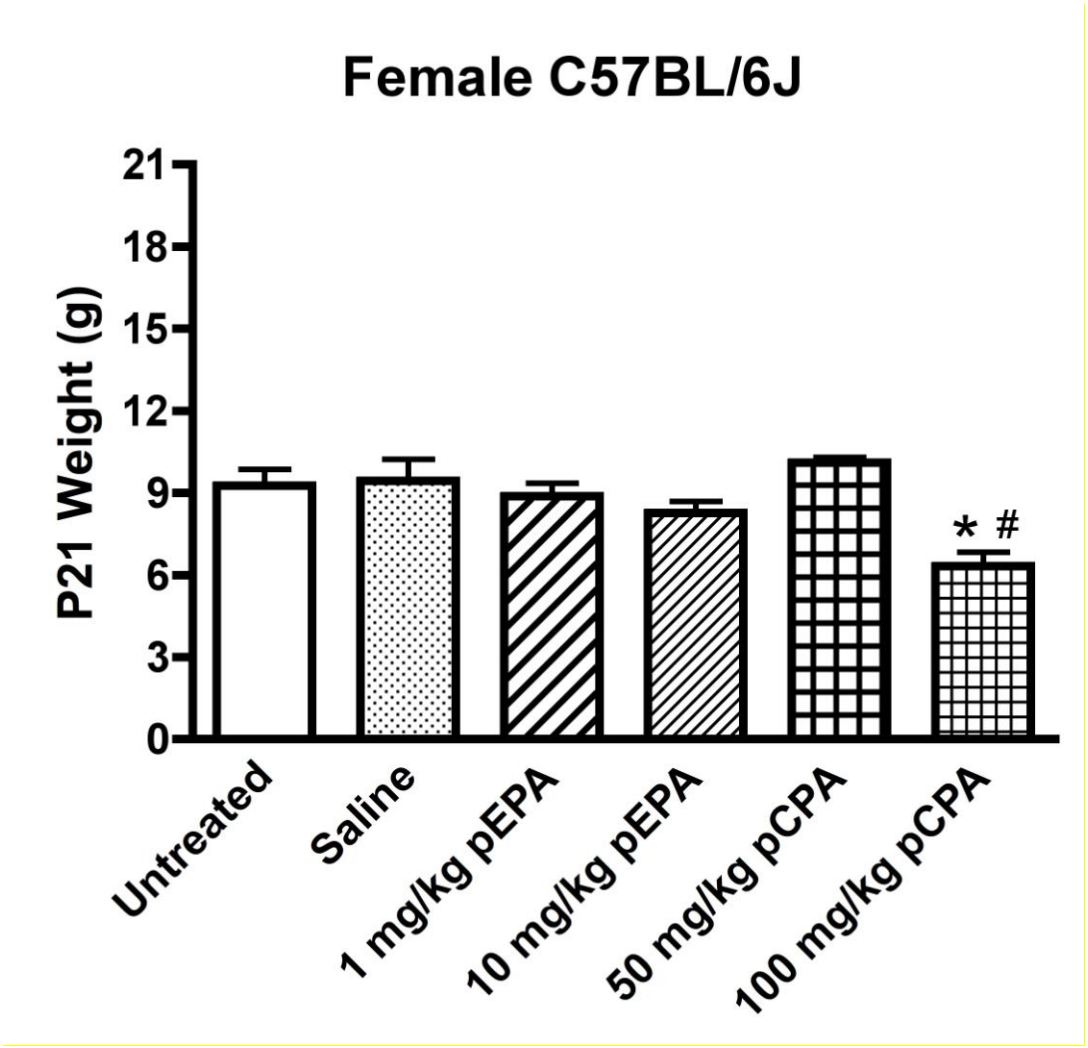

Figure 3-1 Effects of postnatal *p*EPA or *p*CPA on female C57BL/6J P21 weights. Numbers of mice were: Untreated = 10; saline = 7; 1 mg/kg *p*EPA = 9; 10 mg/kg *p*EPA = 4; 50 mg/kg *p*CPA = 2; 100 mg/kg *p*CPA = 11. **P*< 0.05 vs. saline-treated mice and #*P*< 0.05 vs. the lower dose of same drug.

As illustrated in Figure **3-2**, 100 mg/kg *p*CPA had a similar effect on male pup weights such that these were lower at P21 than the male C57BL/6J treated with saline. There was no significant difference between the male C57BL/6J untreated group and the male C57BL/6J saline-treated group, once again indicating no effect of the injection procedure itself. Importantly, none of the C57BL/6J mice administered 10 mg/kg *p*EPA that survived to P21 were male. Otherwise, 1 mg/kg *p*EPA had no statistically significant effect on P21 weights in male C57BL/6J mice.

Different from C57BL/6J mice, there were both significant main effects of treatment ($F(5,77) = 24.8$, $P < 0.001$) and sex ($F(1,77) = 6.19$, $P < 0.001$) for CD-1 pup weights at P21. However, analysis by Tukey *post-hoc* tests showed that there were no significant differences between corresponding treatment groups across the sexes in CD-1 weights at P21. As illustrated in Figure **3-3**, in female CD-1 mice, both the higher 10 mg/kg *p*EPA and higher 100 mg/kg *p*CPA doses were associated with significantly lower P21 weights compared to female saline-treated CD-1 mice. The 10 mg/kg *p*EPA group also had a significantly lower P21 weight compared to female CD-1 mice treated with the lower 1 mg/kg dose of *p*EPA. Once again, there was no significant difference between the female CD-1 untreated group and the corresponding saline-treated group.

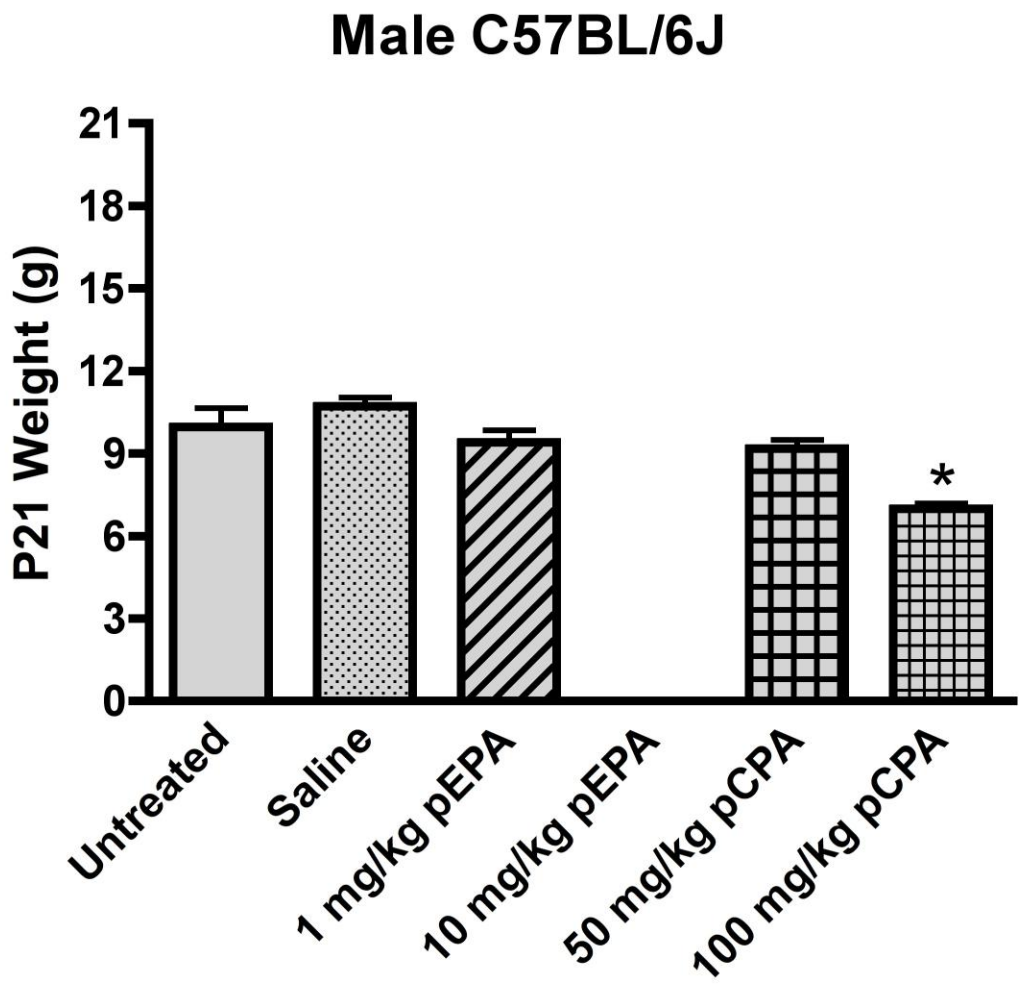

Figure 3-2 Effects of *p*EPA or *p*CPA on P21 weights in male C57BL/6J mice. Numbers of mice treated were: Untreated = 5; saline = 10; 1 mg/kg *p*EPA = 6; 50 mg/kg *p*CPA = 9; 100 mg/kg *p*CPA = 6. **P*< 0.05 vs. saline–treated mice. None of the 10 mg/kg *p*EPA administered C57BL/6J mice that survived to P21 were male.

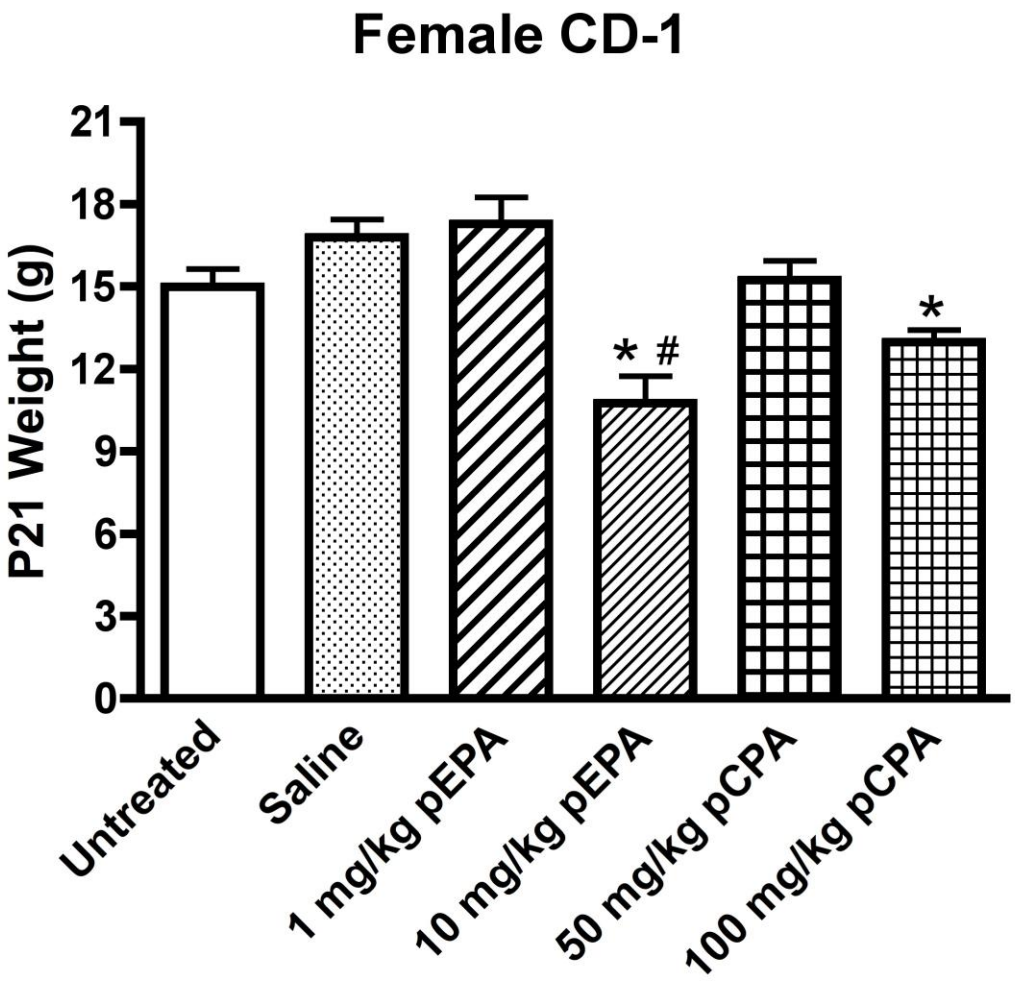

Figure 3-3 Effects of treatment with *p*EPA or *p*CPA on P21 weights in female CD-1 mice. Numbers of mice treated were: Untreated = 6; saline = 12; 1 mg/kg *p*EPA = 7; 10 mg/kg *p*EPA = 5; 50 mg/kg *p*CPA = 8; 100 mg/kg *p*CPA = 9. Statistical significances are indicated by **P*< 0.05 vs. saline-treated mice and #*P*< 0.05 vs. the lower dose of same drug.

Figure **3-4** shows that male CD-1 mice treated with 10 mg/kg *p*EPA and 100 mg/kg *p*CPA similarly had significantly lower P21 weights compared to male CD-1 mice treated with saline. Male CD-1 mice receiving 10 mg/kg *p*EPA also had a significantly lower P21 weight compared to male CD-1 mice administered 1 mg/kg *p*EPA. In addition, the male CD-1 100 mg/kg *p*CPA group showed significantly lower P21 weights compared to male CD-1 mice treated with the lower 50 mg/kg dose of *p*CPA. There was no significant difference between male CD-1 untreated and saline–treated mice.

As illustrated in Figure **3-5**, all treatment groups from the C57BL/6J strain except the 50 mg/kg *p*CPA and the saline-treated groups experienced mortality. Overall survival rates were significantly different across C57BL/6J mice treated with *p*EPA, *p*CPA, or saline [$Chi^2(4)=23.0$; $P<0.001$]. The higher 100 mg/kg dose of *p*CPA in C57BL/6J mice showed a survival rate of 90% by day 8 and 85% by day 16, with the remainder of this group surviving to postnatal day 21. This survival rate was not significantly different from that of saline-treated mice. The higher 10 mg/kg *p*EPA C57BL/6J treatment group had a survival rate of 60% by day 13, losing an additional 10% by day 14, and another 10% by day 19. By postnatal day 21, only 40% of this treatment group survived. In contrast to the higher dose of *p*CPA, the higher dose of *p*EPA in C57BL/6J mice was characterized by a significantly lower survival rate compared to saline-treated mice [$Chi^2(1)=13.61$; $P<0.001$]. Survival rates between the 10 mg/kg and 1 mg/kg pEPA groups were also significantly different [$Chi^2(1)=7.9$; $P<0.05$]. Overall, the higher 10 mg/kg *p*EPA treatment group had the poorest survival rate by postnatal day 21 compared to the other groups in the C57BL/6J strain. The lower dose 1 mg/kg *p*EPA C57BL/6J group had a survival rate of 88% by postnatal day 14, and no deaths occurred during the

remaining 7 days of drug administration. Survival rates in this group were not significantly different from mice treated with saline. In contrast to the mortality observed in C57BL/6J mice treated with *p*EPA or *p*CPA, mice from the CD-1 strain had 100% survival rates throughout the 18 days of treatment regardless of drug or dose (data not shown).

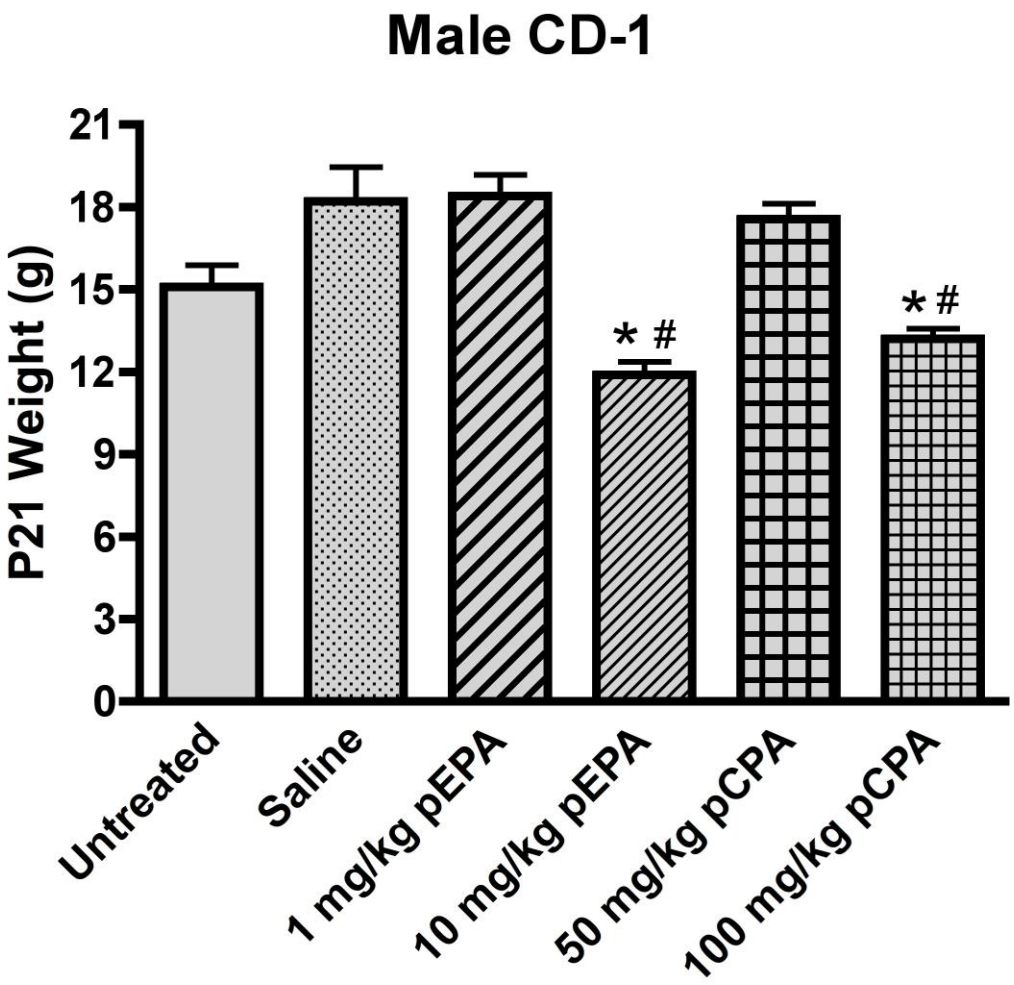

Figure 3-4 Effects of postnatal *p*EPA or *p*CPA on P21 weights of male CD-1 mice. Numbers of mice receiving each treatment were: Untreated = 7; saline = 4; 1 mg/kg *p*EPA = 8; 10 mg/kg *p*EPA = 10; 50 mg/kg *p*CPA = 7; 100 mg/kg *p*CPA = 6. Statistically significant comparisons are indicated by **P*< 0.05 vs. saline-treated mice and #*P*< 0.05, vs. the lower dose of same drug.

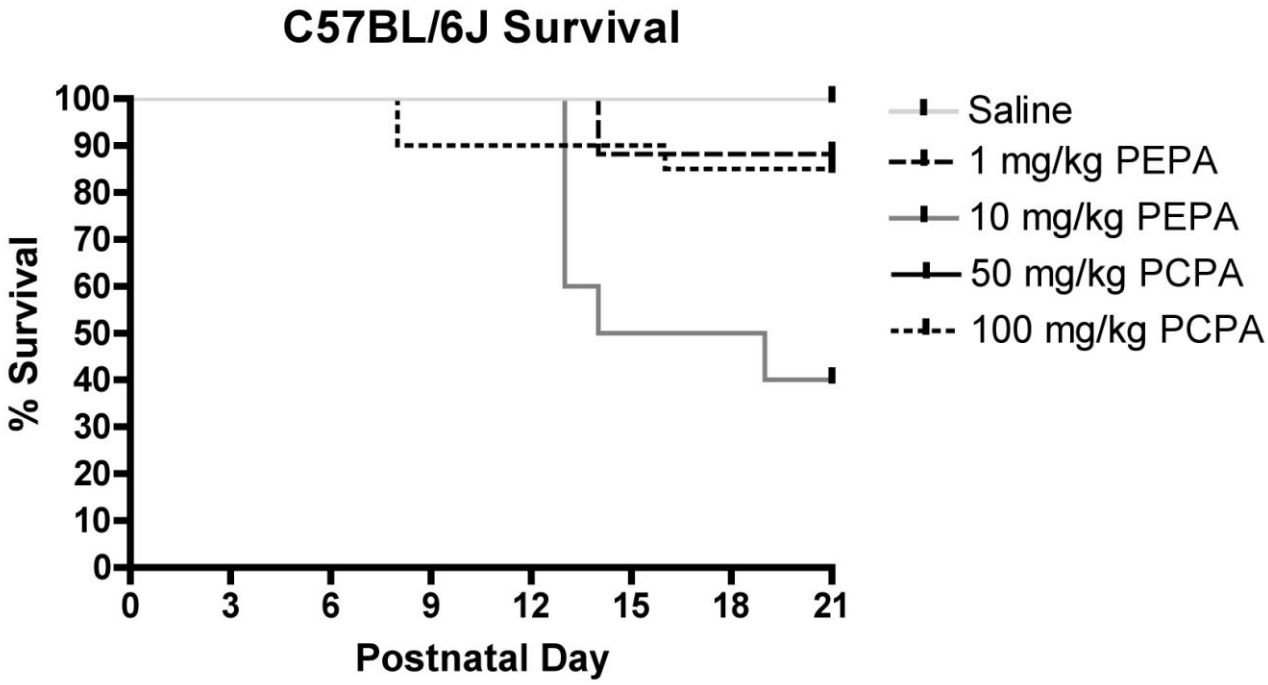

Figure 3-5 Survival rates in C57BL/6J mice treated with *p*EPA or *p*CPA during the early postnatal period. Percent survival with respect to postnatal day for C57BL/6J mice is shown across the treatment period. Numbers of mice treated were: saline = 17; 1 mg/kg *p*EPA = 17; 10 mg/kg *p*EPA = 10; 50 mg/kg *p*CPA = 11; 100 mg/kg *p*CPA = 20. Data for 50 mg/kg *p*CPA line are co-located with saline data.

4. Chapter 4

4.1 Discussion

The results of this study demonstrate that postnatal administration of the higher 100 mg/kg dose of *p*CPA alters normal development in both sexes of C57BL/6J and CD-1 mice. The 100 mg/kg *p*CPA dose had an adverse effect on weight gain in both male and female C57BL/6J and CD-1 mice that was significantly different from the 50 mg/kg dose of *p*CPA. Not only do the data show that postnatal administration of 100 mg/kg *p*CPA elicits a negative physiological response in both strains of mice, but further, movements and sounds made by mice during administration of 100 mg/kg *p*CPA suggest likewise. When mice were injected with the high dose of *p*CPA, they reacted by squirming and vocalizing. The 100 mg/kg *p*CPA treatment group produced more pronounced acute behavioral effects compared to the 50 mg/kg *p*CPA. These observations, which were not quantified, were not observed in *p*EPA-treated mice. Thus, pending further neurochemical analysis, 100 mg/kg *p*CPA appears to be a poor drug/dose for administration to postnatal mice.

Similar to the highest dose of *p*CPA, administration of the higher 10 mg/kg dose of *p*EPA resulted in adverse effects on postnatal weight gain but this was more limited occurring only in both sexes of CD-1 mice. This is indicated by the significantly lower P21 weights of the 10 mg/kg *p*EPA group compared to the saline-treated and 1 mg/kg *p*EPA treatment groups.

Handling, maternal separation, and injecting postnatal mice did not affect weight gain in male or female C57BL/6J or CD-1 mice. Both male and female untreated C57BL/6J mice showed no significant differences in P21 weights compared to saline-treated C57BL/6J mice of the same sex. Moreover, both male and female untreated CD-1 mice showed no significant differences in P21 weights compared to saline-treated CD-1 mice of the same sex.

With respect to postnatal weight gain, the lower 50 mg/kg *p*CPA and 1 mg/kg *p*EPA doses are more advantageous/preferable drugs and doses. When survival rates are considered, however, the more preferable strain would be CD-1 mice as this strain had the highest survival rate (100%) for every treatment group. The C57BL/6J strain experienced mortality after three of the four drug treatment groups (100 mg/kg *p*CPA, 10 mg/kg *p*EPA, and 1 mg/kg *p*EPA), and thus will not be the first choice for future studies unless HPLC analysis to be performed in the near future indicates otherwise.

4.2 What I Learned and Suggested Improvements for Future Studies

The first thing I learned was that when planning studies, sometimes significant pilot work needs to be carried out to determine the conditions under which an experimental study needs to be conducted. Since this was a new project, there was little detailed information from the literature about the best route to decrease postnatal serotonin levels. Thus, we decided to develop new protocols. The second thing I learned was that drawing conclusions about data might not be solely based on easily quantifiable

parameters. I had to take into consideration more subjective animal behavioral responses as well. This is evidenced by the squirming and vocalization behavior that was observed at the higher 100 mg/kg *p*CPA dose. In addition to the analyses of pup weights and survival, acute behavioral observations suggested that this dose of *p*CPA might be poorly tolerated by postnatal mice. However, decreased pup weights and survival were not accompanied by behavioral changes in CD-1 mice.

One way to improve drug administration protocols would be to identify a better labeling tool. Labeling tails with different color markers before drug administration was inefficient since these markings were licked off by the mothers once the pups were returned to their home cages. Thus, it was difficult to identify which pups were supposed to receive which treatment the following day by tail markings alone. For future studies, a non-toxic marker or other way of identifying individual pups that is more permanent, regardless of mother licking, should be identified.

4.3 Future Directions

For future studies, doses lying in between the doses tested in this experiment might need to be considered. In this thesis, two doses of *p*EPA and *p*CPA were evaluated – 1 mg/kg and 10 mg/kg for the former and 50 mg/kg and 100 mg/kg for the latter. The best dose in terms of balancing serotonin depletion with postnatal toxicity might lie between these doses.

In addition, data must be obtained to glean which drug/doses yield large depletions in brain serotonin levels. Once HPLC is performed and the levels of serotonin

and other neurochemicals are analyzed, potential candidates for dosage, drug, and strain may change. For instance, the present results show that administration of 10 mg/kg *p*EPA postnatally to the CD-1 strain is associated with a significantly lower P21 weight compared to saline-treated mice of the same sex and a lower dose of *p*EPA. However, if HPLC analysis shows a 60-70% decrease in serotonin levels at this higher dose and these mice have comparable weights to saline-treated mice at the time of behavioral tests in adulthood, then the 10 mg/kg *p*EPA dose might be the best candidate dose/drug. In addition, HPLC probably should be performed not only at P21 but also at other later time points as it is possible that neurotransmitter levels might remain altered at later ages.

Future behavioral studies in SERT knockout mice will be undertaken once the results of HPLC analysis determines the levels of selective serotonin depletion caused by each drug and dose, as a function of mouse strain. Evaluation of data obtained from behavior tests will show how postnatal administration of serotonin synthesis inhibitors alters anxiety- and depressive-like behaviors in SERT knockout mice during adulthood. In addition, behavior tests, along with data from this thesis and future HPLC analysis will provide new insight into understanding the molecular mechanisms involved in the development of increased anxiety- and depressive-related behaviors, related susceptibility to anxiety and depressive disorders, and how this susceptibility can be reduced or prevented beginning early in life.

REFERENCES

Aghajanian GK, Gallager DW (1975) Raphe origin of serotonergic nerves terminating in the cerebral ventricles. Brain Research 88:221-231.

Alexandre C, Popa D, Fabre V, Bouali S, Venault P, Lesch KP, Hamon M, Adrien J (2006) Early life blockade of 5-hydroxytryptamine 1A receptors normalizes sleep and depression-like behavior in adult knock-out mice lacking the serotonin transporter. Journal of Neuroscience 26:5554-5564.

Ansorge MS, Morelli E, Gingrich JA (2008) Inhibition of serotonin but not norepinephrine transport during development produces delayed, persistent perturbations of emotional behaviors in mice. Journal of Neuroscience 28:199-207.

Ansorge MS, Zhou M, Lira A, Hen R, Gingrich JA (2004) Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice. Science 306:879-881.

Bengel D, Murphy DL, Andrews AM, Wichems CH, Feltner D, Heils A, Mossner R, Westphal H, Lesch KP (1998) Altered brain serotonin homeostasis and locomotor insensitivity to 3, 4-methylenedioxymethamphetamine ("Ecstasy") in serotonin transporter-deficient mice. Molecular Pharmacology 53:649-655.

Blakely RD, De Felice LJ, Hartzell HC (1994) Molecular physiology of norepinephrine and serotonin transporters. Journal of Experimental Biology 196:263-281.

Clarke H, Flint J, Attwood AS, Munafo MR (2010) Association of the 5- HTTLPR genotype and unipolar depression: a meta-analysis. Psychological Medicine:1-12.

DSM-IV-TR (2000) Diagnostic and Statistical Manual of Mental Disorders DSM-IV-TR, Fourth Edition. Washington: American Psychiatric Association.

Farabollini F, Hole DR, Wilson CA (1988) Behavioral effects in adulthood of serotonin depletion by P-chlorophenylalanine given neonatally to male rats. International Journal of Neuroscience 41:187-199.

Gross C, Zhuang X, Stark K, Ramboz S, Oosting R, Kirby L, Santarelli L, Beck S, Hen R (2002) Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. Nature 416:396-400.

Guilloux JP, David DJ, Guiard BP, Chenu F, Reperant C, Toth M, Bourin M, Gardier AM (2006) Blockade of 5-HT1A receptors by (+/-)-pindolol potentiates cortical 5-HT outflow, but not antidepressant-like activity of paroxetine: microdialysis and behavioral approaches in 5-HT1A receptor knockout mice. Neuropsychopharmacology 31:2162-2172.

Heisler LK, Chu HM, Brennan TJ, Danao JA, Bajwa P, Parsons LH, Tecott LH (1998) Elevated anxiety and antidepressant-like responses in serotonin 5-HT1A receptor mutant mice. Proceedings of the National Academy of Science of the United States of America 95:15049-15054.

Holmes A, Yang RJ, Lesch KP, Crawley JN, Murphy DL (2003) Mice lacking the serotonin transporter exhibit 5-HT(1A) receptor-mediated abnormalities in tests for anxiety-like behavior. Neuropsychopharmacology 28:2077-2088.

Jequier E, Lovenberg W, Sjoerdsma A (1967) Tryptophan hydroxylase inhibition: the mechanism by which p-chlorophenylalanine depletes rat brain serotonin. Molecular Pharmacology 3:274-278.

Kalueff AV, Fox MA, Gallagher PS, Murphy DL (2007) Hypolocomotion, anxiety and serotonin syndrome-like behavior contribute to the complex phenotype of serotonin transporter knockout mice. Genes, Brains, and Behavior 6:389-400.

Kessler RC, Chiu WT, Demler O, Merikangas KR, Walters EE (2005) Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. Archives of General Psychiatry 62:617-627.

Kilbey MM, Harris RT (1971) Behavioral, biochemical and maturation effects of early DL-para-chlorophenylalanine treatment. Psychopharmacologia 19:334-346.

Kim DK, Tolliver TJ, Huang SJ, Martin BJ, Andrews AM, Wichems C, Holmes A, Lesch KP, Murphy DL (2005) Altered serotonin synthesis, turnover and dynamic regulation in multiple brain regions of mice lacking the serotonin transporter. Neuropharmacology 49:798-810.

Koe BK, Weissman A (1966) p-Chlorophenylalanine: a specific depletor of brain serotonin. Journal of Pharmacology and Experimental Therapeutics 154:499-516.

Lesch KP, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S, Benjamin J, Muller CR, Hamer DH, Murphy DL (1996) Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. Science 274:1527-1531.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193:265-275.

Mathers CD, Loncar D (2006) Projections of global mortality and burden of disease from 2002 to 2030. Public Library of Science Medicine 3:2011-2030.

Mathews TA, Fedele DE, Coppelli FM, Avila AM, Murphy DL, Andrews AM (2004) Gene dose-dependent alterations in extraneuronal serotonin but not dopamine in mice with reduced serotonin transporter expression. Journal of Neuroscience Methods 140:169-181.

Menard J, Treit D (1999) Effects of centrally administered anxiolytic compounds in animal models of anxiety. Neuroscience and Biobehavioral Reviews 23:591-613.

Murphy DL, Lerner A, Rudnick G, Lesch KP (2004) Serotonin transporter: gene, genetic disorders, and pharmacogenetics. Molecular Interventions 4:109-123.

Numis AL, Unger EL, Sheridan DL, Chisnell AC, Andrews AM (2004) The role of membrane and vesicular monoamine transporters in the neurotoxic and hypothermic effects of 1-methyl-4-(2'-aminophenyl)-1,2,3,6-tetrahydropyridine (2'-NH(2)-MPTP). Molecular Pharmacology 66:718-727.

Ogawa T, Kato H, Mimura Y, Ikeda T, Suzuki MR (1999) para-Chlorophenylalanine induces lenticular opacities by prenatal, neonatal, and juvenile treatments, but not by adult treatment, in rats. Neurotoxicology and Teratology 21:473-477.

Palacios JM, Waeber C, Hoyer D, Mengod G (1990) Distribution of serotonin receptors. Annals of the New York Academy of Science 600:36-52.

Parks CL, Robinson PS, Sibille E, Shenk T, Toth M (1998) Increased anxiety of mice lacking the serotonin1A receptor. Proceedings of the National Academy of Science of the United States of America 95:10734-10739.

Pazos A, Palacios JM (1985) Quantitative autoradiographic mapping of serotonin receptors in the rat brain. I. Serotonin-1 receptors. Brain Research 346:205-230.

Perez XA, Andrews AM (2005) Chronoamperometry to determine differential reductions in uptake in brain synaptosomes from serotonin transporter knockout mice. Analytical Chemistry 77:818-826.

Popa D, Lena C, Alexandre C, Adrien J (2008) Lasting syndrome of depression produced by reduction in serotonin uptake during postnatal development: evidence from sleep, stress, and behavior. Journal of Neuroscience 28:3546-3554.

Ramboz S, Oosting R, Amara DA, Kung HF, Blier P, Mendelsohn M, Mann JJ, Brunner D, Hen R (1998) Serotonin receptor 1A knockout: an animal model of anxiety-related disorder. Proceedings of the National Academy of Science of the United States of America 95:14476-14481.

Raymond JR, Mukhin YV, Gelasco A, Turner J, Collinsworth G, Gettys TW, Grewal JS, Garnovskaya MN (2001) Multiplicity of mechanisms of serotonin receptor signal transduction. Pharmacology and Therapeutics 92:179-212.

Stokes AH, Xu Y, Daunais JA, Tamir H, Gershon MD, Butkerait P, Kayser B, Altman J, Beck W, Vrana KE (2000) p-ethynylphenylalanine: a potent inhibitor of tryptophan hydroxylase. Journal of Neurochemistry 74:2067-2073.

Vinkers CH, Oosting RS, van Bogaert MJ, Olivier B, Groenink L (2010) Early-life blockade of 5-HT(1A) receptors alters adult anxiety behavior and benzodiazepine sensitivity. Biological Psychiatry 67:309-316.

Walther DJ, Bader M (2003) A unique central tryptophan hydroxylase isoform. Biochemical Pharmacology 66:1673-1680.

Wendland JR, Martin BJ, Kruse MR, Lesch KP, Murphy DL (2006) Simultaneous genotyping of four functional loci of human SLC6A4, with a reappraisal of 5-HTTLPR and rs25531. Molecular Psychiatry 11:224-226.

WHO (2008) The Global Burden of Disease 2004 Update. Geneva: World Health Organization.

Zimmer L, Luxen A, Giacomelli F, Pujol JF (2002) Short- and long-term effects of p-ethynylphenylalanine on brain serotonin levels. Neurochemical Research 27:269-275.

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